Serum ethanolamine and hepatocyte proliferation in perinatal and partially hepatectomized rats

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Abstract

It has been shown that the administration of ethanolamine (Etn) to partially hepatectomized rats enhances stimulation of DNA synthesis in regenerating hepatocytes. The present study aimed to test the hypothesis that the level of serum Etn in vivo may be regulated to control the growth of hepatocytes. Concentrations of serum Etn were determined in rats 1) of varying ages (from embryonic-19 (E-19) to 7-week-old), and 2) during regeneration following two-thirds hepatectomy (PH), to investigate whether serum Etn concentration correlates with the rate of proliferation of hepatocytes in growing animals or during regeneration. Serum Etn levels were 3 fold higher in E-19 fetuses and newborns than in adults, and were increased 2 fold 4 h after PH and remained high for at least 24 h. Results in both systems indicated a significant positive correlation between the rate of hepatocyte proliferation and serum Etn levels. Furthermore, Etn supplementation of 0.1 to 1 mmol immediately after PH promoted a significant weight gain and stimulated phosphatidylethanolamine (PE) and phosphatidylcholine (PC) synthesis in the regenerating liver. We also observed that whenever serum Etn levels were elevated, the metabolism of PE and PC in the liver changed dynamically, first by elevating the net synthesis of PE. Taken together, these results suggested that the levels of serum Etn might be regulated based on the physiological state of an animal, which consequently regulates the proliferation of hepatocytes.

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Keywords: Ethanolamine in serum; Phosphatidylethanolamine; Membrane phospholipids; Hepatocyte proliferation; Liver regeneration

Introduction

Mammalian epithelial cells including primary hepatocytes (Ajoika et al., 2002; Kume and Sasaki, 2006; Nelson et al., 1996; Sasaki et al., 1997, 1998) require ethanolamine (Etn) to proliferate in vitro, and stimulation of growth by Etn occurs in a dose-dependent manner (Babcock et al., 1983; Hammond et al., 1984; Kano-Sueoka et al., 1982, 2001; Lechner et al., 1982; Peehl and Starney, 1986; Tsao et al., 1982). Epithelial cells require Etn, because without it, they are unable to synthesize amounts of phosphatidylethanolamine (PE) necessary for sustaining growth (Kano-Sueoka and King, 1988). Without Etn supply, PE content in cell membranes is halved, whereas that of phosphatidylcholine (PC) is increased by 30% (Kano-Sueoka and King, 1988; Kano-Sueoka et al., 1990), and cellular functions associated with cell membranes become abnormal and growth stops (Fisk and Kano-Sueoka, 1992; Kano-Sueoka et al., 1983; Kano-Sueoka and King, 1987; Kano-Sueoka and Nicks, 1993).

In contrast to the in vitro situation, the importance of Etn in vivo remains unclear. We previously found that administration of Etn to rats enhanced stimulation of DNA synthesis in hepatocytes after partial hepatectomy (Sasaki et al., 1997). This finding suggested that Etn might also be an important factor in vivo for the proliferation of epithelial cells including hepatocytes that were Etn-dependent for growth in vitro. We hypothesized that serum Etn plays a role in regulating growth of hepatocytes, and perhaps other epithelial cell types in vivo. To test this hypothesis, we examined whether serum Etn concentration became elevated when there was a large number of proliferating hepatocytes. We also investigated whether administration of Etn promoted proliferation of hepatocytes in vivo in the regenerating liver.

Little is known about how stable or variable serum concentrations of Etn are. Moreover, nothing is known about how or to
what extent serum Etn is regulated. A limited amount of available data suggests that the concentration of serum Etn may be variable. Dickinson et al. (1965, 1970) analyzed concentrations of free amino acids, including that of Etn, in blood plasma in human adults and newborn infants. Their results showed that the average concentration of Etn in adult plasma was 1.6 μM (traces up to 11 μM), whereas in newborn infants it was 52.5 μM (26–92 μM). In contrast, levels of free amino acids in adults and newborns were relatively similar. Houweling et al. (1992) found that serum Etn levels increased from 29 to 50 μM during the first day after two-thirds hepatectomy (PH) in rats. These findings suggested that serum Etn levels can be variable and may be regulated according to demand by cells requiring Etn to grow. Thus, in newborn infants or during liver regeneration, the population of growing Etn-requiring cells would be significantly larger than those in adults or in animals with an intact liver. If the above assumption is correct, it is also possible that supplementation with Etn may further promote the growth of Etn-requiring cells.

In the present study, we used two rat model systems; one consisted of varying ages from E-19 to 7-week-old, and the other model consisted of varying stages of regenerating liver. In both systems, we determined concentrations of serum Etn, rates of hepatocyte proliferation [data for hepatocyte proliferation during regeneration have already been documented (Bucher and Swaffield, 1964; Lieberman and Kane, 1965)] and levels of hepatocyte phospholipids.

Rat and human milk contains high levels of Etn and phosphoethanolamine (pEtn), which can be used for PE synthesis (Jensen, 1995; Kanno et al., 1997). However, it is not known whether their content in milk changes with time after parturition. Therefore, we analyzed the milk of nursing mothers for two weeks after parturition, and compared data with those of serum Etn levels, and with the rate of hepatocyte proliferation in perinatal animals. Our assumption was that milk obtained immediately after parturition would contain higher amounts of Etn and PEtn to meet the demands of the newborn.

Although the process of regeneration of a partially heptatectomized (PHed) liver has been well studied, phospholipid metabolism in the regenerating liver is not fully understood. With regards to PE, Houweling et al. (1992) noted that one day after surgery, concentrations of serum Etn were increased by about 70%, and the rate of PE synthesis was also increased. In the present study, we sought to determine a detailed time course of the changes in the amount of serum Etn and of phospholipids found in the regenerating liver. Were then analyzed results in relation to the way hepatocytes proliferated after PH.

From the results we sought to show that when the rate of hepatocyte proliferation was high, serum Etn levels were also high both in growing animals and in the regenerating liver. In addition, phospholipid profiles in the liver were seen to dynamically change relative to serum Etn levels and the rate of hepatocyte proliferation.

Experiments were carried out to determine whether the administration of Etn promoted the growth of hepatocytes and liver regeneration. We showed that Etn supplementation hastened the recovery process, most likely by stimulating PE and PC synthesis.

We experimentally demonstrate our hypothesis that serum Etn concentrations are regulated in response to demands made by the epithelial cell population. In growing epithelial cells, a sufficient supply of Etn is shown to be necessary to synthesize PE in order to achieve a suitable membrane phospholipid environment for cellular signaling.

**Materials and methods**

**Animals and experimental design**

Sprague-Dawley rats were purchased from Charles River, Japan, and maintained on standard chow (CRF-1, from Oriental Yeast Co., LTD, Japan). Rats were handled according to protocols approved by the Food Science Institute of Meiji Milk Dairies Corporation, which follow the Guide for the Care and Use of Laboratory Animals (NRC1996). Animals bred in the laboratory were used to examine effects of age on serum Etn concentrations and liver phospholipids. Anesthetized animals, E-19, 0, 2, 7, 14, 28, and 49-day-old rats, were sacrificed using diethyl ether after withdrawing blood and removing the liver. Animals older than 4 days were identified by sex, and males and females were analyzed separately. There were no significant differences in serum levels of Etn and amino acids by sex.

Pregnant rats that carried E-19 or day-(d)-20 fetuses were used to examine how Etn (50 mg in hydrochloride salt, Sigma Chemical Co. St. Louis, MO) given by peritoneal injection was transported from mother to fetus. Two, 4, and 6 h after Etn administration, blood was withdrawn from the main artery of the pregnant mother and from the heart of the fetuses. Blood was also withdrawn from the tail vein, plaenema, and umbilical cord of the pregnant rat. Rats that had nursed for 2 to 14 days after parturition were used for analysis of concentrations of Etn and PEtn in milk.

Two-thirds hepatectomy PH was carried out as previously described using 7-week-old male rats (Sasaki et al., 1997). To investigate the effects of Etn supplementation, the hydrochloride salt of Etn dissolved in saline (0.1 mmol or 1 mmol Etn/ml rat) or saline was intraperitoneally injected into rats within a minute after PH. Injection of the same dose of Etn or saline was repeated every 24 h. Anesthetized animals were sacrificed using diethyl ether after withdrawing blood from the main artery and removing the liver at 0, 2, 4, 8, and 24 h after PH for serum Etn and liver phospholipids. Animals were also sacrificed at 1, 2, 3, and 7 days after PH for liver weight.

**Blood collection and amino acid analysis**

Mother rats carrying E-19 fetuses were anesthetized with pentobarbital solution at 50 mg/kg body weight, and blood of E-19, and 0, 2, and 7-day-old rats was withdrawn from the heart. In some cases, blood was collected from the main artery and from the heart of the same animal to compare levels of Etn and amino acids. We found that Etn and amino acid levels were the same from both sources, respectively. Blood was allowed to clot at 4 °C, and serum was collected and stored at −20 °C until ready for analysis. For analysis of E-19 fetuses, blood samples were pooled from two fetuses to allow for duplicate analysis.
Serum was deproteinized by adding two volumes of 5% trichloroacetic acid and centrifuged to remove pelleted particles, and then passed through a 0.45-μm filter. Amino acid analysis including that of Etn was performed using an L-8800 Hitachi High Speed Amino Acid Analyzer. Amino acid concentrations were obtained and expressed against those of Etn in various serum preparations.

**Measurement of hepatocyte proliferation in vivo**

Rates of hepatocyte proliferation in animals of various ages were immunohistochemically determined by detecting 5-bromo deoxyuridine (BrdUrd) incorporated into the nuclei of hepatocytes (Sasaki et al., 1997). Briefly, a BrdUrd solution was peritoneally injected, and 2 h later, the liver was excised and processed for paraffin sectioning and immunostaining.

**Phospholipid analysis**

The liver was immediately excised after sacrifice, cut into small pieces with scissors on ice, and homogenized in 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, and 10 mM NaF with a glass-teflon homogenizer. Lipid extraction from the homogenized liver, and analysis of phospholipids were carried out according to previously described methods (Fisk and Kano-Sueoka, 1992). Components of phospholipids were separated by two-dimensional thin layer chromatography on silica gel G plates. Solvents used were chloroform:Methanol:28% aqueous ammonia (65:35:5 v/v/v) for the first direction, and chloroform:Acetone: Methanol:Acetic acid:Water (10:4:2:2:1 v/v/v/v/v) for the second direction. Phospholipids were located by exposing the dried plates to iodine vapor. Areas of individual phospholipids were scraped from the plates, and the amount of phosphate was measured according to the method of Ames (1966). The amount of protein was determined using BCA assay reagents (Pierce Biototechnology, Rockford, IL).

**Radiolabeling of phospholipids in the regenerating liver**

At the time of PH, 1 ml physiological saline or 0.1 mmol Etn was administered in the abdominal cavity. Twenty μCi [32P]-phosphoric acid (200 μCi/mmol, Amersham Biosciences Corp., Piscataway, NJ) was given by intraperitoneal injection at specific times after surgery. Two hours later, rats were sacrificed and livers were processed to analyze phospholipids as described in the previous section. Radioactivity was detected by exposing the chromatogram on an imaging plate. The exposed spots were cut out, and radioactivity was determined using a Bas 2000 (Fuji Film Corp., Tokyo).

Methylation of Etn moieties in PE was determined by incorporation of [3H]-S-adenosylmethionine in a similar fashion to that of 32P incorporation, except that 10 μCi S-adenosyl-L-methyl-[3H]-methionine (500 mCi/mmol, Amersham Biosciences Corp., Piscataway, NJ) and 100 nmol non-radioactive methionine were given 2 h after surgery. Two hours later, animals were sacrificed and processed as described above. Radioactivity was determined using a Scintillation analyzer.

**Statistical analysis**

All values are expressed as means±SEM. Data were analyzed using StatView (SAS Institute Inc.), and the non-parametric Mann–Whitney test or Student's t-test. Comparison of multiple values was assessed by ANOVA, followed by the Tukey–Kramer test. Differences were considered significant at \( P<0.05 \).

**Results**

**Ontogenical changes of serum Etn**

Blood was collected from 0-, 1-, 2-, 4-, 7-, 14-, 28-, and 49-day-old animals and from E-19 fetuses. The amount of Etn in serum was then determined. As shown in Fig. 1A, Etn levels

![Fig. 1A: Concentrations of Etn in serum of rats of various ages. Serum samples were prepared from E-19, 0-, 1-, 2-, 4-, 7-, 14-, 28-, and 49-day-old animals, and analyzed for Etn and 19 amino acids, as described in the Materials and methods. Values represent means±SEM, \( n=6 \). (B) Rates of proliferation of hepatocytes in animals of different ages. Hepatocyte proliferation was determined by immunohistochemistry by detecting BrdUrd, which was incorporated into cell nuclei of hepatocytes, as described in the Materials and methods. E-19, 2-, 6-, 14-, 24-, 28-, and 49-day-old animals were analyzed. Values represent means±SEM, \( n=4 \). C: Correlation of Etn concentrations with the nuclear labeling index.
were the highest in E-19 fetuses and in 1-day-old animals, and decreased to 1/3 by the 49th day, whereas concentrations of Etn among individual animals of the same age group varied by very little. Animals were weaned 4 weeks after birth, at which time Etn reached adult levels. Serum levels of Etn in pregnant or lactating females were essentially the same as those of non-pregnant adult female or male rats (data not shown).

Supply of Etn from mother to fetus and newborn

The finding that concentrations of serum Etn in the fetus were different from the mother, suggested that the placenta acted as a selective barrier between mother and fetus. Accordingly, we analyzed concentrations of Etn in blood withdrawn from the tail vein, placenta, and umbilical cord in pregnant rats. Fig. 2 shows that concentrations of serum Etn in the placenta and umbilical cord were significantly higher than those in the tail vein, and that Etn levels in the umbilical cord were the same as those in the fetus, indicating that the placenta is a selective barrier for Etn.

To examine the selective nature of the placenta, 30 mg of (approximately 0.5 mmol) Etn was given to pregnant rats by intraperitoneal injection, and temporal changes in serum Etn concentration were examined in mother and fetus (Fig. 3). Etn concentrations in mother and fetus increased 10 fold 2 h after administration of Etn, and gradually decreased as time progressed. However, at any given time point, the ratio of Etn levels in the mother to those in the fetus remained the same at approximately 2:1. Thus, the placenta appeared to maintain a certain concentration gradient for Etn between mother and fetus, irrespective of the absolute amount present in the mother.

Concentrations of Etn and phosphoethanolamine PEtn were measured in the mother’s milk at 2, 7, and 14 days after parturition (Fig. 4). Levels of Etn and PEtn at 2 and 7 days after parturition were twice as high as those at 14 days after parturition, suggesting that levels of Etn and PEtn in milk might be regulated according to the needs of the pups. Although serum contains little PEtn, milk contains a considerable amount of PEtn, which is much higher than that of Etn. PEtn is apparently dephosphorylated before being taken up by cells, and therefore uptake is considerably slower than that of Etn (Kano-Sueoka and King, 1987).

![Fig. 2. Concentrations of Etn in serum taken from an artery, placenta, and umbilical cord of pregnant rats that had E-19 or 20-day fetuses. Values represent means ± SEM, n = 5, P < 0.05 vs. the artery of the pregnant rat.](image)

![Fig. 4. Concentrations of Etn and PEtn in milk 2, 7, and 14 days after parturition. - , Etn; - , PEtn. Values represent means ± SEM, n = 3. Values with different superscripts are significantly different withable P < 0.05.](image)
Ontogenical changes in hepatocyte phospholipids

The amount of total and individual phospholipids in liver per unit amount of protein was compared among rats of varying ages (Fig. 5). The amount of total phospholipids increased ~30% within 1 to 2 weeks after birth, and amounts of most components of phospholipids increased proportionally. However, the amount of PC significantly decreased right after birth, but recovered to the E-19 level 1 week after birth. The ratio of PC/PE in E-19 fetuses was approximately 2.00, but was reduced to approximately 1.6–1.7 and remained at this level for at least 2 weeks after birth because of a decrease in the amount of PC, as described above. This suggested that during rapid hepatocyte growth (E-19 to day 2, Fig. 1B), the amount of Etn in serum, which was 3 fold higher than that in adults, was sufficient for the liver to synthesize PE. However, the rate of PC synthesis might not be sufficient to support growth of the liver and demands from other organs.

Effect of two-thirds (partial) hepatectomy PH on serum Etn concentrations

During liver regeneration, rapid hepatocyte proliferation occurs until the liver reaches its original volume. Therefore, it is conceivable that serum levels of components necessary for regeneration may be elevated during this period, and that Etn may be one such component. To test this possibility, concentrations of Etn in serum were determined over 24 h after PH, and compared with those of sham operated animals. Results are shown in Fig. 6A.

Within 4 h after PH, serum Etn concentrations attained their highest level from 31.4±1.1 to 49.4±2.3 μM, and remained high even 24 h after surgery. These results showed that elevation of serum Etn occurred prior to the onset of the new round of DNA synthesis that occurred 12–24 h after PH (Buchler and Swaffield, 1964; Lieberman and Kane, 1965).

Effect of PH on hepatocyte phospholipids

The liver is important for supplying PC, and perhaps other phospholipids to the other organs in the body (Vance, 1989, 2002); therefore, a PHed liver is expected to be under severe stress. As the remaining liver grows back to its original size, it must also continue supplying phospholipids to other parts of the body. To determine how PH affected phospholipids in the liver, livers were collected up to 24 h after surgery, and amounts of total and individual phospholipid components were determined. Results for PC and PE are shown in Fig. 6B. There was a drastic decrease (by 50%) in the amount of PC 2 h after surgery. However, PE levels gradually recovered to their original values by 24 h after surgery. PE levels were increased 4 h after PH, and remained higher than that in the intact normal liver even 24 h after surgery. This increase might have resulted from an increase in serum Etn concentrations after PH. Due to the decrease in PC levels, the PC/PE ratio significantly decreased immediately after surgery, but returned to normal over 24 h.

A decrease in the PC level and an increase in the PE level after PH have already been shown (Houweling et al., 1991, 1992); and are confirmed by our results. A drastic decrease in PE levels immediately after surgery may be caused by more PC being transported out of the liver than the amount being synthesized, or by PC being degraded in response to PH. An increase in PE levels may in part help restore the original PC levels.
levels by stimulating the synthesis of PC via methylation of PE. Normally, 20–40% of PC in the liver is synthesized via methylation of PE (Sundler and Akesson, 1975). The amount of other phospholipids such as phosphatidylserine, phosphatidylcholine, and sphingomyelin did not significantly change after PH (data not shown). Interestingly, the way in which PC levels decreased and PE levels increased after PH was similar to that in perinatal animals (Fig. 5).

**Effect of Etn administration on weight gain and liver phospholipids in regenerating liver**

The results so far obtained supported our assumption that the Etn present in adult serum was not sufficient when demand for Etn became higher due to a large number of epithelial cell types about to proliferate. The body somehow senses the need for a higher level of serum Etn, and Etn levels become elevated to stimulate PE synthesis. If this assumption is correct, supplying excess Etn during a period of high demand may further promote proliferation of cells such as regenerating hepatocytes. Thus, a series of experiments was carried out to examine the effects of Etn supplementation on the regenerating liver.

The results of hepatocyte nuclear labeling index after PH have already been reported (Lieberman and Kane, 1965; Sasaki et al., 1997). We first tested whether Etn supplementation would affect the weight gain of the regenerating liver. PHed animals received a daily periportal injection of physiological saline as a control or 0.1 mmol Etn for 7 days, and the weight of the regenerating liver was monitored for 10 days. As shown in Fig. 7, the livers of control animals reached their original weight in 7 days. In contrast, the daily administration of Etn significantly stimulated weight gain as early as 1 day after surgery; and at 3 days after surgery liver weight was almost back to normal. These results suggested that the supply of extra Etn helped not only to stimulate DNA synthesis but also to regenerate the liver.

Daily administration of 0.1 mmol Etn significantly raised serum levels (Fig. 6A). This result suggested that Etn supplementation might alter phospholipid metabolism leading to promotion of regeneration. Therefore, animals were given either saline, 0.1 mmol Etn, or saline in the peritoneal cavity at the time of PH; and 24 h later, their livers were excised and the amount of phospholipids was determined. Ratios of PC to PE were compared with that of the normal liver. Values represent means ± SEM, n=3. Values with a and b are significantly different (P<0.05).

**Fig. 8. Effect of Etn administration on the ratio of PC to PE 24 h after PH.** Animals were given either 0.1 mmol Etn, 1 mmol Etn, or saline in the peritoneal cavity at the time of PH; and 24 h later, their liver was excised and its phospholipid components analyzed. Fig. 8 shows the ratio of the amount of PC to PE (PC/PE), which was compared with those of a normal liver. The PC/PE ratio in the control regenerate liver was slightly lower than that of the normal liver, but the difference was not statistically significant. Administration of Etn, particularly at 1 mmol, significantly reduced the PC/PE ratio, presumably by increasing the amount of PE synthesized.

To further examine the effects of Etn administration on phospholipid metabolism in the regenerating liver, a 2-h pulse of [32P]-phosphoric acid was given every 2 h after PH, and the amount of radiolabeled phospholipid components was analyzed (Fig. 9). Etn (0.1 mmol) or saline was given in the abdominal cavity at the time of surgery. In this experiment, the 0 h sample represented normal liver without Etn administration. Interestingly, in normal livers, although PE content was only half that of PC, incorporation of [32P] into PE during the 2 h pulse was 3 fold higher than that of PC. This suggested that the synthetic activity of PE was characteristic high in the liver. In control regenerating livers, the amount of radiolabeled PE remained high, and was unchanged for 6 h after PH, but gradually decreased to less than half of original levels at 24 h. In contrast, the amount of radiolabeled PC steadily increased, and surpassed that of PE 12 h after PH, and still kept increasing. Etn supplementation drastically changed the ratios of radiolabeled PE and PC. The decline in radiolabeled PE started immediately after surgery, and the increase in radiolabeled PE accelerated earlier. These results suggested that supplementation of Etn stimulated PC synthesis by methylation of PE or a base-exchange reaction to replace Etn in [32P]-labeled PE with choline. It is also possible that synthesis of PC via CDP-choline was stimulated by Etn.

A substantial amount of PC is synthesized via methylation of PE by N-methyltransferases in the liver (Delong et al., 1999; Reo et al., 2002; Vance and Ridgway, 1988). To identify whether Etn supplementation stimulated methylation of PE, animals...
were given either saline or 1 mmol Etn at the time of surgery; and 2 h later, $[^3]H$-S-adenosylmethionine was given by an intraperitoneal injection. Two h later, animals were sacrificed, and radioactive methionine incorporated into PC was measured as described in the Materials and methods. Fig. 10 shows that PH by itself slightly increased the level of methylation of PE, although the difference was not statistically significant. However, Etn supplementation stimulated methylation, presumably by increasing the amount of substrate (PE) available for methylation. The results described so far support the idea that Etn supplementation promotes regeneration of the liver by stimulating synthesis of PE, which in turn stimulates synthesis of PC.

**Discussion**

In the present study, we examined (1) whether serum Etn levels in animals were regulated according to demands made by the body; i.e., while Etn concentrations are low in the normal adult, they are elevated when there is a significant population of proliferating Etn-requiring cells, (2) whether supplementing Etn promoted growth of these cells, and (3) whether Etn affected cellular phospholipid metabolism. We used two model systems, one consisted of rats at varying ages from E-19 to 7-week-old, and the other consisted of rats whose livers were at various stages of regeneration.

Using rats of various ages, we found that fetuses and newborns had significantly higher serum Etn levels than mature animals, and that changes in numbers of proliferating hepatocytes *in vivo* correlated well with changes in Etn concentrations in serum. Dickinson et al. (1965, 1970) showed that in humans, plasma Etn concentrations were strikingly different (by approximately 30 fold) between adults and newborns, although those of amino acids were similar. Their data further showed that, although the average Etn level dropped to 1/3 of that of a newborn within 3 days after birth, the level at day 3 was still on an average of 10 times higher than that of the adult. In the rat, we found that serum Etn concentrations of E-19 fetuses and newborns were 2–3 times higher than that of 7-week-old animals. Immediately after birth, levels temporarily decreased. This may be due to the fact that blood of day 0 samples was withdrawn a few hours after birth, but before the animals started nursing. In general, newborns also had higher concentrations of most free amino acids than adults. Examination of Etn content in blood withdrawn from fetuses and from an artery, the placenta, and the umbilical cord of pregnant mothers suggested that the placenta acted as a selective barrier resulting in different Etn concentrations between mother and fetus.

Mother's milk immediately after parturition contains significantly higher concentrations of Etn and P/Etn than those at 2 weeks after parturition. These results suggested that a high Etn level in fetuses might be maintained by a regulatory mechanism.
present in the placenta, and that the high level in newborns might be maintained by consuming milk that contains high levels of Etn and PEtn.

We showed dynamic changes in membrane phospholipids during the perinatal period when liver growth was prominent. In particular, day 2 liver contained significantly less PC than in E-19 fetuses. This may be because the supply of nutrients from the mother is sufficient at around the time of birth, while that from milk immediately after birth is insufficient.

Examination of PHed animals showed that PH increased serum Etn concentrations; 4 h after surgery Etn concentrations were at their highest, and remained high for at least 24 h (Fig. 6A). Further, we found that concomitant with an increase in serum Etn concentrations, the amount of PE also increased in the liver (Fig. 6B). PH also profoundly affected the metabolism of PC. The amount of PC in the liver was decreased by almost a half 2 h after surgery. When hepatocytes are brought into primary culture, degradation of PC is stimulated due to activation of phospholipases C and D. Diacylglycerol and phosphatidic acid (PA) are involved in stimulation of cell proliferation in culture (Exton, 1990, 2000; Jamil et al., 1993). It is not clear how much of the decrease in the amount of PC in PHed animals can be attributed to activation of phosphatases. It is most likely that a decrease in PC levels occurs because synthesis in the remaining liver is not sufficient to meet the increased demand for its own regeneration as well as that by other organs in the body. The above mentioned alteration in hepatocyte phospholipids takes place earlier than the initiation of DNA synthesis, which takes place ~18 h after surgery (Bucher and Swatfield, 1964; Lieberman and Kane, 1965).

We have previously shown (Kume and Sasaki, 2006) that Etn stimulated DNA synthesis in a dose-dependent manner between 0–50 μM. The present study found that serum Etn concentrations in normal adult rats were below 30 μM, which is a suboptimum level. However, it was elevated to 50 μM or higher in perinatal or PHed animals, and these elevated levels were obviously sufficient to support accelerated levels of PE synthesis. Etn supplementation in regenerating livers enhanced the regeneration process by further stimulating PE and PC synthesis. Therefore, the maximum Etn concentration that stimulates hepatocyte proliferation in vivo is likely to be higher than 50 μM (the concentration that stimulates hepatocyte proliferation maximally in vitro).

In many cell types, including hepatocytes, de novo PE synthesis (via decarboxylation of phosphatidylserine) is not sufficient to sustain cell growth unless Etn supplied in the blood is utilized to synthesize PE via CDP-Etn or base-exchange reactions (Kano-Sueoka and King, 1987). We have previously shown that when these cells are starved of Etn, the content of membrane PE is significantly reduced and proliferation slows down because cellular functions involving membranes such as protein kinase C activation or signaling via EGF receptors become abnormal (Kano-Sueoka et al., 1990, 1993; Ajikata et al., 2002; Kume and Sasaki, 2006). Even maintaining cytoskeletal structures requires a proper membrane phospholipid environment (Kano-Sueoka et al., 2001). It is therefore quite possible for the cells to have a regulatory mechanism(s) in vivo to maintain levels of circulating Etn that are suitable for varied physiological conditions.

In both neonatal and PHed animals, the amount of PC found in the membranes was significantly decreased, although only for a short period of time. The relative amount of PE in these membranes was accordingly higher than normal. This is likely to bring about changes in fluidity, surface charge, or hydrophobicity of the membrane bilayer. However, it is not known how the low PC membrane behaves; for example, in cell signaling.

We analyzed Etn contents in commercially available fetal bovine, calf, and adult horse sera, and found that their levels were 8, 3, and 3 μM, respectively (unpublished results). Fetal bovine serum contains 3 times as much Etn as that present in older animals, and calf and adult horse sera contain amounts equivalent to that in adult humans. The adult level of Etn in rats is much higher than that in humans, cows and horses, and this may be because the metabolic rate of the rat is much higher than those of large animals. The adult level may be low because Etn controls the rate of growth of Etn-requiring cells where a population of Etn-requiring cells is at a steady state in healthy individuals.

PE can be taken up by cells in culture, and can replace requirements for Etn (Van der Haegen et al., 1989). Therefore, PE content in serum is also important when considering the regulation of serum Etn levels. We analyzed PE content in the serum of 2-day-old and adult rats. Interestingly, both sera contained little if an PE although they contained substantial amounts of other phospholipids such as PC and sphingomyelin (unpublished results). A moderate degree of starvation but with the addition of supplements to drinking water with up to 1 mM Etn hardly changed the concentration of serum Etn (unpublished results). Taken together, this suggested that the serum Etn level was well regulated in the body, although it remains unclear what regulates it and how. Some signal(s) might stimulate the activation of protein kinase C that in turn, stimulate the activation of phospholipase C and D to decompose PE, finally raising Etn concentration in the blood.

Conclusions

The evidence here supports our belief that the concentration of serum Etn is well regulated, and that it correlates with the numbers of proliferating Etn-requiring cells in vivo as seen in the case of hepatocytes. Furthermore, our findings that Etn supplementation after PH stimulated phospholipid metabolism and promoted regeneration suggests that Etn might be useful in promoting repair of the liver, and perhaps repair of other organs containing significant numbers of Etn-requiring cells.

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