Effects of choline deficiency and methotrexate treatment upon rat liver

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Choline deficiency and treatment with methotrexate (MTX) both are associated with fatty infiltration of the liver. Choline, methionine, and folate metabolism are interrelated and converge at the regeneration of methionine from homocysteine. MTX perturbs folate metabolism, and it is possible that it also influences choline metabolism. We fed rats a choline deficient diet for 2 weeks and/or treated them with methotrexate (MTX; 0.1 mg/kg daily). Choline deficiency lowered hepatic concentrations of choline (to 43% control), phosphocholine (PCho; to 18% control), glycerophosphocholine (GroPCho; to 46% control), betaine (to 30% control), phosphatidylcholine (PtdCho; to 62% control), methionine (to 80% control), and S-adenosylmethionine (AdoMet; to 57% control), while S-adenosylhomocysteine (AdoHcy) and triacylglycerol concentrations increased (to 126% and 319% control, respectively). MTX treatment alone lowered hepatic concentrations of PCho (to 48% control), GroPCho (to 69% control), betaine (to 55% control), and AdoMet (to 75% control). The addition of MTX treatment to choline deficiency resulted in a larger decrease in AdoMet concentrations (to 75% control) and larger increases in AdoHcy and triacylglycerol concentrations (to 150% and 500% control, respectively) than was observed in choline deficiency alone. Livers from MTX-treated animals used radiolabeled choline to make the same metabolites as did livers from controls (most of the label was converted to PCho and betaine). In choline deficient animals, most of the labeled choline was converted to PtdCho. Therefore, MTX depleted hepatic PCho, GroPCho, and betaine by a mechanism that was different from that of choline deficiency. MTX increased the extent of fatty infiltration of the liver in choline deficient rats, and choline deficiency and MTX treatment damaged hepatocytes as measured by leakage of alanine aminotransferase activity. Our data are consistent with the hypothesis that the fatty infiltration of the liver associated with MTX treatment occurs because of a disturbance in choline metabolism.

Keywords: choline deficiency; methotrexate; rat liver

Introduction

Choline is a major dietary source of methyl groups, and it is a precursor for the biosynthesis of the membrane phospholipids phosphatidylcholine (PtdCho), sphingomyelin (SM), and lysophosphatidylcholine (LysoPtdCho), and for the neurotransmitter acetylcholine.[1,2] Choline deficiency is associated with characteristic abnormalities in organ function in the rat. These include fatty infiltration of the liver,[3-9] which occurs because PtdCho availability is limiting at the site of triacylglycerol export from the liver.[10] PtdCho is an essential component of very low density lipoprotein (VLDL): the blood transport molecule for hepatic triacylglycerol.[11] Methotrexate (MTX) treatment is also associated with fatty infiltration of the liver,[12-16] via an unknown mechanism.

The key reactions in the regeneration of methionine from homocysteine lie at the intersection of choline and folate metabolic pathways (*Figure 1*). Betaine, a metabolite of choline, serves as the methyl donor in a reaction converting homocysteine to methionine (catalyzed by betaine:homocysteine methyltransferase {EC 2.1.1.5}.[17] The only alternative methyl-donor for regeneration of methionine is 5-methyltetrahydrofolate, via a reaction catalyzed by 5-methyltetrahydrofolate:homocysteine methyltransferase (EC 2.1.1.13). MTX limits the availability of methyl groups derived from 1-carbon metabolism by competitively inhibiting a key enzyme in intracellular folate metabolism, dihydrofolate reductase (DHFR, EC 1.5.1.4).[18] MTX acts to trap folate in cells which are

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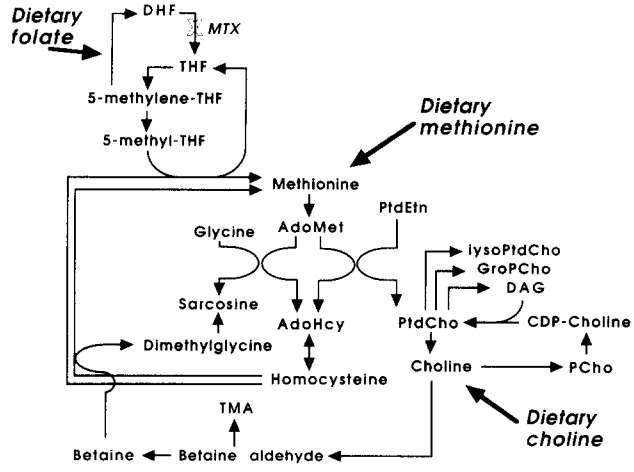

Figure 1 Interrelationships between choline, methionine, and folate metabolism. The key reactions in the generation of methionine from homocysteine lie at the intersection of choline and 1-carbon metabolic pathways. Betaine, a metabolite of choline, serves as the methyl donor in a reaction converting homocysteine to methionine (catalyzed by betaine:homocysteine methyltransferase). The only alternative methyl-donor for regeneration of methionine is 5-methyltetrahydrofolate, via a reaction catalyzed by 5-methyltetrahydrofolate:homocysteine methyltransferase. Methotrexate (MTX) inhibits dihydrofolate reductase.

DHF–dihydrofolate
AdoMet–S-adenosylmethionine
PtdEtn–phosphatidylethanolamine
GroPCho–glycerophosphocholine
CDP-choline–cytidine diphosphocholine
THF–tetrahydrofolate
AdoHcy–S-adenosylhomocysteine
PtdCho–phosphatidylcholine
PCho–phosphocholine
DAG–diacylglycerol

forming dihydrofolate (DHF) during thymidylate synthesis, and this results in the depletion of 5-methyltetrahydrofolate.[19] MTX also competes with folate and reduced folated for entry into the hepatocyte.[20] MTX has been used as a chemotherapeutic agent in the treatment of cancer,[21-23] psoriasis,[24] and rheumatoid arthritis.[25,26]

There are several reasons to believe that a disturbance in folate or methionine metabolism would result in changes in choline metabolism and visa versa. Betaine concentrations in livers of choline deficient rats are markedly diminished,[27-29] as are total folate concentrations.[30] Hepatic AdoMet concentrations are also decreased in animals ingesting diets deficient in choline.[31-36] In part, this is because the 5-methyltetrahydrofolate:homocysteine methyltransferase reaction alone cannot fulfill the total requirement for methionine, and the betaine dependent remethylation of homocysteine is limited by the availability of betaine.[27-29,37] Hepatic AdoMet and betaine concentrations are also diminished after treatment with MTX, probably because betaine supplies are not sufficient for regeneration of all the methionine needed by these animals.[12,27,32-34,36,37] In humans, after intravenous infusion of high doses of MTX, the concentration of methionine decreased by 70% in serum, while concentrations of homocysteine levels increased by 60%.[72]

Many investigators have noted that choline deficiency affects hepatic pools of choline esters.[15,58,73,74] We now describe the effects of both MTX treatment and choline deficiency upon hepatic concentrations of many of the key intermediates in choline and methionine metabolism.

Materials and methods

Animals

Male Sprague-Dawley rats (130 g body weight, Charles River Breeding Laboratories, Wilmington, MA) were housed in suspended stainless-steel wire cages in a climate controlled room at 297 K (24°C), and were exposed to light during 06:00 to 18:00 hours daily. Animals were fed the control diet (ICN Nutritional Biochemicals custom control diet: 10% casein, 10% protein, 20% lard, 56% sucrose, 4% salt mix W, ICN vitamin mix with choline; the diet contained final content of 0.2% choline, 0.27% cystine, and 0.35% methionine) for 1 week prior to use in order to acclimate them to a semi-synthetic diet.

Tissue metabolite studies

Rats were randomly assigned to four treatment groups (N = 5/group) and pair fed a control or choline deficient diet (same as the control diet except we used ICN vitamin mix without choline; contained final content of 0.002% choline) for 2 weeks. Five animals on each diet were treated daily for 2 weeks with MTX (0.1 mg/kg body weight administered intraperitoneally at midday; Behring Diagnostics, La Jolla, CA). This low dose of MTX was selected because, at this dose, hepatic betaine levels decreased while the more severe side-effects of MTX (e.g., gastroenteritis and ulceration[27] did not occur. This dose of MTX is comparable to that used in the treatment of rheumatoid arthritis.[25,26,39] The remaining animals on each diet were treated daily for 2 weeks with 0.9% NaCl (administered intraperitoneally) instead of MTX. On day 15, the rats were anesthetized with diethylether and blood was collected via aortic puncture into a heparinized tube. Blood was immediately placed on ice and subjected to centrifugation at 2,500 × g for 15 minutes at 277 K (4°C). Plasma was aspirated and frozen immediately in liquid nitrogen. Samples of liver were collected by clamping tissue between stainless steel tongs precooled in liquid nitrogen. These were stored at 178 K (−95°C) until analyzed. Other segments of liver were preserved in formalin for histologic examination. These experiments were repeated twice.

Distribution of radiolabeled choline

Additional rats (4 groups, N = 5/group) were treated as described above. On day 15, the animals were restrained and [^{14}C-methyl]-choline chloride dissolved in

0.9% NaCl (59 mCi/mmol; New England Nuclear, Boston, MA) was injected into the lateral tail vein. The syringes were weighed before and after tail vein injection to measure accurately the amount of radiolabel delivered (controls received 5.7 μCi ± 0.30; choline deficients received 5.3 μCi ± 0.1; MTX alone received 5.1 μCi ± 0.3; choline deficient and MTX-treated received 5.1 μCi ± 0.2). After 15 minutes, the rats were anesthetized with diethylether and blood was collected via aortic puncture into a heparinized tube. Blood was immediately placed on ice and subjected to centrifugation at 2,500 × g for 15 minutes at 277 K (4°C). Plasma was aspirated and frozen immediately in liquid nitrogen. Samples of liver were collected by clamping tissue between stainless steel tongs precooled in liquid nitrogen. These were stored at 278 K (−95°C) until analyzed.

Metabolites of choline were isolated from the plasma and liver samples as described below. Peaks from high pressure liquid chromatography (HPLC), or bands from thin layer chromatography plates, which contained metabolites, were collected into scintillation vials. Ten ml of scintillation fluid (ScintiVerse LC; Fisher Chemicals, Medford, MA) was added and the samples were counted using liquid scintillation spectrophotometry (LKB RackBeta; Gaithersburg, MD). Calculations converting cpm to dpm were based upon the external channels ratio.

Histopathology of liver specimens

Sections of liver from each rat were fixed in 10% neutral buffered formalin and processed by standard methods for histological examination. The liver samples were examined by two different pathologists who were not aware of the treatment group from which the sample was derived. Specimens were graded on a scale of 0 to 3 for fatty infiltration (0 = no fatty infiltration; 3 = severe fatty infiltration).

Measurement of liver neutral lipids

Hepatic neutral lipids were extracted using the method of Bligh and Dyer.[40] A portion of the chloroform phase was applied to a silica-gel thin layer chromatography plate (LK5D; Whatman Corp., Clifton, NJ). External standards of free fatty acids (oleic acid: Nu Chek Prep, Inc., Elysian, MN), PtdCho (dipalmitoyl; Sigma Chemicals, St. Louis, MO), and triacylglycerol (trioleic acid: Nu Check Prep, Inc.) were also applied. The plate was developed with hexane/diethylether/acetic acid (50:50:1, v/v) and the segments that cochromatographed with authentic standards of free fatty acids and triacylglycerol were identified under ultraviolet light after spraying the plates with cyclohexatriene. These were scraped and collected into glass tubes. Free fatty acids were assayed as their methyl esters using gas chromatography.[41] Triacylglycerol was hydrolyzed at basic pH to form free fatty acids, and these were measured as their methyl esters using gas chromatography.[41,42]

Isolation and measurement of choline metabolites from liver and plasma

Liver samples were pulverized under liquid nitrogen using a mortar and pestle, forming a homogeneous powder. Plasma samples were used directly. An aliquot of tissue (100 mg) was extracted using the method of Bligh & Dyer.[40] Internal standards labeled with stable isotopes were added to tissues to permit calculation of, and correction for, recovery during the assay (choline-^{15}N chloride and PCho-$^{2}H_9$chloride {N,N,N-trimethyl-$^{2}H_9$; barium salt} were obtained from MSD Isotopes {Merck Chemical Division, St. Louis, Mo}; PtdCho-$^{2}H_9$ {dipalmitoyl; N,N,N-trimethyl$^{2}H_9$: Avanti Polar Lipids, Inc., Birmingham, AL}; GPCho-$^{2}H_9$ and LysoPtdCho-$^{2}H_9$ were prepared from PtdCho-N,N,N-trimethyl-$^{2}H_9$). We attempted to add an amount of internal standard within an order of magnitude of the expected amount of unknown in the tissue. Choline metabolites were isolated using high pressure liquid chromatography (aqueous soluble metabolites) and thin layer chromatography (organic soluble metabolites) methods previously described.[43] After metabolites of choline were isolated, they were hydrolyzed in acid to form unesterified choline using methods previously described.[43] An aliquot of the fraction containing choline, or of the hydrolyzed phosphocholine (PCho), hydrolyzed glycerophosphocholine (GroPCho), hydrolyzed phosphatidylcholine (PtdCho), hydrolyzed sphingomyelin (SM), or hydrolyzed lysophosphatidylcholine (LysoPtdCho) fractions were assayed for choline content according to the method of Freeman et al.[44] as modified by our laboratory.[43] The free choline moiety was converted to the propionyl ester and demethylated using sodium benzenethiolate. This volatile derivative was then isolated using gas chromatography (6 ft. × 2 mm i.d. glass column packed with 3% OV-17 on GC22 precoated with 1% 4-dodecyldiethylene triamine succinamide); with a helium flow of 45 ml/minute, and an isothermal temperature of 373 K (100°C) for 4 minutes. The compounds were fragmented in the mass spectrometer (Hewlett Packard 5970 GC/MSD with model 7673A liquid autosampler) and ion 58 (unique to choline) and 64 (for choline-$^{2}H_9$) were measured.

Betaine was assayed using the method of Barak & Tuma.[28] Betaine periodide was precipitated and then was resuspended in ethylene dichloride. Absorbance was measured at 365 nm. An internal standard labeled with stable isotope was not available for SM, therefore an authentic external standard (2 nmol; from bovine brain; Sigma Chemicals; St. Louis, MO) was always carried through the entire procedure and was used to calculate recovery.

Measurement of methionine

Methionine concentrations were determined using high pressure chromatography with UV detection. This method is a modification of that of Bidlingmeyer[45] and Scholze[46] which we have previously described.[36]

Measurement of S-adenosylmethionine and S-adenosylhomocysteine

AdoMet and AdoHcy concentrations were determined using high pressure chromatography with UV detection. This method is a modification of that of Shivapurker[31] which we have previously described.[36]

Protein assay

Protein was measured using the colorimetric assay of Bradford.[47]

Measurement of hepatic alanine aminotransferase activity

Alanine aminotransferase (ALT) activity was measured using the colorimetric method of Reitman and Frankel[48] (diagnostic test kit; Sigma Chemicals).

Statistical analysis

Data was analyzed using one-way analysis of variance and Scheffe's or Dunnett's test.[49] Data are presented as mean ± standard error of the mean (SE).

Results

Pair-fed rats from all treatment groups gained identical amounts of weight (control animals gained 84 g/2 weeks ± 10, deficients gained 86 g/2 weeks ± 6, those treated with MTX alone gained 80 g/2 weeks ± 8, and deficients treated with MTX gained 77 g/2 weeks ± 2), and none of the animals appeared to be ill. Hepatic protein concentration in the livers of control animals was 149.1 mg protein/g liver (± 0.002) as compared to choline deficient (143.7 mg protein/g liver ± 0.006), MTX alone (150.1 mg protein/g liver ± 0.001), or choline deficient and MTX-treated (142.1 mg protein/g liver ± 0.003) rats. When data were expressed per protein content, the relationships between group's values was not significantly different from those observed when the data were expressed per gram wet weight of tissue.

Rats from all groups, except the control group, had hepatic abnormalities visible in hematoxylin and eosin stained sections examined using the light microscope. The cytoplasm of these hepatocytes was clear with compressed nuclei displaced to the periphery of the cell. Mild to moderate accumulations of cytoplasmic lipid droplets were observed. This was found to occur in a periportal to midzonal distribution with the most severe fatty changes observed in the rats receiving the combined MTX treatment and choline deficient diet. Alanine aminotransferase (ALT) activity in plasma from control rats was 0.55 μkat/l (± 0.05) as compared to MTX-treated rats fed a control diet (0.73 μkat/l ± 0.03). Activity was increased in choline deficient rats (0.92 μkat/l ± 0.08; $P < 0.01$ different from control) and in MTX-treated rats fed a choline deficient diet (1.05 μkat/l ± 0.07; $P < 0.01$ different from control and MTX alone).

Hepatic triacylglycerol concentration was 84 μg/mg protein (± 29) in rats consuming the control diet. In the choline deficient rats, hepatic triacylglycerol concentration increased 3-fold (to 268 μg/mg protein ± 26; $P < 0.01$ different from control). Rats treated with MTX and fed a control diet did not have significantly elevated triacylglycerol concentration (137 μg/mg protein ± 14), but rats treated with MTX therapy and fed the choline deficient diet had a 5-fold increase in hepatic triacylglycerol concentrations (to 421 μg/mg protein ± 32; $P < 0.01$ different from control, choline deficient, and MTX-treated rats). Triacylglycerol was the major constituent of hepatic lipid in all animals. Neither hepatic free fatty acid concentrations nor total phospholipid concentrations changed significantly after any of the treatment regimens (data not shown). The distribution of fatty acid species within hepatic triacylglycerol, free fatty acid, or phospholipid was not altered by either diet or drug (data not shown).

Choline deficiency lowered hepatic concentrations of choline, PCho, GroPCho, betaine, and PtdCho (*Table 1*). MTX treatment of rats fed a control diet lowered hepatic concentrations of PCho, GroPCho, and betaine (*Table 1*). The addition of MTX treatment of choline deficiency did not significantly increase the effects upon choline metabolite concentrations compared to that of choline deficiency alone (*Table 1*).

Choline deficiency lowered hepatic concentrations of methionine and AdoMet, while concentrations of AdoHcy increased (*Table 1*). MTX treatment of rats fed a control diet lowered hepatic concentrations of methionine and AdoMet (*Table 1*). The addition of MTX treatment to choline deficiency resulted in a larger decrease in AdoMet concentration and in a larger increase in AdoHcy concentration in liver (*Table 1*).

Methionine concentrations in control rat plasmas were 50 nmol/ml (± 2), while in choline deficient animals they were 41 nmol/ml (± 2; $P < 0.05$, different from control). MTX treatment did not significantly reduce plasma methionine concentrations in rats fed either the control (47 nmol/ml ± 7) or deficient (40 nmol/ml ± 5) diets.

Less than 1% of the radiolabeled-choline infused was recovered in plasma at the time of sacrifice (control: 1% ± 0.3; choline deficient: 0.7% ± 0.1; MTX alone: 0.7% ± 0.04; choline deficient and MTX: 0.5% ± 0.1; calculated using an estimated plasma volume of 8.5 ml for a 130 g rat). Of this amount, approximately a third was recovered as [^{14}C]-betaine (control: 34% ± 5; choline deficient: 27% ± 5; MTX alone: 29% ± 4; choline deficient and MTX: 22% ± 5), and the remainder was recovered as [^{14}C]-choline. No label was recovered as free methionine. Approximately one tenth of the radiolabeled-choline infused was recovered in liver at the time of sacrifice (control: 10% ± 3; choline deficient: 12% ± 2; MTX alone: 14% ± 2; choline deficient and MTX: 12% ± 1). In control animals, most of the label within liver was in betaine and PCho (*Figure 2*). MTX treatment alone did not significantly alter the distribution of radiolabel (*Figure 2*). In choline deficient rats, most of the radiolabel recovered in liver was in PtdCho (*Figure 2*). The addition of MTX

Table 1 Choline and methionine metabolites after choline deficiency and MTX

Metabolite (nmol/mg protein ± SE)	Control	Deficient	MTX	Deficient & MTX
Choline	0.865 ±0.065	0.368[a,c] ±0.036	0.968 ±0.049	0.389[a,c] ±0.025
Phosphocholine	13 ±1	2.4[a,d] ±0.5	6.2[a] ±0.1	1.5[a,c] ±0.3
Glycerophosphocholine	13 ±1	6[b] ±1	9[a] ±1	3[a,c] ±0.4
Betaine	20 ±3	6[a] ±1	11[a] ±1	6[a] ±1
Phosphatidylcholine	117 ±10	73[a,d] ±3	108 ±8	70[a,d] ±4
Lysophosphatidylcholine	2.3 ±0.3	2.9 ±0.3	2.9 ±0.3	2.1 ±0.1
Sphingomyelin	9 ±2	16 ±1	13 ±2	9 ±1
Methionine	0.382 ±0.022	0.304[a] ±0.016	0.337[b] ±0.017	0.292[a] ±0.009
S-adenosylmethionine	0.528 ±0.019	0.303[a] ±0.032	0.394[b] ±0.003	0.177[a,c,f] ±0.014
S-adenosylhomocysteine	0.223 ±0.014	0.282[b] ±0.010	0.269 ±0.018	0.334[a,d,f] ±0.012
Triacylglycerol (μg/mg protein)	84 ±29	268[a] ±26	137 ±14	421[a,ce] ±32

Rats were fed a control or choline deficient diet for 2 weeks. Five animals on each diet were treated daily for 2 weeks with MTX (0.1 mg/kg body weight administered intraperitoneally). The remaining animals on each diet were treated daily for 2 weeks with 0.9% NaCl (administered intraperitoneally) instead of MTX. On day 15, the rats were anesthetized with diethylether and quick frozen liver samples were collected. Concentrations of compounds were assayed as described in the *Methods* section. Data are expressed as mean nmol/mg protein ± SE, $N = 5$ per group. By 1-way ANOVA and Scheffe's or Dunnett's test: **a** = $P < 0.01$ or **b** = $P < 0.05$ different from control; **c** = $P < 0.01$ or **d** = $P < 0.05$ different from MTX; **e** = $P < 0.01$ or **f** = $P < 0.05$ different from deficient.

treatment to choline deficiency did not further alter the distribution of metabolites (*Figure 2*). Specific activity of choline was much higher in plasma than in liver for all treatment groups (*Table 2*).

Discussion

We found that MTX treatment increased the extent of triacylglycerol accumulation and liver damage associated with choline deficiency. Both treatments perturbed concentrations of choline metabolites within liver. Though others have also observed that betaine concentrations in liver are depleted during treatment with MTX,[27,37,50] this is the first time that an attempt has been made to compare MTX induced changes with choline deficiency induced changes in most of the intermediates of choline and methionine metabolism (*Table 1*).

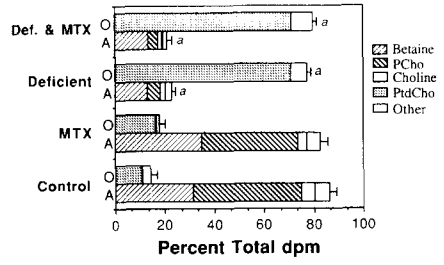

Figure 2 Distribution of radiolabeled choline within choline metabolites of liver. Additional rats (4 groups; **Control,** methotrexate treated {**MTX**}, choline deficient {**Deficient**}, or choline deficient treated with methotrexate {**Def. & MTX**}) were treated as described in *Table 1* legend. On day 15, the animals were restrained and [^{14}C-methyl]-choline chloride dissolved 0.9% NaCl was injected into the lateral tail vein. After 15 minutes, samples of liver were collected by clamping tissue between stainless steel tongs precooled in liquid nitrogen. Organic (**O**) and aqueous (**A**) soluble metabolites of choline were isolated and counted as described in the *Methods* section. Data are expressed as mean percent recovered dpm ± SE; $N = 5$ per group. α = $P < 0.01$ different from control by 1-way ANOVA and Scheffe's test.

Our choline deficient diet contained more methionine than did the diets used by others.[14,30,38] In choline deficient animals, we found that hepatic concentrations of betaine, choline, PCho, PtdCho, and GroPCho were all markedly decreased during choline deficiency (*Table 1*), while triacylglycerol increased. These data confirm earlier observations.[15,58,73,74] In addition, hepatic AdoMet concentrations decreased far more than did hepatic methionine concentrations (*Table 1*). In MTX-treated animals, we observed that betaine, PCho, GroPCho, methionine, and AdoMet concentrations were decreased in liver (*Table 1*). Combined treatment with MTX and choline deficiency resulted in further decreases in AdoMet (*Table 1*), and in further increases in AdoHcy and triacylglycerol concentrations in liver.

Normal liver does not divide rapidly, and therefore does not form much DHF during DNA synthesis. However, liver contains dihydrofolate reductase activity,[20] and thymidylate synthetase activity,[51-53] and during liver growth or regeneration these activities become important.[51,54,55] Hepatocytes in culture are sensitive to MTX because they are proliferating.[53] In

Table 2 Specific activities of choline and its metabolites in plasma and liver

Metabolite	Specific activity (dpm/nmol) ± standard error of mean			
	Control	MTX	Deficient	Deficient & MTX
Plasma				
Choline	723 ± 274	579 ± 97	994 ± 128	839 ± 160
Liver				
Choline	23 ± 4	36 ± 4	9 ± 1	9 ± 3
Betaine	18 ± 5	33 ± 6	17 ± 3	22 ± 7
Phosphocholine	23 ± 6	51 ± 11	7 ± 1	10 ± 5
Phosphatidylcholine	1.3 ± 0.7	1.8 ± 0.4	6.5 ± 1.1	5.5 ± 0.3

Rats were treated as described in *Figure 2*. Concentrations of choline and its metabolites were assayed, and radioactivity was determined, as described in the *Methods* section.

the choline deficient liver, there is a progressive increase in hepatic cell proliferation.[56,57] Thus, choline deficiency should increase hepatic sensitivity to MTX treatment. We observed significant effects of MTX upon normal liver. Perhaps, because the rat is constantly growing, thymidylate synthesis via the folate pathway is significant. Possibly, competition by MTX with folates for entry into the liver was responsible. Finally, we used folic acid as the source of folate in the rat diets. Folic acid must be converted to DHF and then converted to THF; MTX would prevent utilization of dietary folic acid by liver.

We noted that hepatic PtdCho concentrations decreased in choline deficient rats; confirming earlier observations by Haines and Rose[58] and Chen.[15] There are two distinct pathways for the biosynthesis of PtdCho: the CDP-pathway using preformed choline, and the methylation pathway catalyzed by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT), which uses phosphatidylethanolamine (PtdEtn) and AdoMet.[59,60] Choline deficiency, by decreasing the absolute contribution of the CDP-pathway, would increase the requirement for phosphatidylcholine synthesis via the sequential methylation of PtdEtn. It is unclear whether choline deficiency results in an increased synthesis of PtdCho via the PEMT pathway. Much of the existing data were obtained using exogenously added AdoMet, yet hepatic AdoMet concentrations would be expected to limit activity in vivo. When exogenous AdoMet was added in these experiments, choline deficient liver did synthesize PtdCho via PEMT at an accelerated rate.[61-64] This probably occurs because the enzyme is primed with one of its two substrates, since choline deficiency almost doubles the concentrations of PtdEtn in liver endoplasmic reticulum,[64] and increased availability of PtdEtn increases the affinity of PEMT for AdoMet.[65] Thus, for any given available concentration of AdoMet, the activity of PEMT is increased in choline deficiency.

Experiments performed in the presence of high concentrations of AdoMet probably do not reflect in vivo conditions. AdoMet concentrations in liver decreased approximately 50% in choline deficiency (from 75 μmol/l in controls to 40 μmol/l in deficients to 25 μmol/l in deficients treated with MTX). PEMT's K_m for AdoMet is 15-40 μmol/l (depending on which of the three methylations catalyzed by the enzyme are studied.[65] The maximal activity of PEMT (estimated from the V_{max} of the slowest methylation {PtdEtn methylation} with 200 μmol/l AdoMet present) is approximately 1-3 μmol/min/mg purified enzyme protein.[65] We calculated that PEMT activity would be approximately 0.05 μmol/min/mg when liver AdoMet concentrations were 75 μmol/l and 0.025 μmol/min/mg at hepatic AdoMet concentrations of 25 μmol/l (using the data of Ridgeway and Vance for activity in the presence of 0.3 μmol/l PtdEtn[65]). Thus, changes in the availability of AdoMet of the order of magnitude that we observed in choline deficiency (with or without MTX treatment) could cause a several-fold decrease in the rate of methylation of PtdEtn to form PtdCho. This calculation assumes constant PtdEtn concentrations; during choline deficiency the accumulation of PtdEtn should partially compensate for the decreased AdoMet. AdoHcy is an inhibitor of PEMT. We observed that hepatic AdoHcy concentrations increase during choline deficiency (from 30 μmol/l in controls to 40 μmol/l in deficients to 45 μM in deficients treated with MTX). The K_i for AdoHcy inhibition of PEMT is 70 to 280 μmol/l (depending on which of the three methylations catalyzed by the enzyme are studied.[65] Therefore, though increasing AdoHcy will tend to inhibit PEMT, this effect should be small at the concentrations of AdoHcy observed.

Choline specific activity was much lower in liver than in plasma in all groups (*Table 2*). If this were solely due to dilution by endogenous hepatic pools of choline, we would have expected that in the deficient diet groups hepatic choline specific activity would have been higher, not lower, than that of the control diet groups. As discussed earlier, synthesis of choline molecules via the PEMT pathway may be accelerated in choline deficiency.[64] Choline liberated from PtdCho formed by this pathway would dilute the specific activity of choline more in deficient rats. Membrane PtdCho serves as a large reservoir for choline molecules (approximately 120,000 nmol/liver as compared to 1000 nmol unesterified choline/liver; *Table 1*). If the turnover of PtdCho were enhanced in choline

deficiency, large amounts of non-labeled choline would be released from this reservoir and would decrease the specific activity of choline. A 1% breakdown of the PtDCho pool would double the unesterified choline content but would have little effect on the PtdCho content (therefore, we assume in our calculation, that this degradation of PtdCho does not significantly alter PtdCho specific activity).

Our data are consistent with an increased rate of PtdCho turnover in choline deficiency. If we assume that the specific activity of choline within liver near time zero approached that of the specific activity in plasma (probably an underestimate), we calculate (fraction of total pool replaced in one hour = {dpm incorporated into metabolite/μmol/hour}/{dpm/μmol choline at time zero}) that all of the PtdCho pool in liver would be replaced in 138 hours for rats on the control diet, and in 38 hours for rats on the deficient diet (this is reflected in the higher specific activity of PtdCho in the deficient groups; *Table 2*). When we assume the specific activity of choline within liver at time zero to be equivalent to that measured at 15 minutes, we calculate that all of the hepatic PtdCho pool would be replaced in 4.3 hours for rats on the control diet, and in 20 minutes for choline deficient rats. Thus, this second assumption about choline specific activity does not yield physiologically meaningful estimates of hepatic PtdCho turnover. We suggest that the radiolabel within the choline pool of liver was continuously being diluted by non-labeled choline from PtdCho degradation, and the rate of dilution was greatest in deficient animals. However, labeled choline can be incorporated in PtdCho by mechanisms in which PCho is not an obligatory intermediate (base-exchange[77]). Activation of the base-exchange pathway could be one explanation for our observation that radiolabeled choline injected into choline deficient rats was rapidly converted to PtdCho. Choline derived from this pathway would have very low specific activity. Though Beeler and colleagues reported that the base exchange pathway in rat liver is very active,[75] others using time intervals similar to those which we used, observed that the contribution to choline made by the base-exchange pathway was negligible.[76,77]

MTX did not significantly affect the metabolic partitioning of choline (*Figure 2*). Though the total amounts of choline converted to PtdCho were diminished during choline deficiency, the majority of the radiolabeled choline used by choline deficient liver in our experiments was converted to PtdCho, while in control liver this was not the case (*Figure 2*). This is consistent with the specific activity data which suggest that PtdCho turnover (and thus synthesis) was accelerated in choline deficiency. If this shift of choline toward the formation of PtdCho occurred only because choline kinase was activated, we would have expected that PCho would have accumulated; it did not. In addition, choline kinase is induced in the presence of excess choline, not by choline deficiency.[66] Therefore, it is more likely that utilization PCho increased because CDP-choline:diacylglycerol-phosphocholine transferase or phosphocholine cytidylyltransferase increased in activity. Schneider and Vance[61] have reported that CDP-choline:diacylglycerol-phosphocholine transferase activity was normal during choline deficiency. They also observed that cytosolic cytidylyltransferase activity decreased during choline deficiency,[61] and at the time, concluded that enzyme activity had diminished. Since that time, it has been discovered that this enzyme is much more active once it translocates from the cytosol to membranes, and that it is membrane-associated activity which should be measured.[66,67] As the level of cytidylyltransferase remained constant during choline deficiency,[68] decreased cytosolic enzyme activity implies that membrane bound activity increased. Whatever the mechanism, we observed, that during choline deficiency, PtdCho turnover was accelerated and that recently administered choline was shunted towards PtdCho biosynthesis. We suggest that, when choline supplies are limited, increased turnover of PtdCho primes synthetic pathways so that they have the capacity to make PtdCho as soon as any choline is made available.

Fatty infiltration of the liver is a frequently described change associated with choline deficiency or MTX therapy.[5,12-14,16,35,69] We found that MTX treatment increased the amount of triacylglycerol that accumulated in liver during choline deficiency without further decreasing (compared to choline deficiency alone) the total PtdCho levels in liver (*Table 1*). It is possible that changes in a subpool of PtdCho occurred which were not detected as changes in the total hepatic PtdCho pool. The amount of lipid accumulation observed in our experiment was smaller than that seen in studies using choline deficient diets that contain lower concentrations of methionine.[14] Choline supplementation reverses the fatty liver caused by MTX administration.[13,14,35,69,70] We observed increases in serum ALT activity, suggesting that choline deficiency caused hepatocellular damage which was increased by MTX administration. We suggest that a perturbation in choline metabolism contributes to the toxicity of MTX. Clinically, this may be important since a large population of cancer patients are treated with MTX and with intravenous feedings deficient in the nutrient choline.[71] Provision of adequate amounts of choline in the diets of people receiving MTX therapy may be an effective way to diminish the hepatotoxic side-effects of this drug.

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