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Dependence on the Microtubule Network and 90-kDa Heat Shock Protein of Phenobarbital-Induced Nuclear Translocation of the Rat Constitutive Androstane Receptor[S]

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ABSTRACT

The role of the microtubule network in the constitutive androstane receptor (CAR)-mediated transactivation of CYP2B induced by phenobarbital (PB) in rat primary hepatocytes was investigated using the microtubule-disrupting agent nocodazole (NCZ). In human hepatocytes, it was reported that CAR mRNA expression was decreased by a microtubule-disrupting agent through the inhibition of glucocorticoid receptor (GR)-mediated transactivation. However, in the present study, we show that the rat CAR gene was unaffected by the GR-mediated pathway in rat primary hepatocytes treated with NCZ. The PB-induced expression of CYP2B mRNA was repressed in the presence of NCZ for 2 h before and during 4 h of PB treatment, whereas the CAR mRNA and protein expression levels were not affected. Furthermore, the transactivation of the PB-responsive enhancer module-luciferase reporter gene and the nuclear transport of CAR induced by PB were also repressed in the presence of NCZ. Based on these findings, microtubular integrity might be required for PB-induced nuclear translocation of CAR in rat primary hepatocytes. In the same procedures, except that NCZ was replaced with radicicol, the CYP2B mRNA expression induced by PB was also repressed. Taking these into consideration, PB-mediated nuclear translocation of rCAR might be dependent on the 90-kDa heat shock protein as well as the microtubule network.

The constitutive androstane receptor (CAR), a member of the nuclear hormone receptor superfamily of ligand-activated transactivation factors, was originally classified as a xenobiotic-sensing transcription factor along with the pregnane X receptor (Honkakoski et al., 1998). CAR regulates numerous genes, including those encoding CYP2B enzymes in response to any of a large group of xenobiotics and endobiotics represented by phenobarbital (PB) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), and bilirubin, respectively (Sueyoshi et al., 1999; Tzameli et al., 2000). The PB response is mediated by the constitutive androstane receptor (CAR) but they do not bind directly to each other; whereas, TCPOBOP is an agonist ligand of CAR (Sueyoshi et al., 1999; Moore et al., 2000; Tzameli et al., 2000). In addition to CYP2B, CAR regulates the expression of multiple drug and hormone-metabolizing enzymes and transporter proteins such as CYP3A, CYP2C, glutathione transferases, sulfotransferases, UDP-glucuronosyltransferases, and Mrp2 (Sugatani et al., 2001; Ferguson et al., 2002; Goodwin et al., 2002; Kast et al., 2002; Saini et al., 2004).

Unlike other steroid receptors such as the estrogen receptor, which is located in the nucleus in the absence of ligand, the androgen receptor and glucocorticoid receptor (GR) are distributed predominantly in the cytoplasmic compartment. After ligand binding, androgen receptor and GR translocate to the nucleus (Haché et al., 1999; Roy et al., 2001). Nuclear translocation of GR has been well investigated. The GR contains two nuclear localization signals (NLS): NL1, which is located in the area from the DNA binding domain to the hinge region, is similar in sequence to the monopartite SV40 NLS; NL2 is poorly defined and resides in the ligand-binding domain (Picard and Yamamoto, 1987; Cadepond et al., 1992; Savory et al., 1999). GR is complexed with the 90-kDa heat

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ABBREVIATIONS: CAR, constitutive androstane receptor; PB, phenobarbital; NCZ, nocodazole; COL, colchicine; RAD, radicicol; GR, glucocorticoid receptor; Hsp, heat shock protein; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; Dex, dexamethasone; GFP, green fluorescent protein; NLS, nuclear localization signal; PBREM, phenobarbital-responsive enhancer module; GA, geldanamycin; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FK506, tacrolimus; SV40, simian virus 40.

shock protein (Hsp90), Hsp70, and FK506 binding protein 52 in the cytoplasmic compartment (Yang and DeFranco, 1996; Czar et al., 1997). Upon binding of ligands such as cortisol and dexamethasone, GR translocates from the cytoplasm to the nucleus through nuclear pores (Pratt et al., 2004). The movement to the nuclear membrane of GR is dependent on the Hsp90 and dynein motor complex (Harrell et al., 2004). Furthermore, this nuclear transport of GR requires intact microtubules (Galigniana et al., 1998). At the nuclear membrane, the NLS of GR are recognized by transport proteins called importins and undergo facilitated diffusion through nuclear pores.

Dvorak et al. (2003) and Dvorák et al. (2007) showed that the microtubule disrupting reagent colchicine (COL) inhibited PB-inducible CYP2B6 mRNA expression in human primary hepatocytes. Because human CAR is known to be a target gene of GR (Pascussi et al., 2000, 2003), COL might repress GR-mediated mRNA expression of CAR by blocking GR nuclear translocation, affecting the GR-CAR-CYP2B cascade.

CAR is complexed with cytoplasmic CAR retention protein and Hsp90 on microtubules in the cytoplasmic compartment (Kobayashi et al., 2003; Yoshinari et al., 2003). In the livers of mice, pretreatment with the Hsp90 inhibitor geldanamycin (GA) inhibits the nuclear translocation of CAR and cyp2b10 mRNA expression induced by TCPOBOP. Furthermore, we identified two NLS, NLS1 and NLS2, in the DNA binding domain and ligand-binding domain of the rat CAR (rCAR) molecule, respectively (Kanno et al., 2005b, 2007). Based on these observations, the PB-induced nuclear translocation of CAR might require an intact microtubule network, as in the case of GR. In this study, we show that PB-induced nuclear translocation of CAR requires an intact microtubule network and Hsp90.

Materials and Methods

Chemicals and Plasmid Construction. Nocodazole (NCZ) is a product of Calbiochem (San Diego, CA). PB, COL, radicicol (RAD), and dexamethasone (Dex) were purchased from Wako Pure Chemical (Tokyo, Japan). The constructions of pEGFP-rCAR, pcDNA-rCAR, and pGL3-PBREM plasmids have been described previously (Kanno et al., 2005a,b).

Cell Culture. The hepatocytes for primary culture were separated from the livers of 6-week-old male Wistar rats (CLEA, Tokyo, Japan) using the collagenase perfusion method. After enumeration of the cells by Trypan blue dye exclusion, the hepatocytes were cultured in WE medium supplemented with 10% fetal bovine serum. Four hours after plating, the medium was exchanged with WE medium containing 10^{-8} M Dex. To evaluate the effects of glucocorticoid, Dex-free WE medium was used.

Real-Time RT-PCR. Rat primary hepatocytes were seeded in six-well collagen-coated plates. The next day, the cells were treated with 10 μg/ml NCZ or DMSO (vehicle) for 2 h before and for a further 4 h after the addition of 1 mM PB. In the case of Dex treatment, the cells were treated with 1 μM Dex or DMSO for 24 h. Total RNA was isolated with the SV Total RNA Isolation System (Promega, Madison, WI). The RNA concentration was quantified by spectrometry at 260 nm. Reverse transcription was performed with 1 μg of total RNA and the Ready-to-Go T-Primed First-Strand Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer's protocol. Real-time PCR was performed with SYBR Green Realtime PCR Master Mix -Plus (Toyobo Engineering, Osaka, Japan). The oligonucleotide sequences of the primers used for PCR were as follows: CAR: sense, 5′-ACCAGTTTGTGCAGTTCAGG-3′; antisense, 5′-CTTGAGAAGGGAGATCTGGT-3′; CYP2B1/2: sense, 5′-GAGTTCT-TCTCTGGGTTCCTG-3′; antisense, 5′-ACTGTGGGTCATGGA-GAGCTG-3′; CYP3A1: sense, 5′-GTTCACCAGTGGAAGACTCA-3′; antisense, 5′-CTGTAGGCACCAAACACTTC-3′; and GAPDH: sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCT-GTTGCTGTA-3′.

Western Blot Analysis. The cells treated as described above were washed with PBS and harvested with buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.5% Nonidet P-40) supplemented with protease inhibitor cocktail. After being placed on ice for 15 min, the cells were homogenized by pipetting. The cell homogenates were centrifuged (4°C, 15,000*g*) for 10 min, and the pellets were suspended in 100 μl of buffer C (20 mM HEPES, pH 7.9, 0.2M NaCl, 1 mM EDTA, and 1 mM EGTA) after being washed with PBS. The suspended pellets were vortexed for 10 s, placed on ice for 30 min, vortexed for 15 s, and then placed on ice for 10 min. After centrifugation (4°C, 15,000*g*) for 15 min, the supernatants were collected and stored at −80°C until use (nuclear extract). Total protein was extracted with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100) supplemented with protease inhibitor cocktail. The protein concentration was measured with the 2-D Quant Kit (GE Healthcare). Protein samples (5 μg) were separated by SDS-PAGE (gel concentration, 10%). Western blotting was performed with rabbit anti-hCAR antibody (1:1000 dilution; Perseus Proteomics, Tokyo, Japan) or anti-lamin B antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) as a primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 dilution; Cell Signal-

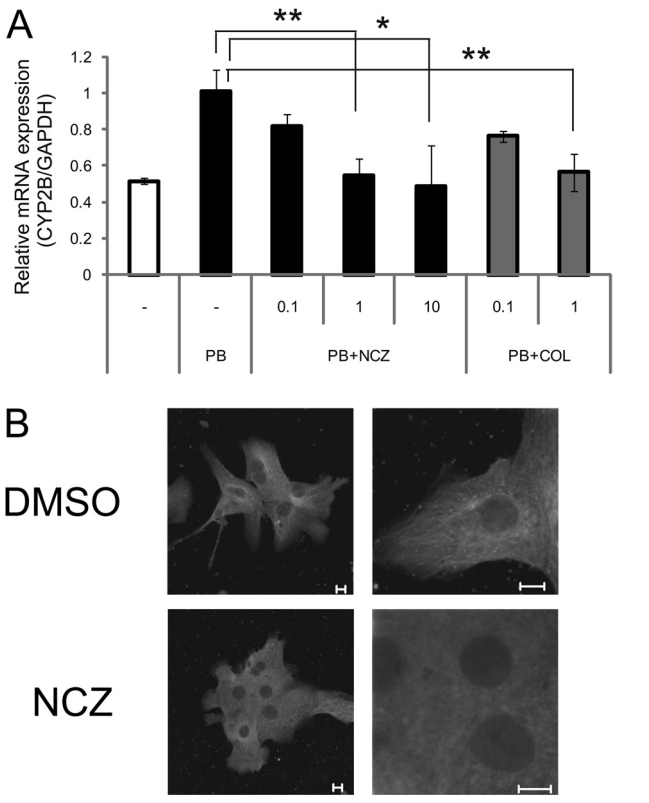

Fig. 1. The effect of NCZ and COL on PB-mediated CYP2B1/2 induction. A, rat primary hepatocytes were treated with 0.1, 1, and 10 μg/ml NCZ and 0.1 and 1 μg/ml COL dissolved in DMSO for 2 h before and during 4 h of PB treatment. Total RNA was extracted, and the mRNA expression of CYP2B was measured by real-time RT-PCR. The results were normalized against those of GAPDH. The results are expressed as the mean ± S.D. (*, $P < 0.05$, $n = 3$). B, microtubule visualization was performed with anti-β-tubulin antibody followed by FITC-conjugated anti-mouse IgG. Fluorescence was observed with a confocal microscopy.

ing Technology, Danvers, MA) as a secondary antibody. Protein bands were visualized using Immobilon Western Detection Reagent (Millipore, Billerica, MA).

Luciferase Reporter Analysis. Rat primary hepatocytes were seeded in 24-well collagen-coated plates and transfected with 500 ng of pGL3-PBREM reporter plasmids and 50 ng of each of CAR-expression and pRL-SV40 plasmids using the TransFectin Transfection Reagent (Bio-Rad Laboratories, Hercules, CA). The next day, the cells were treated with 10 μg/ml NCZ or DMSO (vehicle) for 2 h and for a further 12 h after the addition of 1 mM PB. The cells were harvested, and luciferase activity was measured according to the manufacturer's protocol (Promega).

Immunofluorescence. Rat primary hepatocytes were treated with NCZ or DMSO for 6 h. The cells were fixed with ice-cold methanol and then washed with PBS. The cells were incubated with monoclonal anti-β-tubulin antibody (Sigma-Aldrich, St. Louis, MO) overnight. The FITC-conjugated anti-mouse IgG (Sigma-Aldrich) was used as a secondary antibody. Fluorescence was observed with a Zeiss LSM 510 (Carl Zeiss GmbH, Jena, Germany).

Intracellular Localization Analysis. Rat primary hepatocytes were seeded in four-well collagen-coated chambered coverglasses (Iwaki Corporation, Tokyo, Japan). The cells were transfected with pEGFP-rCAR plasmids on the day of plating using TransFectin Transfection Reagent (Bio-Rad Laboratories). After overnight incubation, the cells were treated with 1 mM PB for 4 h after 2-h pretreatment with NCZ or DMSO. The intracellular expression profiles of GFP-rCAR chimerical proteins were observed with a Zeiss LSM 510. The cells were fractionated into three categories based on their subcellular distribution of fluorescence signals as follows: predominantly nuclear distribution (N > C), even distribution between cytoplasmic and nuclear regions (N = C), and predominantly cytoplasmic distribution (N < C).

Results

Microtubule-Interfering Agent NCZ Inhibits PB-Mediated CYP2B Induction. First, we examined the effect of microtubule-disrupting agents, NCZ and COL, on CYP2B mRNA induction by PB. The enhanced expression of CYP2B mRNA observed after 4 h of PB treatment was repressed in the presence of NCZ or COL for 2 h before PB treatment and during 4-h PB treatment in a dose-dependent manner (Fig. 1A). Next, we tested whether NCZ-treatment disrupted the microtubule network by immunofluorescence staining of tubulin. Figure 1B shows that the microtubule network disruption is caused with 10 μg/ml NCZ for 6 h.

Microtubule Network Disruption Does Not Affect CAR Expression in Rat Primary Hepatocytes. Because disruption of the microtubule network was found to repress human CAR mRNA expression via inhibition of the GR-mediated pathway (Dvorak et al., 2003; Dvorák et al., 2007), we measured the CAR protein and mRNA expression levels with NCZ or COL in rat primary hepatocytes. Rat primary hepatocytes were incubated for 6 or 24 h with either NCZ or

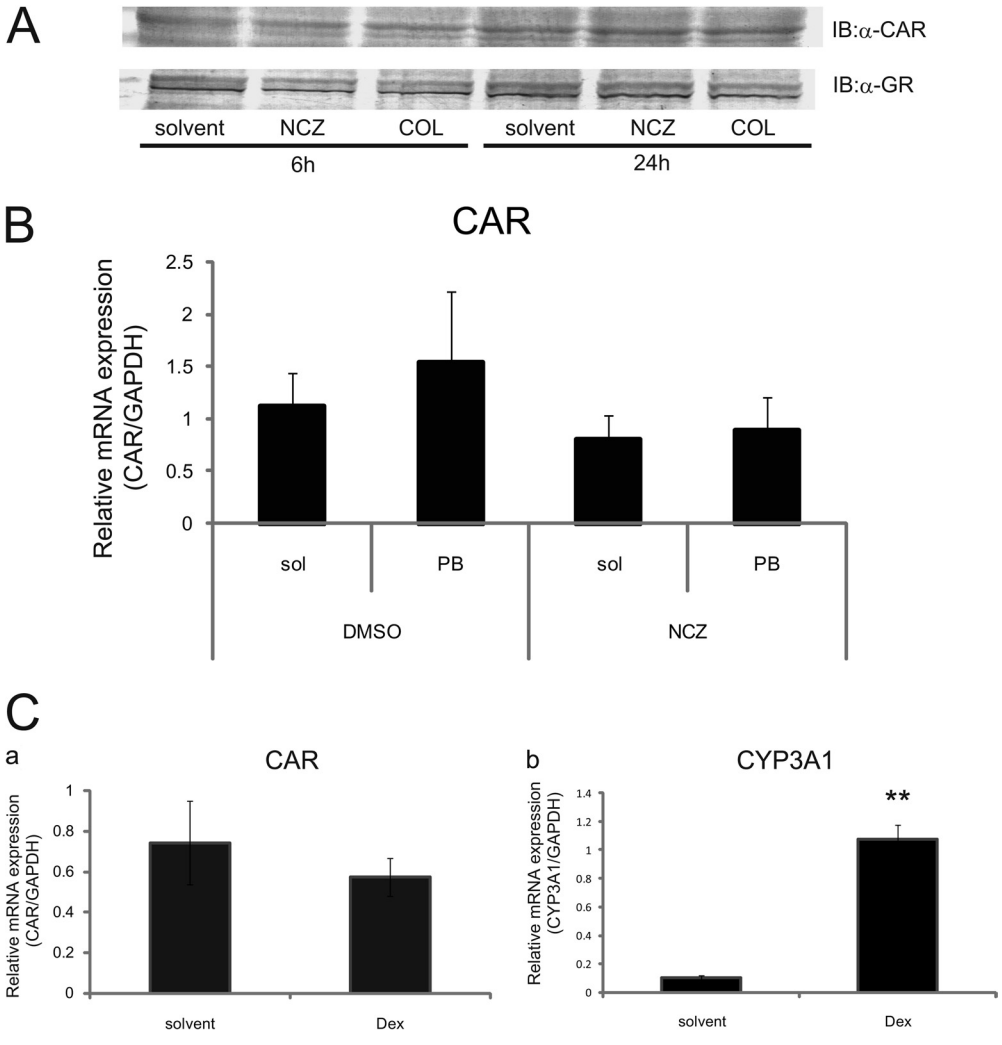

Fig. 2. The effects of NCZ and Dex on the expression of CAR protein and mRNA. A, the rat primary hepatocytes were incubated for 6 or 24 h with 10 μg/ml NCZ or 1 μg/ml COL. Whole-cell lysates (20 μg) were resolved by SDS-PAGE, and the protein bands were detected by immunoblotting using anti-CAR and anti-GR antibodies. B, after 2 h of pretreatment with 10 μg/ml NCZ, the culture of rat primary hepatocytes was continued for a further 4 h in the absence or presence of 1 mM PB. The expression of CAR mRNA was measured by real-time RT-PCR. The results were normalized against those of GAPDH and are expressed as the mean ± S.D. (n = 3). C, rat primary hepatocytes were treated with DMSO or 1 μM Dex for 24 h. Total RNA was extracted, and the mRNA expression of CAR (a) and CYP3A1 (b) was measured by real-time RT-PCR. The results were normalized against those of GAPDH. The results are expressed as the mean ± S.D. (*, $P < 0.05$, n = 3).

COL. The expression levels of CAR and GR proteins detected by Western blot analysis using anti-CAR and anti-GR antibodies, respectively, were not affected by treatment with NCZ or COL (Fig. 2A). Furthermore, CAR mRNA expression was only slightly decreased with NCZ (Fig. 2B). These results suggested that CAR expression was not regulated by GR. Thus, we analyzed whether rat CAR mRNA expression was regulated by GR in rat primary hepatocytes. Although mRNA expression of the GR-target gene (CYP3A1) was increased by treatment with a synthetic GR ligand (Dex) in rat primary hepatocytes (Fig. 2C, b), no simultaneous escalation of CAR mRNA expression was observed (Fig. 2C, a). These observations suggest that the transactivation of rat CAR is not regulated by GR, in contrast with the human CAR. Microtubule network disruption inhibits CAR-mediated induction of CYP2B mRNA in rat primary hepatocytes.

Microtubule Network Disruption Inhibits PBREM-Transactivation in Rat Primary Hepatocytes. To investigate whether CAR mediates the PB-induced CYP2B mRNA expression that was found to be susceptible to NCZ-inhibition, we measured luciferase activity using PBREM-luciferase reporter plasmid. In accordance with the results found for CYP2B mRNA, the enhanced luciferase activity caused by PB treatment was repressed in the presence of NCZ and COL (Fig. 3). These results suggest that disruption of microtubule network interferes directly with the activation of CAR.

PB-Mediated Nuclear Translocation of CAR Requires an Intact Microtubule Network. It is known that an intact microtubule network is required for the nuclear translocation of GR. Therefore, the effect of a disrupted microtubule network on CAR nuclear translocation was investigated using confocal microscopy. Rat primary hepatocytes transiently transfected with the expression plasmids for GFP-tagged rat CAR (GFP-rCAR) were treated with NCZ and PB simultaneously or individually as above 24 h after transfection. A cytoplasmic-dominant distribution of GFP-rCAR was observed in the untreated cells (Fig. 4A). The nuclear accumulation of GFP-rCAR induced by PB treatment was inhibited in the presence of NCZ (Fig. 4A). In addition to these observations, the accumulation of endogenous CAR protein in the nuclear fraction induced by PB treatment was inhibited in the presence of NCZ, without change in total CAR protein levels (Figs. 2A and 4B). These results suggest that an intact microtubule network is a prerequisite for the nuclear translocation of CAR in rat primary hepatocytes.

PB-Mediated Nuclear Translocation of CAR Requires Hsp90. Microtubule-dependent nuclear translocation of GR required Hsp90 (Galigniana et al., 1998; Harrell et al., 2004). Likewise, the nuclear translocation of mouse CAR (mCAR) induced by TCPOBOP was inhibited by GA, suggesting the important role of Hsp90 in the nuclear translocation of ligand-bound mCAR (Yoshinari et al., 2003). Therefore, we examined whether nuclear translocation of CAR induced by PB, which was known as an activator because of the lack of direct binding to CAR, was dependent on Hsp90 using its inhibitor RAD. The short-time treatment with RAD repressed the CYP2B mRNA expression induced by PB (Fig. 5, A and B). PB-dependent nuclear accumulation of rCAR was also inhibited by pretreatment with RAD (Fig. 5, C and D). Taking these observations into consideration, PB-mediated nuclear translocation of rCAR might be dependent on Hsp90 as well as the microtubule network.

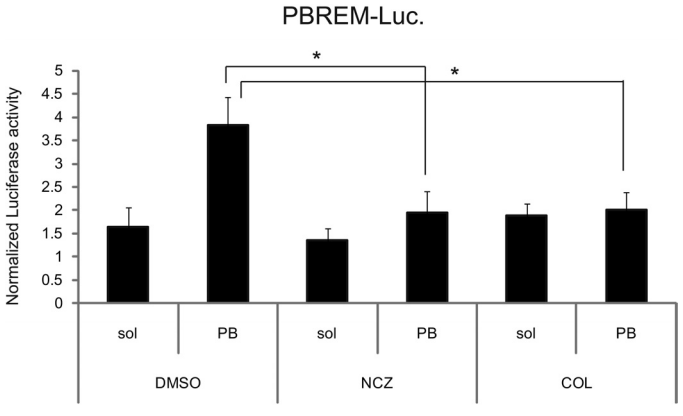

Fig. 3. NCZ and COL inhibits PB-mediated PBREM-transactivation. Rat primary hepatocytes were transfected with PBREM-luc, pRL3-SV40, and pcDNA-rCAR plasmids. Rat primary hepatocytes were treated with 10 μg/ml NCZ and 1 μg/ml COL dissolved in DMSO for 2 h before and during 12 h of PB treatment, and then a luciferase assay was performed. The results are expressed as the mean ± S.D. (**, $P < 0.01$, $n = 4$).

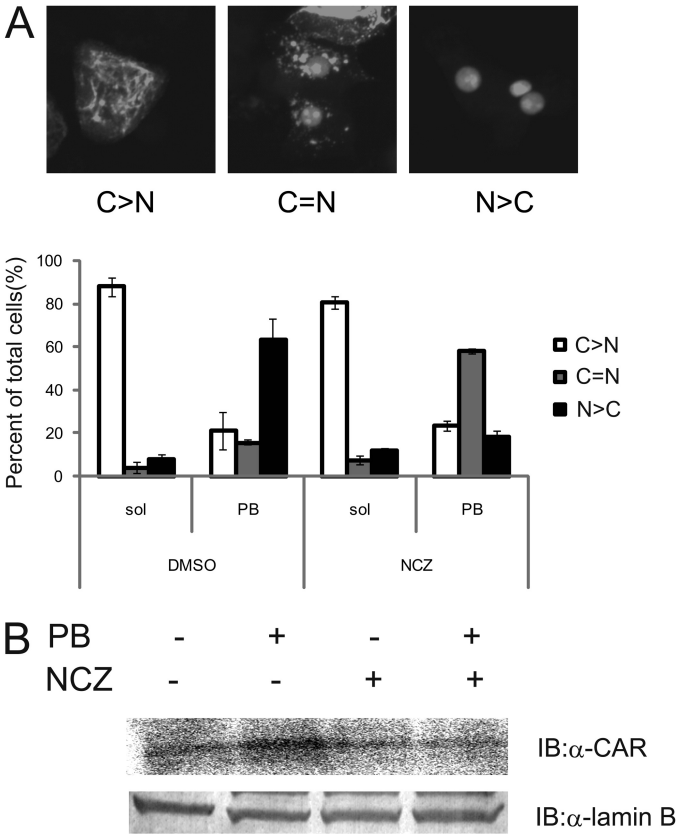

Fig. 4. The effect of NCZ on the PB-induced nuclear translocation of CAR. A, rat primary hepatocytes were transfected with the pEGFP-rCAR plasmid, which codes for GFP-rCAR. Twenty-four hours after the transfection, 10 μg/ml NCZ was added to the culture 2 h before the addition of 1 mM PB. After 4 h of PB treatment, the cells were observed with a confocal laser-scanning microscope. Intracellular localization of fluorescence-positive cells was conducted based on the subcellular distribution of the fluorescence signal as follows: predominantly nuclear fluorescence (N > C), fluorescence evenly distributed between the cytoplasmic and nuclear regions (N = C), and predominantly cytoplasmic fluorescence (N < C). For intracellular localization analysis, at least 200 GFP-positive cells were observed. Error bar shows mean ± S.E. from three independent experiments. B, nuclear extracts (5 μg) of the cells treated as shown in A were resolved by SDS-PAGE, and CAR proteins were detected by Western blot analysis using anti-CAR antibody and anti-lamin B antibody.

Discussion

In the present study, we show that the microtubule-disrupting agent NCZ inhibits PB-elicited CYP2B induction in rat primary hepatocytes via inhibition of the nuclear translocation of CAR. Unlike in human primary hepatocytes, the expression levels of rat CAR mRNA and protein were not markedly changed by NCZ or COL treatment. Rat CAR protein was confirmed by a specific antibody, and the results are shown in supplemental Fig. 1. Disruption of the microtubule network by long-term colchicine treatment for 24 h inhibited PB-mediated CYP2B6 and CYP3A4 induction, repressing CAR and pregnane X receptor expression levels via the inhibition of GR-mediated expression of individual mRNA in human primary hepatocytes (Dvorak et al., 2003). In humans, we speculate that microtubule network disruption is affected by two-step inhibition mechanisms on PB-mediated CYP2B induction. Because the CAR gene was the target of GR-mediated transactivation, the PB-induced expression of CAR target genes such as CYP2B6 and CYP3A4 is considered to be controlled by the microtubule network at dual steps: during nuclear trafficking of GR, and after nuclear trafficking of CAR. Unlike hCAR, rat CAR might not be the target gene of GR. Therefore, in rat primary hepatocytes, the disruption of microtubule network would affect the PB-induced expression of CAR target genes by interfering with nuclear trafficking of CAR, of which the transcription might not be controlled by GR. The divergence in their promoter sequences between human and rat might be responsible for the observed species-specific difference in the control by GR and the susceptibility of expression of CAR to the microtubule network disruption.

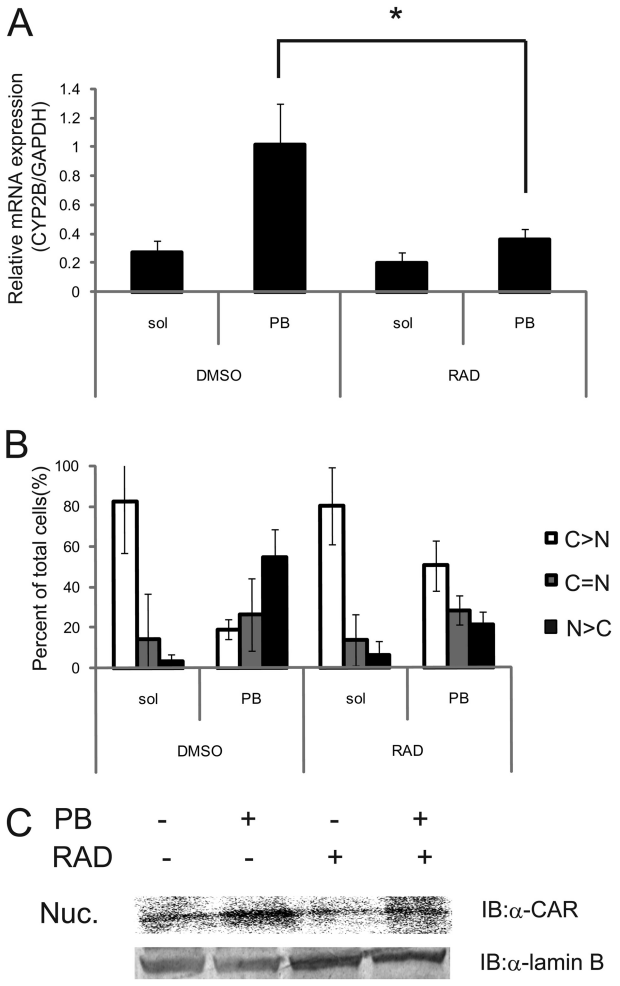

Fig. 5. Effect of RAD on the PB-induced CYP2B mRNA expression and nuclear translocation of CAR. A, after 2 h of pretreatment with 10 μM RAD dissolved in DMSO, the culture of rat primary hepatocytes was continued for a further 4 h in the absence or coexistence of 1 mM PB. Total RNA was extracted from cells, and the mRNA expressions of CYP2B measured by real-time RT-PCR were normalized by those of GAPDH. The results are expressed as the mean ± S.D. (*, $P < 0.05$, $n = 3$). B, rat primary hepatocytes were transfected with plasmids encoding for GFP-rCAR for 24 h. After 2 h of pretreatment with 10 μM RAD dissolved in DMSO, the culture of rat primary hepatocytes was continued for a further 4 h in the absence or coexistence of 1 mM PB. The cells were observed with a confocal laser-scanning microscope. Intracellular localization analysis is described in the legend to Fig. 4. Error bar shows mean ± S.E. from three independent experiments. C, after 2 h of pretreatment with 10 μM RAD dissolved in DMSO, the culture of rat primary hepatocytes was continued for a further 4 h in the absence or coexistence of 1 mM PB. Nuclear extracts (20 μg) of the cells treated as shown in A were resolved by SDS-PAGE, and CAR proteins were detected by Western blot analysis using anti-CAR antibody and anti-lamin B antibody.

Microtubule-dependent nuclear translocation of GR requires Hsp90 (Galigniana et al., 1998; Harrell et al., 2004). A previous report suggested that GA inhibited the induction of Cyp2b10 mRNA expression via the mouse CAR ligand TCPOBOP (Yoshinari et al., 2003). In addition, pretreatment with the Hsp90 inhibitor radicicol inhibited both the expression of CYP2B mRNA and the CAR nuclear translocation induced by PB in rat primary hepatocytes. Thus, intact Hsp90 and an intact microtubule network might be required for the nuclear translocation of CAR induced by CAR activators and ligands as well as the cytoplasmic retention of CAR in the liver and primary hepatocytes in their absence. Although it is known that the GR-Hsp90 complex is linked to cytoplasmic dynein, a molecular motor that processes along microtubular tracks to the nucleus, the equivalent molecule for the CAR-Hsp90 complex has not yet been elucidated. Thus, further investigations are required to elucidate the nuclear translocation mechanism of CAR.

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