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Monooxygenase activity of systems reconstituted with fractions from rats fed standard and low protein diets

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Weanling rats were fed *ad libitum* for 6 weeks either a standard diet containing 22% casein (St) or a low protein diet containing 6% casein (LP). The individual components of the microsomal cytochrome P-450-dependent monooxygenase system such as lipid, cytochrome P-450 and cytochrome P-450 reductase were isolated from the two dietary groups. Then, they were recombined in various combinations and the contributions of the individual components to the monooxygenase activity with respect to benzphetamine and ethylmorphine as substrates were studied. These metabolic activities were strongly decreased (86–95%) when incubations were carried out with fractions from the LP group compared to the St group. This decrease was predominantly (to about 60%) due to changes in cytochrome P-450. A smaller but considerable contribution (about 30%) to the decrease was due to changes in cytochrome P-450 reductase and very little (about 10%) of the decrease was due to changes in phospholipid composition. The observations indicate that low protein diet affects both the specific activities of the individual components and the interaction between cytochrome P-450, cytochrome P-450 reductase and phospholipids.

in the quantity of dietary protein depresses the activity of the cytochrome P-450 dependent microsomal monooxygenase (MO) system towards the substrates investigated [1–4]. These decreases may be accounted for either by decreased levels or by decreased catalytic activities of cytochrome P-450. Another mechanism by which the level of dietary protein intake may influence the specific activity of the MO system is through an effect on phospholipid composition [5, 6]. The work of I.U and COON in 1968 [7] has led to techniques allowing for the fractionation of the various components of the MO system, which when recombined allows for partial reconstitution of activity. With the availability of these procedures we report here the effect of low protein diet on the individual MO components. Moreover, in the present study a method was used which allowed the isolation of cytochrome P-450 at the low levels occuring in non-induced animals on a low protein diet.

Materials and methods

Twenty weanling Sprague–Dawley male rats were fed for one week with a standard (St) diet containing 22% casein and then subdivided into two groups. Group 1 continued on the standard diet and group 2 received a low protein diet (LP) containing 6% casein given *ad libitum* for 6 weeks. Rats were starved overnight before killing and preparation of liver microsomes were carried out as previously described [8].

Purification of cytochrome P-450. Liver microsomal cytochrome P-450 was solubilized with sodium cholate and purified by affinity chromatography on ω-aminooctyl-sepharose 4B prepared as described [9] followed with a hydroxyl appatit column. The cytochrome P-450 was eluted with 50 mM phosphate buffer pH 7.25 from this column and then concentrated with ultra filtration (30 pm). The specific content of this fraction was 17 nmol of cytochrome P-450 per mg protein recovery of the first column was approximately 45% of total microsomal cytochrome P-450, and second column 27%, this fraction had not any contamination by other proteins juged by S.D.S. gel electrophores [10].

Purification of NADPH cytochrome P-450 reductase. The microsomal enzyme was extracted from remaining proteins after the elution of cytochrome P-450 from the Sepharose column as reported by Yasuko and Masters [11]. The specific activity was 21500 units per mg protein assayed by the method of Phillips and Langdon [12]. SDS polyacrylamide gel electrophoresis and transfer to nitrocellulase sheet were carried out as described elsewhere for investigating enzyme hemogenity [13]. Antisera bound to nitrocellulase were visualized by peroxidase-conjugated rabbit antisera and staining with 1-chlor-4-naphtol.

Lipid extraction. Lipids from microsomal membranes were isolated by isopropanol extraction [14]. The lipid content was measured by gravimetry after solvent removal.

Reconstitution of the MO system. The reconstituted monooxygenase system consisted of the following constituents: 0.5 nmol cytochrome P-450, 50 units of cytochrome P-450 reductase and 0.1 mg lipids/ml. A spectrophotometric assay for monooxygenase activity with ethylmorphine and benzphetamine as substrates was used [15].

Results

Table 1 represents the effect of substitution of MO components from rats fed a standard diet (St) by MO components from animals fed a diet low in protein. 6% casein (LP). Monooxygenase activities with ethylmorphine as substrate were markedly lower (in the St group less than 10%) than of those found for benzphetamine. Lu and coworkers [15] using monooxygenase components from a different rat strain also found a lower monoooxygenase activity for ethylmorphine than for benzphetamine. Substitution of the St cytochrome P-450 fraction by LP P-450 caused considerable decreases in ethylmorphine (−63%) and benzphetamine (−73%) metabolism. Substitution of the St cytochrome P-450 reductase by LP reductase caused smaller changes (a 35% and 41% decrease in metabolism of ethylmorphine and benzphetamine, respectively). Substitution of the St lipid fraction by LP lipid caused a very small but still significant decrease in ethylmorphine (−17%, $P < 0.05$) and benzphetamine (−9%, $P < 0.5$)) Metabolism. Substitution of the LP fractions by fractions from the St group is shown in Table 2. The reconstituted system entirely composed of LP components (Table 2) showed an 86% reduction in monooxygenase activity for ethylmorphine and 95% reduction for benzphetamine when compared to the reconstituted system completely composed of St components (Table 1). When individual components of the LP reconstituted system were substituted by St components, again cytochrome P-450 had the greatest effect (4.3–7.5-fold increases in monooxygenase activities for ethylmorphine and benzphetamine, respectively). Substitution of the LP cytochrome P-450 reductase fraction by St reductase increased ethylmorphine metabolism 1.7-fold and benzphetamine metabolism 4.5-fold. Substitution of LP lipid fraction by St lipid showed an 1.4-fold increase in monoooxygenase activity for ethylmorphine and a 3.3-fold increase for benzphetamine as substrate.

Discussion and conclusions

Our previous studies had demonstrated that the depression of hepatic MO activity observed in rats fed low dietary protein is due in part to a depression of the specific enzyme activity [16]. These results are in line with those of several other workers [1, 2]. However, it was not possible to determine whether the decreased monooxygenase activity was due to changes in cytochrome P-450, to changes in cytochrome P-450 reductase, to modifications of microsomal lipids or to a combination of these factors. In the present study reconstituted preparations which contained various

Table 1. Metabolism of ethylmorphine and benzphetamine by a reconstituted microsomal monooxygenase system comprised of lipid, cytochrome P-450 reductase and partially purified cytochrome P-450 derived from the livers of untreated male Sprague–Dawley rats fed a standard diet (St) and the influence of substitutions by components from the livers of rats fed a diet low in protein, 6% casein (LP)*

Substitutions of St fractions	NADPH oxidized nmol/min/ml	
	Ethylmorphine	Benzphetamine
Lipid (St) + Reductase (St) + P-450 (St)	1.01 ± 0.01	16.8 ± 0.21
Lipid (St) + Reductase (St) + P-450 (LP)	0.37 ± 0.02	4.6 ± 0.12
Lipid (St) + Reductase (LP) + P-450 (St)	0.65 ± 0.02	9.9 ± 0.09
Lipid (LP) + Reductase (St) + P-450 (St)	0.84 ± 0.02	15.4 ± 0.10

* Incubation was in 1 ml medium containing 0.5 nmol cytochrome P-450, 50 units cytochrome P-450 reductase and 0.1 mg microsomal lipids. Data represent the mean ± SD of three replicate assays.

Table 2. Metabolism of ethylmorphine and benzphetamine by a reconstituted microsomal monooxygenase system comprised of lipid, cytochrome P-450 reductase and partially purified cytochrome P-450 isolated from the liver of adult male Sprague–Dawley rats fed a diet low in protein, 6% casein (LP) and influence of substitutions by components from the livers of rats fed a standard diet (St)

Substitutions of LP fractions	NADPH oxidized nmol/min/ml	
	Ethylmorphine	Benzphetamine
Lipid (LP) + Reductase (LP) + P-450 (LP)	0.14 ± 0.01	0.88 ± 0.12
Lipid (LP) + Reductase (LP) + P-450 (St)	0.60 ± 0.02	6.62 ± 0.14
Lipid (LP) + Reductase (St) + P-450 (LP)	0.24 ± 0.01	4.02 ± 0.10
Lipid (St) + Reductase (LP) + P-450 (LP)	0.20 ± 0.01	2.9 ± 0.12

See footnote to Table 1.

combinations of components derived from the two dietary groups were constructed so that each individual component was added at equivalent levels. In 1978 Nerurkar *et al.* [17] investigated the effect of low protein diet by using a reconstituted system technique. However, the fractions used were from rats treated with phenobarbital. So the effect observed was not only due to dietary protein. We have shown that induction leads to significant changes not only in cytochrome P-450 isoenzymes but also in lipid composition of microsomes from rats fed a LP diet [18] and that changes of the MO system introduced by LP diet in animals treated with inducers are completely different from those in untreated animals (Amelizad *et al.*, manuscript in preparation). In the present study we have therefore used a more recently developed method which allows the isolation of the very low cytochrome P-450 from animals treated with a LP diet and not treated with any cytochrome P-450 inducers. Moreover Nerurkar and coworkers used weanling rats fed 10 days with unbalanced diets, whilst in this investigation the animals were treated for 60 days with the experimental diets. Substitution of St lipid fraction with the LP lipid decreased *N*-demethylase activity by 10–20% (Table 1). A low protein intake had been demonstrated to alter the phospholipid composition or microsomal membranes [6]. In rats fed 60 days with a 5% casein diet, phosphatidyl ethanolamine and phosphatidyl serine were decreased by 30% and sphingomyelin was increased 2.5 times compared to rats fed a 18% casein diet. Our results can at least in part be explained by a change in phosphatidyl serine content. Haaparanta *et al.* [19] reported that vesicular MO reconstituted systems were up to 6 times more active in deethylation of 7-ethoxycoumarin when prepared with phosphatidyl serine than when prepared with phosphatidyl choline or phosphatidyl ethanolamine. Thus the decrease in demethylation when LP lipids were added to a St system and the increase when St lipids were added to an LP system may in part be explained by changes in phosphatidyl serine content of the microsomal phospholipids.

Substitution of cytochrome P-450 caused higher changes in monooxygenase activity of reconstituted systems compared with other substitutions. Thus the major part in decrease of specific MO activity after treatment with LP diet can be related to changes in cytochrome P-450 (about 60%). However, the change in cytochrome P-450 reductase also plays a considerable role. The contribution of the reductase to the decrease of the MO activities with ethylmorphine and benzphetamine as substrates was about 30%.

Several mechanisms underlying the observed decreases in monooxygenase activities by substituting LP components in the reconstituted system may be envisaged. Besides changes relating only to individual components, changes relating to the interaction between the components may prove to be important. The exact nature of these changes remains to be established.

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Effect of 1-anilino-8-naphthalene sulfonate (ANS) on transferrin and iron uptake by reticulocytes

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Nucleated erythroid cells and reticulocytes are able to acquire iron from plasma transferrin by receptor-mediated endocytosis [1]. Any derangement of membrane structure could markedly affect this iron transport system [2]. A membrane probe, 1-anilino-8-naphthalene sulfonate (ANS), interacts with phospholipid and protein regions of the erythrocyte plasma membrane and creates a negative surface potential of sufficient magnitude to repulse anion transport from the permeation site [3]. In view of the influence of ANS on membrane charges and carrier-mediated transport, the process of transferrin and iron uptake by reticulocytes might be disrupted. The present study investigates this possibility and attempts to find out in what way it could interfere with the transport mechanism.