Placental Thrombosis and Spontaneous Fetal Death in Mice Deficient in Ethanolamine Kinase 2

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Ethanolamine kinase catalyzes the first step in the CDP-ethanolamine pathway for the formation of the major membrane phospholipid phosphatidylethanolamine (PtdEtn). In this work, the predicted Etnk2 cDNA was established as a soluble protein with ethanolamine-specific kinase activity that was most highly expressed in liver. Mice with an inactivated Etnk2 gene were derived, and its absence reduced the rate of PtdEtn synthesis from exogenous ethanolamine in hepatocytes. PtdEtn is a major precursor to phosphatidylcholine in liver; however, Etnk2−/− mice did not have reduced amounts of either PtdEtn or phosphatidylcholine or an altered phospholipid molecular species distribution. The knock-out animals were able to adapt to a choline-deficient diet. The Etnk2−/− mice exhibited a maternal-specific intrauterine growth retardation phenotype that resulted in a 33% reduction in litter size and frequent perinatal death. Histological analysis of pregnant Etnk2−/− females showed that fetal development failed at the late stage of pregnancy in a significant percentage of embryos because of the appearance of extensive placental thrombosis. These results illustrate a non-redundant role for EtnK2 expression in regulating placental hemostasis.

There are two pathways for the synthesis of phosphatidylethanolamine (PtdEtn), a major mammalian membrane phospholipid. The CDP-ethanolamine (Etn) pathway begins with ethanolamine kinase (EtnK; ATP:ethanolamine phosphotransferase, EC 2.7.1.82) followed by CTP:phosphoethanolamine cytidylyltransferase and terminates with a diacylglycerol phosphotransferase (1). The second route to PtdEtn is via the decarboxylation of phosphatidylserine (1, 2). Although the CDP-Etn pathway is considered a major route for PtdEtn in cultured cells (4–7), the phosphatidylserine decarboxylation pathway is the predominant route for PtdEtn in cultured cells (4–7). The focus of our work is the first step in the CDP-Etn pathway, EtnK. There are four related mammalian genes that encode proteins possessing EtnK activity (see Fig. 1). Two of these, choline kinase (ChoK) α and ChoKβ (see Fig. 1), are characterized as ChoKs, but also are able to phosphorylate Etn, a choline (Cho) analog, to a lesser extent (8). Based on this property, both the ChoK and EtnK reactions were considered for some time to be carried out by the same enzyme(s). However, in Saccharomyces cerevisiae, there are two kinases with opposite specificities. The yeast ChoK has a specific activity 3.6-fold higher for Cho compared with Etn, whereas EtnK has a specificity constant 2-fold higher for Etn compared with Cho (9). The discovery of the Drosophila EtnK encoded by the eae gene established the existence of Etn-specific kinases with negligible ChoK activity in higher eukaryotes (10). A defect in the Drosophila EtnK results in a bang-sensitive paralytic phenotype, indicating the potential importance of EtnK to neuronal function (10), and EtnK is also implicated in fly development (11). Mice and humans have two related EtnK genes (see Fig. 1). Human ETNK1 has robust EtnK activity, but ChoK activity is not detected (12). Expression of ETNK1 in cell culture selectively increases Etn uptake and phosphorylation and PtdEtn synthesis (12). A second gene (ETNK2) is identified as a cDNA related to ETNK1 (12). Although bioinformatics analysis suggests that ETNK2 encodes EtnK-specific kinase activity, there are no biochemical data to support this conclusion. ETNK2 is the most highly expressed of the two isoforms and is found primarily in the liver and reproductive tissues of humans (12). A role for EtnK2 in testicular maturation is suggested by the pattern of Etnk2 expression in developing mouse testis (13). Thus, EtnKs can be distinguished from ChoKs, and a number of possible roles from the literature are suggested for mammalian EtnKs.

This investigation establishes mouse EtnK2 as a specific EtnK that is expressed most highly in liver and reproductive tissues. Characterization of phospholipid synthesis in the absence of EtnK2 expression in mice pointed out its dispensability or redundancy in most physiologic processes and cell types. Homozygous disruption of the Etnk2 gene had little effect on liver phospholipid metabolism, neural development, or testicular function. However, a non-redundant role for EtnK2 was indicated by a maternal-specific failure to support late embryonic development, resulting in reduced perinatal size and survival and suggesting compromised placental function. This phenotype was similar to that of the β2-glycoprotein I null mouse, which lacks the principal target antigen for anti-phospholipid antibodies associated with recurrent pregnancy loss.
and fetal growth restriction in women (14). It is thought that the $\beta_2$-glycoprotein I protein complexes with anionic phospholipids and that these complexes can become antigenic and underlie a novel class of autoimmune disorders called antiphospholipid syndrome. Anti-PtdEtn antibodies in particular are prevalent in antiphospholipid syndrome patients with mid- to late pregnancy loss (15). The Etnk2$^{-/-}$ phenotype also resembles the Rho-associated kinase II (ROCK-II) null mouse, which lacks expression of the Rho-associated kinase II isoform, and both male and female embryos exhibit extensive thrombus formation in the placenta, leading to intrauterine growth retardation and low birth weight pups (16). ROCK-II is a serine/threonine kinase that phosphorylates cytoskeletal proteins upon binding to the active form of the Rho GTPase (17). RhoA cycling from the active GTP form to the inactive GDP form can be regulated by the lateral movement of PtdEtn at the cell surface during late stage cytokinesis (18, 19). These new data in this work characterize mammalian EtnK2 for the first time and suggest a link between maternally derived PtdEtn and embryonic signaling across the placental interface.

**EXPERIMENTAL PROCEDURES**

**Cloning of the Mouse Etnk2 cDNA and Construction of the Expression Vector**—The expressed sequence tag data base was searched using the human ENTK1 sequence (12). Two mouse expressed sequence-tagged clones (AI098609 and AA261067) that encoded amino acid 25 through the termination stop codon were identified. The 554-bp EcoRI-NsiI fragment of AI098609 was ligated to the 4352-bp EcoRI-NsiI sequence of AA261067 in the pT7T3D-Pac vector, and these fragments together made up the 3′-end of the cDNA. The 5′-end of the cDNA was amplified from a mouse kidney MarathonReady cDNA kit (Clontech) using forward primer 5′-ACCACTCCACCTCTCTCTCAGG and reverse primer 5′-CATACTACCCGGGACCAAACACG and cloned into the pCR2.1 vector. The 5′-end was sequenced and then subcloned into pcDNA3.1 after XbaI-BamHI digestion. The 3′-end was ligated with the 5′-end in the expression vector at the AfelHindIII sites, resulting in plasmid pPJ184, which carried the 5′-end of the cDNA sequence, the genomic sequence, and the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (ΔC$T$).

**Quantitative Real-time PCR**—The abundance of Etnk2 mRNA was measured by real-time PCR as described previously (20). The oligonucleotide primers and probes were synthesized at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital. The primers and probes were used to assess expression of Etnk2 mRNA (see Table 1) and the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (control reagent kit, Applied Biosystems). Amplification and detection of the real-time PCR products were performed with TaqMan Universal PCR Master Mixes (Applied Biosystems) and an ABI PRISM 7700 sequence system (Applied Biosystems). Experiments were performed with three replicates for each cDNA, and negative reverse transcriptase and no-template controls were run for each reaction on the same 96-well plate. All of the real-time values were compared using the C$T$ method, in which the amount of target mRNA was normalized to the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (ΔC$T$).

**Cell Culture**—Human kidney 293T cells (a gift from Suzanne Baker, Department of Developmental Neurobiology, St. Jude Children’s Research Hospital) were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc.), 2 mm glutamine, and 10 mM HEPES (pH 7.0) in a 5% CO$_2$ humidified atmosphere at 37 °C. Transient transfection of pPJ184 expressing Etnk2 protein, pAL10 expressing human ENTK1, or the pcDNA3.1 empty vector control was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Cells were scraped from the dishes 48 h after transfection after washing with phosphate-buffered saline (PBS) and centrifuged, and the supernatant was removed. Cell pellets were stored frozen at −80 °C.

**Construction of the Mouse Etnk2 Replacement Vector**—In general, the Neo resistance gene cassette was inserted into exon 3 of the gene to replace 66 bp. A negative selection cassette encoding the diphtheria toxin antigen was added at the 3′-end of the mouse genomic DNA insert. Using the mouse Etnk2 cDNA sequence, the genomic Etnk2 sequence (AC034108) was identified in the NCBI mouse genomic data base. The short and long arms of the construct were obtained by PCR using the Advantage® genomic PCR kit (Clontech) following the manufacturer’s instructions. Mouse W9.5 embryonic stem (ES) cell genomic DNA was the template; oligonucleotide primers 5′-TGTCGCCGGCACCAAGTCACCCACATAAGTGC (including a SacII site) and 5′-GCATCTAGATGATGAGATCAGTCCCAGGTATA (with a SalI site) yielded a 1889-bp SacII-XbaI short arm fragment and the 5739-bp ClaI-SalI long arm fragment. The plasmid pMAK6 (pBluescript SK+) containing the diphtheria toxin antigen cassette; a gift from Dr. James Ihle) was inserted into the ClaI and XbaI sites, followed by the 1889-bp SacII-XbaI short arm fragment and the 5739-bp ClaI-SalI long arm fragment. The final plasmid construct was linearized with SacI prior to transfection into mouse AB2.2 ES cells (Lexicon Genetics). The sequences of the long and short arms were confirmed for 650 bases at each end. The plasmid pMAK6 (pBluescript SK+ containing the diphtheria toxin antigen cassette; a gift from Dr. James Ihle, Department of Biochemistry, St. Jude Children’s Research Hospital) was used as the base vector. A 1.2-kb Neo cassette (a gift from Dr. James Ihle) was inserted into the ClaI and XbaI sites, followed by the 1889-bp SacII-XbaI short arm fragment and the 5739-bp ClaI-SalI long arm fragment. The final plasmid construct was linearized with SacI prior to transfection into mouse AB2.2 ES cells (Lexicon Genetics).
Etnk2 Knock-out Mice

More than 20 clones resistant to G418 were selected; genomic DNA was isolated and screened by PCR using primers FP1, RP1, and RN1 (see Table 1). ES cells with one chromosome that had undergone homologous recombination with the replacement vector were identified by a 3366-bp PCR product compared with a 2261-bp product from the wild-type allele obtained using primers FP1 and RP1. Insertion of the Neo cassette was confirmed by a 2479-bp product obtained with primers FP1 and RN1.

Derivation of Etnk2-disrupted Mice—An ES cell colony containing the recombined Etnk2-disrupted locus was injected into C57BL/6J blastocysts, which were then implanted into pseudo-pregnant female mice at the Transgenic Core Facility of St. Jude Children’s Research Hospital. Male offspring with 75–90% agouti color (the coat color contributed by the ES cells) were bred with C57BL/6J females. Pups that were 100% agouti, indicating germ line transmission, were screened. Tail clips from agouti color (the coat color contributed by the ES cells) were collected at 2 days of age and screened for Etnk2 expression by real-time PCR. The DNA was extracted from the tail clips and analyzed by agarose gel electrophoresis. More than 200 clones resistant to G418 were selected; genomic DNA was isolated and screened by PCR using primers FP1, RP1, and RN1 (see Table 1). ES cells with one chromosome that had undergone homologous recombination with the replacement vector were identified by a 3366-bp PCR product compared with a 2261-bp product from the wild-type allele obtained using primers FP1 and RP1. Insertion of the Neo cassette was confirmed by a 2479-bp product obtained with primers FP1 and RN1.

Tissue Histology—Mouse tissues were analyzed using both paraffin sections and frozen sections. Mice were anesthetized by 1,2-14C-choline chloride (specific activity of 50 mCi/mmol, 0.1 mCi/ml exchanged into water; American Radiolabeled Chemicals, Inc., St. Louis, MO) or 40 μM [1,2-14C]choline chloride (specific activity of 50 mCi/mmol, 0.1 mCi/ml exchanged into water; American Radiolabeled Chemicals, Inc.) in a final volume of 50 μl. The sections were stored at −20 °C until used. For paraffin sections, mouse tissues were fixed in 10% formalin and processed by dehydration in 70% ethanol followed by xylene. Tissues were infiltrated and embedded in paraffin, cut into 4-μm sections, mounted on microscope slides, and stored at room temperature until used. Sections were stained with hematoxylin and eosin and viewed under an Olympus BX41 microscope.

Six-micrometer-thick cryosections were used for programmed cell death analysis. Sections were stained based on reactivity determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) using the ApopTag peroxidase in situ apoptosis detection kit (catalog no. S7100, Chemicon) according to the manufacturer’s instructions. In addition, active caspase-3 activity was detected using an antibody that specifically interacts with the cleaved activated fragment of caspase-3. Immunohistochemistry was performed after permeabilization of the cells with 0.2% Triton and blocking in 2.5% horse serum in PBS. Sections were incubated overnight with a 1:200 dilution of a polyclonal antibody against cleaved caspase-3 (Abcam) at 4 °C. The next day, sections were washed with PBS and incubated at room temperature with an Alexa 488-conjugated secondary antibody (Molecular Probes) for 1 h. After washing and mounting, the sections were viewed under an Olympus BX41 fluorescence microscope.

Hormone Determinations—Serum samples were stored at −20 °C until analysis. Coat-A-Count radioimmunoassays (Diagnostic Products Corp.) were performed to quantitatively measure the amounts of progesterone and estradiol in serum samples according to the manufacturer’s instructions. Briefly, 100 μl of duplicate serum samples or known concentrations of hormone standards were added to tubes coated with antibody. 125I-Labeled progesterone or estradiol was then added to each tube and vortexed. Samples were incubated at room temperature for 3 h, decanted, and air-dried. Tubes were counted for 1 min in a Beckman γ-counter.

EtnK and ChoK Assays—Frozen 293T cell pellets transfected with plasmid pPJ184 or pPJ96 (12) or with a control empty vector were thawed on ice and incubated for 1 h in lysis buffer (20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 2 μg/ml apro tinin) on ice. The cells were disrupted by sonication (6 × 30 s) in a cup horn (Microsonic Sonicator 3000), and the particulate fraction was removed by centrifugation at 5000 × g for 5 min at 4 °C. Supernatants were transferred to new tubes, and EtnK or ChoK activities were determined essentially as described previously (12, 21). A flash-frozen tissue slice was thawed on ice, and the wet weight was determined. Two volumes of lysis buffer were added to ~50 mg, and the tissue was broken in a glass homogenizer by 20 strokes and set on ice for 30 min, followed by 15 more strokes. The lysate was centrifuged at 5000 × g for 10 min at 4 °C, and the supernatant was transferred to a new tube prior to assay. The standard assays contained 100 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 10 mM ATP (pH 7.0), and 20 μM [1,2-14C]ethanolamine hydrochloride (specific activity of 110 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO) or 40 μM [1,2-14C]cholesterol chloride (specific activity of 50 mCi/mmol, 0.1 mCi/ml exchanged into water; American Radiolabeled Chemicals, Inc.) in a final volume of 50 μl.
reaction mixtures were incubated at 37 °C for 15 min when using transfected cells or for 30 min when using the mouse tissue lysates. The reaction was stopped by the addition of 10 μl of 0.5 M Na2EDTA, and the tubes were vortexed and placed on ice. Next, 40 μl of each sample were spotted on pre-adsorbent Silica Gel G thin-layer plates, which were developed with 2% ammonium hydroxide and 95% ethanol (5:1, v/v). Phosphoethanolamine and phosphocholine were identified by co-migration with standards, and product formation was quantitated using a Bioscan imaging system. Protein was determined according to the Bradford method (22).

Phospholipid Determinations—Flash-frozen mouse tissues were thawed and weighed, and ~50 μg were extracted by the method of Bligh and Dyer (23). The organic phase containing lipid was concentrated under nitrogen and resuspended in 400 μl of chloroform/methanol (2:1). A 1-μl aliquot was loaded onto a thin-layer silica gel rod, developed first in ether, dried, and then developed in chloroform/methanol/acetic acid/water (50:25:8:3). Lipid mass was detected by flame ionization using an Iatroscan instrument (Iatron Laboratories, Inc., Tokyo, Japan) with PeakSimple software (SRI Instruments), and peaks were identified by co-migration with authentic standards. PtdEtn, phosphatidylcholine (PtdCho), cholesteral, or triglyceride mass was calculated by consulting standard curves for detector response prepared with dioleoyl-PtdEtn, egg PtdCho, PtdEtn, phosphatidylcholine (PtdCho), cholesterol, or triglyceride mass was calculated by consulting standard curves for detector response prepared with dioleoyl-PtdEtn, egg PtdCho, cholesterol, or triglyceride.

Phospholipid Molecular Species Profiling by Mass Spectrometry—Mass spectrometry analysis was performed in the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital using a Finnigan™ TSQ® Quantum triple quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA) equipped with a nanospray ion source. Samples were introduced in 50:50 (v/v) chloroform/methanol and 1% formic acid via static nanoelectrospray using EconoTips™ (New Objective, Inc., Woburn, MA). The instrument was operated in the positive ion mode using parent ion scanning for PtdCho and neutral loss scanning for PtdEtn corresponding to the loss of their respective phosphoryl head groups. Ion source parameters were as follows: spray voltage, 1000 V; capillary temperature, 270 °C; and capillary offset, 35 V. Tube lens offset was set by infusion of the polytyrosine tuning solution (Thermo Electron Corp.) in the electrospray mode. Acquisition parameters for PtdCho were as follows: scan range, m/z 600–900; scan time, 0.3 s; product mass, m/z 184.1; collision energy, 40 V; peak width Q1 and Q3, 0.7 full-width half-maximum; and Q2 collision-induced dissociation gas, 0.5 millitorr. Acquisition parameters for PtdEtn were as follows: scan range, m/z 600–900; scan time, 0.3 s, neutral loss mass, m/z 141.0; collision energy, 30 V; peak width Q1 and Q3, 0.7 full-width half-maximum; and Q2 collision-induced dissociation gas, 0.5 millitorr. Instrument control and data acquisition were performed with Finnigan™ Xcalibur™ software (Version 1.4 SR1, Thermo Electron Corp.).

Preparation and Radiolabeling of Hepatocytes—Whole livers of 2–4-month-old mice were harvested on ice-cold buffer A (each liter contained 3.9 g of NaCl, 0.5 g of KCl, 24 g of HEPES, and 2.7 g of glucose, with the pH adjusted to 7.6 with NaOH). Each liver was chopped with a Vibratome tissue chopper at a setting of 0.5 mm and resuspended in 20 ml of erythrocyte buffer (15 ml of buffer A plus 5 ml of erythrocyte lysis buffer (Qiagen Inc.)) in a 125-ml Erlenmeyer flask, gently mixed in the buffer, and allowed to settle. The supernatant was carefully poured off and discarded, and the procedure with the erythrocyte buffer was repeated twice. The tissue pieces were then resuspended in 30 ml of digestion buffer (buffer A plus 0.7 g/liter CaCl2, 0.5 mg/ml type I collagenase, and 6 μg/ml deoxyribonuclease) and incubated in a water bath shaker at 120 rpm for 20 min at 37 °C. The tissue pieces were allowed to settle on ice, and the supernatant was transferred to a centrifuge tube. The tissue pieces were resuspended in another 30 ml of digestion buffer; the procedures were repeated twice; and the three supernatants were combined. The cell suspension from the supernatants was filtered through Spectra/Mesh nylon (41 μm; Fisher), and liver cells in the filtrate were centrifuged down at 100 × g for 2 min at room temperature. The cell pellet was resuspended in wash buffer (each liter contained 8 g of NaCl, 0.35 g of KCl, 0.16 g of MgSO4, 0.18 g of CaCl2, 2.4 g of HEPES, and 15 g of bovine serum albumin, with the pH adjusted to 7.4 with NaOH) containing 10% (v/v) erythrocyte lysis buffer and centrifuged down three times.

Mouse liver cells were resuspended in PBS containing 0.5% bovine serum albumin to a density of ~1 × 10⁷ cells/ml. For each labeling experiment, 75 μl of the cell suspension were used. The cells were labeled with 2 μM [3H]Etn or [3H]serine (50 Ci/mmol). After a 6-h incubation at 37 °C, cells were harvested and washed twice with PBS. The cells were lysed by sonication in 50 μl of buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 5 mM EDTA, and 50 mM NaF. Cell debris was removed by centrifugation, and aliquots were removed for determination of protein and total [3H]pantothenate incorporated into the cell. The identity of the intracellular metabolites was determined by fractionating the cell lysates by thin-layer chromatography on Silica Gel G plates developed with 2% ammonium hydroxide and 95% ethanol (5:1) using the standards described previously (24). Sections (0.5 cm) were scraped from the plate, and the radioactivity was quantitated in 3 ml of scintillation fluid using a liquid scintillation counter.

RESULTS

Sequence and Distribution of the Murine Etnk2 cDNA—In our previous work with ETNK1, a second human gene called ETK2 was detected by a bioinformatic analysis (12). The mouse Etnk2 gene is located on chromosome 1 and consists of eight exons that span 16.7 kb. The full-length cDNA was assembled as described under “Experimental Procedures,” and the complete 5’-end is contained in expressed sequence tag XM_903670 (reference sequence NT_039192). There is an in-frame stop codon upstream from the predicted ATG initiation codon. The version of ETK2 used throughout this study was isoform 3 (XM_903670) in the NCBI Database. It is important to note that the mouse Etnk2 reference sequence (isoform 1, NP_780652.1) in the NCBI Database does not correspond to the protein we are working with. This sequence lacks 28 amino acid residues at the N terminus and lacks the C-terminal domain that is conserved in all ChoKs and EtnKs. Specifically, the reference sequence lacks the essential aspartate.
residue that coordinates the Mg$^{2+}$ in ATP binding and the conserved structural motif (ILV)$_X$(FWY)$_X$(FWY)$_X$(FWY)$_D$X$_H$, which is critical for catalysis in this protein family (25). Thus, the isoform 1 transcript will not produce catalytically active EtnK.

Isozyme 2 (AAP47267) has a truncated N terminus that begins between isoforms 1 and 4, and isozyme 4 (XM_903847) is even shorter, lacking the first 103 residues at the N terminus of isozyme 3. It is not known if these significant deletions at the N terminus, which remove a significant number of residues conserved in EtnK1 (Fig. 1A), would encode a functional EtnK2. EtnK2 isoform 5 (XM_903848) is a splice variant of isozyme 3 with exon 7 and the conserved Chok/EtnK signature motif deleted and is strongly predicted to lack activity. We selected isozyme 3 as the reference EtnK2 because it has the longest open reading frame.

Northern blot analysis showed that Etnk2 was most highly expressed in liver, followed by kidney and testis (Fig. 1B). The Northern blot signals a transcript of 2.3 kb, which is consistent with the size of our cloned cDNA (Fig. 1A). The distribution of expressed sequence tags is consistent with our Northern analysis and shows that the ovary expresses about the same level of Etnk2 as the testis and that the eye and brain have equivalent expression levels (NCBI EST Profile Viewer). The tissue distribution of mouse Etnk2 mRNA (Fig. 1B) mirrored the distribution of human ETNK2 transcripts (12). A quantitative view of the Etnk2 expression levels in various tissues was obtained by quantitative real-time PCR (Fig. 1C). Kidney Etnk2 transcript levels were 100-fold lower than liver transcript levels, and most of the other tissues were between 1000- and 100,000-fold lower compared with liver.

**Catalytic Activity of EtnK2**—The enzymatic activity of the Etnk2 cDNA clone was analyzed by transient expression in 293T cells (Fig. 2). The lysates from cells transfected with Etnk2 cDNA exhibited significantly higher levels of EtnK activity, but did not possess Chok activity above the background levels detected in control cell lysates.

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Increasing the protein concentration in the assay by 10-fold did not reveal any evidence of increased Chok activity (data not shown), placing the Chok activity at <0.1% of the EtnK activity in EtnK2. The kinetic constants for EtnK2 were estimated in the lysates. The enzyme had an apparent high affinity for both Etn (37 µM) and ATP (26 µM). Thus, the Etnk2 cDNA encoded a robust EtnK activity that lacked detectable Chok activity.
Generation of Etnk2<sup>−/−</sup> Mice—The strategy for the inactivation of the Etnk2 allele is diagramed in Fig. 3. Gene function was eliminated by the introduction of the Neo selection cassette into the third exon of the gene. The Neo insertion into exon 3 eliminated residues 186–207 of the protein sequence, removing a region corresponding to helix E in the ChoK/EtnK structure (25). The truncated protein lacked the ATP-binding site (Brenner’s motif) plus the ChoK/EtnK signature motif and is catalytically inactive. The targeting construct also contained the diphtheria toxin antigen cassette used for negative selection in the isolation of ES clones (Fig. 3). The structure of the knock-out allele was verified by PCR. PCR screening was also the routine method employed to genotype the mice by the DNA extracted from tail clippings (Fig. 3, inset). The ES cells were selected and screened, and germline transmission of the knock-out allele was accomplished as described under “Experimental Procedures.” A line of homozygous Etnk2<sup>−/−</sup> mice were derived from a breeding program.

EtnK Activity in Knock-out Mice—Etnk2 was most highly expressed in liver (Fig. 1B); therefore, our focus with the homozygous knock-out mice was to analyze the effect of ablation of Etnk2 on kinase activities and lipid metabolism in liver. Livers were removed from wild-type and Etnk2<sup>−/−</sup> mice; soluble lysates were prepared; and the EtnK and ChoK activities were determined (Fig. 4). The liver lysates from the Etnk2<sup>−/−</sup> mice possessed 10-fold lower specific activities for EtnK compared with those from the wild-type mice. We were always able to detect EtnK activity in the liver lysates from the knock-out mice. This residual EtnK activity was attributed to a combination of the contributions of liver ChoKs, which have some activity with Etn (8), and the EtnK1 enzyme. The ChoK activities were not appreciably different in any of the tissues derived from the Etnk2<sup>−/−</sup> mice compared with the wild-type animals (Table 2). These enzymatic and expression data show that the liver experienced the most severe reduction in EtnK activity in the knock-out mouse model.

Phospholipid Composition of the Livers of Etnk2<sup>−/−</sup> Mice—If EtnK2 is important for the synthesis of PtdEtn in liver, one possible result
Etnk2 Knock-out Mice

We measured the amount of the two major liver phospholipids (PtdCho and PtdEtn) in the wild-type and Etnk2 knockout mice. We found that there was no difference in the tissue content of these two species (Table 3). We also examined the molecular species fingerprint of liver PtdEtn and PtdCho in the knock-out mice and found that there was no difference in the liver histology in the knock-out and control animals (data not shown), and the hepatic content of PtdCho and PtdEtn was the same (Table 3). Thus, it was clear that sufficient PtdEtn was being produced for PtdCho formation during Cho deprivation in the Etnk2 knockout mice.

Phospholipid Synthesis in Etnk2-deficient Liver—The next step in our analysis was to determine whether the 10-fold reduction in EtnK activity in hepatocytes from Etnk2 knockout mice resulted in a deficiency in PtdEtn synthesis from Etn. The phosphorylation of Etn is the first step in the conversion of extracellular Etn to PtdEtn, and it was clear from our experiments that Etnk2 knockout hepatocytes were defective in PtdEtn synthesis (Fig. 6A). However, the rate of PtdEtn synthesis in Etnk2 knockout hepatocytes was only half the rate observed in the wild-type cells, illustrating that the 10-fold reduction in EtnK activity did not translate into an equivalent reduction in PtdEtn formation by the CDP-Etn pathway. We also performed a labeling experiment with [3H]serine to determine whether there was a difference in the wild-type and knock-out hepatocytes in PtdEtn synthesis from phosphatidylserine (Fig. 6B). These data did not reveal a difference in PtdEtn synthesis from serine in the two hepatocyte preparations.

Reproductive Phenotype in Etnk2-/- Mice—Although there was no discernable phenotype in the adult mutant mice, they did have a clear reproductive defect. Litters from crosses between Etnk2-/- parents resulted in smaller litter sizes and pup mortality (Table 4). Matings between wild-type parents produced eight weaned pups/litter; however matings between Etnk2-/- mice produced 33.6% fewer pups/litter (p < 0.0001). This defective reproductive phenotype was maternal-specific. Matings of Etnk2-/- males with wild-type females resulted in normal litter sizes of Etnk2-/- offspring (Table 4). In contrast, matings of Etnk2-/- females with wild-type males resulted in low numbers of Etnk2-/- offspring (p < 0.0001). A high rate of pup mortality contributed to the low number of pups produced.
There were no pup deaths in the matings between wild-type mice or between wild-type females and Etnk2<sup>−/−</sup> males (Table 4). In contrast, matings between wild-type males and Etnk2<sup>−/−</sup> females (<i>p</i> < 0.0001) and between Etnk2<sup>−/−</sup> males and Etnk2<sup>−/−</sup> females (<i>p</i> < 0.0001) resulted in 10.5 and 16.9% pup mortality, respectively. Matings of Etnk2<sup>+/−</sup> females with Etnk2<sup>+/−</sup> males resulted in normal litter sizes; no pup mortality; and the expected distribution of wild-type, heterozygous, and knock-out animals. The disrupted Etnk2 allele was backcrossed onto the C57BL6/J background for eight generations, and a well organized, and a similar morphology was also observed in Etnk2<sup>+/−</sup> females (data not shown). We also examined the cellular structure of the brains from the wild-type controls, illustrating that disruption of the Etnk2<sup>+/−</sup> females was the same as that of the wild-type females and does not account for the reproductive phenotype.

**Histology of Etnk2<sup>−/−</sup> Mice**—Tissue sections of liver, brain, retina, cochlea, ovary, and uterus from three individual Etnk2<sup>−/−</sup> and wild-type mice at postnatal day 60 were stained with hematoxylin and eosin. The hepatocytes in liver lobes were well organized, and a similar morphology was also observed in Etnk2<sup>−/−</sup> mice. The cellular structure of the brains from the Etnk2<sup>−/−</sup> mice, including the internal granular layer, external granular layer, and Purkinje cell layer, appeared similar to those from the wild-type controls, illustrating that disruption of the cell layers in the cerebral cortex and hippocampus did not occur in Etnk2<sup>−/−</sup> mice (data not shown). We also examined the cochlea and retina structures and observed no differences between Etnk2<sup>−/−</sup> and control mice (data not shown). The mice were also tested for sensorimotor coordination in the accelerating rotarod test, which is sensitive to cerebellar and basal ganglia dysfunction. Etnk2<sup>−/−</sup> mice of both genders showed equal latency to fall as the wild-type controls, suggesting a normal cerebellum (data not shown).

The female reproductive phenotype in Etnk2<sup>−/−</sup> mice could not be attributed to altered morphology or hormone levels. Control mice had several developing follicles, multiple corpora lutea, and a well organized germinal epithelium; interstitial stromal cells and follicles were at various stages of development. The ovaries and uteruses from Etnk2<sup>−/−</sup> mice, dissected at different stages of gestation, were the same as those from the wild-type controls. The progesterone levels in the pregnant Etnk2<sup>−/−</sup> females (32.7 ± 14 ng/ml serum, <i>n</i> = 11) were not different from those in the pregnant wild-type females (31.5 ± 12 ng/ml serum, <i>n</i> = 9). Likewise, there was no difference in the estradiol levels between normal and Etnk2<sup>−/−</sup> mice (24.8 ± 7.0 compared with 26.0 ± 6.2 ng/ml serum). These data illustrate that the hormonal status of the Etnk2<sup>−/−</sup> females was the same as that of the wild-type females and does not account for the reproductive phenotype.

At embryonic day (E) 7.5, embryos from Etnk2<sup>−/−</sup> and control mice were dissected, and transverse sections were stained (Fig. 7A). Both placentas and embryos were at a normal stage of development compared with the wild-type controls. There was no reduction in litter size, and there were no resorbed or empty conceptuses in the Etnk2<sup>−/−</sup> mice. All embryos had similar sized embryonic cavities and decidual swelling, and no appar-

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**FIGURE 5. Analysis of PtdEtn and PtdCho molecular species in wild-type and Etnk2<sup>−/−</sup> mice.** Livers were removed from 3-month-old mice maintained on a standard laboratory diet; the lipids were extracted; and the mass spectra were obtained as described under “Experimental Procedures.” The fatty acid chains that constitute the major molecular species are indicated in shorthand for the number of carbon atom/number of double bonds. A and B, the PtdEtn molecular species profile in wild-type and Etnk2<sup>−/−</sup> mice, respectively; C and D, the PtdCho molecular species distribution in wild-type and Etnk2<sup>−/−</sup> mice, respectively.
ent growth retardation was observed. Sections from \textit{Etnk2}^{−/−} embryos indicated that development was in the process of gastrulation and that all three germ layers were formed and had normal extraembryonic membranes. The morphology, number, and distribution of giant cells appeared normal in the growing placentas. The extraembryonic ectodermal sheet of the chorion was also similar to that in wild-type embryos. These data suggest that implantation and initial growth of placentas were not defective in female \textit{Etnk2}^{−/−} mice. The morphology and number of embryos found at E12.5 were also apparently normal (Fig. 7B), indicating that fetal development was not arrested at this point. At E12.5, the formation of the chorioallantoic connection and the structures of the spongiotrophoblast layer and labyrinth layer in \textit{Etnk2}^{−/−} mice appeared normal in comparison with those in wild-type mice (Fig. 7C). No significant mor-

![FIGURE 6. Metabolic labeling of hepatocytes derived from wild-type and \textit{Etnk2}^{−/−} mice. Hepatocytes were isolated from wild-type and \textit{Etnk2}^{−/−} mouse livers and labeled with either \textsuperscript{14}C\textit{Etn} (A) or \textsuperscript{14}C\textit{serine} (B) for 6 h; the lipids were extracted; and the incorporation into Ptd\textit{Etn} was determined by thin-layer chromatography as described under “Experimental Procedures.” The error bars are S.D. for triplicate determinations.

![FIGURE 7. Normal early embryonic development in \textit{Etnk2}^{−/−} mice. A, section of an E7.5 embryo from an \textit{Etnk2}^{−/−} dam stained with hematoxylin and eosin showing normal histology. A normal number of implanted embryos were observed. B, uterus of a pregnant \textit{Etnk2}^{−/−} mouse at E12.5 showing a normal number of embryos at the same stage of development. C, morphology of the placenta at E12.5 showing the normal development and structures of the chorionic plate (ch), the labyrinth layer (la), and the spongiotrophoblast layer (sp). Scale bars = 100 \textmu m (A and C); 600 \textmu m (B). The images are representative of four \textit{Etnk2}^{−/−} and control mice.

| TABLE 4 | Maternal-specific reduced litter sizes and infant death in \textit{Etnk2}^{−/−} mice |
|---------|-------------------------------|
| Mating (male × female) | Pups/litter$^a$ | Pup mortality$^b$ |
| Wild-type × wild-type | 7.94 ± 0.40 (n = 36)$^c$ | 0/286 (0%) |
| \textit{Etnk2}^{−/−} × \textit{Etnk2}^{−/−} | 5.27 ± 0.38 (n = 37) | 33/195 (16.9%) |
| \textit{Etnk2}^{−/−} × wild-type | 7.60 ± 0.51 (n = 5) | 0/28 (0%) |
| Wild-type × \textit{Etnk2}^{−/−} | 4.80 ± 0.58 (n = 5) | 4/38 (10.5%) |

$^a$ Number of pups weaned.

$^b$ Number of pups dying between birth and weaning.

$^c$ Error ranges as S.D.

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Etnk2 Knock-out Mice

Phenotypic analysis was performed on four viable and nonviable littermate embryos. Large blood clots were clearly visible in the labyrinth layer of the placentas of nonviable embryos at E17.5 (Fig. 8B, right panel), but not in those of normal littermates (Fig. 8B, left panel). Approximately 20% of the total volume of the labyrinth later was occupied by thrombi in the nonviable embryos, and all of the nonviable Etnk2<sup>-/-</sup> placentas showed this striking abnormality. The viable embryos exhibited variable amounts of thrombosis. These thrombotic events were smaller, fewer in number, and located primarily near the edge of the labyrinth layer. Small thrombi occasionally formed in the placentas of wild-type embryos; however, the prevalence and size of thrombi in the Etnk2<sup>-/-</sup> placentas clearly distinguished them from normal mouse placentas. The tissue structures of the maternal decidua layer, chorionic plate, and spongiosotrophoblast layer remained largely unaltered in Etnk2<sup>-/-</sup> mice compared with wild-type females. The functional consequence of the thrombus formation in placentas from Etnk2<sup>-/-</sup> mice, determined by using the TUNEL assay for apoptosis (Fig. 8C) and immunohistochemistry to detect activated caspase-3 (Fig. 8D), revealed clear differences between the nonviable and viable Etnk2<sup>-/-</sup> placentas. Extensive staining of the epithelial cells in the labyrinth layer was observed in nonviable embryos, whereas staining of the spongiosotrophoblast layer and chorionic plate was less pronounced. Little staining with either reagent was observed in viable Etnk2<sup>-/-</sup> placentas (Fig. 8C, left panel) and in wild-type embryos (data not shown). These findings indicate that the placental abnormalities observed in Etnk2<sup>-/-</sup> mice were caused by thrombus formation, leading to restriction of the blood supply to the embryo, extensive apoptosis, and embryonic death.

**DISCUSSION**

This work has identified the second mammalian Etn-specific kinase that is most highly expressed in liver. Like EtnK1 (28), the EtnK2 enzyme is highly selective for Etn and phosphorylates Cho poorly, if at all. The livers of Etnk2<sup>-/-</sup> animals are clearly deficient in EtnK activity, showing that
**EtnK2 Knock-out Mice**

EtnK2 is the major protein responsible for Etn phosphorylation in this tissue. In light of the fact that EtnK2 is clearly the predominate kinase in the CDP-Etn pathway in liver, the finding that hepatocytes are only slightly compromised in the incorporation of exogenous Etn into PtdEtn and that phospholipid content and structure are the same in the wild-type and $Etnk2^{-/-}$ livers is perhaps surprising. Cho deprivation is a nutritional scenario that places a heavy burden on liver PtdEtn synthesis to produce PtdCho (26, 27), but placing the $Etnk2^{-/-}$ mice on a Cho-deficient diet did not have a discernable effect on liver phospholipid composition or pathology. This is in stark contrast to mice lacking PtdEtn N-methyltransferase expression, where liver dysfunction and pathology become apparent after only a few days (11, 26, 27). A deficiency in phosphocholine cytidylyltransferase (29) and pathology become apparent after only a few days (11, 26, 27). A deficiency in phosphocholine cytidylyltransferase (29) and pathology become apparent after only a few days (11, 26, 27).

The brains and testes of $Etnk2^{-/-}$ mice appear normal, contrary to predictions from the study of other systems and the expression pattern of $Etnk2$. The Drosophila eas mutant is a null allele for $EtnK$ that gives rise to flies that become paralyzed when vortexed (10, 31). These flies are not grossly deficient in PtdEtn, but they do have slightly lower levels of PtdEtn in the brain, and their phenotype is thought to arise from an excitability defect caused by altered local membrane phospholipid composition. Although mouse EtnK2 is a homolog of the eas gene product, we did not detect any overt neurological defects in our animals. In contrast to Drosophila, where eas expression is the highest in neural tissue, mice do not express a large amount of the $Etnk2$ transcript in the brain. EtnK1 is the more prominent brain isoform, and $Etnk2$ gene inactivation does not appreciably lower total brain tissue EtnK activity. The finding that $Etnk2$ expression is specifically up-regulated in Sertoli’s cells of the testis during mouse sex determination (13) suggests that the $Etnk2^{-/-}$ knock-out mice would exhibit defects in testicular developement. However, we were unable to detect a morphological abnormality or reproductive failure of $Etnk2^{-/-}$ testis. These considerations led to the conclusion that mammalian tissues have considerable plasticity with regard to PtdEtn synthesis, which is supported by Etn phosphorylation by EtnK1, EtnK2, and the ChoKs and through the operation of the separate phosphatidylethanolamine decarboxylase pathway. Sphinogosine 1-phosphate degradation should be considered as another potential source for phosphoethanolamine (32).

The maternal-specific failure to support late embryonic development in $Etnk2^{-/-}$ mice reveals a non-redundant role for EtnK2 in placental function. $Etnk2^{-/-}$ females give birth to smaller litters, with a mixture of average size pups and low birth weight animals, which often die within 1 week after birth, and a percentage of the embryos die in utero late in gestation. The observation of placental thrombosis and apoptosis associated with the aborted embryos points to the inability of pregnant $Etnk2^{-/-}$ mice to control coagulation in the placental labyrinthis, leading to intrauterine growth retardation and/or death. The maternal components of the placenta derive from the maternal vasculature and uterine decidual cells, which come into close contact with the embryonic cells of the trophoblast lineage in the labyrinth layer (33). There is a delicate balance between clotting and hemorrhage in the placenta (34), and there are multiple factors that impact on maintaining this balance. The estrogen sulfatase knock-out mouse has a similar maternal-specific placental thrombosis phenotype because of the elevation of plasma estradiol (35); however, the comparable estrogen levels in $Etnk2^{-/-}$ and control mice rule out a contribution of estradiol dysregulation to our phenotype. The ROCK-II knock-out phenotype of intrauterine growth retardation and thrombosis is attributed to the inability of embryonic trophoblasts to control coagulation (16). Although this phenotype is similar to the $Etnk2^{-/-}$ phenotype, it differs in being fetal-specific rather than maternal-specific. However, the processes of cell-surface PtdEtn movement and RhoA signaling were recently linked in cultured cells (19); therefore, it is possible that activation of the Rho/ROCK signaling pathway in embryonic trophoblasts may be defective because of the lack of extracellular maternally derived PtdEtn. The protein C anticoagulation pathway is established as critical for placental function (36). A deficiency in protein C gives rise to placental thrombosis and intrauterine fetal death (37) and increases apoptosis in hypoxic endothelial cells (38). PtdEtn is a critical cofactor in the protein C activation pathway (39), raising the possibility that reduced PtdEtn in the placentas of $Etnk2^{-/-}$ females may compromise the generation of activated protein C, leading to thrombosis and hypoxic apoptosis in the placenta. These data indicate the importance of $Etnk2$ expression and, by extension, maternally derived PtdEtn in maintaining hemostasis in the placental labyrinth. Recurrent pregnancy loss and thrombosis are also characteristic of the antiphospholipid syndrome, a multifaceted collection of syndromes associated with antibodies against specific lipid-protein combinations (40). One class of antiphospholipid antibodies inhibits protein C activation in a PtdEtn-dependent manner (41). The lack of maternal PtdEtn or maternal PtdEtn-protein C or PtdEtn-$\beta$-glycoprotein I complexes may create an imbalance in the regulation of coagulation by the embryonic trophoblasts lining the blood vessels. Thus, the hypothesis that EtnK2 has a non-redundant role in producing endothelial cell PtdEtn to support activation of the protein C anticoagulation cascade appears a tenable direction for future work.

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