Potential role of estradiol and progesterone in insulin resistance through constitutive androstane receptor

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Abstract

Normal pregnancy is characterized by insulin resistance, which contributes to the development of gestational diabetes mellitus and preeclampsia by incompletely understood mechanisms. The constitutive androstane receptor (CAR) may participate in insulin resistance in pregnancy, and sex steroids, estradiol (E_2) and progesterone, may also be involved. We applied glucose and insulin tolerance tests and measured the expression of gluconeogenic and lipogenic genes in the livers of oophorectomized mice treated with E_2 and progesterone with or without CAR ligands. We also investigated how E_2 and progesterone affected CAR-mediated signaling and the activity of transcription factors in gluconeogenesis *in vitro*. Mice with the concentrations of E_2 and progesterone within normal physiological range during pregnancy exhibited increased insulin resistance along with increased expression of gluconeogenic and lipogenic genes, and CAR activation rescued the abnormal glucose metabolism. In HepG2 cells, CAR ligands suppressed the gluconeogenic and lipogenic gene expression in the presence of E_2 and/or progesterone. DNA affinity immunoblotting and chromatin immunoprecipitation assay revealed that CAR ligand enhanced the recruitment of the gluconeogenic transcription factors, forkhead box O1 (FOXO1) and hepatocyte nuclear factor 4 α (HNF4 α), but sex steroids suppressed these recruitments on the CAR responsive element. Moreover, CAR ligand suppressed the recruitment of FOXO1 and HNF4 α on their responsive element in gluconeogenic gene promoters and E_2 and progesterone augmented these recruitments on their responsive element. Taken together, these findings suggest that the activation of CAR-mediated signaling may ameliorate insulin resistance under relatively high concentrations of E_2 and progesterone, which were compatible with pregnancy via decreased activities of transcription factors in gluconeogenesis in combination with CAR.

Journal of Molecular Endocrinology (2011) **47**, 229–239

Introduction

Normal human pregnancy is characterized by mild fasting hypoglycemia, postprandial hyperglycemia, and hyperinsulinemia (Cunningham *et al.* 2010). These responses are consistent with a pregnancy-induced state of insulin resistance, the purpose of which is likely to be to ensure a sustained postprandial supply to the fetus (Cunningham *et al.* 2010). Indeed, the insulin sensitivity in normal pregnant women is 45–70% lower during the course of gestation compared with non-pregnant women (Catalano *et al.* 1991, Butte 2000, Freemark 2006). In addition, an increase in insulin resistance contributes to the development of gestational diabetes mellitus and preeclampsia (Kajja *et al.* 1999, Seely & Solomon 2003, Kajja & Greer 2005). However, the mechanism responsible for the insulin resistance during pregnancy is not completely understood. This condition has been attributed to the effects of human placental lactogen, cortisol, human placental GH and prolactin (Ryan & Enns 1988). However, increased levels of free fatty acids, peroxisome proliferator-activated receptors, tumor necrotic factor- α ,

adiponectin, and resistin have all been implicated in insulin resistance in pregnancy (Kajja & Greer 2005). In addition to some placental hormones that decrease insulin sensitivity, estradiol (E_2) and progesterone, could be involved in the gestation-induced insulin resistance because the relatively high concentrations of these hormones, E_2 (100–200 pg/ml) and progesterone (100–200 ng/ml), which are within physiological ranges during pregnancy in their experiments, reduce insulin sensitivity (Ryan & Enns 1988, Livingstone & Collison 2002, Barros *et al.* 2008).

The constitutive androstane receptor (CAR) is an orphan nuclear receptor. It was originally characterized as a nuclear receptor that can activate an empirical set of retinoic acid response elements without retinoic acid (Baes *et al.* 1994, Choi *et al.* 1997), and can be activated in response to xenochemical exposure, including phenobarbital (PB)-stimulated activation of a response element, NR1, found in the human and mouse cytochrome p450 2B (*CYP2B*) genes (Honkakoski *et al.* 1998, Sueyoshi *et al.* 1999). This PB response enhancer module is also located in the upstream region of the uridine diphosphate-5'-glucuronosyltransferase

1A1 (*UGT1A1*) gene and is activated by CAR (Sugatani *et al.* 2001, Masuyama *et al.* 2010). In addition, activation of the bilirubin clearance pathway by CAR ligands is abolished in CAR-null mice (Huang *et al.* 2003), suggesting that the CAR-UGT1A1 pathway may play an important role in bilirubin clearance. Moreover, recent studies have linked CAR to lipid and glucose metabolism. Activation of CAR suppresses lipid metabolism and lowers serum triglyceride by reducing the protein levels of the active form of a lipogenic transcription factor, sterol regulatory element-binding protein 1 (SREBP-1; Roth *et al.* 2008). The key hepatic gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were reported to be repressed in PB-treated mice and these suppressions were CAR-dependent (Ueda *et al.* 2002). Recent *in vivo* studies have demonstrated that activation of CAR improves insulin sensitivity via glucose and lipid metabolic pathways including PEPCK, G6Pase, SREBP-1, and stearoyl-CoA desaturase 1 (SCD-1), a key enzyme for the synthesis of unsaturated fatty acids (Dong *et al.* 2009, Gao *et al.* 2009).

CAR has been shown to participate in insulin resistance, and sex steroids, estrogen and progesterone, may also be involved in insulin resistance during pregnancy (Ryan & Enns 1988, Livingstone & Collison 2002, Barros *et al.* 2008, Dong *et al.* 2009, Gao *et al.* 2009). Therefore, we examined glucose tolerance test (GTT) and insulin tolerance test (ITT) and measured the expression of gluconeogenic and lipogenic genes in the livers of oophorectomized ICR mice treated with E₂ and progesterone in the presence or absence of CAR ligands. We also investigated how E₂ and progesterone affected CAR-mediated signaling and the activity of transcription factors in gluconeogenesis *in vitro*. The mechanisms responsible for the effects of sex steroids, E₂ and progesterone on glucose metabolism and insulin resistance via CAR-mediated signaling, were investigated *in vitro*.

Materials and methods

Materials

E₂, progesterone, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime (CITCO), PB, and pure corn oil were purchased from Sigma-Aldrich. CITCO and TCPOBOP are the only ligands shown to specifically bind to human and mouse CAR and regulate the target genes respectively (Sueyoshi *et al.* 1999, Maglich *et al.* 2003). HepG2 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan).

Animal procedures

Female ICR mice at 8 weeks of age were obtained from Charles River Co. Ltd (Tokyo, Japan), and six mice were examined per group for all *in vivo* experiments. To avoid the effect of menstrual cycle and do experiments under same hormonal conditions, we employed oophorectomized mice for this study. ICR mice were oophorectomized at 8 weeks of age and received once-weekly i.p. injections of TCPOBOP (0.5 mg/kg) or corn oil with or without twice-daily s.c. injections of E₂ (50 µg/kg) and progesterone (3 mg/kg), which were determined by previous reports (Song *et al.* 2001, Barros *et al.* 2008), for 2 weeks from 10 weeks of age. This dose gave plasma concentrations (E₂ = 70.4 ± 6.9 pg/ml, progesterone = 84.2 ± 9.1 ng/ml, *n* = 6) similar to those found in late pregnancy (Masuyama *et al.* 2001, Song *et al.* 2001). Because the concentrations of E₂ and progesterone might change with different strain of mice, age and differences in detection methods of hormones, we also examined plasma concentration of E₂ and progesterone of oophorectomized vehicle-treated control mice (E₂ = 1.4 ± 0.3 pg/ml, progesterone = 3.1 ± 0.7 ng/ml, *n* = 6), pregnant ICR mice on day 18 of gestation in normal pregnant mice (E₂ = 75.9 ± 9.3 pg/ml, progesterone = 80.5 ± 5.9 ng/ml, *n* = 6) and non-pregnant female mice at 8 weeks of age before oophorectomy (E₂ = 7.3 ± 2.4 pg/ml, progesterone = 9.2 ± 1.9 ng/ml, *n* = 6) in ICR mice tested in this study. The animals were killed under ether anesthesia at 12 weeks of age and their livers were removed, immediately frozen and stored at -70 °C until analysis. Total RNA was extracted using TRIzol reagent (Life Technologies, Inc.), according to the manufacturer's instructions. The mice were kept in a temperature- and light-controlled room with free access to normal diet (12% fat, 28% protein, and 60% carbohydrate, Oriental yeast Co., Tokyo, Japan) and water except when undergoing GTTs and ITTs. All procedures performed on the mice were approved by the Institutional Animal Care and Use Committee of Okayama University.

GTT, ITT and measurements of the insulin and total triglyceride levels

Mice at 12 weeks of age were fasted for 16 h before receiving an i.p. injection of D-glucose (2 g/kg body weight) for the GTT or for 4 h before receiving an i.p. injection of human insulin (1.0 U/kg body weight) for the ITT. Blood samples from tail vein were taken before and at 30, 60, 90, and 120 min after the injection. The blood glucose levels were measured by the glucose oxidase method using a Medisafe automated analyzer (Termo, Tokyo, Japan). Fasting insulin, E₂, progesterone, and total triglyceride levels were determined using ELISA kits (Morinaga Institute of Biological

Sciences, Inc., Yokohama, Japan, Cayman Chemicals Co., Ann Arbor, MI, USA). The homeostasis model assessment as an index of insulin resistance (HOMA-IR) was calculated as the fasting insulin concentration ($\mu\text{U}/\text{ml}$) $\times$ fasting glucose concentration (mg/dl)/405 (Matthews *et al.* 1985).

Cell culture and RNA interference

HepG2 cells were cultured in DMEM without phenol red supplemented with 10% charcoal stripped fetal bovine serum. The medium and fetal bovine serum were purchased from Invitrogen Corp. A small interfering RNA (siRNA) cocktail targeting human *CAR* was purchased from B-Bridge International, Inc. (Mountain View, CA, USA) and contained three siRNAs as described previously (Masuyama *et al.* 2010). A negative control cocktail consisting of non-complementary human, mouse, and rat and liposome sequences for siRNA transfection was also purchased from B-Bridge International, Inc. Cells were transfected with the *CAR* siRNAs or control siRNAs using siFECTOR according to the manufacturer's protocol (B-Bridge International, Inc.).

RT-PCR and real-time quantitative PCR

Total RNA was extracted from HepG2 cells and mouse livers using TRIzol reagent (Life Technologies, Inc.). To confirm the downregulation of *CAR* expression by RNA interference, RT-PCR was performed by an RNA PCR kit (TaKaRa Co. Ltd, Kyoto, Japan) according to the manufacturer's protocol. Amplification of the *CAR* sequence was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, Inc., Foster City, CA, USA). The number of PCR cycles required to produce PCR products in the linear logarithmic phase of the amplification curve was determined. The PCR products were electrophoresed in 3% agarose gels and visualized by staining with ethidium bromide. To measure the mRNA levels of *PEPCK*, *G6Pase*, *SREBP-1*, *SCD-1*, *CYP2C9*, and *CYP2B6*, real-time quantitative PCR was performed by a StepOne Real-time PCR System and a TaqMan RNA-to-CT Gene Kit (Applied Biosystems). Specific primers for the human *CAR*, *PEPCK*, *G6Pase*, *SREBP-1*, *SCD-1*, *CYP2C9*, *CYP2B6*, and β -actin and mouse *CAR*, *PEPCK*, *G6Pase*, *SREBP-1*, and *SCD-1* gene sequences were purchased from Applied Biosystems. Aliquots (25 ng) of the RNA samples were assayed using 15 pmol of gene-specific primers and 5 pmol of gene-specific probes in triplicate. As internal controls, the human or mouse β -actin mRNA levels were measured using a pre-developed TaqMan primer and a probe mixture (Applied Biosystems). Normalization of the data was accomplished by quantifying the amount of amplified cDNA products by calculating the ratio of the amount of cDNA relatives to the amount of β -actin cDNA.

Transient transfection studies

The (NR1)³-tk-CAT vector was generated by insertion of three copies of double-stranded oligonucleotides containing the NR1 site (5'-gatcACTGTACTTTCCTGACCTTGgatc-3') as described previously (Sueyoshi *et al.* 1999, Masuyama *et al.* 2010). HepG2 cells were cotransfected with 1 mg of a reporter gene construct, (NR1)³-tk-CAT or tk-CAT. In all experiments, liposome-mediated transfections were accomplished using Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. The transfected cells were treated with ethanol vehicle alone or with the *CAR* ligands PB (1 mM) or CITCO (1 μM), with or without E₂ (100 nM), and/or progesterone (1 μM) for 36 h. Cell extracts were prepared and assayed for CAT activity. The amount of CAT was determined using a CAT ELISA Kit (Roche Diagnostics Co.) according to the manufacturer's instructions.

Western blot analysis

Whole cell extracts were obtained from HepG2 cells using M-PER Mammalian Protein Extraction Reagents (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's protocol, and stored at -80°C until analysis. The protein samples were solubilized in SDS buffer (0.05 M Tris-HCl pH 6.8, 2% SDS, 6% mercaptoethanol, and 10% glycerol) and analyzed by western blotting using a goat polyclonal antibody against *CAR* (1:1000 dilution), a mouse monoclonal antibody against β -actin (1:2000), HRP-conjugated rabbit anti-goat or anti-mouse secondary antibody (1:10 000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), nitrocellulose membranes (Hybond-ECL, GE Healthcare, USA), and chemiluminescent detection reagent (ECL Plus Western blotting detection reagents; GE Healthcare, Amersham, UK) as described previously (Masuyama & MacDonald 1998).

DNA affinity immunoblotting

Nuclear extracts were obtained from HepG2 cells using nuclear and cytoplasmic extraction reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). As previously described (Moore *et al.* 2000), aliquots (200 μg) of the nuclear extracts from HepG2 cells were incubated at 4°C for 30 min with a DNA binding reaction mixture containing 25 nM biotin-labeled NR1, 2 mg of poly (dI/dC), 5 mM dithiothreitol, and 40 μl of $10\times$ DNA binding buffer (200 mM Tris-HCl pH 7.2, 10 mM EDTA, 1% Triton X-100, and 40% glycerol). For rescue experiments, HepG2 cells were cotransfected with various amounts of pcDNA3-steroid receptor coactivator-1 (SRC-1) or pcDNA3 expression vector alone.

A human SRC-1 cDNA was subcloned into the pcDNA3 expression vector (Invitrogen) as described previously (Masuyama *et al.* 2005). DNA/protein complexes were captured with 0.1 mg of magnetic streptavidin beads (Dynabeads; Dynal Biotech, Oslo, Norway) at 4 °C for 30 min. The beads were drawn into a pellet using a magnet and washed three times with DNA binding buffer. The bound proteins were eluted from the magnetic beads by heating at 70 °C in 20 ml of SDS buffer (0.05 M Tris-HCl pH 6.8, 2% SDS, 6% mercaptoethanol, and 10% glycerol) and analyzed by western blotting using rabbit polyclonal antibodies against CAR (1:1000 dilution), SRC-1 (1:500 dilution), forkhead box O1 (FOXO1; 1:500 dilution), hepatocyte nuclear factor 4 α (HNF4 α ; 1:500 dilution), estrogen receptor α (ER α ; 1:1000 dilution) and progesterone receptor (PR; 1:1000 dilution), and HRP-conjugated goat anti-rabbit secondary antibody (1:10 000 dilution; Santa Cruz Biotechnology, Inc.) and chemiluminescent detection reagent (ECL Plus Western blotting detection reagents, GE Healthcare).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed by a ChIP assay kit (Upstate Biotechnology,

Lake Placid, NY, USA) according to the manufacture's protocol. Briefly, HepG2 cells were grown to 90% confluent, were treated with ethanol vehicle alone or with CITCO (1 μ M), with or without E₂ (100 nM), and/or progesterone (1 μ M) for 48 h, were incubated with 1% formaldehyde at room temperature for 10 min and subjected to sonication to reduce DNA length to 0.3–1.0 kb. Chromatin was precleared in the presence of 20 μ l of normal serum, 2 μ g of salmon sperm DNA, and 80 μ l of a 25% protein A-agarose slurry. Precleared chromatin samples were subjected to immunoprecipitation at 4 °C overnight in the presence of 2 μ g of goat polyclonal antibody against CAR, rabbit polyclonal antibodies against SRC-1, FOXO1, HNF4 α , ER α , and PR (Santa Cruz Biotechnology, Inc.), or non-immune rabbit IgG. After collecting the complex by incubation with 60 μ l of a 25% protein A-Sepharose slurry and centrifugation, the beads were washed five times, and the chromatin immune complex was eluted. After reversing the cross-links, DNA was purified and used as a template in PCR. PCR was performed by primer sets specific for the CAR responsive element in the CYP2C9 promoter (Surapureddi *et al.* 2008), the HNF4 α responsive element in the PEPCK promoter (Bhalla *et al.* 2004), and FOXO1 binding site in the G6Pase promoter (Vander Kooi *et al.* 2003).

Figure 1 CAR activation ameliorates sex steroid-induced glucose intolerance and insulin resistance in oophorectomized mice treated with high concentration of sex steroids. (A–E) Treatment with TCPOBOP (0.5 mg/kg per week) ameliorates the glucose intolerance and insulin resistance induced by sex steroid (E₂ + progesterone (P)) treatment in oophorectomized mice, as determined by the GTT (A), ITT (B), fasting insulin level (C), HOMA-IR (D), and total triglyceride level (E). (F) The mRNA expression of genes involved in gluconeogenesis and lipogenesis were measured by real-time PCR analysis of liver extracts from non-pregnant mice treated with corn oil (control), sex steroid or a combination of sex steroid, and TCPOBOP. The results represent means $\pm$ s.d. ($n=6$ per group). * $P<0.01$, ** $P<0.05$ versus corn oil-treated oophorectomized mice.

Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Dunnett's test. Data are presented as the mean $\pm$ s.d. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

CAR activation improves E_2 - and progesterone-induced glucose intolerance and insulin resistance in non-pregnant mice

To test whether relatively high levels of sex steroid, E_2 and progesterone, which are compatible with physiological levels in normal pregnant mice, impair the glucose metabolism and whether CAR activation improves the E_2 - and progesterone-induced glucose intolerance, we performed GTT and ITT in oophorectomized mice treated with E_2 and progesterone. We used TCPOBOP, which was shown to specifically bind to mouse CAR and to regulate the target genes (Sueyoshi *et al.* 1999) as a ligand for CAR. E_2 - and progesterone-treated mice exhibited

significantly poorer glucose tolerance than corn oil-treated (control) mice, while TCPOBOP treatment improved the glucose tolerance in E_2 - and progesterone-treated oophorectomized mice in the GTT (Fig. 1A) and ITT (Fig. 1B). The fasting insulin level, HOMA-IR, and total triglyceride level were increased in E_2 - and progesterone-treated oophorectomized mice, while TCPOBOP treatment decreased the fasting insulin level, HOMA-IR, and total triglyceride level (Fig. 1C–E). The gluconeogenic genes *PEPCK1* and *G6Pase* and the lipogenic genes *SREBP-1* and *SCD-1* were significantly increased in the liver of oophorectomized mice treated with E_2 and progesterone, while TCPOBOP treatment decreased the expression of these genes (Fig. 1F). There were no significant effects of TCPOBOP on insulin sensitivity and the expression of gluconeogenic and lipogenic genes in corn oil-treated control mice (Fig. 1A–F). Moreover, HOMA-IR was significantly increased in E_2 - or progesterone-treated oophorectomized mice (E_2 ; 3.0 ± 0.2 and progesterone; 3.2 ± 0.3) compared with corn oil-treated mice (control; 2.5 ± 0.2) and E_2 or progesterone treatment-induced changes were restored by TCPOBOP. We also observed similar results in

Figure 2 Effects of sex steroid on the expression levels of gluconeogenic and lipogenic genes in the presence or absence of CAR ligands (A and B) and in a concentration-dependent manner (C and D). HepG2 cells were treated with ethanol vehicle (control), phenobarbital (PB, 1 mM or indicated concentration) or 6-(4-chlorophenyl)imidazo [2,1b] [1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO, 1 μ M or indicated concentration), with or without 17 β -estradiol (E_2 , 100 nM or indicated concentration), and/or progesterone (P, 1 μ M or indicated concentration) for 24 h. Total RNA was obtained from the cells and analyzed for the mRNA expression of *PEPCK*, *G6Pase*, *SREBP-1*, *SCD-1*, *CYP2C9*, and *CYP2B6* by real-time quantitative PCR. The mRNA levels were normalized by the β -actin mRNA levels. The results represent means $\pm$ s.d. from three independent experiments. * $P < 0.01$ versus the controls; # $P < 0.01$ versus CITCO or PB.

GTT and ITT and there seems to be robust effects by progesterone compared with E₂, while the combination of E₂ and progesterone had more strong effect on glucose metabolism (data not shown).

Effects of E₂ and/or progesterone on the expression of gluconeogenic and lipogenic genes in the presence or absence of CAR ligands

We evaluated human hepatoma HepG2 cells because native CAR was expressed in this cell line (data not shown). CITCO, which directly interacts with CAR, was used as a CAR ligand and PB, which does not directly interact with CAR, was also used as a CAR activator because they have been shown to activate the CAR-mediated pathway (Sueyoshi *et al.* 1999, Maglich *et al.* 2003, Swales & Negishi 2004). The mRNA expression of the gluconeogenic genes *PEPCK* and *G6Pase* were significantly decreased in the presence of CAR ligands, while the mRNA expression of *CYP2C9* and *CYP2B6*, which is a target gene for human CAR (Kawamoto *et al.* 2000, Ferguson *et al.* 2002), was significantly increased in the presence of PB or CITCO compared with ethanol treatment as a control. In addition, E₂ and/or progesterone treatment significantly increased the *PEPCK* and *G6Pase* mRNA and decreased the *CYP2C9* and *CYP2B6* mRNA expression with PB or CITCO (Fig. 2A). The mRNA expression of *SREBP-1* and *SCD-1* were significantly decreased in the presence of CAR ligands compared with ethanol treatment, while sex steroid treatment significantly increased the *SREBP-1* and *SCD-1* mRNA expression with PB or CITCO (Fig. 2B). And there seem to be robust effects by progesterone compared with E₂, while the combination of E₂ and progesterone had a stronger effect in the presence of PB or CITCO (Fig. 2A and B). The effect of E₂ and/or progesterone on the mRNA expression of *PEPCK* was concentration-dependent (Fig. 2C). We also observed a concentration-dependent effect of CITCO on the *PEPCK* mRNA expression in the presence of E₂ and/or progesterone (Fig. 2D).

Effects of E₂ and/or progesterone on CAR-mediated transcription in the presence or absence of CAR ligands

Next, we introduced the transient reporter assay to examine which stage in CAR-mediated signaling E₂ and progesterone affected. Reporter gene constructs, (NR1)³-tk-CAT or tk-CAT, were introduced into HepG2 cells. Treatment with PB or CITCO significantly activated CAR-mediated transcription in HepG2 cells using (NR1)³-tk-CAT (Fig. 3). E₂ and/or progesterone treatment had no effect on this transcription. There were no non-specific activations with

either ligand in the cells transfected with tk-CAT. In addition, we observed significant decreases in CAR-mediated transcription in the presence of E₂ and/or progesterone with PB or CITCO compared with transcription without sex steroid, while progesterone had more robust effect on transcription.

Effects of CAR siRNAs on the mRNA expression of *G6Pase*, *SREBP-1*, *CYP2C9*, and *CYP2B6* and CAR-mediated transcription in the presence of CAR ligands with or without E₂ or progesterone

To investigate the effects of CAR siRNAs on the mRNA expression levels of *G6Pase*, *SREBP-1*, *CYP2C9*, and *CYP2B6* in the presence of CAR ligands with or without sex steroid, E₂ or progesterone, we qualitatively examined the mRNA levels of these genes in HepG2 cells. We confirmed the efficacy of the CAR siRNAs for knockdown of CAR mRNA expression in HepG2 cells by RT-PCR and western blotting. Neither CAR mRNA nor CAR protein was detected in HepG2 cells transfected with the CAR siRNAs (Fig. 4A). In CAR siRNA-treated cells, there were no significant decreases in the *G6Pase* and *SREBP-1* mRNA levels, nor was there a significant increase in the *CYP2C9* and *CYP2B6* mRNA level in the presence of CAR ligands PB and CITCO, compared with control siRNA-treated cells (Fig. 4B). Suppression of the ligand-induced effects on target gene expression by E₂ or progesterone was not observed in cells transfected with the CAR siRNAs. Next, we examined the effects of the CAR siRNAs on CAR-mediated transcription. The reporter gene constructs, (NR1)³-tk-CAT or tk-CAT, were introduced into HepG2 cells. In

Figure 3 Effects of sex steroid on CAR-mediated transcription in the presence or absence of CAR ligands. HepG2 cells were cotransfected with 1 μ g of a reporter gene construct, (NR1)³-tk-chloramphenicol acetyl transferase (CAT) or tk-CAT. The cells were treated with ethanol (EtOH, control), phenobarbital (PB, 1 mM) or 6-(4-chlorophenyl)imidazo [2,1b] [1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO, 1 μ M), with or without 17 β -estradiol (E₂, 100 nM), or progesterone (P, 1 μ M) for 36 h. The CAT levels were determined using a CAT ELISA kit. The results represent means $\pm$ S.D. from three independent experiments. **P* < 0.01 versus the ethanol control; #*P* < 0.01 versus CITCO or PB.

Figure 4 Effects of CAR siRNAs on the mRNA expression of *G6Pase*, *SREBP-1*, *CYP2C9*, and *CYP2B6*, and on CAR-mediated transcription in the presence of CAR ligands with or without sex steroid. (A) HepG2 cells were transfected with CAR or control siRNAs. Whole cell extracts were prepared, and the CAR protein levels were determined by western blotting using anti-CAR antibodies. Total RNA was also obtained from the cells and analyzed for the expression of *CAR* mRNA by RT-PCR. The PCR products were separated in 3% agarose gels and visualized by ethidium bromide staining. (B) HepG2 cells were transfected with CAR or control siRNAs and treated with ethanol (control), phenobarbital (PB, 1 mM) or 6-(4-chlorophenyl)imidazo [2,1b] [1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO, 1 μ M), with or without 17 β -estradiol (E_2 , 100 nM), or progesterone (P, 1 μ M) for 24 h. Total RNA was obtained from the cells and analyzed for the mRNA expression of *G6Pase*, *SREBP-1*, *CYP2C9*, and *CYP2B6* by real-time quantitative PCR. The mRNA levels were normalized by the β -actin mRNA levels. (C) HepG2 cells were cotransfected with CAR or control siRNAs and 1 μ g of a reporter gene construct, (NR1)³-tk- chloramphenicol acetyl transferase (CAT) or tk-CAT. The cells were treated with ethanol (control), PB (1 mM) or CITCO (1 μ M), with or without E_2 (100 nM), or progesterone (P) (1 μ M) for 36 h. The amount of CAT was determined using a CAT ELISA kit. The results represent means $\pm$ s.d. from three independent experiments. **P*<0.01 versus the controls; #*P*<0.01 versus CITCO or PB.

control siRNA-transfected cells, PB and CITCO significantly activated the native CAR-mediated transcription, and these effects were significantly suppressed by E_2 or progesterone. No CAR-mediated transactivation was observed in the presence of PB or CITCO in the CAR siRNA-transfected cells, even in the presence of E_2 or progesterone. No non-specific effects of the siRNAs on transcription were observed, since there were no differences in basal transcription using the tk-CAT vector between the CAR siRNA- and control siRNA-transfected cells (Fig. 4C).

Effects of E_2 and/or progesterone on the interactions of transcription factors with CAR/NR1 complexes

We examined which transcription factors were recruited by CAR/NR1 complexes and the effects of sex steroid on the interactions of these transcription factors with

CAR/NR1 complexes using a DNA affinity immunoblotting assay. Since CAR-mediated gene regulation has been shown to require SRC-1 (Muangmoonchai *et al.* 2001), we examined whether SRC-1 was associated with CAR/NR1 complexes in the presence of CAR ligands with or without E_2 and/or progesterone. FOXO1 and HNF4 α were also examined because these transcription factors, which play important roles in gluconeogenesis, interact directly with CAR (Kodama *et al.* 2004). SRC-1 was strongly detected on the NR1 element in the presence of CAR ligands, but was much less strongly detected in the presence of CAR ligands with E_2 and/or progesterone. No SRC-1 was detected in controls treated with vehicle alone. In addition, we observed a strong association of FOXO1 and HNF4 α on the NR1 element in the presence of CAR ligands, with a much lower association of these transcription factors in the presence of CAR ligands with E_2 and/or progesterone. No other

Figure 5 Effects of sex steroid on the interactions of transcription factors with CAR/NR1 complexes. (A) Aliquots (200 μg) of nuclear extracts obtained from HepG2 cells were incubated with a DNA binding reaction mixture including biotin-labeled NR1 and treated with ethanol (control), phenobarbital (PB, 1 mM) or 6-(4-chlorophenyl)imidazo [2,1b] [1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO, 1 μM), with or without 17β-estradiol (E₂, 100 nM), and/or progesterone (P, 1 μM). (B) For rescue experiments, various amounts of pcDNA3-steroid receptor coactivator (SRC-1) or pcDNA3 vector were also transfected. DNA/protein complexes were captured with 0.1 mg of magnetic streptavidin beads at 4 °C for 30 min. The beads were drawn into a pellet using a magnet and washed three times with DNA binding buffer. The bound proteins were eluted from the magnetic beads by heating and analyzed by western blotting using a goat polyclonal antibody against CAR and rabbit polyclonal antibodies against SRC-1, FOXO1, HNF4α, ERα, and PR.

nuclear receptor, ERα, was detected in these assays (Fig. 5A). Moreover, overexpression of SRC-1 rescued the decreased association of FOXO1 and HNF4α on the NR1 element in a dose-dependent manner (Fig. 5B).

Recruitment analysis of CAR, FOXO1 and HNF4α on the CAR responsive element and HNF4α and FOXO1 on their responsive element

ChIP assays were performed on the chromatin extracts from the HepG2 cells to examine the effect of CAR ligand and sex steroids, E₂ and progesterone, on the recruitment of CAR, FOXO1 and HNF4α on the CAR responsive element in CYP2C9 promoter (Surapureddi *et al.* 2008), HNF4α on the HNF4α responsive element in the PEPCK promoter (Bhalla *et al.* 2004), and FOXO1 on the FOXO1 binding site in the G6Pase promoter (Vander Kooi *et al.* 2003). CAR ligand CITCO enhanced the recruitment of CAR, FOXO1, HNF4α, and coactivator SRC-1 compared with ethanol treatment, but E₂ and/or progesterone decreased these recruitments on the CAR responsive element (Fig. 6A). Other nuclear receptor, ER and PR, and non-specific binding were not detected in this assay. We also observed the effect of CITCO and sex steroids, E₂ and/or progesterone on the recruitment of HNF4α responsive element in the PEPCK promoter and FOXO1 on the FOXO1 binding site in the G6Pase promoter. The recruitment of HNF4α and FOXO1 on their responsive element was decreased in the presence of CITCO, but the addition of E₂ and/or progesterone

augmented the recruitment of FOXO1 and HNF4α on their responsive element, respectively, and progesterone had more strong effect on the recruitments (Fig. 6B).

Discussion

The sex steroid, E₂ and progesterone may be involved in the gestation-induced insulin resistance, since relatively high concentrations of E₂ (100–200 pg/ml) and progesterone (100–200 ng/ml), which are within physiological ranges during pregnancy in their *in vivo* experiments, reduce insulin sensitivity (Ryan & Enns 1988, Livingstone & Collison 2002, Barros *et al.* 2008). Because the elevations of both E₂ and progesterone were observed during pregnancy, we examined whether treatment of E₂ and progesterone would affect glucose tolerance, insulin sensitivity using GTT, ITT, and HOMA-IR and the expression of the hepatic gluconeogenic enzymes PEPCK and G6Pase (Ueda *et al.* 2002, Gao *et al.* 2009) and lipogenic enzymes SREBP-1 and SCD-1 (Roth *et al.* 2008, Dong *et al.* 2009, Gao *et al.* 2009) in oophorectomized mouse. We also examined whether CAR activation would improve insulin sensitivity and enzyme expression levels in oophorectomized mice treated with E₂ and progesterone because CAR activation has been shown to improve insulin sensitivity in non-pregnant mice using a high-fat diet-induced model of diabetes and in leptin-deficient (*ob/ob*) mice (Dong *et al.* 2009, Gao *et al.* 2009). We observed that relatively high-dose treatment with E₂ and progesterone, which induced the elevated concentration of E₂ and

Figure 6 Recruitment analyses of CAR, FOXO1, and HNF4 α on the CAR responsive element and HNF4 α and FOXO1 on their responsive element by ChIP assay. ChIP assays were performed on the chromatin extracts from the HepG2 cells to examine the effect of CAR ligand and sex steroids, E₂ and progesterone (P), on the recruitment of CAR, FOXO1, and HNF4 α on the CAR responsive element in the *CYP2C9* promoter (A), the HNF4 α responsive element in the *PEPCK* promoter (B) and the FOXO1 binding site in the *G6Pase* promoter (C). HepG2 cells were grown to 90% confluent, were treated with ethanol (EtOH) vehicle alone or with 6-(4-chlorophenyl)imidazo [2,1b] [1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO, 1 μ M), with or without 17 β -estradiol (E₂, 100 nM), and/or progesterone (P, 1 μ M) for 48 h, were incubated with 1% formaldehyde at room temperature for 10 min and subjected to sonication to reduce DNA length to 0.3–1.0 kb. Chromatin was precleared in the presence of 20 μ l of normal serum, 2 μ g of salmon sperm DNA, and 80 μ l of a 25% protein A-agarose slurry. Precleared chromatin samples were subjected to immunoprecipitation in the presence of 2 μ g of goat polyclonal antibody against CAR or rabbit polyclonal antibodies against SRC-1, FOXO1, HNF4 α , ER α or PR, or non-immune rabbit IgG. After collecting the complex by incubation with 60 μ l of a 25% protein A-Sepharose slurry and centrifugation, the beads were washed five times, and the chromatin immune complex was eluted. After reversing the cross-links, DNA was purified and used as a template in PCR. PCR was performed by primer sets specific for CAR responsive element in the *CYP2C9* promoter, HNF4 responsive element in the *PEPCK* promoter and the FOXO1 binding sites in the *G6Pase* promoter.

progesterone within the normal physiological range during pregnancy, induced glucose intolerance and insulin resistance with increased expression levels of gluconeogenic and lipogenic enzymes. Moreover, CAR activation improved the glucose tolerance, insulin sensitivity, and lipid metabolism with suppression of these enzymes. Thus, our data suggested that the relatively high concentrations of sex steroids, E₂ (70–80 pg/ml) and progesterone (80–90 ng/ml), which were compatible with physiological range during pregnancy in ICR mice tested in this study may participate in the induction of insulin resistance via the suppression of CAR-mediated signaling. Our data also demonstrated that CAR activation may serve to improve insulin sensitivity under physiological concentrations of E₂ and progesterone during pregnancy and that the metabolic benefit of CAR activation may have resulted from the combined inhibition of gluconeogenesis and lipogenesis.

Recent data have strongly suggested that CAR activation repressed the gluconeogenic pathway by

interfering with transcription factors or cofactors involved in the transcriptional regulation of gluconeogenic enzymes (Kodama *et al.* 2004, Miao *et al.* 2006). FOXO1 is a key positive regulator of gluconeogenesis. CAR can bind physically to FOXO1 and suppress the association between FOXO1 and the insulin-responsive element in gluconeogenic enzyme promoters such as PEPCK (Kodama *et al.* 2004). HNF4 α is also a positive regulator of gluconeogenesis. CAR inhibits gluconeogenic enzyme gene expression by competing with HNF4 α for binding to the DR1 motif in the promoter region of gluconeogenic enzyme genes (Miao *et al.* 2006). Moreover, CAR has been shown to repress the transcriptional activation of ER α by squelching p160 coactivators including SRC-1 (Min *et al.* 2002). In this study, we observed that high E₂ and/or progesterone conditions enhanced gluconeogenic and lipogenic gene expression levels in a concentration-dependent manner and reduced the associations of SRC-1, FOXO1, and HNF4 α with NR1/CAR complexes in the presence of CAR ligands.

Moreover, overexpression of SRC-1 rescued the decreased associations of FOXO1 and HNF4 α with NR1/CAR complexes with high concentrations of E₂ and/or progesterone. In addition, ChIP assay demonstrated that CAR ligand CITCO enhanced the recruitment of CAR, FOXO1, HNF4 α , and coactivator SRC-1, but E₂ and/or progesterone suppressed the recruitments on the CAR responsive element. We also observed the effect of CITCO and sex steroids, E₂ and/or progesterone on the recruitment of HNF4 α and FOXO1 on their responsive element in the promoter of PEPCK and G6Pase. The recruitment of FOXO1 and HNF4 α on their responsive element was decreased in the presence of CITCO and the addition of E₂ and/or progesterone augmented the recruitment of HNF4 α and FOXO1 on their responsive element. These data suggest that squelching of coactivators including SRC-1 under high E₂ and/or progesterone conditions may inhibit the interactions of CAR with HNF4 α and FOXO1 on CAR responsive element and augment the association of these factors on the promoter of gluconeogenic factors, thereby resulting in increased gluconeogenesis.

We observed that CITCO had more predominant effects on CAR signaling compared with PB. CITCO has high affinity for CAR and directly binds to CAR (Maglich *et al.* 2003), but PB has very low affinity and promotes CAR translocation into nucleus without direct binding (Swales & Negishi 2004), which might result in different levels of effects by CITCO or PB. And there were not significant but robust effects by progesterone compared with E₂, while the combination of E₂ and progesterone had more strong effect in the presence of PB or CITCO. Because different coactivators have been demonstrated to play some roles in ER- and PR-mediated transcription (Glass & Rosenfeld 2000), the different squelching of various coactivators might occur with E₂ and/or progesterone treatment, which results in the differences between E₂ and progesterone. Moreover, Kawamoto *et al.* (2000) demonstrated that 10 μ M of E₂ activated CAR-mediated transcription and enhanced nuclear accumulation in mouse. Because we used relatively lower E₂ concentration in mouse compared with previous report (Kawamoto *et al.* 2000), we might not observed the effect of E₂ on CAR-mediated signaling. In addition, the effects of E₂ and/or progesterone on gluconeogenic and lipogenic genes in HepG2 cells were observed only in the presence of CAR ligand, suggesting that E₂ and progesterone might affect these genes only under CAR activation by endogenous ligand *in vivo*. Because our data included several differences between *in vitro* and *in vivo* experiments in hormone levels and ICR mice and human cell lines, further analysis will be required for these speculations.

In this study, we observed that the relatively high E₂ and progesterone concentrations in the normal physiological range during pregnancy induced glucose

intolerance and insulin resistance in oophorectomized mice, and that the CAR ligand TCPOBOP rescued this abnormal glucose metabolism with decreased expression of gluconeogenic and lipogenic genes. Our data suggest that the activation of CAR may ameliorate the insulin resistance under the relatively high concentrations of sex steroids, E₂ and progesterone, which are compatible with the physiological range during pregnancy, via decreased activity of transcription factors in gluconeogenesis together with CAR and that CAR might be a potential therapeutic target for pregnancy-induced insulin resistance. In addition, because an increase in insulin resistance during pregnancy contributes to the development of gestational diabetes mellitus and preeclampsia (Kaaja *et al.* 1999, Seely & Solomon 2003, Kaaja & Greer 2005), we are examining whether CAR activation can ameliorate the glucose metabolism and signs of preeclampsia by improving insulin resistance using obese pregnant mice.

Declaration of interest

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

Funding

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (17591739 and 22591856).

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Received in final form 8 July 2011

Accepted 18 July 2011

Made available online as an Accepted Preprint 18 July 2011