EFFECT OF TOTAL PARENTERAL NUTRITION AND CHOLINE ON HEPATIC FLAVIN-CONTAINING AND CYTOCHROME P-450 MONOOXYGENASE ACTIVITY IN RATS

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ABSTRACT:
Total parenteral nutrition provides nutrition by infusion into the systemic circulation. By-passing the intestine and processes associated with absorption can cause additional pathophysiological changes to occur. For example, in rats, normal gut and pancreatic cell function may change, absorptive capacity may be altered, and enzyme functional activity including drug metabolism may be affected. The objective of this study was to examine the effects of a control diet or a diet of total parenteral nutrition in the presence or absence of choline on urinary biomarkers and hepatic microsome functional activity from rats. Selective functional markers of cytochrome P-4502E1 (CYP2E1) and flavin-containing monooxygenase (FMO) were examined in vitro. The N-oxygenation of trimethylamine was used as an in vivo selective functional marker for FMO. After the administration of total parenteral nutrition plus choline for 5 days, the urinary excretion of trimethylamine and trimethylamine N-oxide declined approximately 7- and 3-fold, respectively, compared with rats treated with control diet. The concentration of urinary biogenic amines was also significantly affected by total parenteral nutrition. Compared with control animals, rats administered total parenteral nutrition plus choline for 5 days showed a decrease of approximately 5- and 2-fold in urinary dopamine and norepinephrine concentration, respectively. To examine a molecular basis for the influence of total parenteral nutrition ± choline on monooxygenase regulation, hepatic microsomal activity of the FMO and CYP2E1 was examined. Compared with animals treated with a control diet, total parenteral nutrition plus choline in rats caused a 3-fold increase in hepatic microsomal FMO and a 2-fold increase in hepatic cytochrome CYP2E1 functional activity, respectively. Although the data did not reach statistical significance, selective immunoblot studies using hepatic microsomes from rats treated with total parenteral nutrition + choline showed that compared with controls, FMO1 protein was decreased 1.4-fold and FMO3 increased 1.3-fold, respectively. In hepatic microsomes from rats treated with total parenteral nutrition + choline, compared with control animals, FMO4 immunoreactivity was increased 1.6-fold. The data suggest that total parenteral nutrition has a detectable effect on modulating rat FMO3, FMO4, and CYP2E1 monooxygenase functional activity. The clinical relevance of these results is unknown but may be of significance for individuals receiving total parenteral nutrition and those afflicted with trimethylaminuria.

Natural regulation and normal function of the gastrointestinal tract requires direct administration of dietary proteins, fats, and carbohydrates. Total parenteral nutrition (TPN) provides nutrition by direct infusion into the systemic circulation. However, bypassing the intestine and processes associated with absorption can cause additional pathophysiological changes to occur. For example, in rats, normal gut and pancreatic cell function may change, absorptive capacity may be altered, and enzyme functional activity including drug metabolism may be affected within 3 days of initiating TPN (Hughes and Dowling, 1980).

Humans receiving long-term TPN can develop choline deficiency and hepatic steatosis (Buchman et al., 1992). Plasma-free choline is abnormally low in approximately 80% of TPN patients (Buchman et al., 1993). Normally, choline is biosynthesized from methionine. Choline deficiency may arise from 1) defective hepatic trans-sulfuration, 2) deficient phosphatidylcholine utilization and/or abnormal triglyceride transport, or 3) blockage of metabolism of phosphatidylcholine to free choline. Choline is a precursor to trimethylamine (TMA), a dietary amine essentially completely N-oxygenated to trimethylamine N-oxide (TMA N-oxide) and efficiently excreted in humans receiving long-term TPN can develop choline deficiency and hepatic steatosis (Buchman et al., 1992). Plasma-free choline is abnormally low in approximately 80% of TPN patients (Buchman et al., 1993). Normally, choline is biosynthesized from methionine. Choline deficiency may arise from 1) defective hepatic trans-sulfuration, 2) deficient phosphatidylcholine utilization and/or abnormal triglyceride transport, or 3) blockage of metabolism of phosphatidylcholine to free choline. Choline is a precursor to trimethylamine (TMA), a dietary amine essentially completely N-oxygenated to trimethylamine N-oxide (TMA N-oxide) and efficiently excreted in normal animals (Cashman et al., 2003).

Administration of TPN produces effects on drug- and xenobiotic-metabolizing enzyme systems. Continuous infusion of TPN to rats decreased cytochrome P450 (P450) content 25% and produced a 40 to 55% decrease in P450 functional activity (Knodell et al., 1984) compared with control animals fed an identical diet by the enteral route of administration. Infusion of TPN via the jugular vein significantly decreased CYP 3A1 and CYP2C11, although CYP2A1 and...
CYP2C6 were unchanged compared with control animals receiving the same diet by the enteral route of administration (Knodell et al., 1989). In rats (Ross et al., 1983) and humans (Burgess et al., 1987) receiving TPN, a marked decrease in plasma clearance of antipyrine was observed, although in patients receiving TPN in whom lipid emulsion was substituted for some of the carbohydrate, antipyrine clearance was unchanged (Burgess et al., 1987). In another study, rats receiving TPN were observed to possess decreased flavin-containing monooxygenase (FMO) activity (Kaderlik et al., 1991). In addition to changes in monooxygenases, the phase II enzymes mediating glucuronidation, glutathione, and sulfate conjugation were decreased 58%, 29%, and 48%, respectively, in rats receiving lipid-free TPN (Raftogianis et al., 1995, 1996). Feeding male Wistar rats a synthetic diet resulted in a 2-fold decrease in the clearance of TMA (Nnane and Duman, 2001).

In addition to P450, another monooxygenase system has evolved to remove dietary xenobiotics. N- and S-oxygenation of nucleophilic heteroatom-containing compounds by the FMO generally represents a detoxication process (Ziegler, 1990; Cashman, 1995, 2002a). Mammalian FMO comprises a family of five functional enzymes, and FMO1 and FMO3 are the prominent functional enzyme forms in rat liver, although other FMO enzymes including FMO4 also contribute to oxygenation of substrates to polar, more readily excreted metabolites (Lattard et al., 2002).

CYP2E1 plays an important role in the pathogenesis of nonalcoholic steatohepatitis in animal models. In humans, CYP2E1 activity and expression are enhanced in nondiabetic patients with nonalcoholic steatohepatitis (Chalasani et al., 2003) and chronic hepatitis C (Gochee et al., 2003). Because CYP2E1 is toxicologically one of the most important P450 enzymes in steatohepatitis, and because FMO is almost exclusively responsible for the N-oxygenation of TMA, changes in rat liver CYP2E1 and FMO functional activity were examined in control animals and compared with functional activity of rat liver microsomes isolated from animals administered TPN in the presence or absence of choline. In vitro activity was determined by examining the metabolism of selective functional substrate probes of FMO and CYP2E1 in the presence of hepatic microsomes prepared from control rats and rats administered TPN for 5 days in the presence or absence of choline. The in vivo activity of FMO was determined by analysis of urinary TMA and TMA N-oxide concentration. In the present study, compared with controls, a significant increase of FMO and CYP2E1 functional activity was observed in hepatic microsomes obtained from animals treated with TPN + choline. In addition, hepatic FMO immunoreactivity was increased in rats treated with TPN + choline compared with rats receiving a control diet. Changes observed in vitro also were manifested in vivo because TPN + choline significantly modulated urinary concentrations of TMA, TMA N-oxide, and some biogenic amines.

**Materials and Methods**

**Chemicals.** The chemicals used in this study were of the highest purity from commercially available sources. TMA, TMA N-oxide, triethylamine (TEA), methythiolyl sulfide (MTS), 3,4-dihydroxybenzylamine, chloroxazole, and catecholamine standards were purchased from Aldrich Chemical Co. (Milwaukee, WI). 10-[N,N-Dimethylaminopentyl-2-(trifluoromethyl)phenothiazine (5-DPT) and methylthiolyl sulfoxide (MTSO) were synthesized as previously described (Brunelle et al., 1997). The buffers, reagents, and solvents used in this study were obtained from Fisher Scientific (San Jose, CA). All of the compounds of the NADPH-generating system were purchased from Sigma-Aldrich (St. Louis, MO).

**Animal Procedures.** Male Sprague-Dawley rats (180–195 g) from Bantin and Kingman (Seattle, WA) were acclimatized for 3 days before the study. The protocol was approved by the University of Washington Institutional Animal Care and Use Committee and followed the guidelines described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After an overnight fast, the rats were anesthetized with sodium pentobarbital (25 mg/kg i.p.), and intravenous lines were inserted. The right internal jugular line was infused 1 ml/h intravenously, and rats were given chow and water ad libitum for 24 h during the recovery period. Individual rats were housed in metabolic cages and randomized to one of three groups: rat chow plus intravenous saline (n = 4), TPN + choline (n = 4), and TPN − choline (n = 4).

Chow-fed rats were given Rodent Diet 8604 from Harlan Teklad (Madison, WI), and energy content was 16.4 kJ/g with 24% protein, 4.4% fat, and 3.7% fiber. The TPN solution consisted of 25% dextrose, 4.25% amino acids, Trasvalos from Clintec Nutrition (Deerfield, IL), vitamins (5 ml/l), choline (1 g/l), heparin (1000 U/l), minerals, and electrolytes. The energy content was 4.6 kJ/ml and the nitrogen content was 9.6 mg/ml. After a 24-h recovery period, chow-fed rats were started on TPN + choline or TPN − choline at a rate of 1 ml/h for the first 24 h and then increased to 2 ml/h for the remainder of the study. For chow-fed rats, subcutaneous infusion was 1 ml/h. Diets were continuously delivered by 60-ml syringes with infusion pumps. Syringes with diet or saline were changed daily at 8:00 AM.

**Urinary Product Analysis.** TMA. Urine was collected in acidified containers underneath metabolic cages. Acidification of the urine samples prevented loss of the nonvolatile TMA hydrochloride. The concentration of TMA N-oxide and TMA present in each urine sample (taken between 3:00 PM and 7:00 AM the next morning) was analyzed by reverse phase HPLC and gas chromatography (GC), respectively. For determination of TMA levels, a 10-ml sample of acidified urine (i.e., pH = 3) was re-acidified with 0.5 ml of 6 N HCl, transferred to a Reacti-vial, and capped with a Tuf-Bond Teflon silicon septa until the samples were analyzed by GC. Immediately before GC analysis, the samples or the standard solutions were made alkaline by the addition of 0.8 ml of 14 N NaOH at −70°C. The vials were tightly recapped, mixed, and heated in a sand bath at 100−110°C for 1 h. After the sample was brought to room temperature, a sample of head space was injected into the GC. Five injections were made for each determination, and the peak areas were compared with standard curves containing TEA as the internal standard. The amount of TMA in the urine samples was calculated by measuring the ratio of the peak area of the sample compared with the standard curve. The limit of detection was 1.0 nmol TMA/ml of urine (or approximately 0.5 nmol TMA/μmol of creatinine), and the urinary TMA concentration was expressed as nanomoles of TMA per micromole of creatinine ± S.D. Control studies showed the percentage recovery of the internal standard was essentially quantitative, and the sample variability between samples was typically less than 5% from Animals among the same group of animals. Expression of the analyze in terms of nanomoles/micromole of creatinine was a convenient way of normalizing the values for any changes in urine volume.

GC analysis for TMA was carried out on a Hewlett-Packard 5890 Series II gas chromatograph Hewlett Packard (Palo Alto, CA), with a nitrogen-phosphorus detector that was interfaced to a Hewlett-Packard 3396 Series II Integrator. Chromatography was done with a Stabilwax-DB capillary column, 0.32 mm i.d., 0.25 μm df, 30 m, from Alltech Associates Inc. (Deerfield, IL) in a manner similar to that described before (Tjoa and Fennessey, 1991). The conditions for chromatography were: helium carrier flow at 36 ml/min, injector temperature at 200°C, and detector temperature 240°C. The column was operated at 70°C for 2 min postinjection and then programmed to 105°C for 20°C/min. Employing these conditions, the retention times for TMA and TEA were 0.89 min and 0.97 min, respectively.

TMA N-oxide. For determination of TMA N-oxide, 1.0 ml of urine and the sample were concentrated in vacuo at a temperature below 30°C. The residue obtained was taken up in 0.6 ml of MeOH and filtered through a solid phase extraction column. The eluant was used directly in the HPLC analysis. A Hitachi L-6200 HPLC (Hitachi Instruments Inc., San Jose, CA) was used to determine TMA N-oxide concentrations employing a SEDEX 55 evaporative light-scattering detector (Richard Scientific Inc., Novato, CA), interfaced to a Hitachi D-2500 integrator. The reversed phase C18 HPLC column (4.6 × 250 mm, 5-μm Microsorb MV) (Rainin Instruments, Woburn, MA) efficiently separated TMA N-oxide (i.e., retention time 3.2 min) from the solvent front employing a mobile phase of 62% A, 35% B, and 3% C, where A was water,
was acetonitrile, and C was methanol containing 0.4% saturated ammonium hydroxide. An external standard curve of TMA N-oxide was constructed, and the peak area of the sample was compared with the standard curve. The amount of TMA N-oxide was calculated by measuring the peak area of the sample and comparing the value with the standard curve. The limit of detection was 10 nmol/ml of urine (or approximately 5.3 nmol/μmol of creatinine), and the TMA N-oxide concentration was expressed as micromoles of TMA N-oxide per micromole of creatinine ± S.D. Control studies showed that the recovery was >90% and the sample variance was less than 33% for samples from among the same group of animals.

Catecholamines. Each animal was acclimatized, and rat urine was collected at the appropriate time (between 3:00 PM and 9:00 AM the next morning) in acidified vials. The total urine volume was recorded and an aliquot was stored at −70°C until purification by alumina chromatography and analysis using HPLC with electrochemical detection (Anton and Sayer, 1962). The internal standard was 3,4-dihydroxybenzamine. The calculated recoveries were: nor-epinephrine (NorEPI), 55%; epinephrine (EPI), 52%; and dopamine (DA), 50%; and the sample variance was less than 4% for samples from among the same group of animals. Urinary creatinine was measured by a photometric assay with a kit from Sigma-Aldrich. The data were expressed as nanograms of biogenic amine per micromole of creatinine ± S.D.

Liver microsome preparation and enzyme assays. After a 5-day treatment with one of the diets, each animal was used to procure individual liver microsome samples to determine in vitro monoxygenase activity. For preparation of liver microsomes, the infusion was discontinued and the animals were killed by decapitation. Livers were immediately removed, placed in aluminum foil, and rapidly immersed in liquid nitrogen. Livers were stored at −70°C before use. Each liver was rapidly weighed and microsomes were prepared by standard means. Enzyme activities were determined using microsomes stored under a blanket of glycerol at −80°C for 1 month. Protein concentration was determined using bovine serum albumin using the method of Peter et al. (1997). Chlorzoxazone 6-hydroxylation was determined using a 20 μl microsomal suspension containing 1 mg/ml of liver microsomal protein and 20 μl of NADPH generating system before use. Each liver was rapidly weighed and microsomes were prepared by standard means. Enzyme activities were determined using microsomes stored under a blanket of glycerol at −70°C for at least 24 h. 5,6-DPT N-oxide and MTS S-oxidase activity was determined as previously described (Brunelle et al., 1997). Chlorzoxazone 6-hydroxylation was determined using the method of Peter et al. (1990). Protein concentration was determined by bicinchoninic acid (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Cytochromes P450 and b5 content. The concentration of cytochrome b5 and P450 was determined in triplicate from the difference spectrum of NADPH-reduced and oxidized microsomes by the method of Omura and Sato (1964), as described in Pearce et al. (1996).

The same samples used to determine the concentration of cytochrome b5 were also used to determine the concentration of total P450. After the cytochrome b5 spectrum was recorded, 5 μl of 20 mM NADPH was added to the reference cuvette, and a few grains of sodium hydrosulfite were added to both cuvettes. Immediately after the addition of hydrosulfite, the contents of the sample cuvette were saturated with carbon monoxide, and after 2.5 to 3 min, the carbon monoxide difference spectrum of the reduced microsome sample was recorded between 400 and 500 nm. The concentration of P450 was determined from the absorbance difference between 450 and 490 nm, based on an extinction coefficient of 91 m M⁻¹ cm⁻¹.

Expression and Purification of the FMO4 Standard. Carboxyl-terminal-truncated human FMO4 was expressed as an N-terminal maltose-binding C-terminal poly(histidine) protein (i.e., maltose binding protein-FMO4-His₆), purified and used as a standard to quantify FMO4 in rat liver microsomes. The truncated human FMO4 cDNA that encoded 531 amino acids was previously determined and used as a standard to quantify FMO4 in rat liver microsomes. The antisense primer included an 18-nucleotide sequence encoding six histidines, allowing addition of a histidine tag to the truncated FMO4 cDNA. After 35 cycles at 94°C for 20 s, 52°C for 20 s, and 72°C for 2 min, the polymerase chain reaction product was gel-purified, subcloned into the expression vector pMAL-c2, and sequenced on both strands. The sequence data showed that the entire cDNA construct was correct. Escherichia coli JM109 cells were transformed with pMAL-FMO4-His₆, plasmid and grown at 37°C in modified Hanahan’s Broth medium (2% Bacto-tryptone, 0.5% yeast extract, 8 mM NaCl, 10 mM MgCl₂, 2.5 mM KCl, 20 mM glucose) to an absorbance of 0.5 to 0.6 at 600 nm. Isopropyl β-thio-galactopyranoside (0.2 mM), rifampicin (0.05 mM), and 100 μg/ml ampicillin were then added. The cells were incubated overnight with constant shaking at 30°C. Cells were harvested by centrifugation at 6000g for 10 min and resuspended in lysis buffer (50 mM Na₂HPO₄, pH 8.4; 0.5% Triton X-100) containing 0.2% т-α-phosphatidylcholine, and 0.5 mM phenylmethylsulfonylfluoride. After incubation for 30 min at 4°C, the resuspended cells were disrupted by sonication (i.e., three 2-min bursts separated by periods of cooling), then centrifuged at 18,000g for 30 min at 4°C. The resulting supernatant was purified with an amylose column (New England Biolabs, Beverly, MA) as previously described (Brunelle et al., 1997). The amylose-affinity column eluate was then applied to a HisBind Resin (Novagen, Madison, WI) column (5 ml) equilibrated with buffer A (20 mM Tris HCl, pH 7.9; 0.5 M NaCl; and 5 mM imidazole). The column was washed with 5 volumes of buffer A containing 60 mM imidazole. Bound proteins were eluted with buffer A containing 200 mM imidazole according to the manufacturer’s specifications. Eluted fractions were analyzed by SDS-PAGE. The fractions containing the fusion protein were concentrated. The FMO4 concentration was estimated by the bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard.

Determination of FMO Concentration by Immunoquantification. SDS-PAGE and Western blots were done as described by Brunelle et al. (1997). The concentrations of FMO1, FMO3, and FMO4 in rat liver microsomes were determined by immunoquantification using human FMO1, FMO3 Supersomes (BD Gentest, Woburn, MA), or purified truncated human maltose binding protein-FMO4-His₆, respectively, as standards. Anti-rat FMO1 and anti-rat FMO3 antibodies were a generous gift of Prof. E. Benoit (National Veterinary School of Lyon, France) and were used as previously described (Lattard et al., 2002). Anti-human FMO4 antibody was obtained from ProSci, Inc. (Poway, CA) and recognizing antibodies with a synthetic peptide of FMO4 from amino acid residue 417–428. Microsomal proteins (2 or 50 μg) and standard FMO1, 3, or 4 (i.e., 250, 200, 150, 100, and 50 fmol) were resolved by electrophoresis on a 10% polyacrylamide gel under denaturing conditions and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Immunoblots were incubated for 1 h with anti-rat FMO1 or 3 (1:5000) (Lattard et al., 2002) or anti-human FMO4 (1:1000) antibodies. After washing, immunoblots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000). FMO was visualized by enhanced chemiluminescence on X-ray film. Quantification was done by densitometry analysis using a numeric camera employing Scion Image software (public domain, http://www.scion-corp.com/).

Data Analysis. Data were presented as the mean ± standard deviation obtained from at least three independent animals. Statistical analysis was done using StatView 5.0 software. Dunnett and Bonferroni’s t test was used for the pairwise multiple comparisons. A p value <0.02 was considered statistically significant.

Results

The time course of TMA concentration in the urine of rats that received total parenteral nutrition (TPN) alone or TPN + choline declined compared with rats treated with a control diet (Fig. 1). After 5 days of TPN + choline treatment, the decrease in urinary TMA concentration was approximately 7-fold, although even after 3 days of treatment, significant differences were apparent. Under the conditions of the experiment, the rate of diminishment of urinary TMA in the presence of TPN − choline and TPN + choline was approximately 0.029 and 0.013 h⁻¹, respectively (Fig. 1). After 5 days of treating rats with TPN, the presence or absence of choline significantly reduced urinary TMA concentration.

The time course for TMA N-oxide concentration in the urine of rats that received TPN + choline declined compared with rats treated with control diet (Fig. 2). In the presence of TPN + choline, the rate of diminishment for decrease of urinary TMA N-oxide was approximately 0.023 h⁻¹. The concentration of TMA N-oxide in the urine of rats that received TPN alone also showed a decrease, although the decline was much less marked and only reached a maximum difference on day 5 compared with animals treated with TPN + choline.
The concentration of trimethylamine was determined as described under Materials and Methods. Data represent mean (four rats per group) ± S.D. a, the indicated TPN + choline and chow + saline values are statistically different by p < 0.02; b, the indicated TPN + choline and chow + saline values are statistically different by p < 0.02; c, the indicated TPN + choline and TPN = choline values are statistically different by p < 0.02.

Increased 3% (i.e., 90.3% to 93.3% TMA N-oxide formed) for rats treated with TPN + choline. Rats receiving TPN alone increased the mean percentage conversion of urinary TMA to TMA N-oxide 6% (i.e., 84.6% to 90.6% TMA N-oxide formed). That the effect was related to the diet received is seen upon comparison of data from rats receiving a control diet, because they did not detectably increase the mean percentage conversion of urinary TMA to TMA N-oxide (i.e., 87% to 86.8% TMA N-oxide formed). When the ratio of TMA N-oxide divided by TMA concentration was plotted as a function of time, there was no statistically significant difference between rats treated with TPN-choline and animals treated with a control diet. However, the ratio of TMA N-oxide divided by TMA was statistically different between TPN + choline and TPN – choline-treated animals on day 0 (p < 0.001), day 3 (p < 0.01), day 4 (p < 0.02), and day 5 (p < 0.01). The results suggest that TMA N-oxygenation efficiency increased as a consequence of TPN + choline diet treatment.

The analysis of the effect of dietary treatment on amine metabolism and distribution was extended to studying urinary biogenic amines. As shown in Fig. 3, animals receiving TPN + choline or TPN alone over the time course of the 5-day experiment had a marked decrease in urinary DA concentration compared with rats treated with a control diet. For animals treated with TPN alone, notable differences in urinary DA concentration were observed as early as day 2 post-treatment. After 5 days of treatment with either TPN alone or TPN + choline, the decrease in urinary DA was approximately 4-fold. The rate of diminishment over time of urinary DA for animals treated with TPN or TPN + choline was calculated to be approximately 0.035 and 0.019 h⁻¹, respectively.

Compared with DA, the decrease in biogenic amine concentration in the urine of rats followed a distinct time course for EPI (data not shown) and NorEPI (Fig. 4). After treatment with TPN + choline for 5 days, the urinary concentration of EPI increased compared with animals that received a control diet or TPN – choline. The increase in EPI was most pronounced on day 5 post-treatment, but this did not reach statistical significance. In contrast, urinary NorEPI decreased in rats administered TPN ± choline compared with rats administered a control diet (Fig. 4). In agreement with the results for DA and EPI, the effect was most pronounced on day 5 of the treatment.
The concentration of norepinephrine was determined as described under Materials and Methods. Data represent mean (four rats per group) ± S.D. a, the indicated TPN – choline and chow + saline values are statistically different by \( p < 0.02 \); b, the indicated TPN + choline and chow + saline values are statistically different by \( p < 0.02 \).

Animals receiving TPN or TPN + choline gained weight during the 5-day course of treatment (data not shown), suggesting that the infused diet supplied sufficient daily calories for growth. The livers of treated and control animals were weighed to provide information about diet and hepatic content (Table 1). Animals receiving TPN + choline had a mean liver weight of 6.39 ± 1.14 g (range 5.1–7.3 g). Animals receiving TPN – choline averaged 5.87 ± 1.37 g (range 4.2–7.3 g). A liver weight of 8.84 ± 1.24 g (range 7.2–9.9 g) was observed in the animals allowed free access to rat chow (Table 1). The effect of TPN + choline on liver weight and Hepatic Somatic Index paralleled the effect of TPN + choline on the amount of total P450 and cytochrome \( b_6 \) (Table 1).

To examine a molecular basis for the effects of TPN ± choline on TMA, TMA \( N \)-oxide, and biogenic amine urinary concentration described above, we investigated the effects of TPN and the presence or absence of choline on the microsomal activity of selected hepatic monooxygenases obtained from the treated animals. The hepatic microsomal monooxygenase activity observed for liver preparations from TPN + choline-treated animals was compared with liver microsomes from control animals that received free access to standard rat chow. Because TMA is \( N \)-oxygenated by the FMO class of monooxygenase, two selective functional substrates for this enzyme prepare were utilized. In the presence of rat liver microsomes prepared from animals receiving TPN ± choline or control diet, aerobic incubation of 5-DPT resulted in an \( N \)-oxide formation that was linearly dependent on time (i.e., 0–10 min) and protein concentration (i.e., 0–0.4 mg of protein). As shown in Table 2, compared with hepatic microsomes prepared from animals treated with TPN without choline or control diet, animals receiving TPN + choline showed a 2.1-fold and a 3-fold increase in 5-DPT \( N \)-oxide activity, respectively. A similar effect of diet treatment on MTS \( S \)-oxygenase activity was observed in hepatic microsomes prepared from the same animals. In the presence of rat liver microsomes prepared from animals receiving TPN ± choline or control diet, aerobic incubation of MTS resulted in an NADPH-dependent formation of MTSO. In the presence of each type of rat liver microsome preparation, formation of MTSO was linearly dependent on time (i.e., 0–10 min) and protein concentration (i.e., 0–0.4 mg of protein). As shown in Table 2, compared with hepatic microsomes prepared from animals receiving TPN without choline or control diet, animals receiving TPN + choline showed a 2.2-fold and a 3.2-fold increase in microsomal MTS \( S \)-oxygenase activity, respectively. The similar increase in microsomal \( DPTN \)-oxygenase and MTS \( S \)-oxygenase activity as a function of dietary treatment prompted a detailed investigation of the type of FMO present.

The relative amount of each prominent FMO protein was determined by immunquantification. The relative amount of immunoreactive FMO1, 3, and 4 protein was estimated by Western blot analysis using FMO form-selective antibodies (as described under Materials and Methods). Equal amounts of hepatic microsomal protein from animals receiving TPN + choline, TPN alone, or control diet showed a marked difference in the amount of FMO-immunopositive microsomal protein present, and this was dependent on the FMO form examined (Fig. 5). Compared with control diet, rats receiving TPN + choline or TPN – choline decreased the amount of immunoreactive FMO1 35% and 37%, respectively, but the difference did not reach statistical significance. Compared with control diet, the amount of immunoreactive FMO3 was increased 1.6-fold and 1.3-fold in animals receiving TPN + choline and TPN – choline, respectively, but the difference did not reach statistical significance. For FMO4, compared with a TPN – choline diet, rats receiving TPN + choline increased the amount of immunoreactive protein 1.6-fold.

In parallel with the evaluation of FMO quantity and functional activity, CYP2E1 monooxygenase activity was also investigated. As a representative selective functional indicator of a toxicologically important P450 enzyme, the 6-hydroxylation of chlorozoxazone was examined. In the presence of hepatic microsomes prepared from rats receiving TPN ± choline or control diet, formation of 6-hydroxy chlorozoxazone was linearly dependent on time (i.e., 0–30 min) and on protein concentration (i.e., 0–0.4 mg of protein). Hepatic microsomes prepared from rats receiving TPN + choline showed a 2.1-fold increase, respectively, in chlorozoxazone 6-hydroxylase activity, compared with microsomes prepared from rats receiving TPN – choline (Table 1).

### Table 1

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<th>Effect of TPN in the presence or absence of choline or a normal diet on rat hepatic microsomal cytochrome P450 concentration and on chlorozoxazone 6-hydroxylase activity</th>
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<td>Microsome assay (four individual assays per group) were done as described under Materials and Methods. Data represent the mean ± S.D.</td>
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<td>Chlorozoxazone 6-hydroxylase (nmol/min/mg protein)</td>
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* \( p < 0.02 \) versus both TPN – choline and chow + saline.  
* \( p < 0.02 \) versus both TPN + choline and TPN – choline.  
* \( p < 0.02 \) versus both TPN + choline.  
* \( p < 0.02 \) versus both TPN + choline and chow + saline.  

**Fig. 4.** Time-dependent effect of TPN alone (■), TPN plus choline (○), or control diet (▲) on rat urinary norepinephrine as determined by HPLC.
As shown in Table 1, hepatic microsomal protein from rats receiving TPN/H11001 choline or TPN alone gave a 1.2-fold and a 1.3-fold decrease, respectively, in total P450 compared with liver microsomes prepared from animals receiving a control diet. However, the differences did not reach statistical significance. Cytochrome b₅ concentrations were also determined, and cytochrome b₅ concentrations were decreased 1.2-fold and 4.9-fold in microsomes prepared from TPN/H11001 choline or TPN alone compared with nontreated animals, respectively.

**Discussion**

TPN has been observed to decrease hepatic drug and xenobiotic metabolism in animals and humans (Knodell et al., 1984; Burgess et al., 1987). Bypassing the intestine and administration of nutrients via TPN causes jejunal and ileal mucosal hypoplasia and hypofunction. In as few as 3 days after administration of TPN, a whole host of intestinal, hepatobiliary, and gastro-immunological changes result (Klein and Nealon, 1988). In the rat, the morphological changes in the intestine after TPN administration for as few as 3 days resembled that of starvation. The biochemical changes that underlie the physiological response to TPN may also be responsible for alterations in drug metabolism.

Modulation of hepatic enzymes such as FMO by dietary treatments may provide insight into the physiological function of the enzyme. The FMO has apparently evolved to detoxicate highly polarizable soft nucleophiles such as sulfur- and nitrogen-containing xenobiotics present in the environment to nontoxic polar metabolites (Ziegler, 1990). In contrast, the nonphysiological role of P450 is to oxidize hard nucleophiles such as promutagens that also are present in the environment and the diet (Guengerich, 1997).

Previously published studies have shown that hormones increase FMO in vivo (Duffel et al., 1980; Dixit and Roche, 1984). In addition, food restriction has been shown to modulate FMO activity (Dixit and Roche, 1984; Brodfuechren and Zannoni, 1986). Generally, studies to examine the role of xenobiotics or dietary supplementation to induce FMO have proven fruitless. Evidence that rats fed orally with chemically defined semisynthetic diets or with a TPN diet showed a reversible loss of FMO activity compared with chow-fed control animals (Kaderlik et al., 1991).

**Table 2**

<table>
<thead>
<tr>
<th>Activity</th>
<th>TPN + Choline</th>
<th>TPN - Choline</th>
<th>Chow + Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DPT N-oxygenation (nmol/min/mg protein)</strong></td>
<td>1.20 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>MTS S-oxygenation (nmol/min/mg protein)</strong></td>
<td>17.82 ± 8.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.15 ± 6.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.57 ± 1.63&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.02 versus both TPN + choline and chow + saline.

<sup>b</sup> p < 0.02 versus both TPN + choline and chow + saline.
FMO also oxygenates nucleophilic dietary materials in foods. For example, TMA, derived from choline, is $N$-oxygenated by FMO to the detoxication product TMA $N$-oxide. In the present study, based on urinary TMA and TMA $N$-oxide concentrations, FMO-dependent TMA $N$-oxygenation actually increased as a percentage of the available TMA in animals treated for 5 days with TPN + choline or TPN − choline. The increased efficiency of in vivo metabolism of TMA to TMA $N$-oxide is consistent with the increased amount of immunoreactive hepatic FMO3 and FMO4 and the increased functional activity of FMO observed in rats administered TPN + choline. Although the mechanism is uncertain, the results suggest that TPN + choline up-regulates the expression of rat liver FMO4.

Although a considerable number of studies have shown that TPN-treated animals decrease hepatic drug metabolism (Ross et al., 1983; Knodell et al., 1984, 1989; Burgess et al., 1987; Raftergians et al., 1995, 1996), few studies have examined the effect of TPN on the alteration of FMO-related dietary chemical metabolism. As discussed above, FMO contributes to the $N$- and $S$-oxygenation of nucleophilic heteroatom-containing compounds, converting them to $N$- and $S$-oxides that are readily excreted in the urine. It is possible that FMO serves some physiologically important function to detoxicate dietary nucleophiles, particularly for the condition of decreased gut secretory function or steatosis.

A number of factors have been proposed to explain TPN-related effects on gastrointestinal and hepatic function including modulation of gastrointestinal peptide hormones (Johnson et al., 1977). Some gastrointestinal peptide hormones have been observed to increase hepatic P450 (Fang and Strobel, 1981). Depending on the particular form of P450 that is induced, this may result in increased bioactivation or increased detoxication of promutagens. Dietary and pathophysiological factors are capable of inducing CYP2E1, and induction occurs at many regulatory levels including transcription, mRNA stabilization, increases in translational efficiency, and post-translational protein stabilization (Ronis et al., 1996). Many of these separate mechanisms can occur simultaneously. Treatment of hepatocytes with hormones such as EPI that stimulate cAMP-dependent phosphorylation has been shown to decrease CYP2E1 half-life. A high-lipid, low-cholesterol carbohydrate diet and fasting are also associated with an increase in CYP2E1 (Yoo et al., 1991), linked to increased CYP2E1 transcription (Johansson et al., 1988). After 5 days of treating rats with TPN + choline, apparent increases in urinary EPI were observed, although this was not statistically significant. However, urinary DA and NorEPI concentrations were decreased, suggesting that the effect of TPN + choline on these two catecholamines was distinct from their effect on EPI. Based on the fact that tyramine and phenethylamine are good substrates for FMO3 (Lin and Cashman, 1997), we hypothesize that DA and NorEPI are also metabolized by FMO. However, we do not currently know the contribution of FMO-mediated biogenic amine metabolism relative to the metabolism of biogenic amines by other amine oxidases.

The studies reported herein may have some implications for humans suffering from a condition called trimethylaminuria. Trimethylaminuria results from deficient $N$-oxygenation of TMA associated with defective human FMO3 genes (Mitchell and Smith, 2001; Cashman, 2002b; Cashman et al., 2003). Because choline is a dietary source of TMA, severely restricted choline diets may have an adverse impact on biogenic amine excretion and drug or xenobiotic metabolism in addition to the liver problems discussed above. This may be a result of metabolic or other physiological mechanisms. Although choline content in food varies greatly, individuals being treated with a choline-restricted diet should be monitored closely. Dietary choline restriction increases the requirement of folate, a methyl donor that interacts with choline and methionine in metabolic processes (Horne et al., 1989). Women with trimethylaminuria should not restrict dietary intake of choline during pregnancy because choline is very important for normal nervous system and brain development (Cashman et al., 2003). In addition, individuals with severe trimethylaminuria should also avoid tyramine-containing foods because these have been associated with hypertensive conditions (Treacy et al., 1998), and the work herein suggests that an interaction with metabolism or biosynthesis of biogenic amines exists with TPN in the presence or absence of choline.

In summary, we have characterized the effect of TPN in the presence or absence of choline on rat hepatic FMO and CYP2E1 monooxygenase function. The first major conclusion from the data is that compared with a control diet, TPN + choline increases microsomal CYP2E1 and FMO3 and FMO4 functional activity. A second major conclusion from the data is that the in vitro changes were also manifested in vivo. For example, the percentage conversion of urinary TMA to TMA $N$-oxide in rats increased after 5 days on a diet of TPN + choline. A third major conclusion is that biogenic amine metabolism and urinary DA and NorEPI (but not EPI) concentrations were decreased after 5 days of administration of TPN + choline. It is possible that in the rat, dietary modulation of FMO may play a role in the metabolism of drugs and endogenous materials. In humans it is not known what influence dietary modulation has on FMO function, but in view of the dietary changes suggested for individuals with trimethylaminuria, it is important to investigate this further.

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References


