Gastric estradiol-17β (E2) and liver ERα correlate with serum E2 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-17β (E2) level in the portal vein is several times higher than that in the artery. This study aimed to clarify the relationships between gastric E2, hepatic estrogen receptor (ER) α and cholesterol metabolism in cholestatic male rats induced by bile duct ligation (BDL). After BDL, serum E2 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 ERα proteins were determined by immunohistochemistry and western blotting. Portal E2 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 E2 levels was positively correlated with that in the portal vein. Under these conditions, the expression of hepatic Ers1 (ERα) mRNA and protein was significantly reduced in a negative correlation with serum E2 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 E2 levels, which occurs after BDL, results in the reduction of hepatic ERα, the elevation of arterial E2 level and leads to cholesterol metabolism becoming sex steroid dependent.

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

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-17β (E2) levels in the portal vein were much higher than those found in arterial blood. Then it was suggested that about 90% of gastric E2 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 E₂ level is involved in stomach and liver functions.

Cholestasis induced by bile duct ligation (BDL) causes an (up to threefold) increase in serum E₂ levels in male and female rats (Chen et al. 1995, 1998). Furthermore, female rats with both ovariectomy and BDL have increased serum E₂ level compared with ovariectomized rats (Alvaro et al. 2002a). A part, but not all, of serum E₂ originated from ovary E₂ in female rats; however, the origin of the E₂ that increased the portal E₂ level in males remains uncertain. It has been reported that hepatic microsomal cytochrome P450 (CYP)-mediated hydroxylation, 16α-hydroxylase, was reduced with portal bypass and it has been suggested that decreased hepatic 16α-hydroxylation would enhance the accumulation of E₂ in serum (Murray et al. 1988, Cantrill 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 E₂ 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 E₂, 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 E₂ secretion by the rat stomach or the temporal relationships of gastric E₂ to liver functions. This study, therefore, deals with measurement of the E₂ level in the portal vein and the level of E₂ in peripheral plasma, i.e., abdominal aorta. This study is also an attempt to elucidate the effect of BDL on gastric E₂ synthesis and to establish the relationship between gastric E₂ 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 E₂ 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 E₂ concentrations were measured using an E₂ EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA). The assay is based on the competition between free E₂ 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 E₂ were incubated overnight at room temperature in wells of an anti-rabbit IgG-coated plate. Subsequently, the E₂ 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 E₂. We used the normal adult rat serum treated with dextran-coated charcoal for the control solvent to dilute E₂ to make the standard curve. All samples treated without dextran-coated charcoal were measured by an ELISA method just as they were and serum E₂ 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±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>
<thead>
<tr>
<th>Primer sequence</th>
<th>Product</th>
<th>Accession no.</th>
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<tr>
<td>Cyp19a1 (aromatase)</td>
<td>140</td>
<td>M33986</td>
<td>1.14.1</td>
</tr>
<tr>
<td>Forward: ATTGGCATGCAAGAATGG</td>
<td>150</td>
<td>M31681.1</td>
<td>4.1.2.30</td>
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<tr>
<td>Reverse: TGCTGCTGTAGGATCCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp17a1 (17α-hydroxylase)</td>
<td>144</td>
<td>NM054007</td>
<td>1.1.1.62</td>
</tr>
<tr>
<td>Forward: AGTGCATCGGCTACTATC</td>
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<td>AB477039</td>
<td></td>
</tr>
<tr>
<td>Reverse: GAGCTACACGACCTCAGAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsd17b3 (17β-hydroxysteroid dehydrogenase type 3)</td>
<td>114</td>
<td>NM001106409</td>
<td></td>
</tr>
<tr>
<td>Forward: AGAGTGTCATCCACTGCAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse: AGTACAGGCTATACAGAGGC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Esr1 (estrogen receptor α)</td>
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<tr>
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<td>70</td>
<td>BC089790.1</td>
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</tr>
<tr>
<td>Reverse: TTGTAGAGATGCTCCATGCC</td>
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<td></td>
<td></td>
</tr>
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<td>b-actin</td>
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<td>BC089765.1</td>
<td>1.1.1.14.1</td>
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<tr>
<td>Forward: ATGGATCCAGTCCTAGTCCT</td>
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<td>BC089742.2</td>
<td>1.1.13.17</td>
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<tr>
<td>Reverse: AAAGCTCTGTCCTCCAGAGTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp2c55 (steroid 16α- and 2α-hydroxylase)</td>
<td>89</td>
<td>NM_012942.2</td>
<td>1.1.1.53</td>
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<tr>
<td>Forward: ATGGATCCAGTCCTAGTCCT</td>
<td>156</td>
<td>D17310.1</td>
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<tr>
<td>Reverse: AAAGCTCTGTCCTCCAGAGTGG</td>
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</table>

Table 1 Oligonucleotide primers for real-time PCR
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), 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 temporarily 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 E₂ levels in the abdominal artery and portal vein**

The concentrations of E₂ 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 ± S.E.M. of five rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BDL2d</th>
<th>BDL4d</th>
<th>BDL7d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>278.6 ± 4.6</td>
<td>267.0 ± 4.7*</td>
<td>258.2 ± 2.1*</td>
<td>271.9 ± 2.1</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>9.99 ± 0.22</td>
<td>11.4 ± 0.25†</td>
<td>12.0 ± 0.19†</td>
<td>13.3 ± 0.20*†</td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>26.2 ± 1.1</td>
<td>36.7 ± 1.3†</td>
<td>29.9 ± 1.1*§</td>
<td>26.4 ± 0.9†§</td>
</tr>
<tr>
<td>Testis (g)</td>
<td>1.45 ± 0.02</td>
<td>1.43 ± 0.03</td>
<td>1.45 ± 0.08</td>
<td>1.43 ± 0.02</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.34 ± 0.02</td>
<td>5.40 ± 0.34†</td>
<td>8.54 ± 0.41*†</td>
<td>10.01 ± 0.14*†</td>
</tr>
</tbody>
</table>

*P < 0.05, †P < 0.01 vs control. ‡P < 0.05, §P < 0.01 vs BDL2d. †P < 0.05, ‡P < 0.01 vs BDL4d.

Expression of steroid metabolic enzyme mRNAs in the gastric mucosa

Gene expression of mucosal enzyme mRNAs, which encode three metabolic enzymes from progesterone to E2, was determined in control and BDL rats by a real-time RT-PCR method. The results normalized by β-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 E2, 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 E2 levels and that of Cyp17a1 and aromatase (Cyp19a1) mRNA expression after BDL. The change in portal venous E2 levels was negatively correlated with that of one Hsd17b3 expression (P = 0.0058, R² = 0.431).

Immuoquantitation 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 mm²/mm². 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).
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 β-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 7α-hydroxylase (Cyp7a1) that catalyzes cholesterol to 7α-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 3α-hydroxysteroid dehydrogenase Akr1c18, which catalyzes 7α-hydroxycholesterol to 5β-cholestan-3α,7α-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 16α- and 2α-hydroxylase (Cyp2c85), which is a

### 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 β-actin. Each result shows the mean, and the bars represent the s.i.m. of four animals. \( *P<0.05, \ **P<0.01 \) vs control, \( *P<0.05, \ **P<0.01 \) vs BDL2d, and \( *P<0.05, \ **P<0.01 \) vs BDL4d.

### Expression of mRNA levels for ERα 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 β-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

### 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 μm, and the scale bar in Figs C and D indicate 50 μm. 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.i.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.
male-specific monooxygenase, was reduced to <10% of controls after BDL (Fig. 5D), and the reduction was negatively correlated with serum E2 levels in the portal vein (P=0.0041, R²=0.456). The expression of progesterone 6β-hydroxylase (Cyp3a2), which is another male-specific monooxygenase, was reduced by BDL (Fig. 5E), and the reduction was negatively correlated with serum E2 levels in the portal vein (P=0.011, R²=0.380).

In contrast, the expression of steroid disulfate 15β-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 E2 levels in the portal vein (P=0.0162, R²=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, R²=0.562; P=0.0001, R²=0.818; and P=0.02608, R²=0.304 respectively). The expression of Akr1c18, Cyp2c55, and Cyp3a2 mRNA was negatively correlated with both serum E2 and bilirubin levels and that of Cyp7a1 was not correlated with serum E2 or bilirubin levels.

**Immunooquantitation of ERα protein in the liver**

Liver sections were immunostained with the antibody to ERα 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 ERα 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-ERα antibody was reduced to 80% of control levels at BDL7d.

A western blot analysis was performed to detect ERα 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 ERα (Fig. 7A). The levels of ERα protein normalized by β-actin decreased to 68% of controls at BDL2d and gradually

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**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.

**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 β-actin expression. Each column and bar shows the mean and s.e.m. respectively of four animals. *P<0.05, **P<0.01 vs control, *P<0.05, **P<0.01 vs BDL2d, and ***P<0.05, ****P<0.01 vs BDL4d.
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 E2 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 E2 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 E2 level in the portal vein increased after BDL, it is suggested that gastric E2 may participate in the increase in liver weight.

As gastric E2 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 6**

Light photomicrographs of liver ERα cells in BDL and control rats. Liver from control (A) and BDL (B) rats were immunostained with the antibody to ERα and the number of immunostained ERα cells and their immunostainabilities decreased in BDL rats. The population of immunoreactive ERα cells was measured by image analysis (C). BDL significantly decreased the number of ERα cells in comparison with the controls. Scale bars indicate 50 μm. 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 ERα protein was negatively correlated with that of portal venous E2 ($P<0.0098$, $R^2=0.389$), but not with that of arterial E2 and serum bilirubin. The results revealed that BDL causes the reduction of the ERα protein expression in correlation with portal venous E2 level.

**Discussion**

These results show that E2 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 E2 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 E2 in systemic circulation in the male rats is derived from gastric E2 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 E2 level in the artery is attributable to the increase in gastric E2 in the portal vein after BDL because E2 levels in the systemic circulation are positively correlated with those in the portal vein.

**Figure 7**

Western blot analysis of hepatic ERα protein in BDL rats. Liver lysates in control and BDL rats were loaded on an SDS–PAGE, and ERα (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. ERα protein decreased by BDL (B). The ERα expression was normalized by β-actin expression. Each column and bar shows the mean and s.e.m. respectively of four animals. *$P<0.05$, **$P<0.01$ vs control and ***$P<0.05$ 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 E2 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 E2 is negatively correlated with the expression of Hsd17b3 mRNA but not with Cyp17a1 and Cyp19a1 mRNAs. It also shows that the portal venous E2 level after BDL might be, if anything, related to a certain extent to E2 synthesis in the stomach. Furthermore, the portal E2 levels were positively correlated with the arterial E2 levels in this study, and it has been reported that the portal venous and arterial E2 levels were increased in partially hepatectomized rats (Kobayashi et al. 2013a). It is assumed that the gastric E2 in the portal vein flows into the systemic circulation because of the reduction in hepatic ERx, and some gastric E2 then returns to the portal vein via the gastric circulation to increase portal venous E2.

The expression of Ers1 mRNA decreased to one third of control levels at BDL2d and BDL4d (Fig. 5A), and the reduction of ERx 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 E2 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 ERx protein in hepatocytes (Alvaro et al. 2000, 2002b) and that E2 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, Stavréus-Evans et al. 2001). The present results indicate, therefore, that some gastric E2 in the portal vein passes through the liver to the systemic circulation without binding to hepatocytes and that the serum E2 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 E2, whereas female-specific Cyp2c12 is up-regulated by E2. 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 E2 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 E2 and its receptor, Ers1; however the expression of Cyp2c12 mRNA is regulated only by serum E2 and not by Ers1 or serum total bilirubin. ERx is present in both hepatocytes and cholangiocytes in the liver, and the reduction rate of ERx 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 E2 increased when returning from artery, is closely related to not only E2 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 ERx and CYP gene expression.

BDL increases the serum total bilirubin as well as E2. The major cholesterol degradation pathway is conversion to bile acids in the liver. Cholesterol metabolized to 7α-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 E2 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 7α-hydroxycholesterol to 5β-cholesterol-3α,7α-diole, was reduced to half by BDL (Fig. 5F). Thus, the bile acid synthesis is likely to be regulated by bile acids and/or E2.

The present results in BDL rats show that the elevation of arterial E2 levels is caused by the increase in gastric and portal venous E2 levels and the reduction of hepatic ERx 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 E2 levels, which
occurs after BDL, results in the reduction of hepatic ERα 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 Streng (Tokei 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