# Transcriptional Determination of Sexually Dimorphic Expression of Nuclear Receptor Constitutively Active Receptor (CAR) in Wistar Rats

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The mammalian constitutively active receptor (CAR) is a ligand-activated transcription factor that participates in controlling the expression of *cytochrome P450 2B (CYP2B)* genes in response to phenobarbital (PB) in rodents. CYP2B forms are highly inducible in liver and intestine in male Wistar rats. In contrast, PB-dependent increases in CYP2B activity are rarely observed in female Wistar rats. The profiles of CAR mRNA expression measured by RT-PCR in various organs in male and female Wistar rats were consistent with those of PB-dependent expression of *CYP2B* genes. The sex-related dimorphic PB-responses of *CYP2B* genes in Wistar rats might be determined transcriptionally as are the organospecific PB-responses in both males and females.

**Key words** —— constitutively active receptor, sexual dimorphism, Wistar rat, CYP2B1

#### INTRODUCTION

Constitutively active receptor (CAR) predominantly expressed in the liver and intestine<sup>1)</sup> is a member of the nuclear hormone receptor superfamily and is responsible for the phenobarbital induction of UDP-glucuronosyltransferase (UGT1A1),<sup>2)</sup> CYP2B1/2, CYP2B6, CYP3A1/2 and CYP3A4<sup>3,4)</sup> both *in vivo* and *in vitro*. CAR interacts with its cognate response element through DR4 motifs in the 5'-flanking region of target genes such as the phenobarbital (PB)-responsive enhancer module (PBREM) in the mouse, rat and human *CYP2B* genes, by forming a heterodimer with the 9-*cis*-retinoic acid receptor alpha (RXR $\alpha$ ). In our previous study using male Wistar rats, simultaneous expression of CYP2B1 and CYP2B2 was observed in the liver to a far less extent than the exclusive expression of CYP2B1 in the lung. Expression of the hepatic CYP2B forms could be significantly enhanced in the presence of PB, while the pulmonary forms were unresponsive to the PB-induction.<sup>5</sup>

Male-specific induction of *CYP2B* genes by PB has been reported in certain rodent strains.<sup>6,7)</sup> It has been reported that PB induces CYP2B in the livers of male but not female Wistar rats, whereas PB induces CYP2B proteins in both male and female Fisher rats.<sup>8,9)</sup> The regulatory function of CAR appeared to be responsible for the sexually dimorphic response of CYP2B to the PB-treatment at the translational and/or posttranslational steps in Wistar rats and the sex-unrelated response in Fisher rats.<sup>10)</sup>

In this study, we measured the amounts of CAR transcripts in various organs of both male and female Wistar rats and obtained findings in both liver and gastrointestinal organs that contradict those reported previously.

## MATERIALS AND METHODS

Animals and Treatment — Seven-week-old male and female Wistar rats (Clea Japan) were housed under a 12-hr light-dark cycle and allowed free access to food and water. The liver, lung, spleen, kidney, heart, stomach, and intestinal tract segments were removed and processed for the isolation of to-tal RNA.

**RT-PCR Analysis of the CAR mRNA** — Total RNA was extracted from the homogenate of 25 mg of each of the rat organs using an RNeasy Kit (Qiagen, Hilden, Germany). After incubation at 65°C for 10 min, the extracts were quickly placed in an ice-cold water bath. The total RNA and oligo-dT primer were added to RTG You-Prime First-Strand Beads (Amersham Biosciences, NJ, U.S.A.), and left at room temperature for 1 min. Reverse transcription was then performed at 37°C for 1 hr to obtain cDNA, to which a primer pair and puReTaq<sup>TM</sup>Ready-To-Go<sup>TM</sup>PCR Beads (Amersham Biosciences) were

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Fig. 1. Expression of the CAR mRNA in Various Organs in Male and Female Wistar Rats

CAR mRNA was amplified by RT-PCR (35 cycles) as described in the "MATERIALS AND METHODS" using G3PDH cDNA, which was amplified by RT-PCR (25 cycles) using the same total RNA sample, as an internal standard.

added. After adjusting the total volume to 25  $\mu$ l, the cDNA was amplified for 25 or 35 cycles of denaturation at 95°C for 15 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min in a thermal cycler. The following oligonucleotides were used as CAR-specific sense and antisense primers, respectively: 5'-ACCAGTTTGTGCAGTTCAGG-3' (Accession No. AB104736; bases 621-640) and 5'-CTTGAGAAGGGAGATCTGGT-3' (Accession No. AB104736; bases 798-817)<sup>11)</sup> and G3PDH-specific sense and antisense primers, 5'-ACCACAGT CCATGCCATCAC-3' and 5'-TCCACCACCCT GTTGCTGTA-3'. The reaction products were separated by agarose gel electrophoresis and analyzed using a Fluor Imager (Amersham Biosciences) after staining with ethidium bromide. The efficiency of the reverse transcription was controlled by RT-PCR with glyceraldehyde 3-phosphate dehydrogenase (G3PDH)-specific primers.

Subcloning of PCR Products —— The PCRamplified hepatic CAR cDNA fragment was recovered from the agarose gel using a GFX<sup>TM</sup> PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and ligated to the Sma I site of pBluescript II after treatment with a Blunting Kination Ligation Kit (Takara, Japan) to blunt and dephosphorylate its ends. Competent Escherichia coli (E. coli) cells (XL-1 Blue) transformed with 100 ng $-1 \mu g$  of plasmid DNA were plated on LB/Amp plates and incubated at 37°C overnight. White single colonies were selected from the plates and incubated in Luria-Broth supplemented with ampicillin and tetracycline overnight. Plasmids harboring the RT-PCR products were purified using a GFX<sup>TM</sup> Micro Plasmid Prep Kit (Amersham Biosciences).

**Sequencing** — The nucleotide sequencing of PCR products was carried out using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit and GeneRapid (Amersham Biosciences,

Model Seq4x4). The conditions for PCR were as follows: 30 cycles of denaturation at 95°C for 30 sec, annealing at the individual annealing temperatures for 30 sec, and extension at 72°C for 90 sec. The electrophoresis was run under conditions of 1250 V and 30 mA, at 50°C for 60 min using Tris-Borate-EDTA as a running buffer. The pBluescript II-specific sequence primers were as follows with the annealing temperatures in parentheses:

T3 HT sense primer (54°C), 5'-AATTAACCCT CACTAAAGGGAAC-3';

T7 HT antisense primer (58°C), 5'-GTAATACGA CTCACTATAGGGCGA-3'.

#### **RESULTS AND DISCUSSION**

The expression of CAR mRNA in various organs of male and female Wistar rats was determined by the RT-PCR method (Fig. 1). In both sexes, the expression of CAR mRNA was observed exclusively in the liver and intestine. The male-specific PB-induction of CYP2B proteins in the livers of Wistar rats was attributed to the sexually dimorphic expression of regulatory CAR protein.<sup>10)</sup> Furthermore, based on the lack of a sexual difference at the mRNA level, posttranscriptional modifications such as a poor translation of CAR mRNA or rapid turnover of CAR protein were proposed by the same authors to explain the diminished expression of CAR protein in female rats. In contrast, a marked sexual divergence in the expression of CAR mRNA in both liver and intestine was observed in the present study. We must note that the transcriptional control underlying the CAR-dependent sexual dimorphism in the PB-induction of CYP2B mRNA in Wistar rats was deduced by RT-PCR. The expression of CAR mRNA was not inducible by the PB-treatment (data not shown). The lack of PB response is explained by



Fig. 2. Longitudinal Expression of CAR mRNA in Gastrointestinal Organs in Male and Female Wistar Rats

Gastrointestinal organs were excised, segmented longitudinally into two stomach, duodenal, four small intestinal, cecum and two colon pieces and processed to obtain total RNA preparations. CAR mRNA and G3PDH mRNA were amplified by RT-PCR (35 cycles and 25 cycles, respectively) using the same total RNA samples (Fig. 2A). The band intensities of CAR cDNA normalized to those of G3PDH cDNA are shown in Fig. 2B.

the absence of PBREM in the promoter region of the *CAR* gene. The PB-dependent induction of CYP2B depends on the nuclear translocation of CAR but not the *de novo* expression of CAR. Yoshinari *et al.* reported that the strong sex-dependent difference in the CAR mRNA levels could not be observed in both WKY and F344 rats. Their observation might result from the RT-PCR band intensities, which were close to the plateau level.

CAR controls phase I (*CYP2B*, *CYP2C*, *CYP3A*), phase II (*UGT1A1*), and transporter (*SLC21A6*, *MRP2*) genes involved in drug metabolism and bilirubin clearance in the liver. The small intestine is known to contain small but significant amounts of a

large number of predominantly hepatic enzymes and the nuclear receptors. If CAR plays an important role in bilirubin metabolism and fecal excretion in the digestive tract, the expression of CAR might be determined by the bilirubin distribution in the intestine. The expression of CAR mRNA was analyzed along the entire length of the gastrointestinal organs using two segments of stomach, duodenum, four segments of small intestine, cecum and two segments of colon. Although male-dominant sexual dimorphic expression of CAR mRNA is conserved in all the segmented gastrointestinal samples, the longitudinal expression profile of CAR mRNA was shared by both sexes as shown in Fig. 2 with the second intestinal segment showing the highest level of CAR mRNA. The results could be partly explained by the cumulative exposure level of bilirubin to the epithelial cells of gastrointestinal organs.

Studying the effects of PB in wild-type and CAR-knockout mice, Ueda et al.12) identified 138 genes that were differentially expressed. Of these, 52 genes were either induced or repressed by PB in a CAR-dependent manner. Although CAR knockout rats are not available, the male-dominant responses to the test compounds in Wistar rats are conveniently regarded as candidates for CAR-dependent episodes, except the hormone-dependent ones. The hepatic expression of CYP2B and UGT1A1 (or *bilirubin UGT1*) genes with PBREMs was inducible when male but not female Wistar rats were treated with PB.<sup>10,13)</sup> In contrast, multi-drug resistance protein MRP3 is inducible in the presence of PB in both male and female Wistar rats, implying a CAR-independent nature.<sup>14)</sup> DDT and its analogue methxychlor (MXC) are well-known inducers of CYP2B1 in rodents, being classified as PB-type inducers.<sup>15)</sup> When the effect of MXC on liver cytochrome P450s was investigated in adult male and female Wistar rats, however, females were found to be more responsive to MXC than males in terms of CYP2B-related activities, suggesting the endocrine disrupting potential of MXC.<sup>16)</sup>

The sexual dimorphism of CYP2B-induction by PB might be caused by the sexual difference in the constitutive expression of CAR regulated either transcriptionally or posttranscriptionally. The mechanism by which the expression of the *CAR* gene is controlled remains to be elucidated.

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