Postnatal development of gastric aromatase and portal venous estradiol-17β levels in male rats

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Abstract

Gastric parietal cells synthesize and secrete estradiol-17β (E_2) into gastric veins joining the portal vein, and a large amount of gastric E_2 first binds to its receptors in the liver. However, the role of the gastric E_2 is not entirely clear during postnatal development. The objective of this study was to reveal the onset of aromatase and other steroid-synthesizing enzymes in the gastric mucosa; to determine the period of rising E_2 levels in the portal vein; and to further understand the relationship between gastric E_2 and liver estrogen receptor α (ERα). The immunoblot bands and the immunohistochemistry of gastric mucosa revealed that aromatase protein began to express itself at 20 days and then increased in the levels of aromatase protein from 20 days onward. Expression of mRNAs for gastric aromatase (*Cyp19a1*) and other steroid-synthesizing enzymes, 17α-Hydroxylase (*Cyp17a1*) and 17β-hydroxysteroid dehydrogenase (*HSD17b3*), also increased similar to the increment of aromatase protein. Portal venous E_2 levels were elevated after 20 days and increased remarkably between 23 and 30 days, similar to gastric aromatase mRNA levels. The E_2 level was approximately three times higher at 40 days than that at 20 days. The liver weight and *Esr1* levels began to increase after 20 days and the increment was positively correlated with the change of portal venous E_2 levels. These findings suggest that some changes may occur around 20 days to regulate the gastric E_2 synthesis and secretion.

Key Words

- development
- stomach
- aromatase
- estradiol-17β

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Introduction

Estrogen is an important factor to regulate the postnatal sexual maturation in the rat. For half a century, it has been reported that the steroid hormone evokes functions of uterine tissue, the brain, and the pituitary during postnatal development (Ramirez & Sawyer 1965, Smith & Davidson 1968, Baker & Kragt 1969, Döhler & Wuttke 1975, Andrews *et al.* 1981, Döcke *et al.* 1984). The enhanced expression of estrogenic effects during postnatal maturation processes could be due to a change in estrogen target tissues in response to the amount of plasma estrogen. However, it has been shown that the estrogen target tissues are maturated by an increased plasma estrogen that is secreted from uncertain tissues.

In the metabolic pathway of steroidogenesis, progesterone is converted into estradiol-17β (E_2) via androgens, enzymes, 17α-hydroxylase, 17β-hydroxysteroid dehydrogenase (17β-HSD), and aromatase. Though the aromatase activity in testicular Sertoli cells and granulose

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cells of the ovary is higher than other tissues in adult rats (Dorrington & Armstrong 1975, Erickson & Hsueh 1978, Fukuda *et al.* 1979), the specific activity of aromatization in testis and ovary is very low in neonatal and immature rats. The activity in the hypothalamus is several times higher than that in any other tissue including the testis and ovaries of fetal rats (George & Ojeda 1982). The fact that neural E_2 can stimulate the growth and balancing of hypothalamic neurons suggests that locally formed estrogen may be involved not only in sexual differentiation but also in more general aspects of brain development (Toran-Allerand 1976, Arai & Matsumoto 1978, George & Ojeda 1982), but may not be enough to secrete into the systemic circulation to maturate estrogen target tissues. It has been reported that the gastric parietal cells aromatize androgens to estrogens by the aromatase and that the specific aromatase activity in gastric mucosa is corresponding to that in the ovary in adult rats (Ueyama *et al.* 2002). Gastric parietal cells secrete a large amount of estrogens into the portal vein and then about 90% of the gastric estrogen is bound to estrogen receptors (ERs) in the liver. Therefore, the amount of gastric estrogen flow into the systemic circulation is dependent on the liver function (Kobayashi *et al.* 2013). It is widely accepted that estrogens are rendered biologically inactive when they are bound to plasma estrogen binding protein or α-fetoprotein in fetal and neonatal animals (Michel *et al.* 1974, Radanyi *et al.* 1977). The amount of main plasma estrogen binding protein, a α-fetoprotein being produced from yolk sac and liver, is highest at the end of fetal periods and exponentially declines in concentration during a few days of postnatal life (Masseyeff *et al.* 1975, Germain *et al.* 1978). In this study, it was hypothesized that the stomach might be a main organ to produce E_2 after diminishing plasma estrogen binding proteins and that the gastric E_2 might have an important role for liver function during postnatal development.

The research described in this article was designed to define the role of gastric estrogen regulating serum estrogen levels during postnatal development of male rats. The results of this study suggest that gastric E_2 and liver function are important factors for the serum estrogen levels in the systemic circulation during the postnatal development of rats.

Materials and methods

Animals

Wistar male rats (Japan SLC, Inc., Shizuoka, Japan) from 15 to 40 days of age were used. All animals were housed in air-conditioned quarters with a 12 h light:12 h darkness cycle (light 0700 to 1900 h) and given a standard pellet diet and tap water *ad libitum*. All animals were killed at 0800 h at each day under sodium pentobarbital anesthesia (40 mg/kg, Abbott Co Ltd). Blood was taken from the portal vein and abdominal artery. The liver and gastric mucosae were then removed; each animal group consisted of four rats. All procedures were performed in accordance with the institutional guidelines and approved by the animal research ethics committee at Yamagata University.

Western blotting

Gastric mucosa were homogenized in a lysis buffer containing a protease inhibitor (1 mM phenylmethylsulphonyl fluoride, Sigma), and the protein concentration was estimated using the Bromopyrogallol Red-molybdenum (VI) complex protein determination kit (Wako Pure Chem, Osaka, Japan). For immunoblotting, 20 μg total protein were separated on 10% SDS–PAGE. Protein samples were transferred onto PVDF membranes, blocked with 4% non-fat dry milk in TBST, and incubated with the primary antibody against aromatase (1:100 000, AbD Serotec, Oxford, UK) and β-actin (1:10 000, Santa Cruz Biotechnology, Inc.). Followed by reaction with anti-mouse IgG coupled to peroxidase (Nichirei, Tokyo, Japan), blots were revealed with ImmunoStar LD (Wako Pure Chem). Average exposure time was 1 min.

Immunohistochemistry

For immunohistochemical staining, the stomach was fixed overnight at 4 °C in Bouin's solution without acetic acid. Tissues were then dehydrated in a graded ethanol series and embedded in Paraplast embedding media (Sigma). Serial sections at a thickness of 5 μm were prepared. Sections at every 50 μm interval were mounted onto glass slides. The sections were immunostained by the peroxidase-labeled antibody method using antibodies against the aromatase (1:10 000, AbD Serotec), and stomach sections from adult male rats were used as positive specimens. The immunostained areas were then expressed in terms of mm^2/mm^2.

Estimation of mRNA levels by real-time RT-PCR

Total RNAs were prepared from the gastric mucosa and the liver by an RNA Isolation Kit (RNAspin mini, GE Healthcare UK Ltd, Buckinghamshire, UK), which included a genomic DNA digestive step by DNase I

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treatment. Expression of steroid-synthesizing enzyme mRNAs in the stomach and of *Esr1* in the liver was determined by real-time RT-PCR. Total RNA (0.5 μg) was converted into cDNA by reverse transcription using a High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems). All primers at a length of 20 bps were designed in-house following sequences based on the nucleotide sequences of the rat and were intron-spanning. Primer sequences used were as follows: cytochrome P450 aromatase (EC: 1.14.14.1, *Cyp19a1*, GenBank accession no. M33986) forward: 5′-ATTGGCATGCACGAGAATGG-3′, reverse: 5′-TGCTGCTTGATGGATTCCAC-3′, 17α-hydroxylase (EC: 4.1.2.30, *Cyp17a1*, GenBank accession no. M31681) forward: 5′-AGTGATCATCGGCCACTATC-3′, reverse: 5′-GAGCTACCAGCATCTGCAAA-3′, 17β-HSD(3) (EC: 1.1.1.62, *HSD17b3*, GenBank accession no. NM054007) forward: 5′-AGAGTGTCATCCACTGCAAC-3′, reverse: 5′-AGTACAGGCTATACAGAGGC-3′, ERα (*Esr1*, GenBank accession no. AB477039) forward: 5′-TCCACTTGATGGCCAAAGCT-3′, reverse: 5′-TTGTAGAGATGCTCCATGCC-3′, and β-actin (GenBank accession no. NM001106409) forward: 5′-TGACAGGATGCAGAAGGAGA-3′, reverse: 5′-TAGAGCCACCAATCCACACA-3′.

Real-time PCR was performed in the Applied Biosystems 7300 Fast Real-Time PCR System with a Power SYBR Green PCR Master Mix according to the manufacturer's protocol. PCR conditions were 10 min at 95 °C, with 40 cycles of denaturation for 15 s at 95 °C and an annealing–extension for 60 s at 60 °C. According to the computational method of Livak & Schmitten (2001), a relative amount of each gene in each animal including β-actin was calculated based on a threshold of cycles and compared with that found in the controls. To confirm the absence of contamination of genomic DNA in total RNAs, PCR was carried out without the RT step and none of the total RNAs could detect PCR products of either primer set within 40 PCR cycles.

Measurement of serum E_2 concentrations

Serum E_2 concentrations were measured using an E_2 EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA). The assay is based on the competition between free E_2 and an estradiol tracer, which is estradiol linked to an acetylcholinesterase molecule at a limited number of estradiol-specific rabbit antiserum binding sites. Briefly, plasma samples and antiserum to E_2 were incubated overnight at room temperature in wells of an anti-rabbit IgG-coated plate. Subsequently, the E_2 linked to acetylcholinesterase was added and kept for 1 h at room temperature. After washing the plate, Ellman's reagent, which contains the substrate to acetylcholinesterase, was added to develop the color for 1.5 h in the dark. The amount of reaction product was measured at 405 nm with the detection range of the system being 0.3–3000 pg/ml E_2. To eliminate nonspecific binding, plasma samples were pre-absorbed by dextran-coated charcoal and measured at the same time.

Statistical analysis

The data were statistically analyzed by one-way ANOVA followed by the Bonferroni/Dunn test and linear regression, as appropriate, using StatView software (Hulinks, Inc., Tokyo, Japan). The *P* value was set at either <0.05 or <0.01.

Results

Western blots of aromatase in gastric mucosa

To detect aromatase, western blot analysis was performed, and the gastric mucosa showed a single band with a molecular mass of 55 kDa, which matched the molecular mass of aromatase (Fig. 1). The immunoblot bands revealed that aromatase expression started at 20 days, and the levels of gastric aromatase protein increased with the advancing age of animals. The expression of aromatase protein reached a plateau at 30 days and maintained high levels onward.

Change of the population of stomach cells immunostained with antibodies to aromatase

Light micrographs of immunostained sections at various ages are shown in Fig. 2A, B, C, D, E, F, G, H, and I. None of stomach cells in gastric mucosa were immunostained with aromatase antibody at 15 days (Fig. 2A), and a few cells

Figure 1
Western blot analysis of gastric aromatase protein during the postnatal developmental rat. Lysates of gastric mucosa at 15-, 20-, 23-, 25-, 27-, 30-, 35-, and 40-day rats were loaded on SDS–PAGE, and aromatase (upper lane) and β-actin (lower lane) were detected by immunoblotting. The experiments were conducted by loading equal amounts of gastric mucosal proteins in each lane. Aromatase protein began to increase after 20 days stepwise.

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appeared to be slightly stained at 20 days (Fig. 2B and C). The number of aromatase cells and their staining intensities were increased with advancing ages after 20 days (Fig. 2D, E, F, G, H, and I). Furthermore, it was observed that the height of mucosal epithelium was thickened twice from 15 to 40 days. Figure 2J shows the change in the population of aromatase cells in gastric mucosa, and its value is expressed in terms of mm^2/mm^2. The number of aromatase cells started increasing around 20 days, and it greatly increased until day 40 in accordance with the increment in epithelial thickness and stomach size.

Expression of steroid metabolic enzyme mRNAs in the gastric mucosa

Gene expression of mucosal enzyme mRNAs, which encode three metabolic enzymes from progesterone to E_2, was determined by a real-time PCR method from 15 to 40 days (Fig. 3). Comparing to the expression of *Cyp17a1* at 15 days, it increased significantly at 20 days and then gradually increased toward the peripubertal period at 40 days (Fig. 3A). The expressions of *HSD17b3* and *Cyp19a1* were low during the first 3 weeks of life but then started to increase significantly at 23 days keeping their elevation until 40 days (Fig. 3B and C respectively). The expressions of *Cyp17a1*, *HSD17b3*, and *Cyp19a1* at 40 days were 25, 10, and 20 times higher than those at 15 days respectively ($P<0.01$). The age-dependent increase in *Cyp19a1* expression was consistent with the results of western blot and immunohistochemistry, and the expression of *Cyp19a1* was positively correlated with that of *Cyp17a1* mRNA ($P<0.0001$, $R^2=0.723$) and *HSD17b3* ($P<0.0001$, $R^2=0.824$). Therefore, E_2 synthesis from progesterone increased age dependently after 23 days.

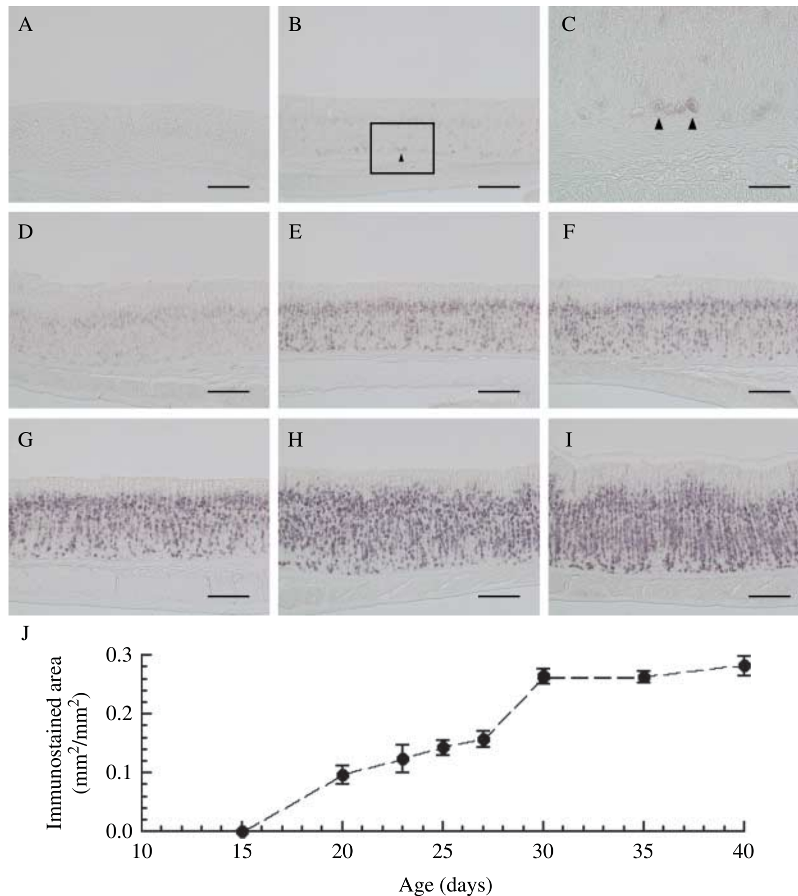

Figure 2
Light photomicrographs of developmental gastric mucosa. Stomach sections at 15 (A), 20 (B, C is the denoted area of B), 23 (D), 25 (E), 27 (F), 30 (G), 35 (H), and 40 (I) days were immunostained with antibodies to aromatase. A few numbers of cells were slightly stained at 20 days (B and C, arrowheads). The number of immunostained cells and their immunostainabilities were increased after 23 days, and the immunostained area reached a plateau from 30 days onward (J). Scale bars in A, B, D, E, F, G, H, and I indicate 200 μm, and the scale bar in C indicates 50 μm. Values show the immunostained area (mm^2/mm^2) and the standard error of over 30 sections in each group.

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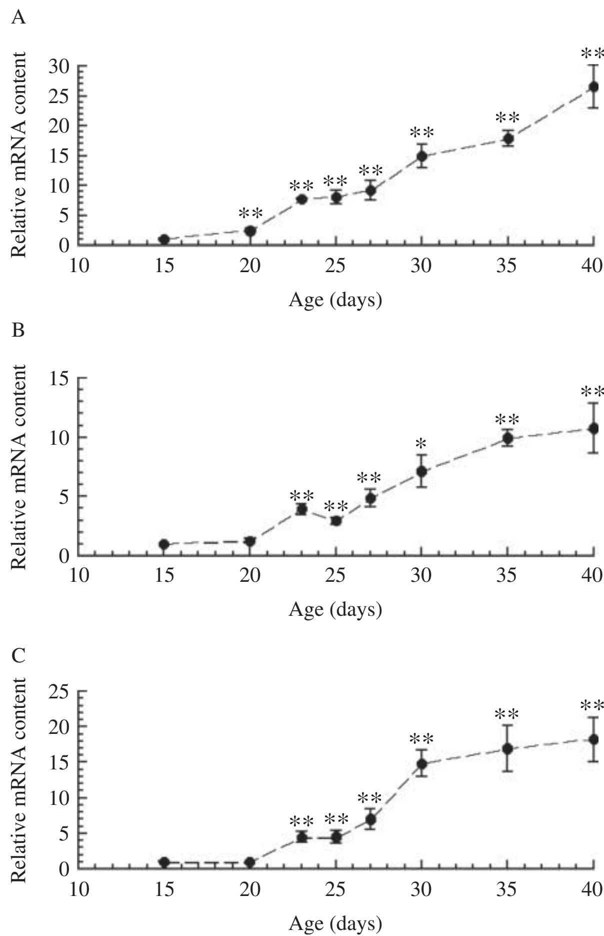

Figure 3
Gene expression level of aromatase (*Cyp19a1*), 17α-hydroxylase (*CYP17a1*), and 17β-HSD(3) (*HSD17b3*) in the gastric mucosa. Expression of estradiol-17β metabolic enzyme mRNAs was quantitatively evaluated by real-time PCR from 15 to 40 days rats. It was observed that the expression of *Cyp17a1* (A), *HSD17b3* (B), and *Cyp19a1* (C) levels started to increase after 20–23 days, and positive correlations of *Cyp19a1* levels with *Cyp17a1* and *HSD17b3* were clarified. Expression of *Cyp19a1* normalized by β-actin. Each point shows the mean, and the bars represent the standard error of four animals. $*P<0.05$, $**P<0.01$ vs 15 days.

Change of serum E_2 levels in the portal vein and abdominal artery

The concentration of serum E_2 in the portal vein and abdominal artery was measured chronologically, and the results are shown in Fig. 4. The concentrations of serum E_2 in the portal vein and artery were 15–35 pg/ml between 15 and 23 days and then those in the portal vein started to elevate gradually from 25 days until 40 days. At 40 days, the value of serum E_2 in the portal vein was 2.3 times higher than that at 23 days ($P<0.05$). On the other hand, the concentration of arterial E_2 decreased form 23 to 27 days and kept constant levels at 10–27 pg/ml between 27 and 40 days. The regression analysis showed that the change of E_2 levels in the portal vein was positively correlated with that of the *Cyp19a1* expression ($P<0.0001$, $R^2=0.599$); however, the change of arterial E_2 levels was not correlative with that of the *Cyp19a1* expression.

Liver growth and expression of ERα mRNA in the liver

Liver weight increased with age from 15 to 40 days (Fig. 5A), and the increment of liver weight was positively correlated with that of serum E_2 in the portal vein ($P<0.0001$, $R^2=0.426$). Gene expression of liver *Esr1* was measured by a real-time PCR method, and the result is shown in Fig. 5B. The expression of *Esr1* increased gradually with age until 27 days and drastically increased until 40 days. The expression of *Esr1* at 40 days was 46 times higher than that at 15 days. The age-dependent change of liver *Esr1* was positively correlated with that of serum E_2 in portal vein ($P=0.0003$, $R^2=0.373$).

Discussion

Most estrogens are believed to be synthesized depending on the expression of aromatase in the ovary, uterus, and placenta in premenopausal females, and there are many reports on the extraovarian tissues and synthesized and secreted estrogens in adults (Nelson & Bulun 2001). In

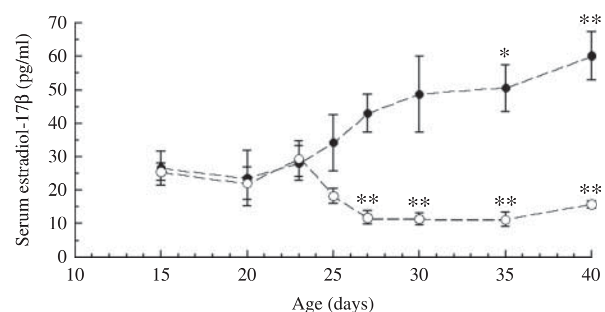

Figure 4
Change of estradiol-17β (E_2) levels in portal vein and artery. The serum E_2 levels were determined by enzyme immunoassay as shown in the Materials and method section. Portal venous E_2 levels (closed circle) started to increase after 23 days and then continued to increase gradually thereafter. Arterial E_2 (open circle) maintained the same level as portal venous E_2 by 23 days and decreased to maintain the constant levels from 27 to 40 days. Changes in E_2 levels in the portal vein showed positive correlations with gastric *Cyp19a1* levels significantly. Each point shows the mean, and the bars represent the standard error of four animals. $*P<0.05$, $**P<0.01$ vs 15 days.

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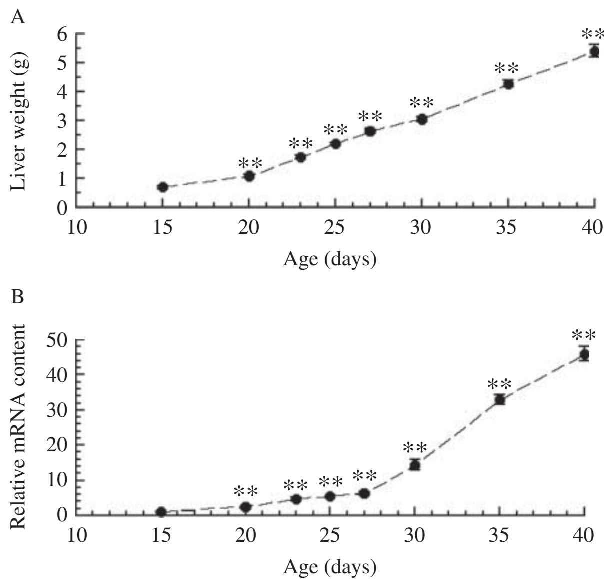

Figure 5
Change of the liver weight and the expression of liver *Esr1*. Liver weight was increased with advancing age of rats (A). The expression of *Esr1* was measured by real-time PCR from 15 to 40 days, and *Esr1* levels began to elevate at 20 days and increased after 30 days considerably (B). Changes of liver weight and *Esr1* level showed positive correlation with portal venous estradiol-17β levels. The expression of *Esr1* was normalized by β-actin expression. Each point shows the mean, and the bars represent the standard error of four animals. $^{**}P<0.01$ vs 15 days.

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immature rats, the ovary in females (Meijs-Roelofs *et al.* 1973) and the adrenal gland in females and males (Baird *et al.* 1969, Weisz & Gunsalus 1973) have been postulated for a long time as the source of E_2; however, it is difficult to explain the developmental change of serum E_2 levels. Recently, it was reported that the stomach is one of the extraovarian tissues synthesized and secreted by E_2, and the E_2 levels in the portal vein are over ten times higher than those in the artery in normal conditions of adult male rats (Ueyama *et al.* 2002, 2004, Kobayashi *et al.* 2013). It is considered that much, if not all, of E_2 in the systemic circulation is derived from the stomach in immature rats. In this study, it becomes apparent that E_2 synthesis and secretion in the stomach begins to elevate after 20 days from aromatase protein investigated by western blotting and immunohistochemistry (Figs 1 and 2), aromatase mRNA levels (Fig. 3), and E_2 levels in the portal vein (Fig. 4). These results are the first findings that focused on the E_2 synthesized and secreted from parietal cells in gastric mucosa during the postnatal development, and it is considered that these findings are associated with previous reports on the serum E_2 level (Germain *et al.* 1978, Greenstein 1992). It is well known that the stomach functions not only as a gastrointestinal system but also as an endocrine system, and gastrin (Thompson 1969), somatostatin (Lucey 1986), and ghrelin (Kojima *et al.* 1999) can be enumerated as the major gastric peptide hormones. These peptide hormones are observed in the stomach in the fetal period by immunohistochemistry (Braaten *et al.* 1976, Hayashida *et al.* 2002), and the speculated onset mechanism of gastric E_2 synthesis is different from that of other gastric endocrine systems. Parietal cells, which also synthesize and secrete gastric acid, are changed ultrastructurally and histochemically after weaning around 20 days (Ekelund *et al.* 1985). Before 20 days, the synthesis and secretion of E_2 is very low, and after 20 days, it increases by some regulatory mechanism of E_2 synthesis and secretion in the stomach.

As the gastric E_2 is synthesized from progesterone by metabolic enzymes, 17α-hydroxylase, 17β-HSD(3), and aromatase (Ueyama *et al.* 2004), the change in serum E_2 in the portal vein is significantly correlated with the expression of the enzyme mRNAs (Fig. 4). A large amount of gastric E_2 binding to liver ERs is normal; however, the level of arterial E_2 is dependent on the pathophysiological conditions of the liver (Kobayashi *et al.* 2013). The present results show that gastric E_2 was correlated with the expression of liver *Esr1* and the liver weight (Fig. 5) and that the serum E_2 levels of systemic circulation, which passes through the liver, was reduced to keep the constant level of arterial E_2 levels. However, the E_2 level in the portal vein at 15 days tends to be slightly higher than that at 20 days when some of the epithelial cells in the stomach start to synthesize E_2. Döhler & Wuttke (1975) reported that serum E_2 levels were high for the first 2 days in newborn male and female rats and were decreased to one fourth and then increased again to two thirds of those in newborns between 9 and 19 days. Furthermore, they described that the question arises why serum E_2 levels are high in newborn rats and increase again after a transient drop in both sexes during the second and third weeks of life. It is considered that the serum E_2 in newborn rats may be maternal and that the high levels of serum E_2 decrease gradually by onset of E_2 synthesis in the stomach. As a result of the combined effects of elevated *Esr1* expression and of liver weight, several hundred-fold increases in the total ERα per liver were observed during this period (Fig. 5). Thus, the serum E_2 levels in the artery decreased with the increase in liver ERα contents in marked contrast to those in the portal vein.

It is well known that serum E_2 in perinatal rats is maternal and binds to plasma estrogen binding proteins (Michel *et al.* 1974). One of them, α-fetoprotein, is well

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known as the major fetal serum protein and has a very high affinity for estrogens (Lai *et al*. 1976, Mizejewski 2004). Although α-fetoprotein is synthesized in fetal liver and yolk sacs abundantly, it reduces exponentially postnatally and regresses by 20 days (Lai *et al*. 1976, Nayak & Mital 1977, Greenstein 1992). On the other hand, the aromatase protein and mRNAs levels reached a plateau per unit at 30 days, it is indicated that E_2 levels in 40 days tend to keep increasing. In Fig. 2A, B, C, D, E, F, G, H, and I, light micrographs of gastric mucosa show a thickening height of the gastric mucosal epithelium. It is feasible to surmise that the elevation is induced by the growth of gastric mucosa and/or the stomach gradually increasing blood flow.

This is the first study to report the changes of gastric E_2 levels in the portal vein during the postnatal development; however, the mechanism of regulation of gastric E_2 is unknown and needs further studies to clarify the overview of gastric E_2.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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