The FASEB Journal express article 10.1096/fj.01-0564fje. Published online February 25, 2002.

Perturbations in choline metabolism cause neural tube defects in mouse embryos *in vitro*

Melanie C. Fisher*, Steven H. Zeisel†, Mei-Heng Mar†, and Thomas W. Sadler*

*Department of Cell Biology and Anatomy, School of Medicine, and †Department of Nutrition, School of Public Health and School of Medicine, University of North Carolina, Chapel Hill, North Carolina

Corresponding author: Thomas W. Sadler, University of North Carolina, Department of Cell Biology and Anatomy, CB# 7090, Chapel Hill, NC 27599-7090. E-mail: tsadler@med.unc.edu

ABSTRACT

A role for choline during early stages of mammalian embryogenesis has not been established, although recent studies show that inhibitors of choline uptake and metabolism, 2-dimethylaminoethanol (DMAE), and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3), produce neural tube defects in mouse embryos grown *in vitro*. To determine potential mechanisms responsible for these abnormalities, choline metabolism in the presence or absence of these inhibitors was evaluated in cultured, neurulating mouse embryos by using chromatographic techniques. Results showed that 90%–95% of ^{14}C-choline was incorporated into phosphocholine and phosphatidylcholine (PtdCho), which was metabolized to sphingomyelin. Choline was oxidized to betaine, and betaine homocysteine methyltransferase was expressed. Acetylcholine was synthesized in yolk sacs, but 70 kDa choline acetyltransferase was undetectable by immunoblot. DMAE reduced embryonic choline uptake and inhibited phosphocholine, PtdCho, phosphatidylethanolamine (PtdEtn), and sphingomyelin synthesis. ET-18-OCH_3 also inhibited PtdCho synthesis. In embryos and yolk sacs incubated with ^{3}H-ethanolamine, 95% of recovered label was PtdEtn, but PtdEtn was not converted to PtdCho, which suggested that phosphatidylethanolamine methyltransferase (PeMT) activity was absent. In ET-18-OCH_3 treated yolk sacs, PtdEtn was increased, but PtdCho was still not generated through PeMT. Results suggest that endogenous PtdCho synthesis is important during neurulation and that perturbed choline metabolism contributes to neural tube defects produced by DMAE and ET-18-OCH_3.

Key Words: neurulation • dimethylaminoethanol • ET-18-OCH_3 • embryo culture

In adult tissues, choline is an essential nutrient and its metabolites are important for methylation, acetylcholine and phospholipid biosynthesis, and cell signaling (1, 2). However, how choline and its metabolites are used during gastrulation and neurulation stages of mammalian embryogenesis has not been determined. In a rapidly growing organism, such as an embryo, it is expected that phosphatidylcholine (PtdCho) and sphingomyelin synthesis through the CDP-Choline pathway (Fig. 1) would be required for cell membrane assembly and that these molecules may also have regulatory roles when hydrolyzed into signaling molecules, such as

diacylglycerol and ceramide. Additional PtdCho may also be synthesized through phosphatidylethanolamine-N-methyltransferase (PeMT) (Fig. 1). Acetylcholine could function as a growth factor or morphogen during gastrulation, as has been indicated in chick and sea urchin embryos (3–8). Finally, the choline and folic acid metabolic pathways are linked through betaine (Fig. 1). Folic acid is important in the prevention of neural tube defects, possibly by maintaining the balance of homocysteine and methionine (9–13). Therefore, as suggested by Klein (12), choline may also be important in preventing neural tube defects by contributing methyl groups through betaine and lowering homocysteine concentrations.

Previously, we demonstrated that exposure of neurulating mouse embryos in culture to an inhibitor of choline uptake, 2-dimethylaminoethanol (DMAE), or an inhibitor of PtdCho synthesis, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3), caused growth and developmental abnormalities (14), which suggests that choline and its metabolites are important for normal embryogenesis. Therefore, to delineate how gastrulation/neurulation stage embryos use choline and to determine biochemical mechanisms associated with the developmentally toxic effects of DMAE and ET-18-OCH_3, control and inhibitor-treated mouse embryos were incubated *in vitro* with radiolabeled choline or ethanolamine. High-pressure liquid and thin-layer chromatography were used to evaluate choline uptake and the metabolism of choline or ethanolamine by the embryo and visceral yolk sac. Reverse transcription (RT) polymerase chain reaction and immunoblotting were used to analyze mRNA expression of betaine homocysteine methyltransferase and presence of choline acetyltransferase, respectively. Inhibitor effects on diacylglycerol and ceramide were also studied.

MATERIALS AND METHODS

Whole embryo culture and sample collection for chromatography

ICR mice (Harlan Laboratories, Indianapolis, IN) were mated overnight. Mating was confirmed by the presence of a sperm plug, which was designated gestational day (GD) 1. On GD 9, embryos were prepared for culture as described previously (15). The uterine decidua, parietal yolk sac, and Reichert's membrane were removed; 4–5 somite stage embryos, with the visceral yolk sac, ectoplacental cone, and amniotic membrane intact, were incubated in immediately centrifuged rat serum (16) and tyrodes buffer (Sigma, St. Louis, MO) (3:1) at 37°C, rotating at 30 rpm. Before inhibitors were added to the culture medium, 2-dimethylaminoethanol (Sigma) was mixed with tyrodes and ET-18-OCH_3 (Kamiya Biomedical Co., Seattle, WA) was sonicated in rat serum for 10 min at 37°C.

All embryos were gassed with 5% O_2, 5% CO_2, and 90% N_2 at the start of culture, and, in 24-h studies, after 10 h. At 7 or 21 h of culture, 3.0 μCi /ml of methyl ^{14}C-choline chloride (200 μCi/ml, 50–62 mCi/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) or ^{3}H-ethanolamine hydrochloride (1 mCi/ml, 15–40 Ci/mmol; Amersham) was added, and cultures were gassed with 5% O_2, 5% CO_2, and 90% N_2 (at 7 h) or 20% O_2, 5% CO_2, and 75% N_2 (at 21 h) and incubated for another 3 h. Embryos were then terminated in cold tyrodes buffer, the visceral yolk sac was removed, and both embryos and yolk sacs were rinsed three times in cold tyrodes and stored separately on ice until 6 (24-h culture) or 12 (10-h culture) of each were pooled. Samples were then frozen in liquid nitrogen and stored at –80°C. In the samples in which acetylcholine

synthesis was analyzed, the tissue was suspended immediately in 1N formic acid and tissue extraction was begun.

High-pressure liquid and thin layer chromatography

Tissue extraction

To separate aqueous and organic choline metabolites, we extracted collected tissue by using a modified version of a method established by Bligh and Dyer (17), as described by Pomfret et al (18). We added 125 µl cold distilled water (dH_20) or, for acetylcholine analysis, cold 1N formic acid, to each sample of pooled embryos or visceral yolk sacs. Tissues were then sonicated in ice water, and 25 µl was removed and stored at –80°C for protein assay. We added 400 µl of cold methanol (MeOH): chloroform ($CHCl_3$) (2:1) to the remaining 100 µl sample. Samples were placed on ice for 1 h. They were vortexed every 15 min and stored overnight at –20°C. Samples were then vortexed and centrifuged at 4°C, 6,000 rpm, for 5 min. The supernatant was transferred to a fresh tube, the pellet was re-extracted with 250 µl MeOH:$CHCl_3$:dH_2O (2:1:0.8), and supernatants were combined. We then added 100 µl $CHCl_3$ and 100 µl dH_20 added to the supernatant, and samples were vortexed and centrifuged at room temperature (6,000 rpm) for 2 min to separate the aqueous and organic phases. The aqueous phase was then transferred to a fresh tube, and both phases were dried in a Savant SpeedVac Concentrator (Savant Instruments, Inc., Holbrook, NY).

High-pressure liquid chromatography (HPLC)

HPLC was carried out by using the method established by Pomfret et al (18). The aqueous phase, containing betaine, choline, acetylcholine, glycerophosphocholine, and phosphocholine, was resuspended in 130 µl MeOH:dH_2O (12:1), injected into a Pecosphere-3C Si Cartridge (Perkin Elmer, Norwalk, CT), and eluted with a binary, nonlinear gradient of acetonitrile:ethanol:acetic acid:1 mol/L ammonium acetate:dH_20:0.1 mol/L sodium phosphate (800:68:2:3:127:10, v/v changing to 400:68:44:88:400:10, v/v). Metabolite peaks were detected by an online radiometric detector (LB506C, Berthold Inc., Nashua, NH).

Thin-layer chromatography (TLC)

The method used for thin-layer chromatography was modified from Pomfret and colleagues (18). The organic phase, containing PtdCho, phosphatidylethanolamine (PtdEtn), lysophosphatidylethanolamine (LPE), and sphingomyelin, was suspended in 30 µl MeOH:$CHCl_3$ (2:1) and spotted on a "Baker Analyzed" SI 250 PA silica gel plate (VWR Scientific Products, West Chester, PA). To ensure that all of the organic phase was included, we rinsed the tube with 20 µl MeOH:$CHCl_3$ (2:1), which was added to the original spot on the plate. PtdCho, PtdEtn, LPE, and sphingomyelin standards (10 mM each) were also included on the plate. After drying, the TLC plate was developed in $CHCl_3$:MeOH:40% methylamine (60:20:5) for 40 min. We used diphenylhexatriene (0.03%) in $CHCl_3$ was used to visualize the lipid products. PtdCho, PtdEtn, LPE, and sphingomyelin bands were marked and scraped into scintillation vials. Distilled water (500 µl) was added, samples were vortexed, and, after 1 h, scintillation fluid was added. After incubating overnight at room temperature, samples were then counted in a Wallace 1410 liquid

scintillation counter. Choline concentration in the media at the time-labeled choline was added and, at the end of culture, was measured as described by Pomfret and colleagues (18). The specific activity of choline was then calculated, and all ^{14}C-choline data were adjusted accordingly.

Protein assay

To adjust for variability in sample size, we determined total protein for each sample by using a Bio-Rad protein assay kit (Bio-Rad Life Science Research, Hercules, CA). In duplicate, 10 µl of each sonicated sample was combined in a glass test tube with 90 µl dH_2O and 5 ml of 1:4 diluted Bio-Rad dye reagent. After being vortexed, the tubes were incubated for 15 min at room temperature. Absorbance was measured at 595 nm, and total protein was calculated by using a standard curve created with bovine serum albumin.

Reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR was used to determine whether betaine homocysteine methyltransferase (BHMT) was expressed in GD 9, 9.5, or GD 10 mouse embryos and visceral yolk sacs. Adult liver and kidney were used as BHMT positive controls, and actin primers were used to verify successful RNA isolation.

RNA isolation and DNase treatment

Adult liver; kidney; or GD 9, 9.5, or 10 mouse embryos and their visceral yolk sacs were isolated in cold tyrodes buffer, collected in TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH), and stored at –80°C. In order to obtain a significant quantity of mRNA, we pooled approximately 20 GD 9, 10 GD 9.5, and 6 GD 10 embryos or yolk sacs. The tissue was homogenized in TRI-REAGENT and extracted with chloroform. Total RNA was precipitated from the aqueous phase with isopropanol and then washed with 75% ethanol made with diethyl pyrocarbonate treated water (DEPC H_20). The RNA pellet was dried and suspended in 20 µl DEPC H_20.

In order to eliminate the possibility of DNA contamination, we treated RNA with RQ1 RNase-free DNase (Promega Corporation, Madison, WI). To each 20 µl RNA sample, 5 units (5 µl) DNase, 3 µl 10× reaction buffer (400 mM Tris-HCl [pH 8.0], 100 mM $MgSO_4$, and 10 mM $CaCl_2$), and 2 µl DEPC H_20 were added. The reaction mix was incubated at 37°C for 30 min and stopped with RQ1 DNase Stop Solution (20 mM EGTA, pH 8.0), and the DNase was inactivated with a 10-min incubation at 65°C. The RNA was then re-isolated with phenol/chloroform extraction and ethanol precipitation with 3 M sodium acetate. The precipitated pellet was washed with 75% ethanol, dried, and resuspended in 20 µl DEPC H_20.

Reverse-transcription, PCR amplification, and agarose gel electrophoresis

We used a GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA) for mRNA transcription and cDNA amplification. DEPC H_2O (6 µl), $MgCl_2$ (25 mM; 4 µl), 10× PCR buffer (500 mM KCl, 100 mM Tris-HCL, pH 8.3; 2 µl), deoxynucleotides (2 µl each), RNase inhibitor (1 µl), MuLV

reverse transcriptase (1 μl), and random hexamers (1 μl) were added to 3 μl of the DNase-treated RNA preparation for a total volume of 20 μl. To allow for primer extension, we incubated the mixture for 10 min at room temperature. The reaction was then incubated in a Perkin Elmer DNA Thermal Cycler at 42°C for 1 h, 95°C for 5 min (heat inactivation), and 5°C for 5 min. We split the RT reaction mixture in order to amplify both BHMT and actin cDNA for each sample. For the PCR reaction, primers, additional $MgCl_2$ and 10× PCR buffer, dH_2O, and AmpliTaq DNA polymerase were added to 10 μl of the cDNA generated in the RT reaction. The primers used were as follows:

BHMT: lower primer (bases 58–82) 5' ATGCCGGAGAAGTTGTGATTGGAGA 3'
upper primer (bases 526–550) 5' GCTACGGGCTTACCAGATGCTTTTA 3'

β-Actin: lower primer (bases 521–539) 5' TACCACAGGCATTGTGATGG 3'
upper primer (bases 811–831) 5' AATAGTGATGACCTGGCCGT 3'

The conditions for BHMT cDNA amplification were: cycle 1, 94°C for 5 min, 57°C for 1 min, 72°C for 1 min; cycles 2–34, 94°C for 1 min, 57°C for 1 min, 72°C for 1 min; cycle 35, 94°C for 1 min, 60°C for 10 min. For β-actin cDNA the conditions were cycle 1, 94°C for 5 min, 57°C for 1 min, 72°C for 1 min; cycles 2–24, 94°C for 1 min, 57°C for 1 min, 72°C for 1 min; and cycle 25, 94°C for 1 min, 60°C for 10 min. PCR products and a molecular-weight standard were run on a 3% agarose gel containing 0.5 μg/ml ethidium bromide for 30 min at 105 volts.

Immunoblot

Western blotting was used to determine whether choline acetyltransferase (ChAT) was present in GD 9, 9.5, or 10 mouse embryos and visceral yolk sacs. Adult and fetal (GD 15) brain were selected as positive controls. Samples were collected and homogenized on ice in 0.0625M Tris-HCl, pH 6.8, and 2% SDS. We used a Bio-Rad DC protein assay kit (Bio-Rad Life Science Research) to determine protein concentration according to the manufacturer's instructions. Six microliters of a modified version of 5X Laemmli sample buffer (19) (50% glycerol, 25% β-mercaptoethanol, 0.5% bromophenyl blue, 0.0625 M Tris-HCl, 2% SDS) were added to 50 μg of protein, and 0.0625M Tris-HCl -2% SDS was used to bring the volume to 30 μl. Bio-Rad Prestained Precision Protein Standards (Bio-Rad Life Science Research) and protein samples were loaded onto Novex™ (San Diego, CA) 10% polyacrylamide tris-glycine pre-cast mini gels. Two gels were run simultaneously with 1X Novex™ Tris-Glycine running buffer for 2 h at 40 mA. Proteins were transferred to pre-wet (3 min MeOH, 5× dH_20 rinse, 5 min Towbin transfer buffer) polyvinylidene difluoride membranes in cold (4°C) Towbin transfer buffer (192 mM glycine, 25 mM Tris base (pH 8.3), 0.5% SDS, 15% methanol) (20) for 1 h at 400 mA. The membranes were blocked for 1 h at room temperature in 5% milk in 1× PBS-0.1% Tween (PBS-T) and then were incubated overnight at 4°C with 1:5,000 goat anti-ChAT antibody (Chemicon International, Temecula, CA) diluted in 1% milk in PBS-T. Membranes were washed six times (10–15 min each) with PBS-T and then were incubated 1 h at room temperature with 1:50,000 peroxidase conjugated rabbit anti-goat IgG (Pierce, Rockford, IL) diluted in 1% milk in PBS-T. After being washed six times (10–15 min each) in PBS-T, membranes were washed twice (15 min each) in PBS and treated with SuperSignal West Dura Extended Duration Substrate (Pierce) for 5 min at room temperature. Excess SuperSignal was removed and blots were exposed to film.

All membrane incubations were done on a shaker at 50 rpm. Gels were stained with Coomassie blue to confirm transfer.

Diacylglycerol and ceramide assay

After 24 h of culture, control and inhibitor treated embryos and their visceral yolk sacs were collected and rinsed in cold tyrodes, pooled (12 embryos or yolk sacs per sample), frozen in liquid nitrogen, and stored at –80°C. Diacylglycerol and ceramide levels were measured according to the method described by Previati and colleagues (21). Samples were homogenized in 250 µl cold dH_2O by sonication on ice, and an aliquot (50 µl) was removed for a Lowry protein assay (22) in order to normalize sample size variability. The remaining sample (200 µl) was extracted with 4 ml MeOH:$CHCl_3$ (1:2) for 30 min. Distilled water (1 ml) was added, and the samples were centrifuged at 1,000 rpm for 5 min. The aqueous phase was transferred to a fresh tube and washed with $CHCl_3$ (2 ml). After centrifugation, the aqueous phase was removed to a new tube. The organic phases from both extractions were pooled, dried in a Savant SpeedVac Concentrator (Savant Instruments, Inc.), and stored in a small volume of chloroform at –20°C.

The lipid phase was solubilized in 100 µl anhydrous chloroform and 10 µl each of 100 mM NAP ((S)-6-methoxy-methyl-2-naphthaleneacetic acid), 100 mM DCC (N,N'-dicyclohexylcarbodiimide), and 100 mM DMAP (4-dimethylaminopyridine) in anhydrous $CHCl_3$ was added. Samples were mixed and incubated at –20°C for at least 3 h. Drying in the SpeedVac stopped the reaction. Samples were then resuspended in $CHCl_3$ (15 µl), extracted with hexane (2 ml), and centrifuged. The supernatant was transferred to a new tube and mixed with 5 ml MeOH:dH_20 (4:1). Following centrifugation, the organic (top) phase was removed and the aqueous phase was re-extracted as above. The organic phases were pooled, dried in the SpeedVac, and dissolved in hexane (1 ml), and 50 µl was injected onto an Econosphere CN HPLC column (Alltech, Deerfield, IL). Diacylglycerol and ceramide were eluted with a gradient of hexane and 3% 2-propanol in hexane (90:10 to 0:100 to 98:2 v/v) and detected with a Prostar 360 fluorescent detector (Varian Instruments, Walnut Creek, CA).

Statistical analysis

Repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to compare diacylglycerol and ceramide levels in control and inhibitor-treated embryos and yolk sacs after 24 h of culture. Labeled choline and ethanolamine metabolites measured after 10 h embryo culture were compared between control and inhibitor treated embryos or yolk sacs by using ANOVA followed by Dunnett's multiple comparisons test. A metabolite comparison between control and ET-18-OCH_3 treated embryos or yolk sacs cultured 24 h was made by using a two-tailed *t*-test. A *P*-value less than or equal to 0.05 was considered significant.

RESULTS

Choline utilization by gastrulation and neurulation stage mouse embryos and abnormal choline metabolism in inhibitor treated embryos and visceral yolk sacs

To evaluate choline metabolism and to determine biochemical mechanisms associated with previously observed teratogenic effects of DMAE and ET-18-OCH_3, we used HPLC and TLC to measure the uptake and incorporation of ^{14}C-choline into choline metabolites in control and inhibitor-treated embryos and their visceral yolk sacs (Figs. 2–4). In addition, PtdCho synthesis through the PeMT pathway was evaluated by using ^{3}H-ethanolamine (Figs. 5, 6). Embryos were exposed to previously determined teratogenic doses of DMAE (375 μM) or ET-18-OCH_3 (275 μM), which had been shown to cause a greater than 90% malformation rate (14), for 7 or 21 h before the addition of ^{14}C-choline or ^{3}H-ethanolamine. Control cultures received only vehicle for the same incubation period. After a 3-h incubation with the labeled metabolite, cultures were terminated and tissue was collected for HPLC and TLC.

After 10 h of culture, 95% of ^{14}C-choline detected in control embryos was in the form of phosphocholine (69%) and PtdCho (26%) (Fig. 2). In control visceral yolk sacs, 78% of labeled choline was in phosphocholine and 14% in PtdCho. In embryos and yolk sacs cultured for 24 h, ~90% of ^{14}C-choline was detected in phosphocholine and PtdCho (Fig. 4). The distribution of these metabolites in 24-h visceral yolk sacs was similar to the pattern at 10 h (72% phosphocholine, 17% PtdCho); whereas in the 24-h embryo, 40% of the ^{14}C-choline was in the form of phosphocholine and 57% PtdCho. At both time points, the remaining ^{14}C-choline detected was free choline, betaine, glycerophosphocholine (not shown), or sphingomyelin. In a separate experiment in which samples were treated with formic acid in order to prevent acetylcholine degradation, approximately 1% of ^{14}C-choline could be detected as acetylcholine (ACh) in visceral yolk sacs from both 10- and 24-h cultures, but no labeled ACh was observed in cultured embryos (data not shown). Finally, in both embryos and yolk sacs cultured 10 or 24 h and incubated with ^{3}H-ethanolamine, approximately 95% of radiolabel detected in the lipid phase was in the form of phosphatidylethanolamine (PtdEtn) and the remainder was found in lysophosphatidylethanolamine (LPE), a PtdEtn metabolite (Figs. 5, 6). No ^{3}H-label was detected in PtdCho.

After 10 h of embryo culture with 375 μM DMAE, the amount of labeled choline in the embryo was reduced by 60% (Fig. 2). However, choline uptake by the visceral yolk sac appeared to be unaffected. In both yolk sacs and embryos treated with DMAE, the incorporation of labeled choline into phosphocholine, PtdCho, and sphingomyelin was reduced to approximately 25%, 35%, and 50% of control values, respectively. In the DMAE-treated embryos, the amount of labeled choline in betaine was approximately threefold higher than in controls. Treatment with DMAE also caused a reduction in PtdEtn and LPE synthesis from labeled ethanolamine in both embryos and visceral yolk sacs (Fig. 5).

The effects of 275 μM ET-18-OCH_3 on choline uptake and metabolism were evaluated after both 10 and 24 h of whole embryo culture (Figs. 3, 4). We had described previously the effects of ET-18-OCH_3 on phosphocholine and PtdCho synthesis after 10 h of embryo culture (14), and, for continuity, these data are also reported in Figure 3. In visceral yolk sacs after 10 or 24 h and, in embryos after 24 h, treatment with ET-18-OCH_3 resulted in almost twice the amount of labeled phosphocholine compared with controls. After 10 h of culture, PtdCho and sphingomyelin accumulation was reduced by approximately 50% in embryos exposed to ET-18-OCH_3. A trend toward reduced PtdCho ($P = 0.0675$) and reduced sphingomyelin ($P \leq 0.05$) accumulation was

also observed in 24-h ET-18-OCH_3 treated embryos. Treatment with ET-18-OCH_3 also appeared to produce a pattern of elevated labeled betaine in embryos after 10- and 24-h culture, and a significant increase in betaine was observed in visceral yolk sacs after 24 h. Additionally, free ^{14}C-choline levels were found to be twice as high as control values in embryos treated with ET-18-OCH_3 for 24 h. In visceral yolk sacs from embryos incubated with ^{3}H-ethanolamine, ET-18-OCH_3 treatment resulted in a 25%–30% increase in PtdEtn synthesis, but PtdCho synthesis through the PeMT pathway was not observed (Figs. 5, 6).

Betaine homocysteine methyltransferase was expressed at later neurulation stages, but 70 kDa choline acetyltransferase was absent from neurulating mouse embryos

We found that betaine could be synthesized from choline and that the activity of this oxidative pathway appeared to be elevated in DMAE and ET-18-OCH_3 inhibitor treated embryos (Figs. 2–4). To determine whether betaine can contribute to methionine synthesis during these stages of development, RT-PCR was used to determine whether betaine homocysteine methyltransferase was expressed in GD 9, 9.5, and 10 embryos and their visceral yolk sacs (Fig. 7). Results showed that BHMT was not expressed in either yolk sac or embryonic tissue until GD 10, when neurulation was nearly complete.

The biochemical studies described above also indicated that acetylcholine was synthesized in the visceral yolk sac after 10 and 24 h of embryo culture. Immunoblotting was used to determine whether choline acetyltransferase could be detected in GD 9, 9.5, and 10 embryos and yolk sacs, using adult and fetal (GD 15) brain tissue as controls (Fig. 8). In adult brain, the anti-ChAT antibody labeled two protein bands of 70 kDa and 45 kDa. However, 70 kDa ChAT was undetectable in fetal brain and absent in GD 9-10 embryos and yolk sacs. In fetal brain and embryos, the antibody instead labeled a 50 kDa protein band. In embryos, yolk sacs, and fetal brain, a 40 kDa anti-ChAT labeled protein band was also observed.

Diacylglycerol and ceramide levels were altered in inhibitor treated embryos

Diacylglycerol (DAG) and ceramide (CER) are important in the synthesis of PtdCho and sphingomyelin (Fig. 1) and are released when these phospholipids are hydrolyzed (23). Inhibiting choline uptake and metabolism with DMAE or ET-18-OCH_3 could alter the relative proportion of DAG and CER available to embryonic cells for cell signaling and the synthesis of PtdCho and sphingomyelin. Therefore, HPLC was used to evaluate the effects of these inhibitors on DAG and CER after 24 h of whole embryo culture (Fig. 9). Compared with controls, diacylglycerol concentrations were elevated by 17% in ET-18-OCH_3 treated embryos, but remained similar to control values in embryos treated with DMAE. In addition, treatment with either DMAE or ET-18-OCH_3 resulted in a 15% and 25% increase, respectively, in embryonic ceramide concentrations. Compared with controls, there was no significant difference in the levels of DAG or CER in the visceral yolk sacs from ET-18-OCH_3 or DMAE treated conceptuses.

DISCUSSION

Choline metabolism by gastrulation and neurulation stage mouse embryos and their visceral yolk sacs was investigated by tracing the metabolism of labeled choline or ethanolamine with HPLC and TLC. Previous studies demonstrated choline kinase and CTP: phosphocholine cytidylyltransferase activity in preimplantation stage mouse embryos (24, 25). In our experiments, the synthesis of phosphocholine and PtdCho from ^{14}C-choline in cultured embryos and yolk sacs indicates that these enzymes are also present and active during gastrulation and neurulation stages of development (Figs. 2–4). In adult tissues, PtdCho is usually the most prominent choline metabolite (18, 26). Therefore, the high ratio of phosphocholine to PtdCho observed in embryos after 10 h of culture and in visceral yolk sacs after either 10 h or 24 h of culture, suggests that, at these stages of development, the CDP-Choline pathway is still developing and may not be as efficient as in adult tissues in converting phosphocholine to PtdCho. After 24 h of culture, there appears to be an increase in PtdCho synthesis in the embryo, suggesting continued maturation of this pathway. However, it should be noted that it is difficult to distinguish whether radioactive counts in the embryo resulted from labeled metabolite being transported from visceral yolk sac to embryo or whether the embryo itself has the capacity to metabolize choline to PtdCho. The visceral yolk sac has been shown previously to possess metabolic activities at these stages of morphogenesis (27) and therefore it is possible that PtdCho is synthesized at this site and then transported to the embryo. In this respect, an increase in labeled PtdCho in the embryo after 24 h of culture may represent more efficient visceral yolk sac synthesis and transfer.

Additional metabolites generated from ^{14}C-choline in embryos and visceral yolk sacs included betaine, glycerophosphocholine, and sphingomyelin. In visceral yolk sacs, small quantities of labeled acetylcholine were also observed. These results suggest that choline oxidase, PtdCho: ceramide choline phosphotransferase, and, in yolk sacs, choline acetyltransferase are active during neurulation stages of development (Fig. 1). Betaine has the potential to play an important role during neurulation as a methyl donor for the production of methionine and as a means of lowering homocysteine concentration. Recent studies show that methionine and folic acid can prevent neural tube defects, such as spina bifida and exencephaly, possibly by altering methyl group metabolism and/or reducing elevated homocysteine (9–13, 28–30). In the present study, however, RT-PCR analysis indicated that betaine homocysteine methyltransferase was not expressed until neural tube closure was nearly complete. Hence, choline would not be an acceptable substitute for folic acid as a methyl donor in the prevention of neural tube defects and betaine synthesis may serve only as a shunt for excess choline during gastrulation and neurulation. This conclusion is consistent with results showing that choline could only partially rescue rat embryos grown in methionine deficient medium (12). However, as this pathway matures, betaine could become important in maintaining the balance between homocysteine and methionine by providing additional methyl groups to the methylation cycle.

Although a small amount of acetylcholine was synthesized from labeled choline in the visceral yolk sac, immunoblot revealed that the typical 70 kDa choline acetyltransferase was not detectable in fetal brain, embryos, or visceral yolk sacs. Instead the ChAT antibody labeled lower molecular weight bands (40–50 kDa) in these tissues. It has been documented that the ChAT gene generates multiple mRNA splice variants (31–34). One such variant gives rise to a

50 kDa protein, pChAT, which appears to be localized to the peripheral nervous system (34) and could account for the 50 kDa band observed in fetal brain and embryos. Whether or not the 40-kDa band represents another as yet unidentified splice variant of ChAT remains to be determined. It is also not clear whether these proposed splice variants of ChAT are active in converting choline to acetylcholine. However, the existence of labeled acetylcholine in the visceral yolk sac is indicative of at least some ChAT activity. It is possible that acetylcholine in the visceral yolk sac could act as a growth factor or morphogen during early mammalian embryogenesis, as has been proposed in other species (3–8). Other neurotransmitters, such as serotonin, have also been found to have regulatory roles during gastrulation, neurulation, craniofacial, and heart development (reviewed in ref. 8).

One question that arose from our studies was whether embryos or their surrounding membranes could generate additional PtdCho through the PeMT pathway in order to overcome a block in the CDP-choline pathway for PtdCho synthesis. Our results showed that, although control embryos could synthesize phosphatidylethanolamine (PtdEtn) from labeled ethanolamine, they did not convert PtdEtn to PtdCho via the PeMT pathway. Furthermore, inhibition of the CDP-choline pathway with ET-18-OCH_3 led to increased levels of PtdEtn, but PtdCho was still not generated through PeMT. Therefore, we concluded that gastrulation and neurulation stage mouse embryos must use the CDP-choline pathway to synthesize PtdCho. Consistent with these results, targeted gene disruption of the PeMT gene, *pempt*, had no observable effect on embryonic growth and development (35). In addition, PeMT activity was not detectable in the liver until later stages of fetal development (36, 37).

Considering the potential importance of choline and its metabolites in embryonic development, we hypothesized that alterations in choline metabolism would cause developmental defects during the early stages of embryogenesis. This hypothesis was substantiated in a previous study (14) in which the application of DMAE or ET-18-OCH_3 to embryo culture resulted in neural tube and craniofacial abnormalities. The present study indicates that reduced PtdCho availability due to decreased choline transport and/or an inhibition of PtdCho synthesis could be responsible for the observed defects. For example, free choline levels were lowered in DMAE-treated embryos, but not in visceral yolk sacs, which suggests that DMAE may have inhibited the transport of choline from yolk sac to embryo (Fig. 2). However, we also observed reduced ^{14}C-labeled phosphocholine, PtdCho, and sphingomyelin levels in the visceral yolk sac, which suggests that DMAE could be inhibiting choline kinase in this tissue (Fig. 1). Inhibiting choline kinase could, in turn, allow free choline levels to be maintained in the yolk sac, even if choline uptake from the media was diminished. DMAE treatment also resulted in reduced incorporation of ^{14}C-choline into phosphocholine, PtdCho, and sphingomyelin in the embryo. It is not clear whether this effect was the result of reduced choline uptake and/or transfer of choline and its metabolites from the visceral yolk sac, or whether there was direct inhibition of embryonic choline kinase by DMAE. Nevertheless, it is apparent that reduced PtdCho availability caused by an inhibition of choline transport and/or repressed choline kinase by DMAE can be teratogenic.

Our results with DMAE are consistent with previous reports that demonstrate that DMAE is a competitive inhibitor of choline uptake and transport in the kidney and placenta (38, 39) and an inhibitor of choline kinase in the kidney and liver (40). DMAE also caused a reduction in phosphatidylethanolamine synthesis from ^{3}H-ethanolamine, reflecting that, in addition to being a

choline analog, DMAE is related structurally to ethanolamine. It was also interesting that betaine levels were elevated in DMAE-treated embryos, a result that is in contrast to reports in the literature that suggest DMAE is an inhibitor of choline oxidase (39–42). Instead, during embryogenesis, it appears that the oxidative pathway provided an alternate route for choline that may have accumulated when choline kinase was inhibited.

ET-18-OCH_3 is an antineoplastic agent that has been shown to inhibit PtdCho synthesis at the level of CTP: phosphocholine cytidylyltransferase in cell culture (Fig. 1) (43, 44). The pattern of reduced embryonic PtdCho and increased phosphocholine (the immediate precursor of CDP-choline) in visceral yolk sacs at 10 h and 24 h and in embryos after 24 h culture, indicates that ET-18-OCH_3 inhibited the CDP-choline pathway in the conceptus (Figs. 3, 4). This result also suggests that, at least at the 10 h timepoint, the primary effect on ET-18-OCH_3 on PtdCho synthesis was on the visceral yolk sac. Elevated phosphocholine in 24-h ET-18-OCH_3-treated embryos is consistent with increased activity of the CDP-choline pathway observed in control embryos cultured for the same length of time. There also appeared to be a trend toward increased betaine synthesis in ET-18-OCH_3-treated embryos at both time points and in the yolk sac after 24 h culture. Because we demonstrated that BHMT is first expressed on GD 10, a stage equivalent to after 24 h of whole embryo culture, additional betaine could be used for methylation at later stages of development.

Diacylglycerol and ceramide are second messengers involved in signal transduction pathways that regulate apoptosis (45–49). In our experiments, compared with controls, diacylglycerol and ceramide levels were higher in ET-18-OCH_3-treated embryos. Elevated DAG and CER concomitant with reduced PtdCho and sphingomyelin have previously been associated with apoptosis (49, 50). In addition, an inhibition of PtdCho synthesis by ET-18-OCH_3 has been associated with apoptosis in cell culture (44, 51–55) and in developing embryos (14). ET-18-OCH_3 is also an inhibitor of phosphatidylinositol specific phospholipase C and protein kinase C (56, 57). Therefore, in addition to an inhibition of PtdCho synthesis through the CDP-choline pathway, the effects of ET-18-OCH_3 on embryonic development could also reflect abnormal cell signaling.

To conclude, the majority of choline used by cultured embryos and their visceral yolk sacs is phosphorylated to phosphocholine, which is then used for PtdCho synthesis through CDP-choline. An inhibition of the CDP-Choline pathway and reduced choline availability for PtdCho synthesis resulted in increased cell death and developmental defects (14), which could be the result of inadequate PtdCho for membrane biosynthesis or aberrant cell signaling through PtdCho metabolites. Hence, it is apparent that an endogenous pathway for PtdCho synthesis is required for normal growth and development.

Pregnancy has been shown to deplete choline pools (26, 58), and the demand for choline by a developing fetus could put the mother and, subsequently, the conceptus at risk for choline deficiency (26). Prenatal choline deficiency has been associated with modified spatial and temporal memory, shortened attention spans (59, 60), and resulted in higher levels of apoptosis, reduced cell proliferation, and altered differentiation in the fetal hippocampal region (61, 62). In adults, choline deficiency and the ensuing abnormalities in choline metabolism caused liver damage in humans and hepatocarcinomas in rats (63–69). The current work establishes a critical

role for choline during the early stages of organogenesis, a stage when many birth defects are induced. Because pregnant women may be at risk for choline deficiency, and reduced choline availability has been shown to have a negative impact on embryonic and fetal development as well as adult liver, it has been suggested that an increase in dietary choline intake during pregnancy may be beneficial (26, 70).

The potential teratogenic effects of dimethylaminoethanol are of special concern, as it is currently sold as a nutrient supplement that claims to enhance acetylcholine-related functions, such as memory and learning (71–74). A related molecule found in many commercial products, diethanolamine (DEA), has also been shown to disrupt choline metabolism and has caused tumor formation in mice (75, 76). Thus, compounds that alter choline metabolism in adult (DMAE and DEA) and embryonic (DMAE) tissues are readily accessible to pregnant women and have the potential to be teratogenic in humans.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health grants HD29495 (T.W.S.) and AG09525 (S.H.Z.).

REFERENCES

1. Zeisel, S. H. (1981) Dietary choline: biochemistry, physiology, and pharmacology. *Annu Rev Nutr* 1, 95-121

2. Zeisel, S. H., and Blusztajn, J. K. (1994) Choline and human nutrition. *Annu Rev Nutr* 14, 269-296

3. Augustinsson, K-B., and Gustafson, T. (1949) Cholinesterase in developing sea-urchin eggs. *J Cell Comp Physiol* 34, 311-321

4. Gustafson, T., and Toneby, M. (1970) On the role of serotonin and acetylcholine in sea urchin morphogenesis. *Exp Cell Res* 62, 102-117

5. Valinsky, J. E., and Loomis, C. (1984) The cholinergic system of the primitive streak chick embryo. *Cell Differ* 14, 287-294

6. Laasberg, T., Pedak, A., and Neuman, T. (1987) The muscarinic receptors - mediated action of acetylcholine in the gastrulating chick embryo. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 86C, 313-316

7. Falugi, C. (1993) Localization and possible role of molecules associated with the cholinergic system during “non-nervous” developmental events. *Eur J Histochem* 37, 287-294

8. Buznikov, G. A., Shmukler, Y. B., and Lauder, J. M. (1996) From oocyte to neuron: do neurotransmitters function in the same way throughout development. *Cell Mol Neurobiol* 16, 533-559

9. Czeizel, A. E. (1995) Folic acid in the prevention of neural tube defects. *J Pediatr Gastroenterol Nutr* 20, 4-16

10. Oakley, G. P. J., Adams, M. J., and Dickinson, C. M. (1996) More folic acid for everyone, now. *J Nutr* 126, 751S-755S

11. Johnston, R. B. J. (1997) Folic acid: new dimensions of an old friendship. *Adv Pediatr* 44, 231-261

12. Klein, N. W. (1997) The use of whole rat embryo cultures to identify and characterize causes of reproductive failure. *Int J Dev Biol* 41, 267-273

13. Eskes, T. K. A. B. (1998) Neural tube defects, vitamins and homocysteine. *Eur J Pediatr* 157, S139-S141

14. Fisher, M. C., Mar, M-H., Zeisel, S. H., and Sadler, T. W. (2001) Inhibitors of choline uptake and metabolism cause developmental abnormalities in neurulating mouse embryos. *Teratology* 64, 114-122

15. Sadler, T. W. (1979) Culture of early somite mouse embryos during organogenesis. *J Embryol Exp Morph* 49, 17-25

16. Steele, C. E., and New, D. A. (1974) Serum variants causing the formation of double hearts and other abnormalities in explanted rat embryos. *J Emb Exp Morph* 31, 707-719

17. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37, 911-917

18. Pomfret, E. A., da Costa, K-A., Schurman, L. L., and Zeisel, S. H. (1989) Measurement of choline and choline metabolite concentrations using high-pressure liquid chromatography and gas chromatography-mass spectrometry. *Anal Biochem* 180, 85-90

19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*London*) 227, 680-685

20. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76, 4350-4354

21. Previati, M., Bertolaso, L., Tramarin, M., Bertagnolo, V., and Capitani, S. (1996) Low nanogram range quantitation of diglycerides and ceramide by high-performance liquid chromatography. *Anal Biochem* 233, 108-14

22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193, 265-275

23. Zeisel, S. H. (1993) Choline phospholipids: signal transduction and carcinogenesis. *FASEB J* 7, 551-557

24. Pratt, H. P. (1980) Phospholipid synthesis in the preimplantation mouse embryo. *J Reprod Fertil* 58, 237-248

25. Moon, E. A., and O'Neill, C. (1997) CTP:phosphocholine cytidylyltransferase activity in the preimplantation mouse embryo. *J Reprod Fertil* 110, 213-218

26. Zeisel, S. H., Mar, M-H., Zhou, Z., and da Costa, K-A. (1995) Pregnancy and lactation are associated with diminished concentrations of choline and its metabolites in rat liver. *J Nutr* 125, 3049-3054

27. Lloyd, J. B. (1990) Cell physiology of the rat visceral yolk sac: a study of pinocytosis and lysosome function. *Teratology* 41, 383-393

28. Essien, F. B. (1992) Maternal methionine supplementation promotes the remediation of axial defects in *Axd* mouse neural tube mutants. *Teratology* 45, 205-212

29. Nosel, P. G., and Klein, N. W. (1992) Methionine decreases the embryotoxicity of sodium valproate in the rat: in vivo and in vitro observations. *Teratology* 46, 499-507

30. Shaw, G. M., Velie, E. M., and Schaffer, D. M. (1997) Is dietary intake of methionine associated with a reduction in risk for neural tube defect-affected pregnancies? *Teratology* 56, 295-299

31. Misawa, H., Ishii, K., and Deguchi, T. (1992) Gene expression of mouse choline acetyltransferase: alternative splicing and identification of a highly active promoter region. *J Biol Chem* 267, 20392-20399

32. Lorenzi, M. V., Trinidad, A. C., Zhang, R., and Strauss, W. L. (1992) Two mRNAs are transcribed from the human gene for choline acetyltransferase. *DNA Cell Biol* 11, 593-603

33. Grosman, D. D., Lorenzi, M. V., Trinidad, A. C., and Strauss, W. L. (1995) The human choline acetyltransferase gene encodes two proteins. *J Neurochem* 65, 484-491

34. Tooyama, I., and Kimura, H. (2000) A protein encoded by an alternative splice variant of choline acetyltransferase mRNA is localized preferentially in peripheral nerve cells and fibers. *J Chem Neuroanat* 17, 217-226

35. Walkey, C. J., Donohue, L. R., Bronson, R., Agellon, L. B., and Vance, D. E. (1997) Disruption of the murine gene encoding phosphatidylethanolamine *N*-methyltransferase. *Proc Natl Acad Sci USA* 94, 12880-12885

36. Pelech, S. L., Power, E., and Vance, D. E. (1983) Activities of the phosphatidylcholine biosynthetic enzymes in rat liver during development. *Can J Biochem Cell Biol* 61, 1147-1152

37. Cui, Z., Shen, Y-J., and Vance, D. E. (1997) Inverse correlation between expression of phosphatidylethanolamine-*N*-methyltransferase-2 and growth rate of perinatal rat livers. *Biochim Biophys Acta* 1346, 10-16

38. Sweiry, J. H., and Yudilevich, D. L. (1985) Characterization of choline transport at maternal and fetal interfaces of the perfused guinea-pig placenta. *J Physiol (Lond)* 366, 251-266

39. Lohr, J., and Acara, M. (1990) Effect of dimethylaminoethanol, an inhibitor of betaine production, on the disposition of choline in the rat kidney. *J Pharmacol Exp Ther* 252, 154-158

40. Haubrich, D. R., Gerber, N. H., and Pflueger, A. B. (1981) Deanol affects choline metabolism in peripheral tissues of mice. *J Neurochem* 37, 476-482

41. Wells, I. C. (1954) Oxidation of choline-like substances by rat liver preparations. Inhibitors of choline oxidase. *J Biol Chem* 207, 575-583

42. Haubrich, D. R., and Gerber, N. H. (1981) Choline dehydrogenase: assay, properties and inhibitors. *Biochem Pharmacol* 30, 2993-3000

43. Boggs, K. P., Rock, C. O., and Jackowski, S. (1995) Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP:phosphocholine cytidylyltransferase step. *J Biol Chem* 270, 7757-7764

44. Boggs, K., Rock, C. O., and Jackowski, S. (1998) The antiproliferative effect of hexadecylphosphocholine toward HL60 cells is prevented by exogenous lysophosphatidylcholine. *Biochim Biophys Acta* 1389, 1-12

45. Obeid, L. M., Linardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) Programmed cell death induced by ceramide. *Science* 259, 1769-1771

46. Lucas, M., and Sanchez-Margalet, V. (1995) Protein kinase C involvement in apoptosis. *Gen Pharmacol* 26, 881-887

47. Mizushima, N., Koike, R., Kohsaka, H., Kushi, Y., Handa, S., Yagita, H., and Miyasaka, N. (1996) Ceramide induces apoptosis via CPP32 activation. *FEBS Lett* 395, 267-271

48. Yoshimura, S-I., Banno, Y., Nakashima, S., Takenaka, K., Sakai, H., Nishimura, Y., Sakai, N., Shimizu, S., Eguchi, Y., Tsujimoto, Y., and Nozawa, Y. (1998) Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death: inhibitory effects of Bcl-2 on ceramide formation and caspase-3 activation. *J Biol Chem* 273, 6921-6927

49. Yen, C-L. E., Mar, M-H., and Zeisel, S. H. (1999) Choline deficiency-induced apoptosis in PC12 cells is associated with diminished membrane phosphatidylcholine and sphingomyelin, accumulation of ceramide and diacylglycerol, and activation of a caspase. *FASEB J* 13, 135-142

50. Yen, C-L. E., Mar, M-H., Meeker, R. B., Fernandes, A., and Zeisel, S. H. (2001) Choline deficiency induces apoptosis in primary cultures of fetal neurons. *FASEB J* 15, 1704-1710

51. Modolell, M., Andreesen, R., Pahlke, W., Brugger, U., and Munder, P. G. (1979) Disturbance of phospholipid metabolism during the selective destruction of tumor cells induced by alkyl-lysophospholipids. *Cancer Res* 39, 4681-4686

52. Diomede, L., Colotta, F., Piovani, B., Re, F., Modest, E. J., and Salmona, M. (1993) Induction of apoptosis in human leukemic cells by the ether lipid 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine. A possible basis for its selective action. *Int J Cancer* 53, 124-130

53. Mollinedo, F., Martinez-Dalmau, R., and Modolell, M. (1993) Early and selective induction of apoptosis in human leukemic cells by the alkyl-lysophospholipid ET-18-OCH_3. *Biochem Biophys Res Commun* 192, 603-609

54. Boggs, K. P., Rock, C. O., and Jackowski, S. (1995) Lysophosphatidylcholine attenuates the cytotoxic effects of the antineoplastic phospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine. *J Biol Chem* 270, 11612-11618

55. Vrablic, A. S., Albright, C. D., Craciunescu, C. N., Salganik, R. I., and Zeisel, S. H. (2001) Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-O-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine in p53-defective hepatocytes. *FASEB J* 15, 1739-1744

56. Daniel, L. W. (1993) Ether lipids in experimental cancer therapy. In *Cancer Chemotherapy* (Hickman, J. A., and Tritton, T. R., eds.) pp. 146-178, Blackwell Scientific Publications Ltd.: Oxford, England

57. Arthur, G., and Bittman, R. (1998) The inhibition of cell signaling pathways by antitumor ether lipids. *Biochim Biophys Acta* 1390, 85-102

58. Gwee, M. C. E., and Sim, M. K. (1978) Free choline concentration and cephalin-N-methyltransferase activity in the maternal and foetal liver and placenta of pregnant rats. *Clin Exp Pharmacol Physiol* 5, 649-53

59. Meck, W. H., and Williams, C. L. (1997) Simultaneous temporal processing is sensitive to prenatal choline availability in mature and aged rats. *NeuroReport* 8, 3045-3051

60. Meck, W. H., and Williams, C. L. (1999) Choline supplementation during prenatal development reduces proactive interference in spatial memory. *Brain Res Dev Brain Res* 118, 51-59

61. Holmes-McNary, M. Q., Loy, R., Mar, M-H., Albright, C. D., and Zeisel, S. H. (1997) Apoptosis is induced by choline deficiency in fetal brain and in PC12 cells. *Brain Res Dev Brain Res* 101, 9-16

62. Albright, C. D., Tsai, A. Y., Friedrich, C. B., Mar, M-H., and Zeisel, S. H. (1999) Choline availability alters embryonic development of the hippocampus and septum in the rat. *Brain Res Dev Brain Res* 113, 13-20

63. Copeland, D. H., and Salmon, W. D. (1946) The occurrence of neoplasms in the liver, lungs, and other tissues of rats as a result of prolonged choline deficiency. *Am J Pathol* 22, 1059-67

64. Locker, J., Reddy, T. V., and Lombardi, B. (1986) DNA methylation and hepatocarcinogenesis in rats fed a choline-devoid diet. *Carcinogenesis* 7, 1309-1312

65. Chandar, N., and Lombardi, B. (1988) Liver cell proliferation and incidence of hepatocellular carcinomas in rats fed consecutively a choline-devoid and a choline-supplemented diet. *Carcinogenesis* 9, 259-263

66. Zeisel, S. H., Da Costa, K-A., Franklin, P. D., Alexander, E. A., Lamont, J. T., Sheard, N. F., and Beiser, A. (1991) Choline, an essential nutrient for humans. *FASEB J* 5, 2093-2098

67. Buchman, A. L., Dubin, M., Jenden, D., Moukarzel, A., Roch, M. H., Rice, K., Gornbein, J., Ament, M. E., and Eckhert, C. D. (1992) Lecithin increases plasma free choline and decreases hepatic steatosis in long-term total parenteral nutrition patients. *Gastroenterology* 102, 1363-1370

68. Buchman, A. L., Dubin, M. D., Moukarzel, A. A., Jenden, D. J., Roch, M., Rice, K. M., Gornbein, J., and Ament, M. E. (1995) Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. *Hepatology* 22, 1399-1403

69. da Costa, K-A., Cochary, E. F., Blustajn, J. K., Garner, S. C., and Zeisel, S. H. (1993) Accumulation of 1,2-*sn*-diradylglycerol with increased membrane-associated protein kinase C may be the mechanism for spontaneous hepatocarcinogenesis in choline deficient rats. *J Biol Chem* 268, 2100-2105

70. Institute of Medicine Report, National Academy of Sciences, USA. (1998) *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B_6, Folate, Vitamin B_{12}, Pantothenic Acid, Biotin, and Choline*. pp. 255-274, National Academy Press: Washington, D.C.

71. Pfeiffer, C. C., Jenney, E. H., Gallagher, W., Smith, R. P., Bevan, W., Killam, K. F., Killam, E. K., and Blackmore, W. (1957) Stimulant effect of 2-dimethylaminoethanol - possible precursor of brain acetylcholine. *Science* 126, 610-611

72. Haubrich, D. R., Wang, P. F. L., Clody, D. E., and Wedeking, P. W. (1975) Increase in rat brain acetylcholine induced by choline or deanol. *Life Sci* 17, 975-980

73. Dean, W., and Morgenthaler, J. (1990) Smart drugs and nutrients: How to improve your memory and increase your intelligence using the latest discoveries in neuroscience. pp. 99-101, Health Freedom Publications: Menlo Park, CA

74. Huemer, R. P. (1999) Smart drugs and orthomolecular substances for aging minds. *J Adv Med* 12, 119-131

75. National Toxicology Program (1999) NTP technical report on the toxicology and carcinogenesis studies of diethanolamine (CAS No. 111-42-2) in F344/N rats and B6C3F$_1$ mice (dermal studies). NTP TR 478, NIH Publication No. 99-3968

76. Stott, W. T., Bartels, M. J., Brzak, K. A., Mar, M-H., Markham, D. A., Thornton, C. M., and Zeisel, S. H. (2000) Potential mechanisms of tumorigenic action of diethanolamine in mice. *Toxicol Lett* 114, 67-75

Received October 10, 2001; accepted January 3, 2002.

Fig. 1

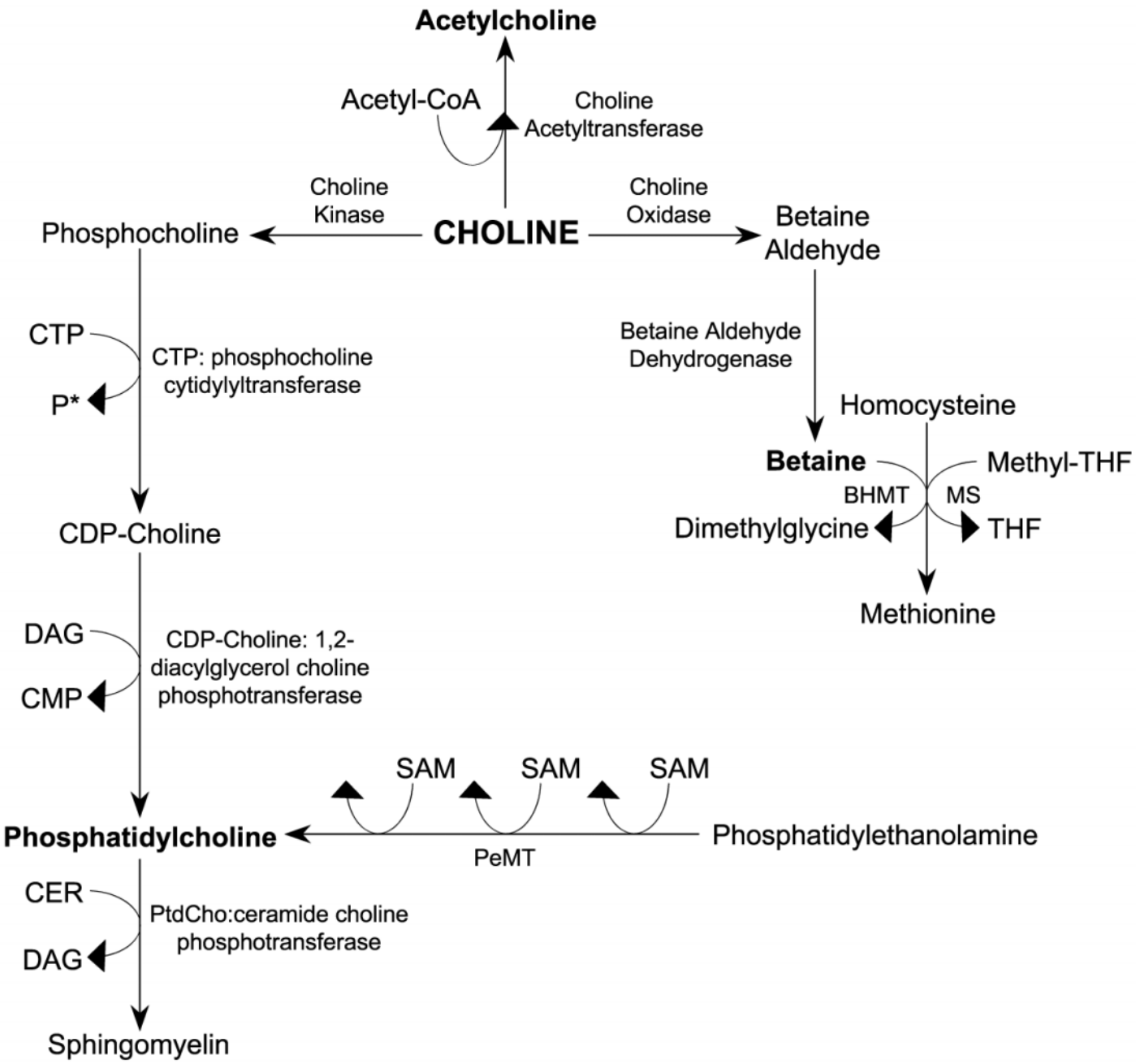

Figure 1. Choline metabolism results in the formation of acetylcholine, betaine, and phosphatidylcholine. BHMT, betaine homocysteine methyltransferase; CDP-Choline, cytidine diphosphocholine; CER, ceramide; CMP, cytidine monophosphate; CTP, cytidine triphosphate; DAG, 1, 2-diacylglycerol; Methyl-THF, methyltetrahydrofolate; MS, methionine synthase; P*, phosphate; PeMT, phosphatidylethanolamine-N-methyltransferase; PtdCho, phosphatidylcholine; SAM, S-adenosyl methionine; THF, tetrahydrofolate.

Fig. 2

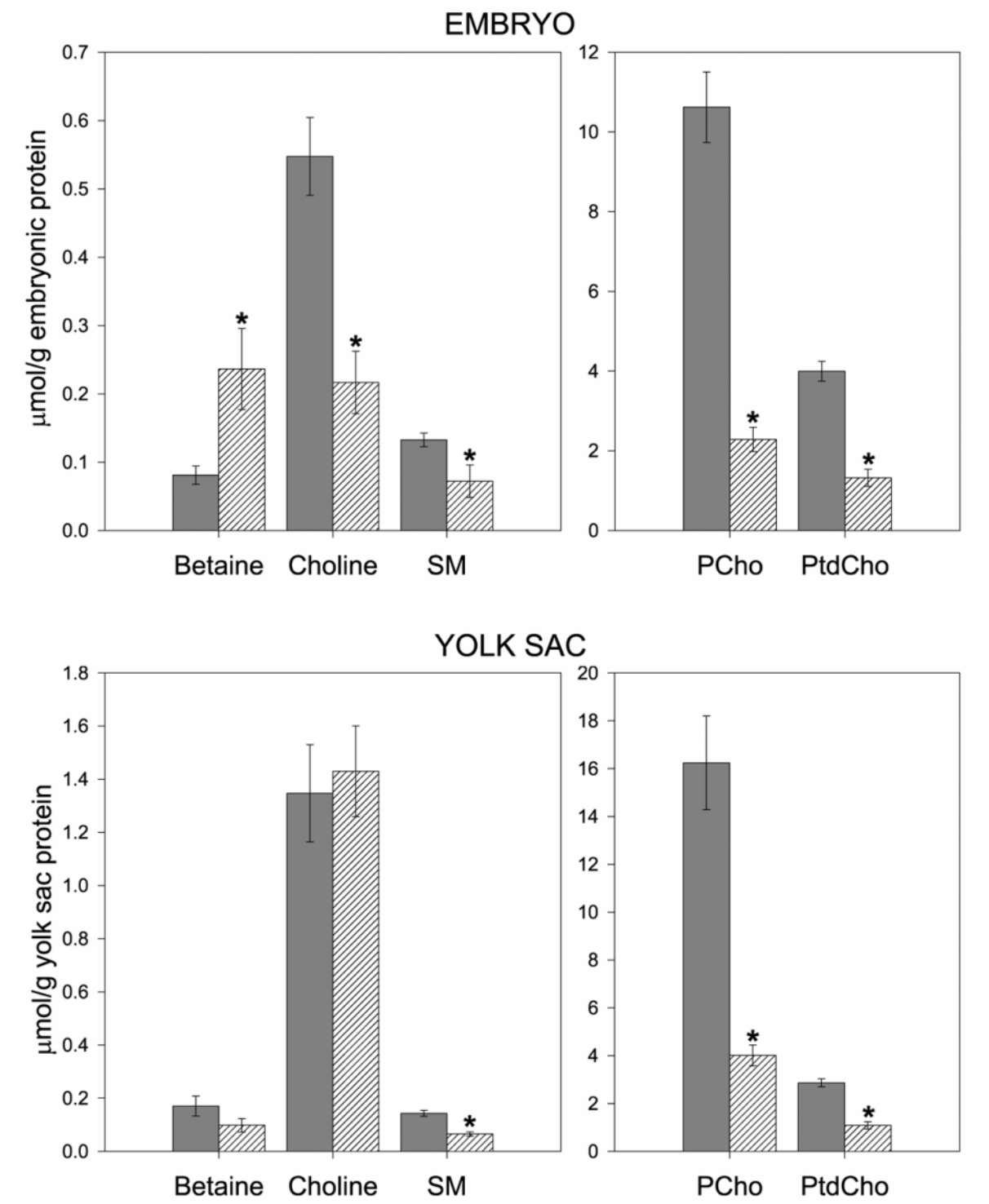

Figure 2. HPLC and TLC were used to evaluate the metabolism of ^{14}C-choline by GD 9 mouse embryos and yolk sacs exposed to control (gray bars) or 375 µM DMAE treated (diagonally striped bars) medium for 10 h in embryo culture. In control embryos and yolk sacs, choline was incorporated into phosphocholine (PCho), phosphatidylcholine (PtdCho), sphingomyelin (SM), and betaine. DMAE inhibited choline transport and the metabolism of choline to PCho, PtdCho and SM. Betaine levels increased in DMAE treated embryos. N = 8–9 (control) or 6 (DMAE) per point (12 embryos/yolk sacs per N); ± SEM; *$P < 0.05$ compared with control group (ANOVA).

Fig. 3

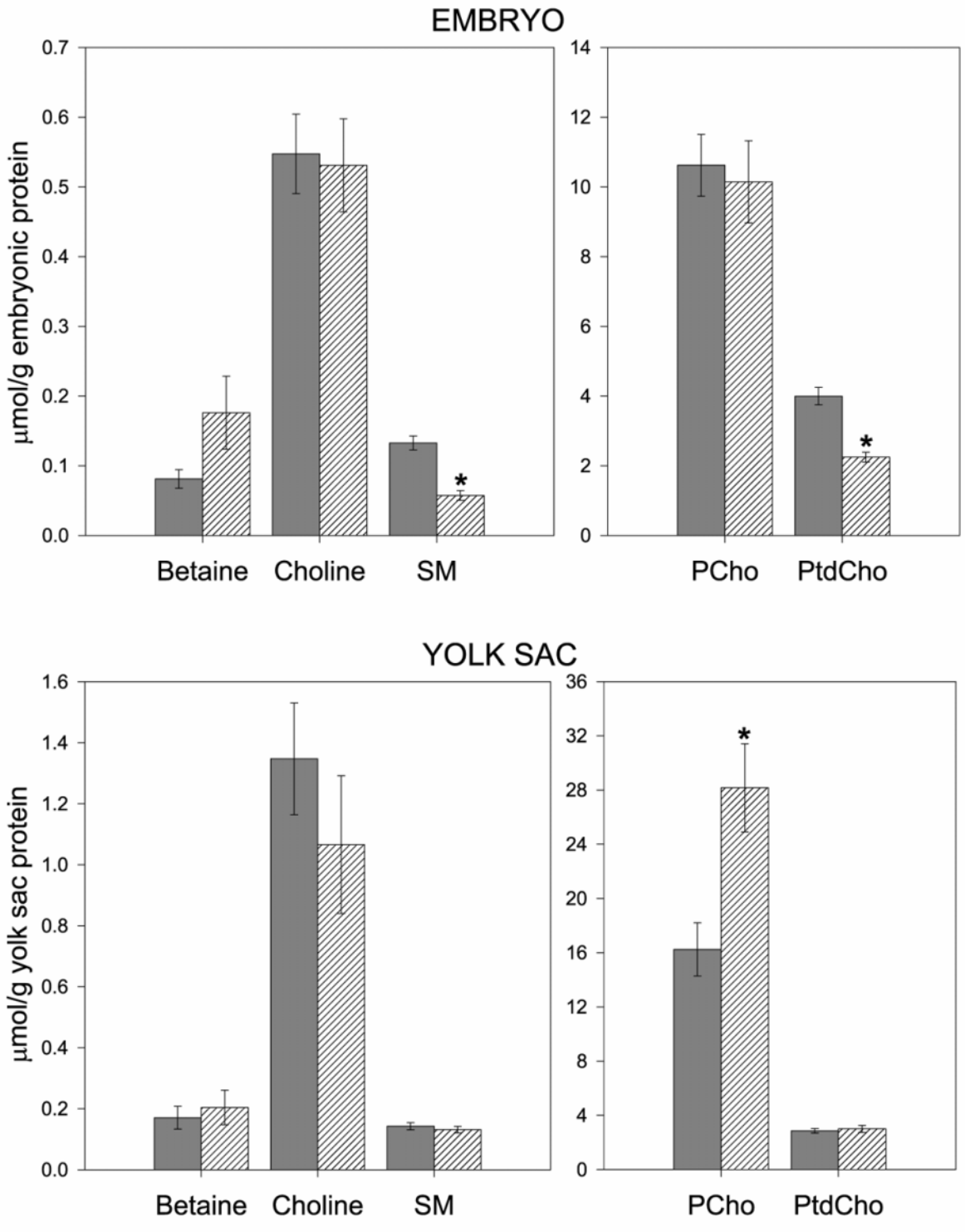

Figure 3. The effects of ET-18-OCH_3 on embryonic and yolk sac ^{14}C-choline metabolism after 10 h of whole embryo culture. Compared with controls (gray bars), treatment with 275 µM ET-18-OCH_3 (diagonally striped bars) resulted in reduced embryonic phosphatidylcholine (PtdCho) and sphingomyelin (SM). Phosphocholine (PCho) was elevated in ET-18-OCH_3 treated yolk sacs. N = 8–9 (control) or 6 (ET-18-OCH_3) per point (12 embryos/yolk sacs per N); ± SEM; *$P < 0.05$ compared with control group (ANOVA).

Fig. 4

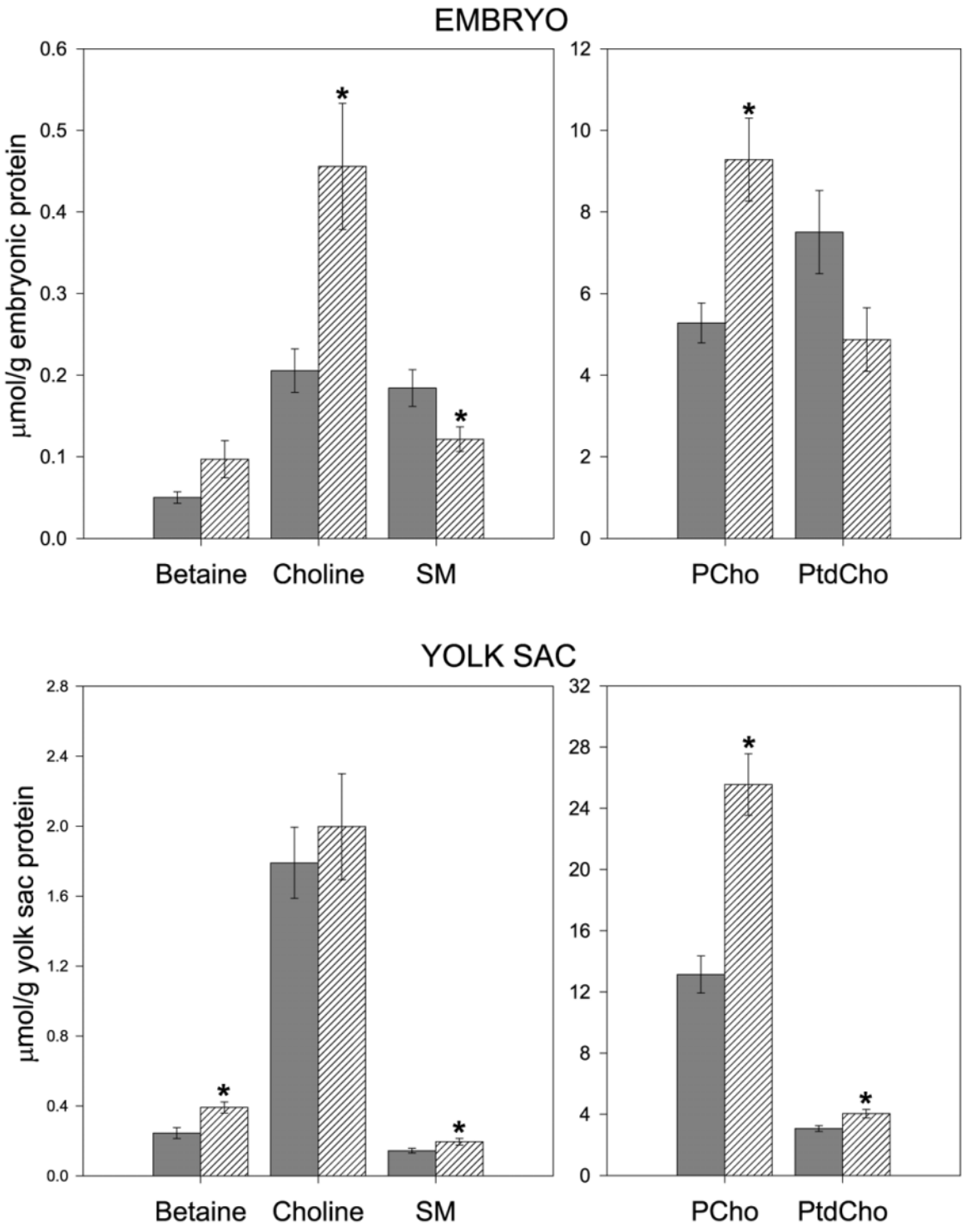

Figure 4. ^{14}C-Choline metabolism in control (gray bars) or 275 µM ET-18-OCH$_3$ (diagonally striped bars) treated embryos and yolk sacs after 24 hours of embryo culture. In control embryos and yolk sacs, labeled choline was incorporated into phosphocholine (PCho), phosphatidylcholine (PtdCho), sphingomyelin (SM), and betaine. Treatment with ET-18-OCH$_3$ resulted in increased embryonic and yolk sac PCho and reduced embryonic PtdCho and SM, (P=0.0675, $P < 0.05$, respectively). N = 5–6 (control) or 6 (ET-18-OCH$_3$) per point (6 embryos/yolk sacs per N); ± SEM; *$P < 0.05$ compared with control group (two-tailed t-test).

Fig. 5

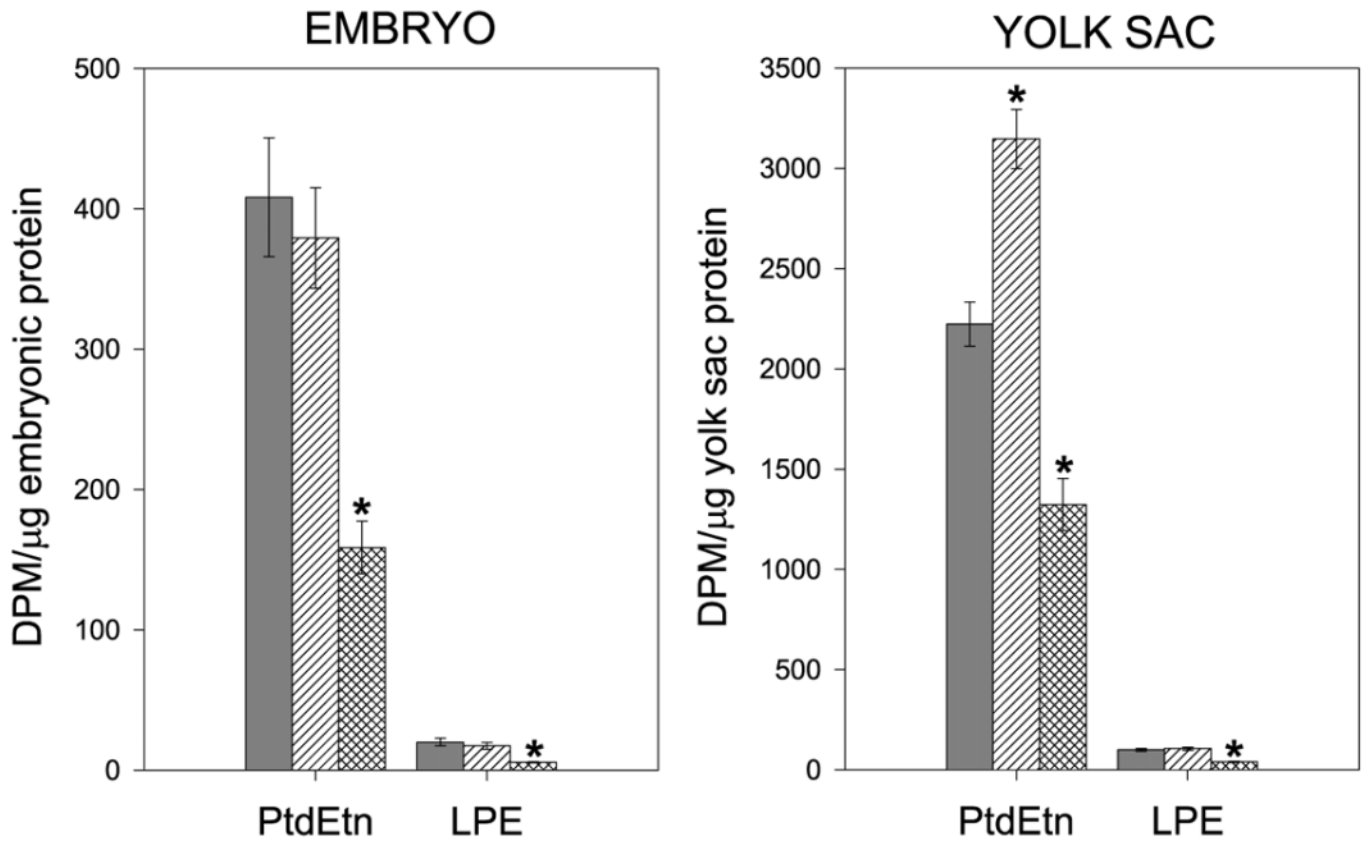

Figure 5. HPLC and TLC were used to study the metabolism of ^{3}H-ethanolamine by control and inhibitor treated embryos and yolk sacs incubated for 10 h in culture. In controls (gray bars), labeled ethanolamine was incorporated into phosphatidylethanolamine (PtdEtn) and lysophosphatidylethanolamine (LPE), but not phosphatidylcholine. Treatment with 375 µM DMAE (cross-hatched bars) resulted in reduced levels of PtdEtn and LPE. PtdEtn synthesis was elevated in 275 µM ET-18-OCH_3 (diagonally striped bars) treated yolk sacs. N = 6 (control; ET-18-OCH_3) or 4 (DMAE) per point (12 embryos/yolk sacs per N); ± SEM; * $P < 0.05$ compared with control group (ANOVA).

Fig. 6

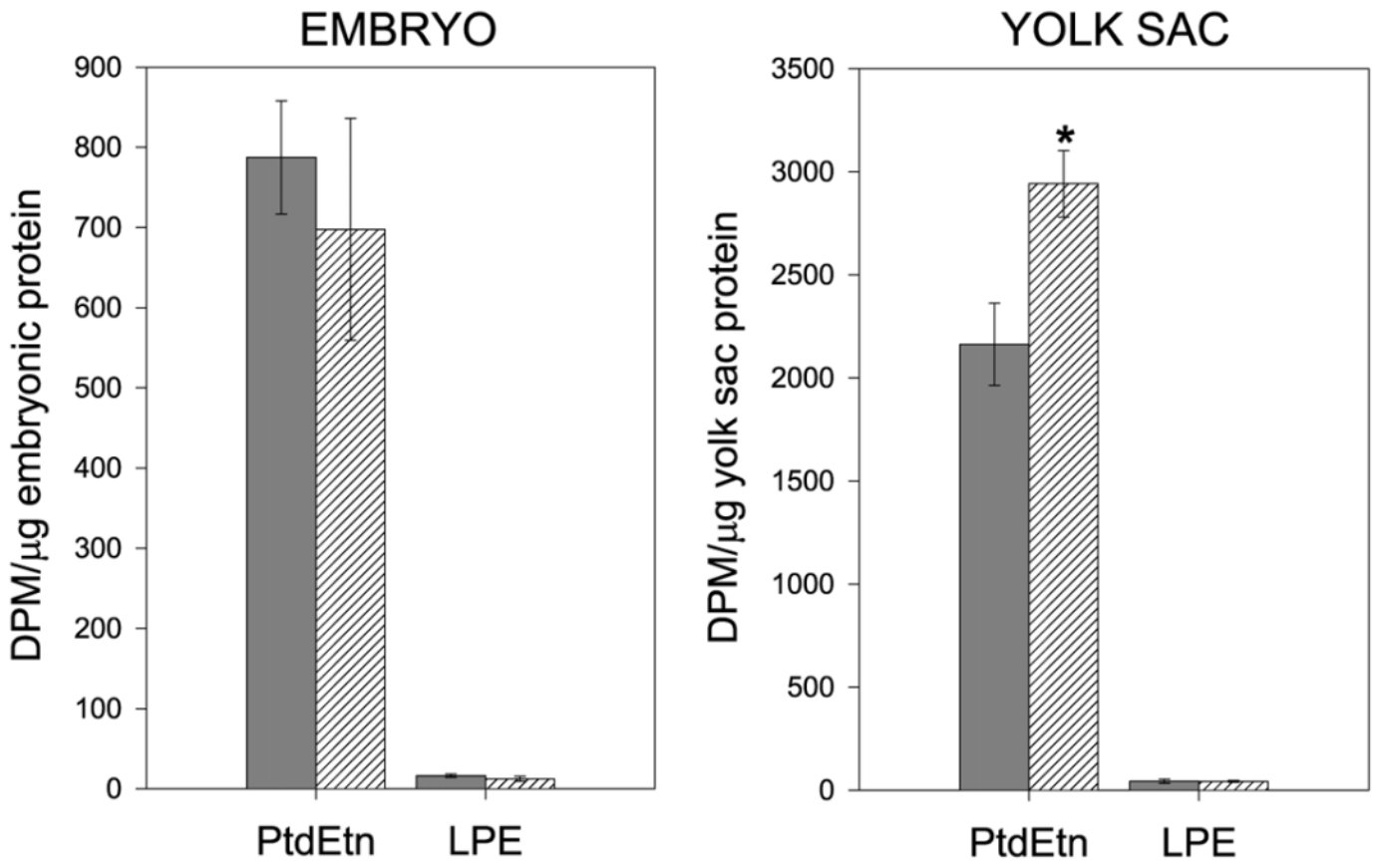

Figure 6. The metabolism of ^{3}H-ethanolamine by embryos and yolk sacs exposed to control or 275 μ M ET-18-OCH_3 treated medium for 24 h. Compared with controls (gray bars), treatment with ET-18-OCH_3 (diagonally striped bars) resulted in elevated yolk sac phosphatidylethanolamine (PtdEtn). LPE, lysophosphatidylethanolamine; N = 4 per point (6 embryos/yolk sacs per N); ± SEM; * $P < 0.05$ compared with control group (two-tailed *t*-test).

Fig. 7

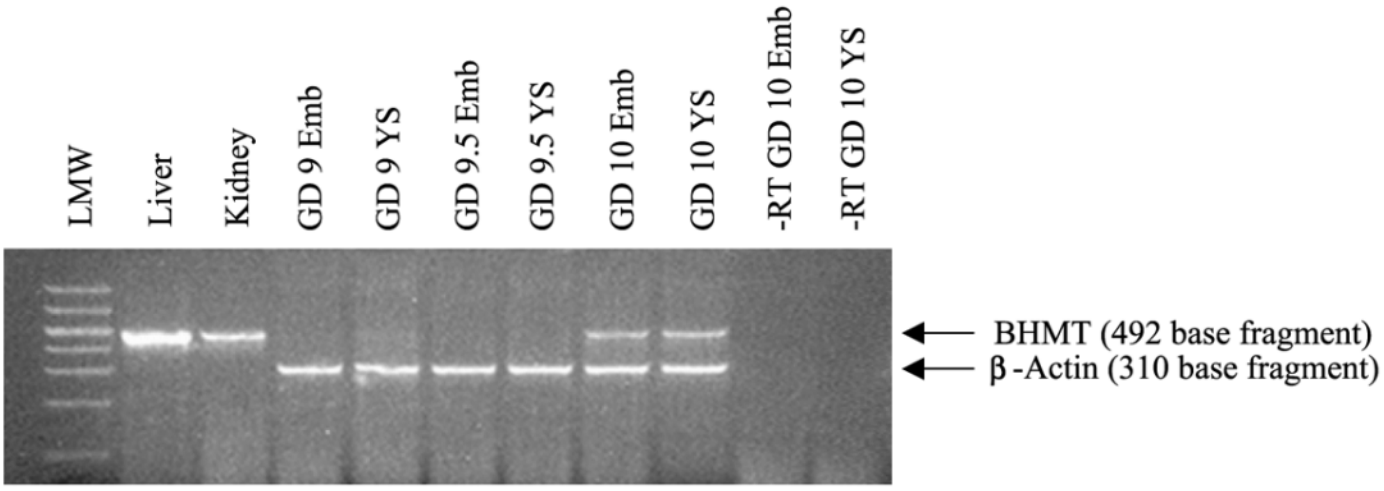

Figure 7. The expression of betaine homocysteine methyltransferase (BHMT) in GD 9, 9.5, and 10 embryos and yolk sacs. BHMT mRNA was expressed in GD 10 embryos (Emb) and yolk sacs (YS). Adult liver and kidney were positive controls for BHMT. β-actin was a positive mRNA control and negative control samples were processed without reverse transcriptase (-RT). LMW, low-molecular-weight marker.

Fig. 8

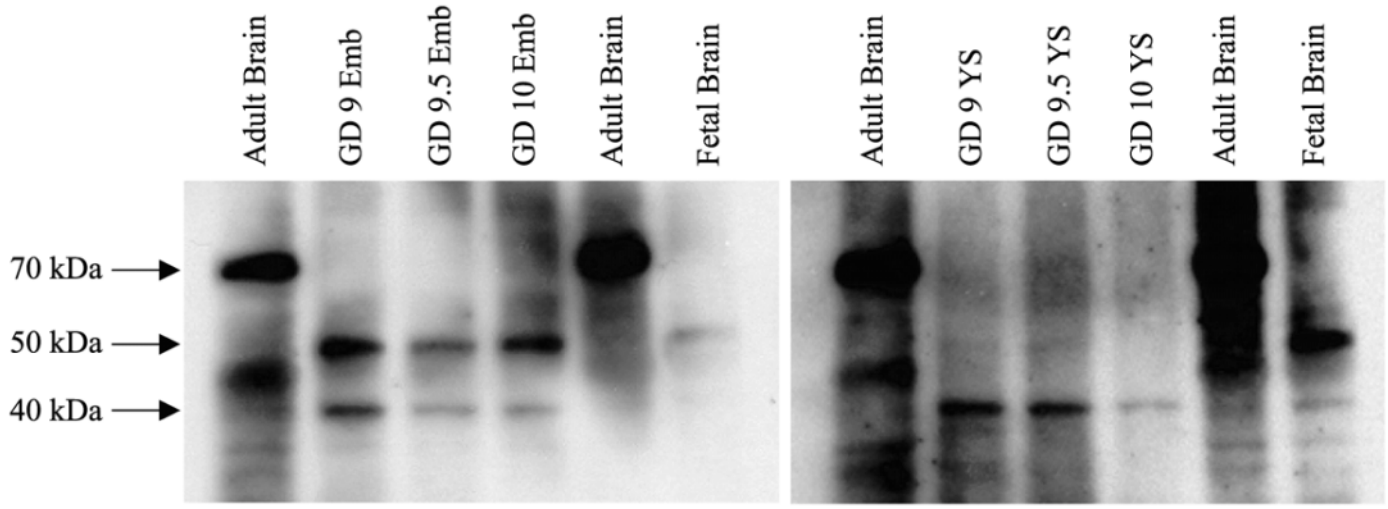

Figure 8. Immunoblot of proteins from GD 9, 9.5, and 10 embryos and yolk sacs with an antibody to choline acetyltransferase (ChAT). Adult and fetal (GD 15) brain were positive controls for ChAT protein. ChAT (70 kDa) was found in adult brain, but not in the other tissues studied. Instead, alternatively spliced forms of ChAT may be present in fetal brain and GD 9-10 embryos (Emb) and yolk sacs (YS).

Fig. 9

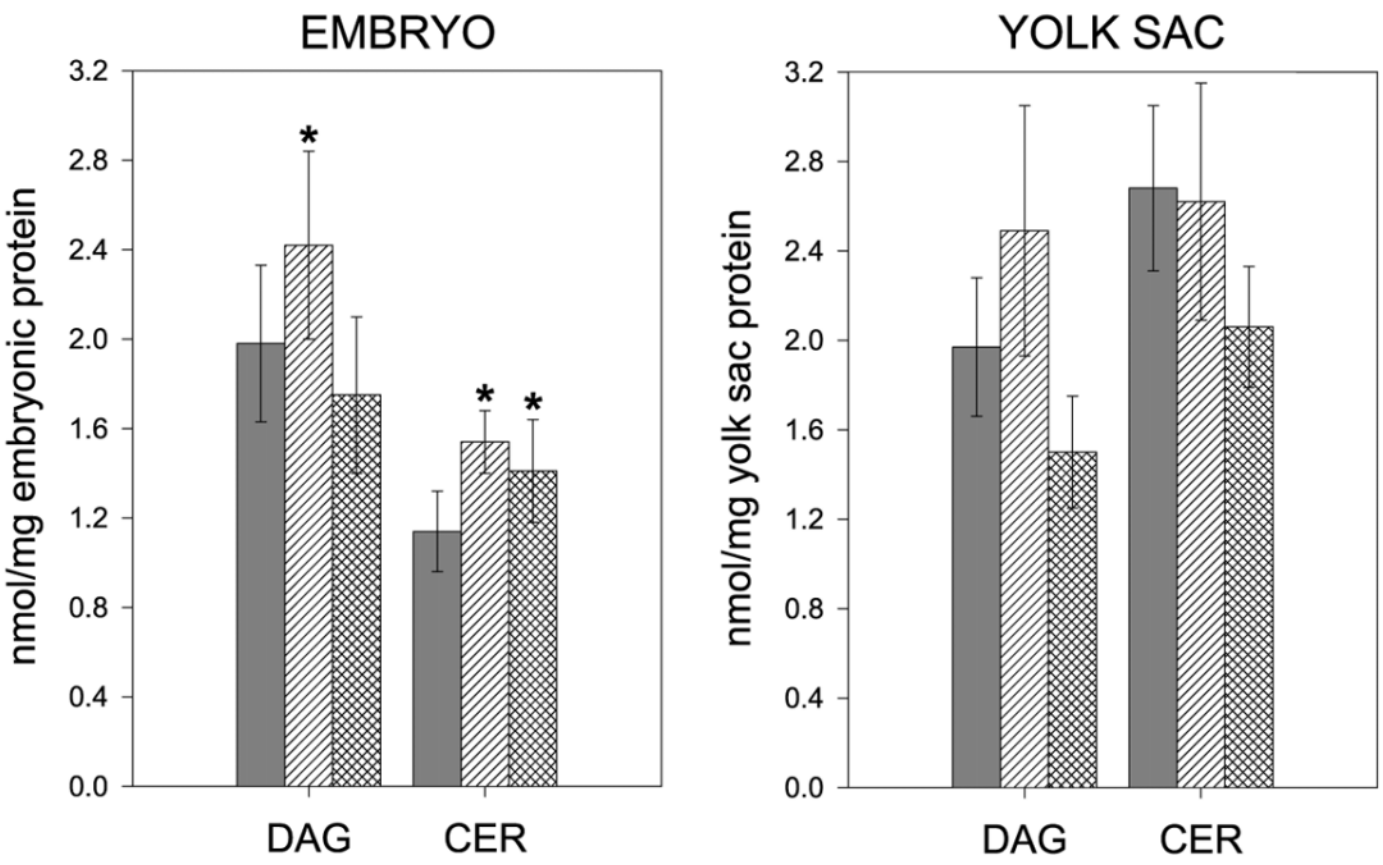

Figure 9. Diacylglycerol and ceramide levels in control (gray bars) and inhibitor treated embryos and yolk sacs after 24 h of culture. An increase in diacylglycerol (DAG) was observed in 275 μM ET-18-OCH_3 (diagonally striped bars) treated embryos. Ceramide (CER) was elevated in embryos treated with either 375 μM DMAE (cross-hatched bars) or ET-18-OCH_3. N = 4 per point (12 embryos/yolk sacs per N); ± SEM; *$P < 0.05$ compared with control group (Repeated Measures ANOVA).