DIFFERENTIAL INHIBITION OF DRUG METABOLISM BY HEPATIC MICROSOMAL LIPIDS OF NEONATAL AND ADULT RATS

Michael M. Iba, Lester F. Soyka[1] and Martin P. Schulman

Dept. of Pharmacology, University of Illinois College of Medicine, Chicago, IL. 60612

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SUMMARY. When hepatic microsomes from adult male rats were incubated with microsomal lipids (ML) extracted from the microsomes of neonatal rats, ethylmorphine N-demethylase activity was inhibited by 55%, while aniline hydroxylase activity was not. ML produced a type I spectral change in microsomes of adult rats as did ethylmorphine. The inhibitory component of ML resided in the phospholipid fraction and was destroyed by exposure to O_2. ML from adult male rats contained 20% unsaturated fatty acids and were only one-half as potent as those from neonates (38% unsaturated). These results suggest that phospholipid(s) containing unsaturated fatty acids inhibit the metabolism of type I drugs by combining with their binding site, and may be responsible, at least in part, for the low activity of drug metabolism in the neonate.

The mixed-function oxidase system (MFOS) responsible for the metabolism of xenobiotics, steroids and fatty acids is intimately associated with the endoplasmic reticulum of the hepatocyte. Its activity is markedly decreased in neonatal rodents (1). From solubilized microsomes an active system has been reconstituted containing cytochrome P-450, an NADPH-dependent flavoprotein reductase and phosphatidylcholine (2). Increasing the amount of cytochrome P-450 or reductase led to increased enzymic activity while excess phospholipids were inhibitory in this system.

The phospholipid content of hepatic lipids as well as phospholipid synthesis is greater in the neonatal rodent than in the adult (3, 4). It seemed plausible therefore, that microsomal phospholipids could contribute to the low activity of the MFOS of the neonate.

EXPERIMENTAL PROCEDURES

Preparation of Microsomes. Male rats of Sprague-Dawley descent, raised on

[1] Present address: Dept. of Pharmacol., U. of Vt., Burlington, Vt. 05401.

standard Purina laboratory chow and weighing 150-200 g, were decapitated after an overnight fast. Neonates were five days old at sacrifice and were not fasted. Livers were perfused in situ with ice-cold 1.15% KCl, excised and homogenized in 5 volumes of 1.15% KCl. Hemoglobin-free microsomes were isolated as described by Omura and Sato (5) and resuspended in buffer A(0.05M Tris-HCl in .92% KCl, pH7.5). Protein was determined by the method of Lowry et al. (6).

Lipid Extraction. Total lipids were extracted according to a modification of the method of Folch et al. (7), in which N_2-saturated solvents were employed at -5 to 0°. Phospholipids were prepared from the total lipids according to Norred and Wade (8) omitting α-D-tocopherol. Aqueous suspensions (10 mg/ml) of lipids (liposomes) were prepared in buffer A by the method of Pederson et al. (9) and used within 12 hours. Unsaturation of the total lipid extracts was estimated by a modification (10) of the lipoxidase method of MacGee (11).

Microsomal Enzyme Assays. Ethylmorphine and aminopyrine N-demethylase activities were measured by formaldehyde production (12) but with semicarbazide omitted from the reaction mixture. Aniline hydroxylase activity was assayed by p-aminophenol production (12). Lipid peroxidation was measured by the thiobarbituric acid procedure (13). Rates of NADPH generation and disappearance were measured as follows: For the generation assay, a mixture of 4 mg microsomal protein ± lipids extracted from 4 mg microsomal protein, 12 mM $MgCl_2$, 18 mM Na_3 isocitrate, and 0.4 units isocitrate dehydrogenase, in 6 ml of buffer A, was preincubated for 5 min at 37° and then added in equal amounts in 3 ml sample and reference cuvettes. After establishing a baseline, the reaction was initiated by adding 3.6 µmoles NADP (in .01 ml buffer A) to the sample cuvette and .01 ml buffer A to the reference cuvette. The increase in absorbance at 340 nm was then recorded, and an extinction coefficient of $6.22 mM^{-1}cm^{-1}$ used to estimate the amounts of NADPH formed. NADPH disappearance (i.e., NADPH oxidase) was assayed as described above except that isocitrate, isocitrate dehydrogenase, $MgCl_2$ and NADP were omitted and the decrease in absorbance at 340 nm was recorded following the addition of 3.6 µmoles NADPH to the sample cuvette.

RESULTS. Addition of the microsomal lipid extracted from microsomes of adults or neonates inhibited the N-demethylase activity of hepatic microsomes from adult rats, using the type I substrates, ethylmorphine (Fig. 1) and aminopyrine (data not shown). Lipid extracts on a per mg weight basis from the microsomes of adults were only one-half as inhibitory as those from the neonates. Conversely, extracts from both adult and neonates slightly stimulated the hydroxylation of the type II substrate, aniline (Fig. 1).

The following experiments were performed to characterize the inhibition by the lipid extract. (a) Since enhanced lipid peroxidation inhibits microsomal drug metabolism (14), the effect of the added lipids on malondialdehyde (MDA) formation was measured. MDA production was unchanged by the presence of lipid extracts from neonates and

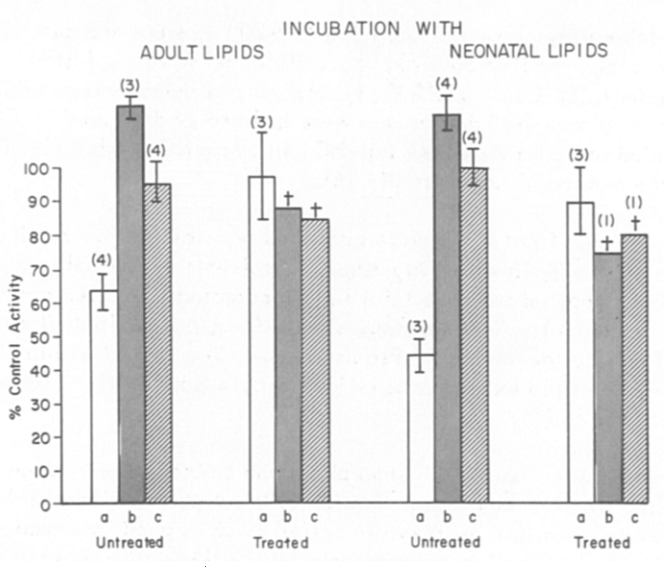

Figure 1. Effect of addition of fresh or treated lipid extracts from microsomes of adult or neonatal rats on the (a) ethylmorphine N-demethylase, (b) aniline hydroxylase and (c) lipid peroxidase activity of microsomes from adult male rats.

Reaction vessels containing 2 mg microsomal protein ± microsomal lipids (1 mg) were pre-incubated in air for 5 min. with an NADPH-generating system (1.2 mM NADP, 6.0 mM $MgCl_2$, 9.0 mM Na_3 isocitrate, and 0.1 units isocitrate dehydrogenase per ml) and drug (4 mM ethylmorphine or 8 mM aniline). The incubation volume was 1 ml and contained 50 mM Tris-KCl buffer. The reaction was initiated by the addition of substrate. Incubations were in duplicate in 10 ml open Erlenmeyer flasks at 37° for 10 min. in air with constant shaking. Treated lipids were prepared by subjecting a suspension of the lipids in a small flask to a stream of oxygen for two minutes. The flask was stoppered and incubated at 40° for 60 mins. and cooled in ice prior to addition to the reaction mixture. The number of experiments is shown in the parentheses.

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adults (Fig. 1). (b) NADPH generation (265 μmoles/min/mg protein) and oxidation (13 μmoles/min/mg protein) were the same in the presence or absence of the lipids; therefore, reduced availability of the cofactor was not responsible for the inhibition. (c) The inhibitory component resided in the phospholipid fraction of the lipid extracts; the neutral lipid fraction from either adult or newborn was inactive (data not shown). (d) Analysis of the lipid extracts revealed that the degree of unsaturation in neonatal lipids (38%) was about twice that of lipids extracted from microsomes of adult rats (20%). (e) When the lipid extracts were exposed to O_2 at 40° for 60 min., they lost their inhibitory action on

ethylmorphine N-demethylase activity (Fig. 1). These inactive extracts were virtually devoid of unsaturation (0.2%). (f) The addition of lipid extracts from adults or neonates to microsomal suspensions from adults caused a concentration-dependent spectral change characteristic of type I substrates (Fig. 2). The maximum spectral change induced by total lipid extracts from adults was only about one-half that obtained by the same weight of lipids from neonates. The oxygen exposed "treated lipids" failed to induce a spectral change. After attainment of the maximum spectral change with the lipid extracts, addition of ethylmorphine caused no further change. Moreover, the spectral change induced by aniline (type II) as well as the carbon monoxide (CO) and ethylisocyanide (EtNC) difference spectra of reduced cytochrome P-450 were not altered by the addition of lipid extracts. These data suggest that the binding site(s) for the unsaturated lipids

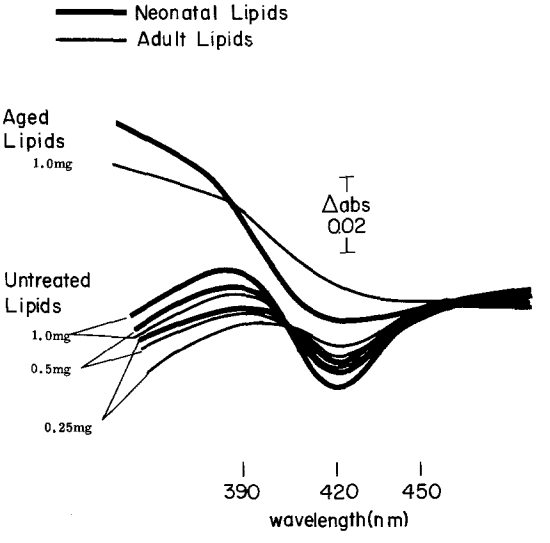

Figure 2. Spectral changes induced in microsomes by lipid extracts.

Hepatic microsomes (2 mg/ml), suspended in Tris-KCl buffer, pH 7.5, were added to the sample and reference cuvettes of a Shimadzu MPS-50L split-beam spectrophotometer. After establishing a baseline, lipid extracts were sequentially added to the sample cuvette to total amounts of 0.25, 0.75, and 1.0 mg. The reference cuvette was balanced with an equivalent volume of buffer. With treated lipids, only 1.0 mg was added to the sample cuvette.

were other than the heme of cytochrome P-450, which ligands with aniline, CO and EtNC. The neutral lipid (anhydrous acetone extract) subfraction did not produce a type I spectral shift. Treated lipids induced the anomalous spectra shown in Fig. 2.

DISCUSSION. Several mechanisms have been suggested to account for the impaired drug metabolizing activity of the neonatal rodent. These include: high levels of gestational steroids of maternal origin (15), androgen deficiency (16) and high levels of growth hormone (17).

The inhibitory factor described herein appears to differ from these and to be due to unsaturated fatty acids present in phospholipids. The ratio of the inhibitory potency of extracts from microsomes of neonates vs. those from adults correlated well with the relative degree of unsaturation of the lipid extracts. Treatment with heat and oxygen resulted in loss of unsaturation and destruction of the inhibitory activity. That such unsaturated fatty acids interact at the binding site for type I substrates was evidenced by the induction of a type I spectrum.

Interactions of fatty acids with hepatic microsomes, e. g., the induction of type I difference spectra, have been observed with laurate, which is hydroxylated by the cytochrome P-450 system (2), and with prostaglandins (18) which are oxidation products of unsaturated fatty acids and, possibly, substrates for the P-450 MFOS. Since the type I spectral shift occurred immediately upon addition of lipids to microsomes, it is improbable that an active material, such as prostaglandin, was formed or released from the lipid fraction during mixing.

Our results, plus indirect evidence in the literature, led us to propose that unsaturation of the constituent fatty acids of microsomal membranes may inhibit the activity of enzymes associated with these membranes. For example, enhanced unsaturation of phospholipids induced by dietary lipids altered the spectral characteristics (19) and inhibited the enzymic activities of phospholipid requiring inner mitochondrial membrane proteins (20). Depletion of microsomal lipids by detergent resulted in enhanced activities

of microsomal glucuronyl transferase and glucose-6-phosphatase (21). Solvent extraction of microsomes enhanced the maximum inducible spectral change by type I compounds (22) and the magnitude of this spectral change usually correlated with the magnitude of metabolism of a compound (23). Finally, although essential for maximum activity, the phosphatidylcholine fraction of solubilized microsomes was inhibitory at increased concentrations (2).

Inhibition of the MFOS by unsaturated phospholipids may account in part for the low activity (relative to the male) of female, pregnant and lactating rats as well as the age-dependent deficiency. Females and neonates may have enhanced lipogenesis as a result of their higher levels of estrogenic hormones. Indeed, studies in progress appear to indicate a greater degree of unsaturation of the phospholipids derived from lipid extracts of the microsomes of such rats.

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