Gastric estradiol-17 β (E_2) and liver ER α correlate with serum E_2 in the cholestatic male rat

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Abstract

Cholestasis is associated with changes in hepatic cholesterol metabolism and serum estrogen levels. Ueyama and colleagues reported that the gastric estradiol-17 β (E_2) level in the portal vein is several times higher than that in the artery. This study aimed to clarify the relationships between gastric E_2 , hepatic estrogen receptor (ER) α and cholesterol metabolism in cholestatic male rats induced by bile duct ligation (BDL). After BDL, serum E_2 levels in the portal vein and artery were measured by ELISA. The gene expression of gastric estrogen-synthesizing enzymes and various hepatic enzymes for cholesterol metabolism were measured by real-time RT-PCR, and gastric aromatase and hepatic ER α proteins were determined by immunohistochemistry and western blotting. Portal E_2 levels increased by 4.9, 5.0, and 3.6 times that of controls at 2 days after BDL (BDL2d, BDL4d, and BDL7d respectively). The change in arterial E_2 levels was positively correlated with that in the portal vein. Under these conditions, the expression of hepatic *Ers1* (ER α) mRNA and protein was significantly reduced in a negative correlation with serum E_2 levels in the portal vein after BDL. The expression of hepatic male-specific cytochrome P450 (CYP) genes *Cyp2c55* and *Cyp3a2* decreased and female-specific *Cyp2c12* increased after BDL. It is postulated that the increase in gastric E_2 levels, which occurs after BDL, results in the reduction of hepatic ER α , the elevation of arterial E_2 level and leads to cholesterol metabolism becoming sex steroid dependent.

Key Words

- bile duct ligation
- estradiol
- estrogen receptor
- stomach
- liver

Journal of Endocrinology
(2013) 219, 39–49

Introduction

It has been widely accepted that the main sources of estrogen in circulation are testicular Sertoli cells in male rats and granulosa cells of the ovary in female rats (Erickson & Hsueh 1978, Fukuda *et al.* 1979, Dorrington & Armstrong 1995). Among liver disease, cirrhosis, pylemphraxis, and biliary atresia, there is an increase in the serum estrogen levels in males; however, the origin of serum estrogens is unknown. In recent years, Ueyama *et al.* (2002, 2004)

reported the presence of estrogen-synthesizing enzymes including aromatase in gastric parietal cells using immunohistochemistry, *in situ* hybridization, and RT-PCR. Furthermore, they also reported that the gastric aromatase activity in both adult males and female rats is equivalent to that in the ovary, and the estradiol-17 β (E_2) levels in the portal vein were much higher than those found in arterial blood. Then it was suggested that about 90% of gastric E_2 is bound

to estrogen receptors (ER) in the liver. Our recent study reports that the amount of gastric estrogen flow into the systemic circulation depends on the pathophysiological conditions of the liver in portal vein-ligated or partial hepatectomized rats (Kobayashi *et al.* 2013a). Therefore, it is considered that the serum E_2 level is involved in stomach and liver functions.

Cholestasis induced by bile duct ligation (BDL) causes an (up to threefold) increase in serum E_2 levels in male and female rats (Chen *et al.* 1995, 1998). Furthermore, female rats with both ovariectomy and BDL have increased serum E_2 level compared with ovariectomized rats (Alvaro *et al.* 2002a). A part, but not all, of serum E_2 originated from ovary E_2 in female rats; however, the origin of the E_2 that increased the portal E_2 level in males remains uncertain. It has been reported that hepatic microsomal cytochrome P450 (CYP)-mediated hydroxylation, 16 α -hydroxylase, was reduced with portal bypass and it has been suggested that decreased hepatic 16 α -hydroxylation would enhance the accumulation of E_2 in serum (Murray *et al.* 1988, Cantrill *et al.* 1989). Hepatic CYP proteins have an important role in the metabolism of steroid hormones and bile acids, and some of them are expressed in a sex-specific manner. In CYP genes of the male rat liver, for instance, *Cyp2c55* and *Cyp3a2* are the predominant forms of CYP, and they are expressed at very low levels in females (Waxman 1988, Chen *et al.* 1998). Conversely, *Cyp2c12* is the major form in the female liver and is expressed minimally in males (Waxman 1988, Mode *et al.* 1989, Chen *et al.* 1998). *Cyp2c55* is regulated by androgens, and hepatic levels are maintained in the adult male rat by serum androgen (Einarsson *et al.* 1973, Kamataki *et al.* 1983, Mode *et al.* 1989). Chen *et al.* (1995, 1998) reported the downregulation of male-specific genes, *Cyp2c55* and *Cyp3a2*, together with a decrease in serum testosterone levels in male rats with BDL and supported the suggestion that the elevation of serum E_2 levels was caused by dysregulation of the constitutive expression of hepatic CYP genes in portal vein ligation (PVL) and BDL rats. The gastrohepatic axis of E_2 , however, was not considered in that study a decade ago.

In spite of all this evidence indicating the role of the stomach in the gastrohepatic system, studies to date have not included measurements of E_2 secretion by the rat stomach or the temporal relationships of gastric E_2 to liver functions. This study, therefore, deals with measurement of the E_2 level in the portal vein and the level of E_2 in peripheral plasma, i.e., abdominal aorta. This study is also an attempt to elucidate the effect of BDL on gastric E_2

synthesis and to establish the relationship between gastric E_2 synthesis and hepatic cholesterol metabolism.

Materials and methods

Animals

Wistar male rats (Japan SLC, Inc., Shizuoka, Japan) were housed in air-conditioned quarters with a 12 h light:12 h darkness cycle (light 0700 to 1900) and given a standard pellet diet and tap water *ad libitum*. All procedures were performed in accordance with the institutional guidelines and approved by the animal research ethics committee at Yamagata University.

Surgery

BDL was performed on two groups according to the method of Chen *et al.* (1995, 1998). To produce biliary obstruction, animals were anesthetized with sodium pentobarbital (40 mg/kg, Abbott Co. Ltd.), the abdomen was opened by a midline incision, and the common bile duct was ligated twice and transected at 2 (BDL2d), 4 (BDL4d), and 7 days (BDL7d) before termination. After surgery, animals were allowed to recover and were given free access to food and water as required. Experiments were performed at 0800 h at 12 weeks of age under sodium pentobarbital anesthesia. Control animals were sham operated on and otherwise handled in an identical fashion. Blood was taken from the portal vein and the abdominal artery, and all animals were killed by exsanguination. The gastric mucosa and liver were then removed; each animal group consisted of four rats.

Measurement of serum total bilirubin and E_2 concentrations

Total bilirubin concentrations in the serum were measured by the clinical chemical analysis Spotchem (ARKRAY, Inc., Tokyo, Japan). Total bilirubin in serum samples react with a diazonium salt in reagent and produces azobilirubin which produces a red pigment on the reagent strip. This red pigment was analyzed by colorimetric determination at 550 nm. Controls were calculated by an already-known concentration in the apparatus, and total bilirubin value was calculated based on that control. 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, for 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 . We used the normal adult rat serum treated with dextran-coated charcoal for the control solvent to dilute E_2 to make the standard curve. All samples treated without dextran-coated charcoal were measured by an ELISA method just as they were and serum E_2 concentration was calculated from the standard curve. The intra- and inter-assay coefficient of variation (CV) were determined at multiple points on the standard curve. The average of %CV intra-assay variation is 14.26 ± 3.08 and inter-assay variation is 11.29 ± 3.59 . %CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

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 treatment. Expressions of steroid-synthesizing enzyme mRNAs in the stomach as well as of *Ers1* and cholesterol metabolic enzymes in the liver were determined by real-time RT-PCR. Total RNA (0.5 µg) was converted into cDNA by RT using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). All primers at a length of 20 bps were designed in the laboratory following sequences based on the nucleotide sequences of the rat and were intron-spanning. Primer sequences are shown in Table 1. The nomenclature of CYP genes was used according to the report of Nelson *et al.* (1996). After designing and preparing the primer sets, it was confirmed that each primer set acts for the target gene analysis. We also investigated the efficiency of amplification of *b-actin* is $94.8 \pm 0.14\%$ in gastric mucosa or liver, and the efficiency of amplification was almost equal in both the house-keeping gene and target genes.

Table 1 Oligonucleotide primers for real-time PCR

Primer sequence	Product	Accession no.	EC
<i>Cyp19a1</i> (aromatase) Forward: ATTGGCATGCACGAGAATGG Reverse: TGCTGCTTGATGGATTCCAC	140	M33986	1.14.14.1
<i>Cyp17a1</i> (17 α -hydroxylase) Forward: AGTGATCATCGGCACTATC Reverse: GAGCTACCAGCATCTGCAAA	150	M31681.1	4.1.2.30
<i>Hsd17b3</i> (17 β -hydroxysteroid dehydrogenase type 3) Forward: AGAGTGTCATCCACTGCAAC Reverse: AGTACAGGCTATACAGAGGC	144	NM054007	1.1.1.62
<i>Esr1</i> (estrogen receptor α) Forward: TCCAATTGATGGCCAAAGCT Reverse: TTGTAGAGATGCTCCATGCC	96	AB477039	
<i>b-actin</i> Forward: TGACAGGATGCAGAAGGAGA Reverse: TAGAGCCACCAATCCACACA	114	NM001106409	
<i>Cyp2c55</i> (steroid 16 α -and 2 α -hydroxylase) Forward: ATGGATCCAGTCCTAGTCCT Reverse: AAAGCTCTGTCTCCAGAGTGAG	82	BC088146.1	1.14.14.1
<i>Cyp2c12</i> (steroid disulfate 15 β -hydroxylase) Forward: GGATCCATTTGTGGTCTTGG Reverse: AGGGCTTGGTCTCCATAGAT	70	BC089790.1	1.14.14.1
<i>Cyp3a2</i> (progesterone 6 β -hydroxylase) Forward: ACCTGCTTTCAGCTCTCAC Reverse: GTTCCAAGTCGGTAGAGAAG	76	BC089765.1	1.14.14.1
<i>Cyp7a1</i> (cholesterol 7 α -hydroxylase) Forward: GGATCCATTTGTGGTCTTGG Reverse: AGGGCTTGGTCTCCATAGAT	89	NM_012942.2	1.14.13.17
<i>Akr1c18</i> (3 α -hydroxysteroid dehydrogenase) Forward: TATCTGTGACACATGGGAGG Reverse: AGGTGACATTCCACCTGGTT	156	D17310.1	1.1.1.53

Real-time RT-PCR was performed in the Applied Biosystems 7300 Fast real-time RT-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 & Schmittgen (2001), a relative amount of each gene in each animal including *b-actin* was calculated based on a threshold cycle (Ct) and compared with that found in the controls. Ct of *b-actin* displayed stable values in all groups. 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.

Determination of antibody-positive area by immunohistochemistry

For immunohistochemical staining, the stomach and liver were 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 from sham-operated controls and from BDL7d rats. The sections were reacted by the peroxidase-labeled antibody method using antibodies against aromatase (1:10 000, MCA2077S, AbD Serotec, Oxford, UK) and ERα (1:300, sc-542, Santa Cruz Biotechnology, Inc.) and incubated overnight at 37 °C. An antibody with anti-mouse or anti-rabbit IgG coupled to peroxidase (Nichirei, Tokyo, Japan) was then reacted for 1 h at 37 °C. Over 30 sections from four animals in each group (eight to ten sections from a single animal on an average) were examined. The immunostained areas were then expressed in terms of mm²/mm² and cells/mm². As a negative control of immunohistochemistry, nearly adjacent sections were incubated without the primary antibody and then incubated with anti-mouse IgG coupled to peroxidase as the second antibody. No labeling was found in these control sections.

Immunoquantitation of gastric aromatase and hepatic ERα by western blotting

Gastric mucosa and livers were homogenized in a lysis buffer containing a protease inhibitor (1 mM phenyl-methylsulphonyl 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 was separated on 10% SDS-PAGE. Protein samples were transferred onto PVDF membranes and blocked with 4% non-fat dry milk in TBST. The membrane was incubated with the primary antibody against aromatase (1:100 000), ERα (1:10 000) and β-actin (1:10 000, sc-47778, Santa Cruz Biotechnology, Inc.). Following reaction with anti-mouse IgG coupled to peroxidase (Nichirei), blots were revealed with ImmunoStar LD (Wako Pure Chem). Average exposure time was 1 min. Western blotting using PBS without first antibody as negative controls as well as immunohistochemistry were examined. All antibodies have been validated by several references, and these antibodies were used in our previous study in gastric mucosa of rats (Turner *et al.* 2002, Omoto *et al.* 2005, Firestein *et al.* 2008, Kobayashi *et al.* 2013b).

Statistical analysis

The data were statistically analyzed by one-way ANOVA followed by the Student's *t*-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

Effects of BDL on liver weight and serum total bilirubin

After the complete obstruction of the bile duct, the body weight was temporally reduced at BDL2d and BDL4d and recovered to that of sham-operated control rats at BDL7d (Table 2). The control rats gained about 28.6 g of their body weight in the last 7 days. In contrast adrenal weight temporally increased within 7 days of BDL and then reduced to the control levels. Liver weight was gradually increased by BDL and reached 133% of control liver weight at BDL7d. Testis' weight, however, did not change within 7 days of BDL.

Furthermore, BDL rats clearly showed jaundice, as demonstrated by an increased arterial serum total bilirubin level of more than 29-fold (*P*<0.01, Table 2). The serum total bilirubin levels in the artery were the same as those in the portal vein (data not shown).

Changes in serum E_2 levels in the abdominal artery and portal vein

The concentrations of E_2 in the abdominal artery and portal vein were measured after BDL, and the results are

Table 2 Changes of body, organ weight, and serum total bilirubin after BDL. Body, liver, adrenal gland, and testis weight at 2 days (BDL2d), 3 days (BDL3d), and 7 days (BDL7d) after BDL. Serum total bilirubin level in abdominal artery increased by BDL7d. Values are expressed as the mean $\pm$ S.E.M. of five rats

	Control	BDL2d	BDL4d	BDL7d
Body (g)	278.6 $\pm$ 4.6	267.0 $\pm$ 4.7*	258.2 $\pm$ 2.1 [†]	271.9 $\pm$ 2.1
Liver (g)	9.99 $\pm$ 0.22	11.4 $\pm$ 0.25 [†]	12.0 $\pm$ 0.19 [†]	13.3 $\pm$ 0.20 ^{†,§,}
Adrenal (mg)	26.2 $\pm$ 1.1	36.7 $\pm$ 1.3 [†]	29.9 $\pm$ 1.1* [§]	26.4 $\pm$ 0.9 ^{§,}
Testis (g)	1.45 $\pm$ 0.02	1.43 $\pm$ 0.03	1.45 $\pm$ 0.08	1.43 $\pm$ 0.02
Total bilirubin (mg/dl)	0.34 $\pm$ 0.02	5.40 $\pm$ 0.34 [†]	8.54 $\pm$ 0.41 ^{†,§}	10.01 $\pm$ 0.14 ^{†,§,}

* $P < 0.05$, [†] $P < 0.01$ vs control. [‡] $P < 0.05$, [§] $P < 0.01$ vs BDL2d. ^{||} $P < 0.05$, ^{††} $P < 0.01$ vs BDL4d.

shown in Fig. 1. The arterial E_2 level was increased ten fold compared to controls at BDL2d and remained at high levels after the BDL operation (Fig. 1A). The level of serum E_2 in the portal vein was 5.4 times higher than that in the artery of sham-operated control rats. The portal venous E_2 level was also increased 4.9 times higher at BDL2d than that of controls and remained high until 7 days after BDL (Fig. 1B). The level of arterial E_2 positively corresponded to that of portal venous E_2 ($P < 0.0001$, $R^2 = 0.484$). A change in total bilirubin levels in the abdominal artery was also positively correlated with that of portal venous and arterial E_2 ($P < 0.036$, $R^2 = 0.277$ and $P < 0.0002$, $R^2 = 0.635$ respectively).

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 in control and BDL rats by a real-time RT-PCR method. The results normalized by *b-actin* expression are shown in Fig. 2. The expression of 17α -hydroxylase (*Cyp17a1*), which catalyzes progesterone to androstenedione, mRNA increased at BDL2d, decreased at BDL4d, and then returned nearly to control levels at BDL7d (Fig. 2A). In the expression of 17β -hydroxysteroid dehydrogenase type 3 (*Hsd17b3*), which catalyzes androstenedione to testosterone, mRNA significantly decreased at BDL2d and then gradually returned to control levels at BDL7d (Fig. 2B). The expression of aromatase, which catalyzes testosterone to E_2 , mRNA significantly decreased at BDL2d and BDL4d and then increased back to control levels at BDL7d (Fig. 2C). There was no correlation between the change in portal venous E_2 levels and that of *Cyp17a1* and aromatase (*Cyp19a1*) mRNA expression after BDL. The change in portal venous E_2 levels was negatively correlated with that of one *Hsd17b3* expression ($P = 0.0058$, $R^2 = 0.431$).

Immunoquantitation of aromatase protein in the gastric mucosa

Aromatase protein in the stomach at BDL7d was analyzed by the methods of immunohistochemistry and western blotting. Light micrographs of immunostained sections are shown in Fig. 3A, B, C, and D. Staining intensities and the number of immunostained aromatase cells increased with BDL. Figure 3E 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-positive cells increased to 113% at BDL7d. There is no difference in the epithelial thickness and stomach size (data not shown).

To detect aromatase protein, western blot analysis was performed, and the results are shown in Fig. 4. The gastric mucosa showed a single band with a molecular mass of 55 kDa, which matched the molecular mass of aromatase (Fig. 4A). The immunoblot bands revealed that the levels of gastric aromatase protein normalized by β -actin increased to 152% of control levels at BDL7d ($P < 0.05$, Fig. 4B).

Figure 1 Changes in arterial and portal venous estradiol-17 β (E_2) levels after BDL. Arterial E_2 level increased ten fold compared to that of controls at BDL2d, and high levels remained after the BDL operation (A). Portal venous E_2 level also increased to 4.9 times higher at BDL2d than that of controls and remained high until 7 days after BDL (B). Each column shows the mean, and the bars represent the S.E.M. of four animals. ^a $P < 0.05$, ^b $P < 0.01$ vs control, ^c $P < 0.05$, ^d $P < 0.01$ vs BDL2d, and ^e $P < 0.05$, ^f $P < 0.01$ vs BDL4d.

Figure 2

Gene expression levels of *Cyp17a1*, *Hsd17b3*, and *Cyp19a1* in the gastric mucosa. Expression of E₂ metabolic enzyme mRNAs was quantitatively evaluated by the real-time RT-PCR method. The expression of *Cyp17a1* mRNA increased at BDL2d, decreased at BDL4d, and then returned to control levels at BDL7d (A). The expression of *Hsd17b3* mRNA significantly decreased at BDL2d and then gradually returned to control levels at BDL7d (B). The expression of *Cyp19a1* mRNA significantly decreased at BDL2d and BDL4d and then increased to control levels at BDL7d (C). Expressions of steroid metabolic enzyme mRNAs were normalized by *b-actin*. Each result shows the mean, and the bars represent the s.e.m. of four animals. ^a $P < 0.05$, ^b $P < 0.01$ vs control, ^c $P < 0.05$, ^d $P < 0.01$ vs BDL2d, and ^e $P < 0.05$, ^f $P < 0.01$ vs BDL4d.

Expression of mRNA levels for ER α and cholesterol metabolic enzymes in liver

Gene expression of hepatic *Ers1* and cholesterol metabolic enzymes was measured by a real-time RT-PCR method in BDL rats, and the results normalized by *b-actin* expression are shown in Fig. 3. The expression of hepatic *Ers1* reduced to 39 and 29% of control levels at BDL2d and BDL4d respectively, recovering to 91% of control level at BDL7d

(Fig. 5A). The change by BDL negatively correlated with that of E₂ levels in the portal vein ($P = 0.0152$, $R^2 = 0.353$); however, it was not correlated with that of E₂ levels in the abdominal artery and serum total bilirubin levels.

One of the cholesterol metabolic enzymes in the liver, cholesterol 7 α -hydroxylase (*Cyp7a1*) that catalyzes cholesterol to 7 α -hydroxycholesterol through bile acid synthesis, reduced its mRNA at BDL2d and BDL4d and then increased by 176% of control levels at BDL7d (Fig. 5B). The change of *Cyp7a1* was not correlated with that of E₂ levels in the portal vein. The gene expression of 3 α -hydroxysteroid dehydrogenase *Akr1c18*, which catalyzes 7 α -hydroxycholesterol to 5 β -cholestane-3 α ,7 α -diole, was reduced to 29–51% of control levels in BDL rats (Fig. 5C), and the reduction was negatively correlated with serum E₂ levels in the portal vein ($P = 0.0073$, $R^2 = 0.413$). The expression of steroid 16 α - and 2 α -hydroxylase (*Cyp2c55*), which is a

Figure 3

Light photomicrographs of the gastric mucosa. Stomach sections from control (A, C is the boxed area of A) and BDL7d (B, D is the boxed area of B) were immunostained with antibodies to aromatase. The area of immunostained cells and their immunostainabilities increased in BDL. Scale bars in Figs A and B indicate 200 μ m, and the scale bar in Figs C and D indicate 50 μ m. The immunostained area occupied with aromatase cells was measured and is shown in values in terms of mm²/mm² (E). Each column and bar shows the mean and s.e.m. respectively of over 30 sections coming from four animals in each group (eight to ten sections from a single animal on average). ** $P < 0.01$ vs control.

Figure 4

Western blot analysis of gastric aromatase protein in BDL rats. Lysates of gastric mucosa in control and BDL7d rats were loaded on an SDS-PAGE and aromatase (upper lane) and β -actin (lower lane) were detected by immunoblotting (A). The experiments were conducted by loading equal amounts of gastric mucosal proteins in each lane. Aromatase protein increased at BDL7d (B). The aromatase expression was normalized by β -actin expression. Each column and bar shows the mean and s.e.m. respectively of four animals. * $P < 0.05$ vs control.

male-specific monooxygenase, was reduced to $<10\%$ of controls after BDL (Fig. 5D), and the reduction was negatively correlated with serum E_2 levels in the portal vein ($P = 0.0041$, $R^2 = 0.456$). The expression of progesterone 6 β -hydroxylase (*Cyp3a2*), which is another male-specific monooxygenase, was reduced by BDL (Fig. 5E), and the reduction was negatively correlated with serum E_2 levels in the portal vein ($P = 0.011$, $R^2 = 0.380$).

In contrast, the expression of steroid disulfate 15 β -hydroxylase (*Cyp2c12*), which is a female-specific monooxygenase, increased suddenly to 757% of control levels at BDL2d and dropped to 196 and 333% at BDL4d and BDL7d respectively (Fig. 5F). The expression of *Cyp2c12* was positively correlated with serum E_2 levels in the portal vein ($P = 0.0162$, $R^2 = 0.348$). Among the above enzymes, the expression of *Akr1c18*, *Cyp2c55*, and *Cyp3a2* mRNA was negatively correlated with the change in serum total bilirubin levels ($P = 0.0008$, $R^2 = 0.562$; $P = 0.0001$, $R^2 = 0.818$; and $P = 0.02608$, $R^2 = 0.304$ respectively). The expression of *Akr1c18*, *Cyp2c55*, and *Cyp3a2* mRNA was negatively correlated with both serum E_2 and bilirubin levels and that of *Cyp7a1* was not correlated with serum E_2 or bilirubin levels.

Immunoquantitation of ER α protein in the liver

Liver sections were immunostained with the antibody to ER α and compared with sham-operated and BDL rats at 7 days after the operation (Fig. 6A and B). A number of nuclei in hepatocytes were well stained in the sham-operated control rats; the staining intensities and the number of immunostained nuclei were decreased at BDL7d. The population of nuclei immunostained with

ER α antibody was measured, and the result was expressed in terms of cells/mm² (Fig. 6C). The population of immunostained nuclei was decreased to 84% of the control level at BDL7d. The number of immunostained nuclei with anti-ER α antibody was reduced to 80% of control levels at BDL7d.

A western blot analysis was performed to detect ER α protein, and the results are shown in Fig. 7. The gastric mucosa showed a single band with a molecular mass of 67 kDa, which matched the molecular mass of ER α (Fig. 7A). The levels of ER α protein normalized by β -actin decreased to 68% of controls at BDL2d and gradually

Figure 5

Gene expression levels of *Ers1*, *Cyp7a1*, *Akr1c18*, *Cyp2c55*, *Cyp2c12* and *Cyp3a2* in the liver. Expression of *Ers1* and cholesterol metabolic enzyme, *Cyp7a1*, *Akr1c18*, *Cyp2c55*, *Cyp2c12* and *Cyp3a2* mRNAs were quantitatively evaluated by a real-time RT-PCR method. Liver *Ers1* mRNA reduced to one third of control levels at BDL2d and BDL4d and recovered to 91% of control levels at BDL7d (A). *Cyp7a1* mRNA reduced to 54 and 71% at BDL2d and BDL4d and then increased to 176% of control levels at BDL7d (B). The expression of *Akr1c18* mRNA reduced to 29–51% of control levels in BDL rats (C). Male-specific *Cyp2c55* and *Cyp3a2* mRNAs decreased (D and E respectively), and female-specific *Cyp2c12* mRNA increased by BDL (F). The expression of mRNAs was normalized by *b-actin* expression. Each column and bar shows the mean and s.e.m. respectively of four animals. ^a $P < 0.05$, ^b $P < 0.01$ vs control, ^c $P < 0.05$, ^d $P < 0.01$ vs BDL2d, and ^e $P < 0.05$, ^f $P < 0.01$ vs BDL4d.

Figure 6

Light photomicrographs of liver ER α cells in BDL and control rats. Liver from control (A) and BDL (B) rats were immunostained with the antibody to ER α and the number of immunostained ER α cells and their immunostainabilities decreased in BDL rats. The population of immunoreactive ER α cells was measured by image analysis (C). BDL significantly decreased the number of ER α cells in comparison with the controls. Scale bars indicate 50 μ m. Values show the immunostained area (cells/mm²), and the s.e.m. of over 30 sections from four animals in each group (eight to ten sections from a single animal on average). ** $P < 0.01$ vs control.

recovered to 73 and 85% of controls at BDL4d and BDL7d respectively after BDL (Fig. 7B). The change in hepatic ER α protein was negatively correlated with that of portal venous E_2 ($P < 0.0098$, $R^2 = 0.389$), but not with that of arterial E_2 and serum bilirubin. The results revealed that BDL causes the reduction of the ER α protein expression in correlation with portal venous E_2 level.

Discussion

These results show that E_2 levels in the abdominal artery increased in male rats after BDL (Fig. 1A), this increase in the systemic circulation level is in agreement with previous reports (Chen *et al.* 1995, 1998). The results also show for the first time that the level of E_2 in the portal vein increased to 490% of control levels at BDL2d (Fig. 1B), and the increased level was 2.6 times higher than that of the systemic circulation. It has been considered that a large part, if not all, of E_2 in systemic circulation in the male rats is derived from gastric E_2 synthesized in parietal cells of the gastric mucosa (Ueyama *et al.* 2002, 2004, Kobayashi *et al.* 2013a,b). Thus, it is a feasible explanation that the increase in E_2 level in the artery is attributable to the increase in gastric E_2 in the portal vein after BDL because E_2 levels in the systemic circulation are positively correlated with those in the portal vein.

It is known that estrogens are involved in liver regeneration, development, and/or growth (Francavilla *et al.* 1986, 1989a,b), and that the chronic administration of estrogens for pharmacological purposes in adults results in an enlargement of liver mass (Eagon *et al.* 1985, Alvaro *et al.* 2000). It has been reported that E_2 synthesis in the stomach starts to elevate after about 20 days in postnatal development male rats and these changes are positively correlated with the liver weight and the expression of *Ers1* mRNA in the liver (Kobayashi *et al.* 2013b). Thus, it is considered that gastric aromatase and portal E_2 play an important role in liver growth and/or function as well as in the BDL study. In addition, it has been reported that BDL induced significant increases in liver weight (Dueland *et al.* 1991). In this study, although the body weight reduced at BDL2d and BDL4d, the liver weight significantly increased after the BDL operation (Table 2). As the E_2 level in the portal vein increased after BDL, it is suggested that gastric E_2 may participate in the increase in liver weight.

As gastric E_2 is synthesized from progesterone by metabolic enzymes (Ueyama *et al.* 2004), this study analyzed the expression of *Cyp17a1*, *Hsd17b3*, and *Cyp19a1* mRNAs in the gastric mucosa after BDL (Fig. 2).

Figure 7

Western blot analysis of hepatic ER α protein in BDL rats. Liver lysates in control and BDL rats were loaded on an SDS-PAGE, and ER α (upper lane) and β -actin (lower lane) were detected by immunoblotting (A). The experiments were conducted by loading equal amounts of liver proteins in each lane. ER α protein decreased by BDL (B). The ER α expression was normalized by β -actin expression. Each column and bar shows the mean and s.e.m. respectively of four animals. ^a $P < 0.05$, ^b $P < 0.01$ vs control and ^c $P < 0.05$ vs BDL2d.

In these metabolic enzymes, the expression of *Hsd17b3* mRNA reduced at BDL2d and both *Cyp17a1* and *Hsd17b3* mRNA reduced at BDL4d. The results, therefore, show that the reduced production of testosterone in the stomach may occur to some extent by 4 days after BDL. It is suggested that BDL reduces conversion from progesterone to testosterone rather than increasing aromatization of testosterone to E_2 in the stomach. This event is in agreement with the report that serum testosterone is reduced to 25–80% of control levels within 7 days after BDL in male rats (Chen *et al.* 1995, Yang *et al.* 2011). At BDL7d, the expression of *Hsd17b3* mRNA returned to the control level while *Cyp19a1* mRNA and aromatase protein increased compared with those in controls. The results of this study show that the change in levels of portal venous E_2 is negatively correlated with the expression of *Hsd17b3* mRNA but not with *Cyp17a1* and *Cyp19a1* mRNAs. It also shows that the portal venous E_2 level after BDL might be, if anything, related to a certain extent to E_2 synthesis in the stomach. Furthermore, the portal E_2 levels were positively correlated with the arterial E_2 levels in this study, and it has been reported that the portal venous and arterial E_2 levels were increased in partially hepatectomized rats (Kobayashi *et al.* 2013a). It is assumed that the gastric E_2 in the portal vein flows into the systemic circulation because of the reduction in hepatic ER α , and some gastric E_2 then returns to the portal vein via the gastric circulation to increase portal venous E_2 .

The expression of *Ers1* mRNA decreased to one third of control levels at BDL2d and BDL4d (Fig. 5A), and the reduction of ER α protein expression was observed in BDL rats (Figs 6 and 7). The reduction of hepatic *Ers1* mRNA and protein is negatively correlated with the increased E_2 level in the portal vein, but not with that in the artery or serum bilirubin level. These results are in agreement with the reports that BDL induces a decrease in ER α protein in hepatocytes (Alvaro *et al.* 2000, 2002b) and that E_2 increases the hepatic ER levels dose-dependently within low (physiological) ranges and decreases the ER levels with reduction of the hepatic enzyme activities at high (pharmacological) doses (Parini *et al.* 2000, Stavrus-Evans *et al.* 2001). The present results indicate, therefore, that some gastric E_2 in the portal vein passes through the liver to the systemic circulation without binding to hepatocytes and that the serum E_2 in the artery is then elevated by BDL.

Some liver microsomal CYPs are maintained by serum sex steroid hormones (Dueland *et al.* 1991, Chen *et al.* 1995, Chico *et al.* 1996). Male-specific CYP genes, *Cyp2c55* and *Cyp3a2*, are down-regulated by E_2 , whereas female-specific *Cyp2c12* is up-regulated by E_2 . The present results

show that the expression of *Cyp2c55* and *Cyp3a2* mRNA decreased, while *Cyp2c12* mRNA was increased by BDL in correlation with the change in E_2 levels in the portal vein. The expression of *Cyp2c55* and *Cyp3a2* mRNA is also correlated with that of *Ers1* mRNA and with the level of serum total bilirubin; however, the expression of *Cyp2c12* is neither correlated with that of *Ers1* mRNA nor with the level of serum total bilirubin.

These results indicate that the expression of *Cyp2c55* and *Cyp3a2* mRNA is regulated by serum E_2 and its receptor, *Ers1*; however the expression of *Cyp2c12* mRNA is regulated only by serum E_2 and not by *Ers1* or serum total bilirubin. ER α is present in both hepatocytes and cholangiocytes in the liver, and the reduction rate of ER α protein by BDL is a few times higher in hepatocytes than in cholangiocytes (Alvaro *et al.* 2000). In conclusion, these results raise the possibility that decreasing hepatic ER, although portal E_2 increased when returning from artery, is closely related to not only E_2 levels but also to CYP genes for cholesterol metabolism in the liver. However, all the details of the hepatic cholesterol including estrogen metabolism have not yet been brought to light. Further study is needed to clarify the relationship between ER α and CYP gene expression.

BDL increases the serum total bilirubin as well as E_2 . The major cholesterol degradation pathway is conversion to bile acids in the liver. Cholesterol metabolized to 7 α -hydroxylated bile acids is a principle pathway, and *Cyp7a1* is the initial and rate-determining enzyme in this classic bile acid synthesis pathway. There are contradictory reports that E_2 enhances short-term bile acid synthesis in rat hepatocytes *in vivo* (Chico *et al.* 1996) and that the specific activity of *Cyp7a1* and its mRNA are suppressed by feeding with the bile acids cholic acid, chenodeoxycholic acid, or deoxycholic acid in rats (Ren *et al.* 2003). This study shows that the expression of *Cyp7a1* reduced to 54–71% at BDL2d and BDL4d respectively and was over-expressed to 176% of control levels at BDL7d (Fig. 5B). The gene expression of *Akr1c18*, which acts in the next step of CYP7a1 and catalyzes 7 α -hydroxycholesterol to 5 β -cholestane-3 α ,7 α -diole, was reduced to half by BDL (Fig. 5F). Thus, the bile acid synthesis is likely to be regulated by bile acids and/or E_2 .

The present results in BDL rats show that the elevation of arterial E_2 levels is caused by the increase in gastric and portal venous E_2 levels and the reduction of hepatic ER α expression, and these changes make alterations to the expression of sex-specific CYP genes, *Cyp2c55*, *Cyp2c12*, and *Cyp3a2*, for cholesterol metabolism in the liver. It is postulated that the increase in gastric E_2 levels, which

occurs after BDL, results in the reduction of hepatic ER α and the elevation of arterial E₂ levels, which leads to cholesterol metabolism becoming sex steroid dependent, and that the elevated serum E₂ levels in the systemic circulation is dependent on the pathological state of the liver.

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.

Funding

This work was supported by the Life Science Laboratory Research Grant (grant numbers K10G05, K11G05), the Yamagata Health Support (grant number H21012), and the Yuki Plan of Yamagata University (grant number DAY0010).

Acknowledgements

The authors acknowledged Mr Nathan Strenge (Tokai University Yamagata Senior High School) for his suggestions and critically reading the manuscript.

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Received in final form 18 July 2013

Accepted 22 July 2013

Accepted Preprint published online 22 July 2013