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MULTIPLE DRUG METABOLISM IN ISOLATED HEPATOCYTES: ENHANCEMENT OF ANILINE HYDROXYLATION

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Summary

A number of agents were tested for their ability to enhance the p-hydroxylation of aniline using isolated hepatocytes as a model system. Although the observed stimulation or inhibition was not concentration dependent, various substrates for the hepatic mixed-function oxygenase (MFO) system (p-nitroanisole, 7-ethoxycoumarin, biphenyl, N,N'-dimethylaminoazobenzene, and benzphetamine) stimulated the hydroxylation at a concentration of 0.5 mM. This effect was not seen with all substrates. In general, aniline hydroxylation was not affected by the other agents tested (steroids, metabolic inhibitors and MFO inhibitors). However, enhancement was noticed with testosterone and progesterone at the lowest concentration (0.05 mM), with 2,6-dichloro-4-nitrophenol and salicylamide at 0.05 mM and 0.5 mM and with 7,8-benzoflavone at 5.0 mM.

Introduction

Enhancement of drug metabolism *in vitro* has been demonstrated with various agents (1). The increased rate of metabolism occurs within minutes after the addition of the enhancing agent. The mechanism by which this enhancement occurs is to date unresolved. Prior administration of many drugs *in vivo* causes an increased enzyme activity for many substrates. In general, this is associated with an increase in the total amount of enzyme (for a specific substrate) and is termed induction. Induction should not be confused with *in vitro* enhancement, which also shows an increase in the rate of product formation but does not show changes in the quantity of enzyme.

Oxidative metabolism of drug substrates by the liver requires the terminal oxidase cytochrome P_{450}, molecular oxygen, and reducing equivalents from NADPH transferred by the flavoprotein cytochrome P_{450} reductase. Importantly, cytochrome P_{450} has been shown to exist as a family of hemoproteins (2) having

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different substrate specificities. Indirectly, this accounts for the diverse number of endogenous, as well as exogenous, compounds which are actively metabolized by this system. In addition, reducing equivalents from NADH may be transferred through cytochrome b_5 reductase and cytochrome b_5 to participate in hepatic mixed-function oxidation of certain drugs (3).

Since these two electron transport systems are located in the endoplasmic reticulum, liver homogenates, isolated microsomes, reconstituted systems and perfused livers have been widely employed in the study of hepatic drug metabolism. More recently the use of isolated hepatocytes has become a prominent model for study. Enhancement *in vitro* has been noted previously in the former systems (4-6) and we have recently reported enhancement of aniline hydroxylation in isolated hepatocytes using p-nitroanisole as the stimulating agent (7). In this communication we report that a number of compounds can stimulate aniline hydroxylation in the isolated hepatocyte.

Methods

Male Sprague Dawley rats (200-250 gms) were maintained on Purina Rat Chow and allowed to drink *ad libitum* a 0.1% phenobarbital solution in their drinking water (7-10 days). Isolated hepatocytes were routinely prepared from two livers perfused simultaneously, checked for cell count and viability (greater than 85% using trypan blue exclusion) and then pooled. The isolation procedure is our modification of the method of Ingebretsen *et al.* (8) and has been reported previously.

Incubations were carried out at 37°C (with shaking) in stoppered 25 ml reaction flasks. Reactions were initiated by the addition of cells and the atmosphere above the cells was flushed briefly with O_2:CO_2, 95:5. Final volumes of 2.0 ml contained 5×10^6 cells and aniline either alone (10 mM) or in combination with one of the number of compounds tested. Drugs prepared in incubation medium were added directly, while use of acetone or ethanol as solvents required evaporation of the solvent prior to initiation of the reaction. After thirty minutes the reactions were terminated with 1.0 ml of ice-cold 20% TCA and the

cell precipitate was centrifuged at 1500 x g for 20 min. Aliquots of the cell-free supernatant were then assayed for free p-aminophenol as described before (7). When benzphetamine and N,N'-dimethylaminoazobenzene were used as enhancing agents, interference from the parent drug was removed by 3 extractions (3 ml per extraction) with water-saturated ethyl acetate.

Results

Table I illustrates the effects of various compounds on the p-hydroxylation of aniline. Drug substrates and products commonly employed in the study of drug metabolism are listed in Group I. Several of these compounds (p-nitroanisole, 7-ethoxycoumarin, biphenyl, N,N'-dimethylaminoazobenzene and benzphetamine) caused a very significant enhancement of aniline hydroxylation at 0.5 mM, displaying approximately a 100% increase over the control value of aniline alone. Although the concentration needed to distinguish between enhancement and inhibition varied among these drugs, the drugs which caused enhancement were evident at a concentration of 0.5 mM. It is also of interest that p-nitrophenol and 7-hydroxycoumarin, both products of reactions, elicited a stimulation at the lower concentrations while two other products, 4-aminoantipyrine and formaldehyde, had no effect. However, aminopyrine (substrate for 4-aminoantipyrine formation) did not show as large a stimulation as some of the other drugs tested. In contrast, 3,4-benzo[a]pyrene, hexobarbital and phenobarbital were analogous to 4-aminoantipyrine and formaldehyde in that the enhancement of aniline metabolism was not seen at any concentration.

The five steroids in Group II neither altered nor caused an inhibition of aniline metabolism with the notable exceptions of testosterone and progesterone. These two steroids only stimulated aniline metabolism (20-40%) at a concentration of 0.05 mM.

The compounds in Group III showed similar results as the compounds in Group II. In general, either no change or no inhibition was noted except for 2,6-dichloro-4-nitrophenol and salicylamide. These two compounds produced a stimulation in aniline metabolism (40-50%) at concentrations of 0.05 mM and

TABLE I: EFFECT OF VARIOUS AGENTS ON ANILINE HYDROXYLATION IN ISOLATED HEPATOCYTES

		Aniline Hydroxylation (% of Control)[a]		
		Concentration of Drug Added		
	Drug Addition	(0.05 mM)	(0.5 mM)	(5.0 mM)
Group I:	p-Nitroanisole	119	231	122
	p-Nitrophenol	112	126	50
	7-Ethoxycoumarin	120	219	76
	7-Hydroxycoumarin	134	126	58
	Biphenyl	102	213	130
	N,N-Dimethylaminoazobenzene	139	204	178
	Benzphetamine	135	186	95
	Aminopyrine	101	120	125
	4-Aminoantipyrine	103	102	88
	3,4-Benzo(a)pyrene	98	100	109
	Hexobarbital	88	85	59
	Phenobarbital	101	85	51
	Formaldehyde	103	103	102
Group II:	Testosterone	143	107	90
	Progesterone	124	108	95
	17 β-Estradiol	102	95	82
	Cortisol	102	76	80
	Cholesterol	107	76	82
Group III:	2,6-Dichloro-4-Nitrophenol	154	139	78
	Salicylamide	144	143	93
	Rotenone	108	100	86
	Potassium Cyanide	101	94	83
	Ethanol	105	104	103
Group IV:	SKF-525A	85	75	54
	Metyrapone	98	103	90
	3-Amino-1,2,4-Triazole	105	105	101
	Imidazole	105	99	57
	7,8-Benzoflavone	112	116	152

a) Each point represents an N of 2 experiments, where each N consists of 2 pooled livers. Male Sprague Dawley rats (200-250 gms.) were maintained on 0.1% phenobarbital in their drinking water for 7-10 days. The mean value for aniline alone was 40.2 nmoles p-aminophenol formed/$5x10^6$ cells/30'.

0.5 mM but not at 5.0 mM.

Group IV is composed of agents which have been used to inhibit cytochrome P_{450}-mediated reactions. SKF-525A appeared to be the only compound to

be an effective inhibitor at all three concentrations. Metyrapone, 3-amino-1,2,4-triazole and imidazole showed essentially no change at 0.05 mM and 0.5 mM, while 7,8-benzoflavone produced a slight stimulation at these lower concentrations. At 5.0 mM, 7,8-benzoflavone gave a 50% enhancement of aniline metabolism, while SKF-525A and imidazole caused an approximate 50% inhibition. 3-Amino-1,2,4-triazole and metyrapone were not effective inhibitors at this concentration.

Discussion

Classically, mutual inclusion of two drug substrates *in vitro* leads to competitive inhibition of at least one or both of the substrates utilized (9). This concept is very useful in partially explaining the prolongation of drug action *in vivo* when combination drug therapy is employed. However, the stimulation of drug metabolism *in vitro* has been shown to be a real phenomenon occurring primarily with aromatic hydroxylation reactions. Agents that enhance aromatic hydroxylation include acetone, 2,2'-bipyridine, o-phenanthroline and metyrapone (10), as well as volatile anesthetics (11), ethylisocyanide (12), paraoxon (13) and certain carcinogens (14). Very few generalizations can be made concerning the mechanism by which these agents elicit their enhancing effect. Most of these agents are not only structurally dissimilar but also differ in their requirement for metabolic conversion (13,14). Indeed, upon review of the nature and conditions for enhancement, insight towards a common mechanism appears to be very perplexing.

As shown in Table I, a variety of structurally unrelated compounds stimulate the p-hydroxylation of aniline to a very significant extent in the isolated hepatocyte. Substrates for mixed-function oxidase reactions, as well as their products, can cause this stimulation. Interestingly enough, 7,8-benzoflavone, a differential effector of aryl hydrocarbon hydroxylase (P448) (15), shows enhancement of aniline metabolism at a concentration causing 50% inhibition by other mixed-function oxidase inhibitors. Agents used to compromise the metabolic status in the cell (rotenone, KCN and ethanol) had no effect

while 2,6-dichloro-4-nitrophenol and salicylamide were ascertained to be enhancing agents.

Clearly it is not yet possible to make a general statement in regard to the mechanism of enhanced aniline metabolism in the isolated cell. The present communication does point out that many factors must be considered when one studies hepatocyte drug metabolism. In the isolated hepatocyte, as in vivo, there are many competing reactions which are concurrent with oxidative metabolism. These disposition reactions may in turn regulate the rate of drug metabolism (i.e., by removal of product inhibition). 2,6-Dichloro-4-nitrophenol and salicylamide implicate the effects of competition reactions, since these agents have been used to compete with sulfation (16) and glucuronidation (17). Although the inhibitors of cellular metabolic processes proved of no significance in this system, energy processes and redox changes cannot be overlooked as points of control in the cell. Changes in reduced pyridine nucleotide levels may play a role as evidenced by the synergistic increase in certain drug biotransformations in the presence of both NADPH and NADH (3). Preliminary microsomal data tends to rule out this type of enhancement at least when p-nitroanisole and aniline are metabolized simultaneously in the presence of both pyridine nucleotides (18). Alterations in cellular uptake of drugs could influence the rate of substrate appearance at the enzyme site, even though it has been shown that drug uptake is probably a non-energy requiring diffusion process (19). Hepatocytes from control and phenobarbital pretreated animals produce the same percentage stimulation of aniline metabolism even though the basal rate was higher in the phenobarbital animals (7). This may argue for a direct or localized effect of these agents on the biotransformation or disposition pathways. It must also be borne in mind that the interaction between the different P_{450} species with the other components of the microsomal electron transport system and conjugation reactions may not be equivocal in microsomes and isolated hepatocytes. Without question, the enhancement in isolated hepatocytes appears to be complex and remains to be elucidated. Further work

is currently in progress to discern the mechanism by which this enhancement occurs in the isolated hepatocyte.

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