RESEARCH ARTICLE

Capsaicin induces CYP3A4 expression via pregnane X receptor and CCAAT/enhancer-binding protein β activation

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Scope: Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the principal pungent ingredient in hot red and chili peppers. Many studies have focused on the anticarcinogenic or chemopreventive activities of capsaicin. However, the influence of capsaicin on CYP3A4, its involvement in drug metabolism, and the underlying mechanisms remain unclear.

Methods and results: Here, we examined the effect of capsaicin on CYP3A4 expression and the metabolism of CYP3A1 substrate, nifedipine in male Sprague–Dawley rats. Capsaicin induced the enzymatic activity and expression of CYP3A4 in HepG2 cells. Treatment with a human pregnane X receptor (hPXR) inhibitor reduced the inductive effects of capsaicin on CYP3A4 expression. Capsaicin also induced the activation of CCAAT/enhancer-binding protein β (C/EBPβ). Moreover, capsaicin increased the activation of the transient receptor potential vanilloid type-1 receptor downstream signaling components Ca^{2+}/calmodulin-dependent protein kinase and Akt. Capsaicin elevated the level of CYP3A1 in rat liver and significantly increased the biotransformation of nifedipine to dehydronifedipine.

Conclusion: From these data, we conclude that capsaicin induces CYP3A4 expression in vitro and in vivo. This induction was achieved by the activation of hPXR and C/EBPβ. Our results suggest that capsaicin might induce CYP3A4 expression; thus, exposure to capsaicin may increase the metabolism of CYP3A4 substrate and potentially cause food–drug interactions.

Keywords:
Capsaicin / C/EBPβ / CYP3A4 / Food–drug interactions / PXR

1 Introduction

Several foods have been reported to interact with medications, leading to clinically relevant adverse drug reactions. These interactions increase the oral bioavailability of a number of drugs, including cyclosporin, midazolam, and triazolam [1]. Food–drug interactions can arise from the modulation of cytochrome P450 (CYP) isoenzymes such as CYP3A4. In this regard, several herbs and foods have already been reported to induce or inhibit CYP3A4 [1]. It is estimated that CYP3A4 is responsible for the metabolism of > 50% of medications,

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Abbreviations: AC/EBP, constitutively active C/EBP-specific dominant-negative mutant; **C/EBPβ**, CCAAT/enhancer-binding protein β; **CaMK**, Ca^{2+}/calmodulin (CaM)-dependent protein kinase; **CPS**, capsaicin; **CPZ**, capsazepine; **hPXR**, human pregnane X receptor; **LAP**, liver-enriched transcription activating protein; **LIP**, liver-enriched transcription inhibitory protein; **NIF**, nifedipine; **PCN**, pregnenolone 16α-carbonitrile; **PXR**, pregnane X receptor; **RIF**, rifampicin; **SFN**, sulforaphane; **TRPV1**, transient receptor potential vanilloid type-1 receptor

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many of which are either metabolically activated and/or metabolically inactivated (detoxified). Therefore, transcriptional and posttranscriptional alterations in CYP3A4 activity have direct effects on the efficacy of these medications [2, 3]. Recent molecular and pharmacological studies have demonstrated that the transcriptional activation of CYP3A4 is mediated by pregnane X receptor (PXR) or CCAAT/enhancer-binding protein (C/EBP). Human PXR (hPXR, NR1I2) is essential for the regulation of a large and growing array of drug disposition genes corresponding to all phases of drug metabolism. hPXR regulates the expression of CYP3A4 by forming a heterodimer with its obligate partner, retinoid X receptor (RXR), and the complex binds to the nuclear receptor response elements found in the regulatory regions of these genes. Celia et al. [4] highlighted the critical involvement of C/EBPβ, which binds to the 5′-flanking region (−5950/−5663 bp) of CYP3A4 in human hepatocytes. C/EBP transcription factors are critical for normal cellular differentiation and function in a variety of tissues. The prototypic C/EBP contains a basic region involved in DNA binding followed by a leucine zipper motif involved in homodimerization and heterodimerization with other family members. C/EBPα and C/EBPβ are the predominant C/EBP isoforms expressed by adult hepatocytes in the healthy liver [5, 6].

Capsaicin stimulates transient receptor potential vanilloid type-1 receptor (TRPV1), located primarily on polymodal C-fibers, and initiates a complex cascade of events, including neuronal excitation and the release of proinflammatory mediators, desensitization of the receptor, and neuronal toxicity [7–10]. Capsaicin is converted to phenoxy radical intermediates, including reactive species by CYP2E1, which have chemoprotective activity against specific chemical carcinogens and mutagens [11]. We recently reported that capsaicin weakly induced CYP1A1 expression and that 3-MC-induced CYP1A1 levels were suppressed by capsaicin; C/EBPβ activation and 3-MC inhibition induced AhR transactivation by capsaicin, contributing to the suppression of CYP1A1 expression [12]. However, the influence of capsaicin on CYP3A4 in hepatocytes and the interaction between capsaicin-induced CYP3A4 and transcriptional regulators such as hPXR and C/EBPβ are unclear. Thus, we investigated the effect of capsaicin on CYP3A4 expression in HepG2 cells. In this study, we demonstrate that capsaicin regulates the expression of CYP3A4 through hPXR- and C/EBPβ-dependent mechanisms.

2 Materials and methods

2.1 Materials

The chemicals and cell culture materials used in this study were obtained from the following sources: capsaicin (purity, > 99.8%), rifampicin, capsazepine (CPZ), SB-366791, sulforaphane (SFN), pregnenolone 16α-carbonitrile (PCN), nifedipine (NIF), dehydronifedipine (DNIF), and methaqualone (internal standard, IS) were obtained from Sigma Chemical Co. (St. Louis, MO); the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay kit was obtained from Roche Co. (London, UK); the CYP3A4 enzymatic activity kit was obtained from Promega (Madison, WI); Lipofectamine™ 2000, the SYBR® Safe DNA Gel Stain kit, and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Invitrogen Co. (Carlsbad, CA); fetal bovine serum (FBS), penicillin-streptomycin solution, and trypsin were obtained from Life Technologies, Inc. (Carlsbad, CA); pCMV-β-gal was obtained from Clontech (Palo Alto, CA); the protein assay kit was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA); the antibodies used [against CYP3A4, CYP3A1, C/EBPβ (C-19), phospho-CaMKIα (Thr177), and β-actin (C4)] were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-Akt/phospho-Akt (Ser473), phospho-C/EBPβ (Thr188/Thr37) (C-19), and the secondary antibodies (HRP-linked anti-rabbit and -mouse IgG) were obtained from Cell Signaling Technology (Danvers, MA); W7 (N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide) and LY 294002 were obtained from Calbiochem (La Jolla, CA); acetonitrile was obtained from J.T. Baker (Seoul, Korea); and the ECL chemiluminescence system and polyvinylidene difluoride membranes were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All chemicals were of the highest grade commercially available.

2.2 Cell culture and treatment

The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. Capsaicin and rifampicin were dissolved in ethanol and DMSO, respectively; stock solutions were added directly to the culture media. The control cells were treated with DMSO alone; the final concentration of DMSO was kept at < 0.2%.

2.3 Cytotoxicity assay

Cell cytotoxicity was examined using a WST-1-based assay kit according to the manufacturer's instructions. Capsaicin (1–100 μM) was added to the wells and the plates were incubated at 37°C. After 24 h, the cells were treated with 10 μL of WST-1 solution. Relative cytotoxicity was quantified by measuring the absorbance at 550 nm using a spectrophotometer (Varioskan; Thermo Electron Co., Marietta, OH). Capsaicin does not interfere at this wavelength.

2.4 Measurement of CYP3A4 enzymatic activity

HepG2 cells were plated in 24-well plates and cultured overnight. The cells were then transfected with plasmids containing the hPXR (pPXR) gene and treated with capsaicin (1–20 μM) for 48 h. CYP3A4 enzymatic activity was measured using a P450-Glo CYP3A4 enzymatic activity kit (Promega) according to the manufacturer's instructions.

2.5 Quantitative polymerase chain reaction (PCR)

pPXR-transfected HepG2 cells were incubated with rifampicin (10 μM) or capsaicin (1–20 μM) for 24 h. Total RNA was extracted from the treated cells using RNAiso reagent (Takara, Shiga, Japan) according to the manufacturer's instructions. PCR was performed for 35 cycles under the following conditions: denaturation at 98°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 30 s. The band intensities of the amplified DNA were visualized using a SYBR® Safe DNA gel stain kit.

2.6 Real-time PCR

pPXR-transfected HepG2 cells were incubated with rifampicin (10 μM) or capsaicin (1–20 μM) for 24 h. To detect CYP3A4, total RNA was extracted after stimulation and treatment with RNAiso reagent (Takara) according to the manufacturer's instructions. Product formation was monitored continuously during PCR using Sequence Detection System software (ver. 1.7; Applied Biosystems, Foster City, CA). The accumulated PCR products were detected directly by monitoring the increase in the reporter dye (SYBR®). The expression levels of CYP3A4 in the exposed cells were compared to those in control cells at each time point using the comparative cycle threshold method. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of actin, a housekeeping gene.

2.7 Chromatin immunoprecipitation (ChIP) assay

pPXR-transfected HepG2 cells that had been incubated with capsaicin (1–20 μM) were cross-linked with formaldehyde in PBS at room temperature for 10 min then sonicated. The sonicated chromatin-DNA complexes were precipitated with antibodies against hPXR or C/EBPβ or nonspecific mouse IgG. PCR analysis was performed using 2 μL of purified DNA, oligonucleotide primers, and Platinum Taq DNA polymerase. After 40 cycles, the products were analyzed using a SYBR® Safe DNA gel stain kit.

2.8 Luciferase and β-galactosidase assays

Cells were transfected with 1 μg of pCYP3A4-Luc, pC/EBP-Luc, pPXR plasmid, AC/EBP plasmid, and/or 0.2 μg of pCMV-β-gal per well using Lipofectamine™ 2000. A chimeric CYP3A4 luciferase reporter plasmid was created by inserting the CYP3A4 upstream regulatory region (−7836 to −6093) PCR product into the pGL3 basic vector [13]. pC/EBP-Luc was purchased from Stratagene (La Jolla, CA); the pPXR expression vector was kindly provided by Dr. Sang Seop Lee (Inje University College of Medicine, Busan, South Korea). The AC/EBP expression vector, which was a gift from Dr. Keon Wook Kang (Chosun University, Gwangju, South Korea), encoded a mutant form of the C/EBP region. After transfection for 4 h, fresh medium containing 10% FBS was added to the cells. The cells were incubated with capsaicin (1–20 μM) for 18 h then lysed. The lysed cell preparations were centrifuged at 12 000 rpm for 10 min, and the supernatant assayed for both luciferase and β-galactosidase activity. Luciferase activity was measured using a luciferase assay system (Promega) with a luminometer, according to the manufacturer's instructions. The β-galactosidase assay was performed in 250 μL of assay buffer containing 0.12 M Na_2HPO_4, 0.08 M NaH_2PO_4, 0.02 M KCl, 0.002 M $MgCl_2$, 0.1 M β-mercaptoethanol, and 50 μg of o-nitrophenyl-β-galactoside. Luciferase activity was normalized to β-galactosidase activity and expressed as the proportion of activity detected, relative to the vehicle control.

2.9 Western blotting

Cell lysates were prepared after capsaicin treatment and resolved by 10% SDS-PAGE. The membranes were probed with the appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were visualized using an ECL Western blot kit, according to the manufacturer's instructions.

2.10 Animal treatment and preparation of microsomal fractions

Five-week-old male Sprague–Dawley rats were obtained from Daehan Biolink (Chungbuk, Korea). The rats were allowed access to Purina rodent chow and tap water ad libitum. The animals were maintained in a controlled environment at 21 ± 2°C and 50 ± 5% relative humidity with a 12-h dark/light cycle and acclimatized for at least 1 week. All experiments were performed following the rules and regulations of the Animal Ethics Committee of Chungnam National University. The rats were divided into five groups. Groups of rats were orally administered 1, 3, and 5 mg/kg/day of capsaicin dissolved in a vehicle consisting of ethanol, Tween 80 and tap water (5:5:90%, v/v/v) for 7 days and intraperitoneally

injected with 1 mg/kg/day of PCN dissolved in olive oil for 3 days. The rats were sacrificed, their livers rapidly removed, and hepatic microsomal fractions prepared by centrifugation, as described previously [14].

2.11 Pharmacokinetic studies

The rats were randomly divided into three groups, with five rats in each group. Capsaicin dissolved in the vehicle was orally administered at 1 and 3 mg/kg/day for 7 consecutive days to the treatment groups; the vehicle was administered to the control group. The rats were allowed access to Purina rodent chow and tap water ad libitum. On day 8, all three groups were gavaged with 30 mg/kg of NIF. Heparinized blood samples (100 μL) were serially taken from the subclavicular vein at 0, 15, 30, 45, 60, 90, 120, 180, 240, 360, and 480 min, and plasma was obtained by centrifugation at 10 000×*g* for 10 min at 4°C.

2.12 Determination of the plasma NIF and DNIF concentrations

The precursor ions $[M+H]^+$ and patterns of fragmentation of NIF, DNIF, and the IS were monitored using the positive ion mode. The plasma concentrations of NIF and DNIF were quantified using an API 4000 LC/MS/MS system (Applied Biosystems) equipped with an electrospray ionization interface. The compounds were separated on a reversed-phase column (Kinetex, 150 × 2.0 mm internal diameter, 2.6 μm particle size; Phenomenex, Torrence, CA), and the mobile phase in isocratic conditions was 0.1% formic acid-ACN (1:3, v/v). The column was heated to 30°C, and the mobile phase was eluted at 0.2 mL/min using an HP 1100 series pump (Agilent, Wilmington, DE). NIF, DNIF, and the IS produced mainly protonated molecules at *m/z* 347.2, 345.0, and 251.4, respectively. The product ions were scanned in Q3 after collision with nitrogen in Q2 at *m/z* 315.0, 284.2, and 131.7 for NIF, DNIF, and the IS, respectively. Quantitation was performed by selected reaction monitoring of the protonated precursor ions and the related product ions using the ratio of the area under the peak for each solution. The analytical data were processed with Analyst software (version 1.4.1, Applied Biosystems). The IS (60 μL, 10 ng/mL in ACN) was added to 20 μL of rat plasma, vortexed for 10 s, and centrifuged at 13 200 rpm for 10 min. Finally, a 3-μL aliquot of the supernatant was injected into the column.

2.13 Pharmacokinetic analysis

Area under the plasma concentration-time curves for NIF and DNIF (AUC_{8h}) in the presence and absence of capsaicin were determined using the trapezoidal rule. AUC_{8h} ratios for DNIF (AUC_{DNIF}) and NIF (AUC_{NIF}) were calculated to measure the effect of capsaicin. Peak concentration (C_{max}) and the time (T_{max}) to C_{max} were directly derived from the curve, and the elimination rate constant (k_e) was calculated using the slope of the log-linear regression of the terminal elimination phase.

2.14 Statistical analysis

The values represent the means from three independent experiments, each performed in triplicate. One-way analysis of variance (ANOVA) was used to determine the significance of differences between the treatment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for p-values < 0.01. The ratio of AUC_{DNIF} to AUC_{NIF} was performed using ANOVA followed by the Tukey test (SPSS 10.0; SPSS Inc., Chicago, IL). In this case, $p < 0.05$ was considered to be statistically significant.

3 Results

3.1 Capsaicin regulates CYP3A4 protein, mRNA expression, and promoter activity

To determine the optimal concentrations for use in our studies, the potential cytotoxicity of capsaicin was tested in HepG2 cells. Figure 1A shows that 1, 10, 25, and 50 μM capsaicin did not affect cell viability; however, 100 μM, the highest concentration tested, caused a 49% decrease in cell viability in HepG2 cells. Thus, all subsequent studies were conducted using 1–20 μM capsaicin.

The induction of CYP3A4 in human-derived cell lines has been studied in models of human tissues [15]. HepG2 cells are one of the most useful cell lines to represent human liver due to the differentiated function of this human hepatoblastoma. However, the constitutive expression of hPXR is low or undetectable in HepG2 cells [16]. hPXR expression was achieved by transfecting a stable human PXR (hPXR) expression plasmid. Thus, we examined the influence of capsaicin on CYP3A4 expression using HepG2 cells as a tool. To confirm hPXR expression, its level was compared between HepG2 and pPXR-transfected HepG2 cells by Western blot analysis, which showed hPXR overexpression in the pPXR-transfected HepG2 cells (data not shown). To examine the effect of capsaicin on CYP3A4, we tested CYP3A4 activity in pPXR-transfected HepG2 cells; rifampicin, a hPXR agonist, was used as a positive control. When pPXR-transfected cells were treated with rifampicin or capsaicin for 24 h, there was a significant increase in CYP3A4 enzymatic activity (Fig. 1B). To confirm the effect of capsaicin, we measured the protein level of CYP3A4 by immunoblotting using pPXR-transfected HepG2 cells. Capsaicin and rifampicin induced CYP3A4 protein expression (Fig. 1C). The effects of capsaicin on CYP3A4 gene expression was further investigated using quantitative and real-time PCR assays. Capsaicin and rifampicin increased the expression of CYP3A4 mRNA in

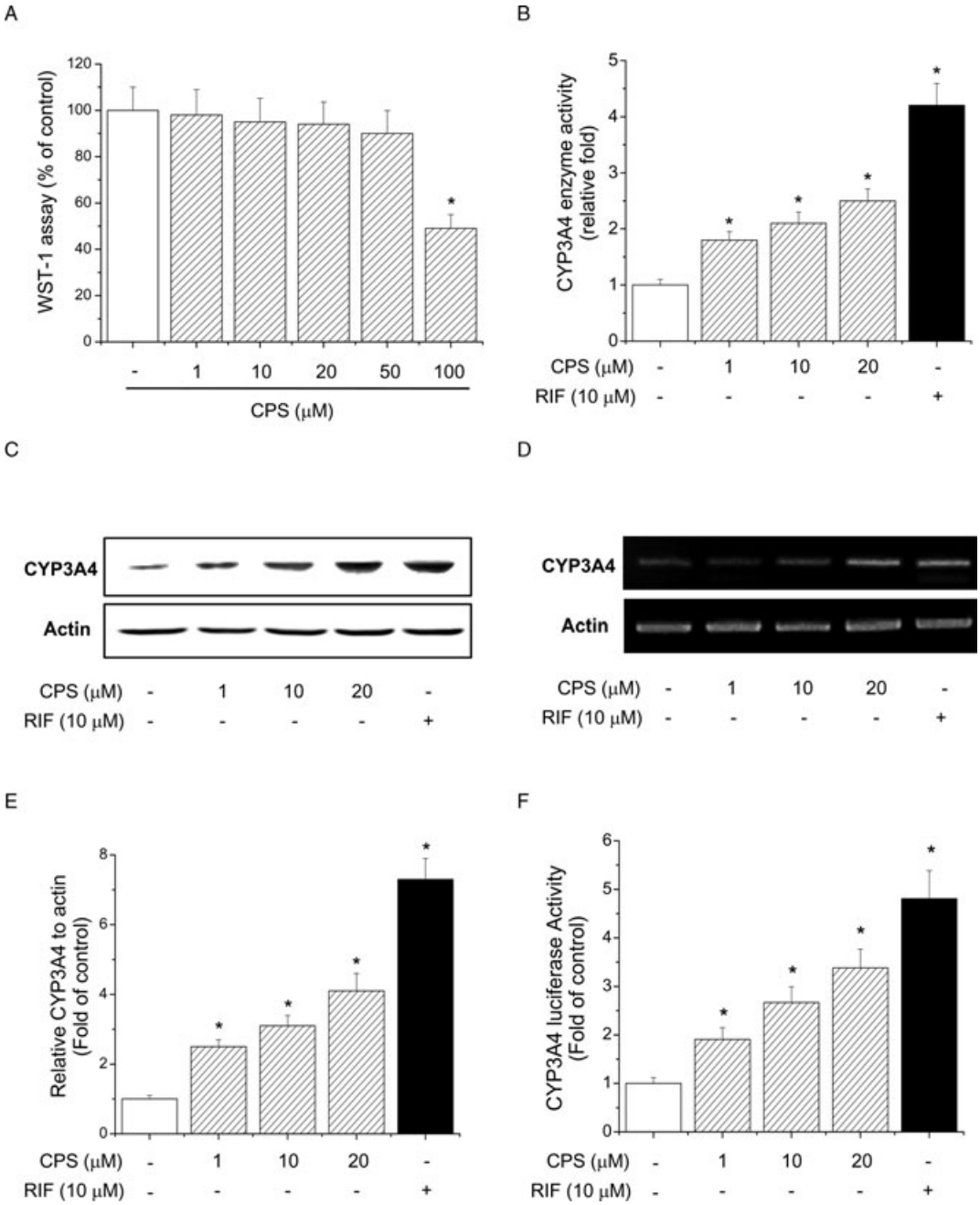

Figure 1. Effect of capsaicin (CPS) on CYP3A4 expression. (A) Effect of CPS on cytotoxicity. Cells were seeded in 96-well plates and treated with differing concentrations of CPS (1–100 μM) for 24 h. Cell viability was assessed using the WST-1 assay for the rapid detection of cytotoxicity. (B) Effect of CPS on CYP3A4 enzymatic activity. Cells were transfected with 0.1 μg of PXR expression vector for 4 h followed by treatment with CPS (1–20 μM) or rifampicin (RIF; 10 μM) for 48 h. CYP3A4 enzymatic activity was measured using a P450-Glo CYP3A4 enzymatic activity kit (Promega). (C) Effect of CPS on CYP3A4 protein expression. Transfection was performed as described in (B). pPXR-transfected cells were incubated with CPS (1–20 μM) or RIF (10 μM) for 24 h. The CYP3A4 protein level was analyzed by the immunoblotting of cell lysates with anti-hCYP3A4 antibodies. The CYP3A4 protein level was normalized using actin. Each blot is representative of three independent experiments. (D, E) Effect of CPS on CYP3A4 gene expression. pPXR-transfected cells were incubated with CPS (1–20 μM) or RIF (10 μM) for 24 h. The cells were then lysed, and total RNA was prepared for the RT-PCR and real-time PCR analysis of CYP3A4 mRNA expression, relative to actin expression. CYP3A4 mRNA expression was compared between the treated and untreated cells. (F) Effect of CPS on CYP3A4 promoter activity. Cells were cotransfected with CYP3A4-Luc and pPXR and cultured with CPS (1–20 μM) or RIF (10 μM) for 24 h. The cells were then harvested and assayed for luciferase activity. $*p < 0.01$, significantly different from the control.

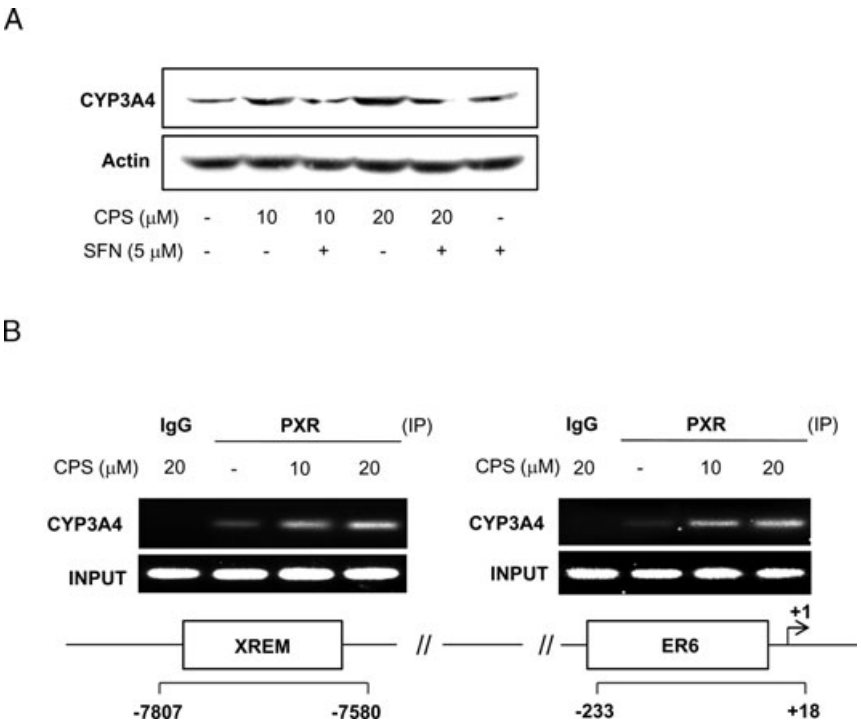

Figure 2. The role of hPXR in capsaicin (CPS)-mediated CYP3A4 expression. (A) pPXR-transfected cells were pretreated with the hPXR inhibitor sulforaphane (SFN; 5 μM) and then treated with 10 or 20 μM CPS for 24 h. The CYP3A4 protein level was analyzed by the immunoblotting of cell lysates with anti-hCYP3A4 antibodies. Each blot is representative of three independent experiments. (B) ChIP analysis of hPXR binding to the CYP3A4 5′-flanking region by CPS. pPXR-transfected cells were incubated with CPS (10 and 20 μM) for 12 h. The cells were then formaldehyde cross-linked, and the association of hPXR with DNA sequences was determined by a ChIP assay. The regions amplified by PCR are indicated in the lower panel. Three independent ChIP assays were performed; typical results are shown. The PCR products were analyzed using a SYBR® Safe DNA gel stain kit. IP, immunoprecipitation. $*p < 0.01$, significantly different from the control.

pPXR-transfected HepG2 cells (Fig. 1D and E). Additionally, we transfected HepG2 cells with pPXR and a CYP3A4-Luc reporter construct. Capsaicin induced CYP3A4 luciferase activity, similar to the pattern of results obtained for enzyme activity, the protein level, and gene expression (Fig. 1F). Thus, capsaicin seems to regulate the transcriptional activation of CYP3A4 in pPXR-transfected HepG2 cells.

3.2 Role of hPXR in capsaicin-mediated CYP3A4 expression

To demonstrate that capsaicin affects the expression of CYP3A4 by regulating hPXR, we assayed pPXR-transfected HepG2 cells that were treated with capsaicin and a hPXR antagonist by Western blotting. Sulforaphane (SFN) is an antagonist of hPXR [17]. Therefore, to investigate the role of hPXR in the induction of CYP3A4 gene expression by capsaicin, pPXR-transfected HepG2 cells were pretreated with SFN for 1 h and then treated with capsaicin for 24 h. SFN attenuated the increase in CYP3A4 protein expression by capsaicin (Fig. 2A). To further test this effect, we performed a ChIP assay on the pPXR-transfected HepG2 cells. The induction of CYP3A4 by hPXR depended on two specific responsive elements: a proximal everted repeat sequence (ER6) located at −160 bp [18–21] and a distal xenobiotic-responsive enhancer module (XREM) located between −7.8 and −7.2 kb upstream of the CYP3A4 transcription start site [13]. Capsaicin treatment induced the association of hPXR with the regulatory regions of CYP3A4 (Fig. 2B), supporting the hypothesis that the effect of capsaicin is enhanced by the binding of hPXR to regulatory sequences in the CYP3A4 gene, thereby inducing hPXR-regulated gene expression.

3.3 Capsaicin induces C/EBPβ activation

Recently, it was reported that CYP3A4 is modulated by C/EBP factors (i.e. C/EBPβ and C/EBPα) [22] and that C/EBPβ specifically binds C/EBP regulatory element(s) within CYP3A4 promoter/enhancer sequences [4]. A truncated form having a molecular weight close to that of full-length C/EBPβ was originally shown to activate transcription in liver cells and was named liver-enriched transcription activating protein (LAP), while a lower molecular weight form was shown to repress transcription in liver cells and was named liver-enriched transcription inhibitory protein (LIP) [23]. To further validate the role of capsaicin in regulating the function of CYP3A4, we examined the effect of capsaicin on the function of C/EBPβ. Capsaicin significantly induced LAP and LIP C/EBPβ protein expression (Fig. 3A, left panel). Capsaicin-induced LAP protein production was approximately two-fold higher than LIP protein expression (Fig. 3A, right panel). Previous studies reported that C/EBPβ-LAP activates transcription whereas the truncated form, C/EBPβ-LIP, antagonizes LAP activity leading to gene repression [12]. Thus, capsaicin may induce CYP3A4 expression-mediated C/EBPβ LAP activation. The β-actin

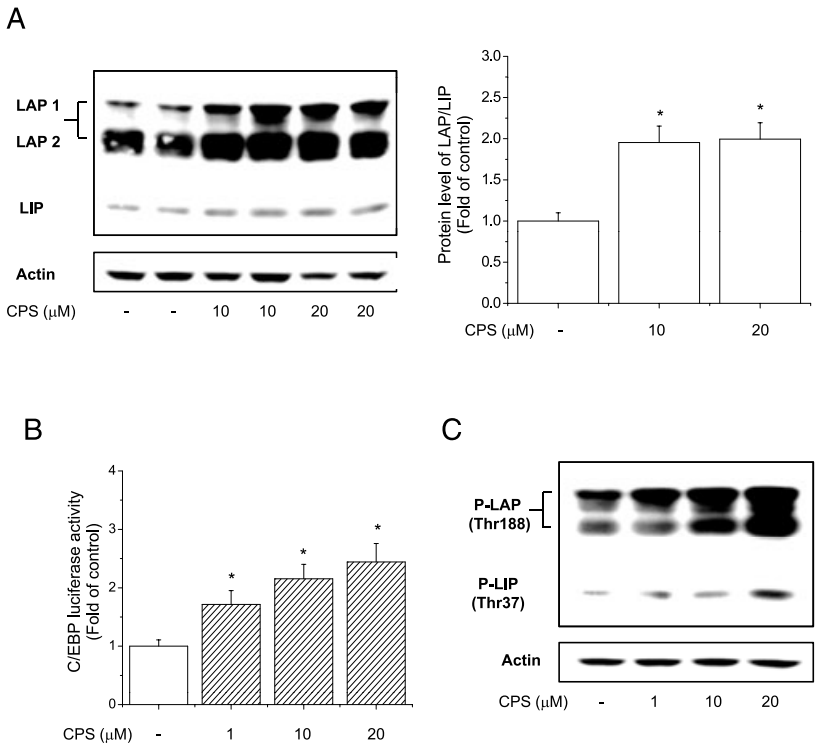

Figure 3. Effect of capsaicin (CPS) on C/EBP expression in HepG2 cells. (A) Effect of CPS on C/EBP protein expression. Cells were cultured with CPS (1–20 μM) for 24 h. The C/EBPβ protein level was analyzed by the immunoblotting of cell lysates with anti-C/EBPβ antibodies (upper). The C/EBPβ protein level was normalized to actin. Each blot is representative of three independent experiments with similar results. The protein expression data presented below the bands are the fold-change compared with LAP and LIP after normalization with the respective loading control value (lower). (B) Effect of CPS on C/EBP promoter activity. Cells were transfected with C/EBP-Luc and then incubated with CPS (1–20 μM) for 24 h, harvested, and assayed for luciferase activity. $*p < 0.01$, significantly different from the control. (C) Effect of CPS on C/EBPβ activation. Cells were cultured with CPS (1–20 μM) for 6 h.

control was expressed constitutively and unaffected by the presence of capsaicin. To clarify the mechanism of C/EBPβ protein expression, we transfected cells with C/EBP-Luc reporter constructs. Capsaicin increased C/EBP luciferase activity in a dose-dependent manner (Fig. 3B). Thus, capsaicin may regulate the transcriptional activation of C/EBP. A possible mechanism by which capsaicin regulates CYP3A4 is by directly phosphorylating C/EBPβ. As expected, in the immunoblot assay, capsaicin directly phosphorylated C/EBPβ (Fig. 3C), suggesting that capsaicin can directly induce the expression and phosphorylation of C/EBPβ. Therefore, we anticipated that the capsaicin-mediated induction of CYP3A4 would lead to activation of C/EBPβ.

3.4 C/EBPβ activation is required for the capsaicin-mediated upregulation of CYP3A4

To correlate the activation of C/EBP with the induction of capsaicin-mediated CYP3A4 levels, a constitutively active C/EBP-specific dominant-negative mutant (AC/EBP) was expressed in HepG2 cells. AC/EBP expression blocked the ability of capsaicin to induce the increase in CYP3A4 mRNA and protein levels in pPXR-transfected HepG2 cells (Fig. 4A and B). Thus, CYP3A4 activation was enhanced by capsaicin-mediated C/EBPβ transcriptional activation. To determine whether the capsaicin-mediated upregulation of C/EBPβ expression was correlated with CYP3A4 expression, we performed a ChIP assay on HepG2 cells transfected with pPXR. Capsaicin induced C/EBPβ binding to the −5950/−5663-bp CYP3A4 5′-flanking region in pPXR-transfected HepG2 cells (Fig. 4C). Therefore, capsaicin-inducible CYP3A4 expression may regulate C/EBPβ activation directly.

3.5 Effect of TRPV1 on the capsaicin-mediated upregulation of CYP3A4

To determine whether the capsaicin-mediated upregulation of CYP3A4 expression was correlated with TRPV1 signaling, we examined the effect of a TRPV1 antagonist. Capsaicin exerts its effect by binding to TRPV1 [24, 25]. Capsazepine (CPZ), a synthetic competitive antagonist of capsaicin [26], is a TRPV1 antagonist capable of blocking the activation induced by capsaicin, heat, and protons [27]. CPZ reduced the capsaicin-mediated increase in CYP3A4 gene and protein expression, and luciferase activity in HepG2 cells (Fig. 5A–C). Also, another TRPV1 antagonist SB-366791 [28] reduced the capsaicin-mediated increase of CYP3A4 gene expression as well as luciferase activity (see Supporting Information Fig. S1). Thus, the induction of CYP3A4 expression by capsaicin may involve a TRPV1-dependent signaling pathway.

3.6 Downstream signaling pathways in the capsaicin-mediated upregulation of CYP3A4 in HepG2 cells

We investigated changes in the TRPV1 downstream signaling pathway caused by capsaicin. Capsaicin enhanced the phosphorylation of the TRPV1 downstream signaling component

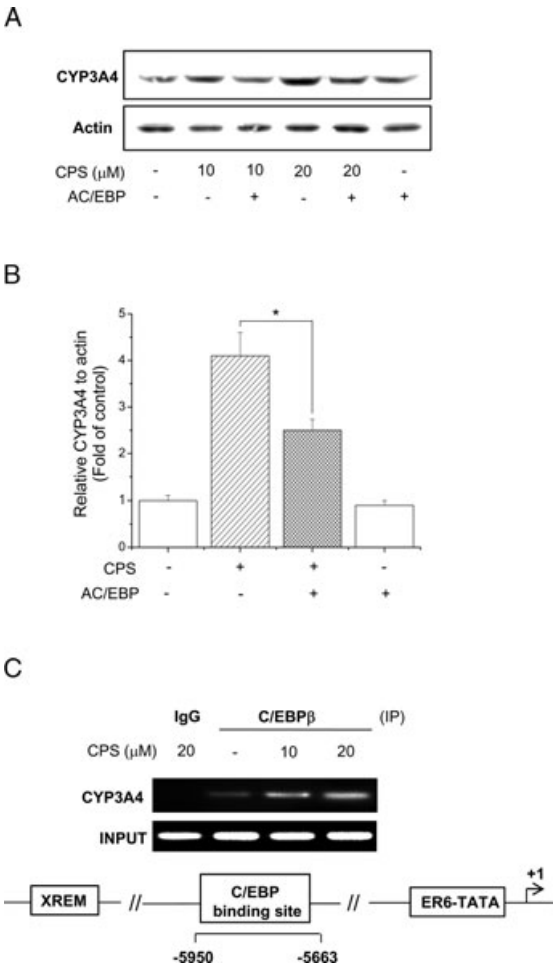

Figure 4. The role of C/EBP in capsaicin (CPS)-mediated CYP3A4 expression in HepG2 cells. (A) Effect of AC/EBP on CPS-induced CYP3A4 protein expression. Cells were transfected with AC/EBP vector, a plasmid with mutations in the C/EBP region, for 24 h and then incubated with 10 or 20 μM CPS for 24 h. The CYP3A4 protein level was analyzed by the immunoblotting of cell lysates with anti-CYP3A4 antibodies. (B) Effect of AC/EBP on CPS-suppressed CYP3A4 promoter activity. Cells were transfected with pCYP3A4-Luc and/or AC/EBP for 24 h and then treated with 20 μM CPS for 24 h. The cells were then harvested and assayed for luciferase activity. $*p$ and $**p < 0.01$, significantly different from the CPS and AC/EBP+CPS groups, respectively. (C) ChIP analysis of C/EBP binding to the CYP3A4 5′-flanking region in the presence of CPS. Cells were incubated with CPS (10 and 20 μM) for 12 h. The cells were then formaldehyde cross-linked, and the associations of C/EBPβ with DNA sequences were determined by a ChIP assay. The regions amplified by PCR are indicated in the lower panel. Three independent ChIP assays were performed; typical results are shown. The PCR products were analyzed using a SYBR® Safe DNA gel stain kit. IP, immunoprecipitation. $*p < 0.01$, significantly different from the control.

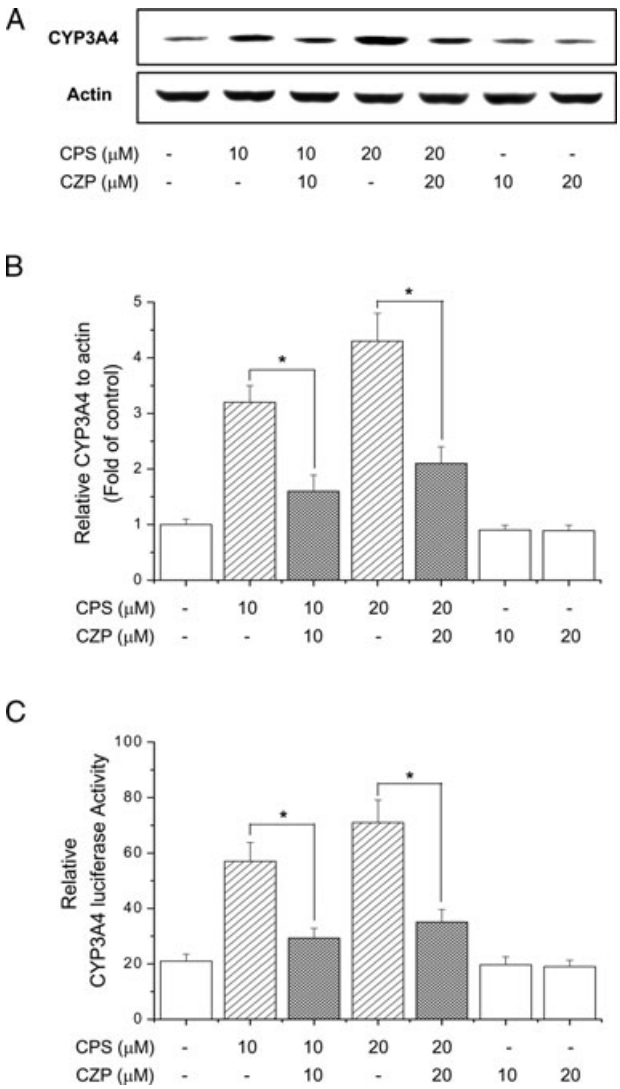

Figure 5. Effect of TRPV1 on capsaicin (CPS)-mediated CYP3A4 expression. Cells were pretreated with a TRPV1 antagonist, capsazepine (CPZ; 10 or 20 μM), followed by treatment with 10 or 20 μM CPS for 24 h. The cells were then lysed, and the CYP3A4 protein level was analyzed by immunoblotting using anti-hCYP3A4 antibodies (A) or total RNA was prepared for the real-time PCR analysis of CYP3A4 mRNA expression (B). $*p$ and $**p < 0.01$, significantly different from the control and CPS, respectively, by the Newman–Keuls test. (C) Cells were transfected with CYP3A4-Luc and then cultured with CPZ (10 or 20 μM) and/or CPS (10 or 20 μM) for 24 h. The cells were then harvested and assayed for luciferase activity. $*p$ and $**p < 0.01$, significantly different from the control and CPS-treated cells, respectively, by the Newman–Keuls test.

Ca^{2+}/calmodulin (CaM)-dependent protein kinase I (Thr177) (CaMKI) in a dose-dependent manner (Fig. 6A). The TRPV1 antagonist (CPZ) and CaM antagonist W7 reduced capsaicin-induced CaMKI (Thr177) phosphorylation (Fig. 6A). However, the Akt inhibitor LY294002 did not affect capsaicin-induced CaMKI (Thr177) phosphorylation (Fig. 6A). TRPV1 mediates intracellular calcium release activity, and calcium regulates the PI3-kinase/Akt pathway [29, 30]. Capsaicin, in a concentration-dependent manner, induced Akt phosphorylation (Fig. 6B). Furthermore, the TRPV1 antagonists CPZ, W7, and LY294002 inhibited the capsaicin-induced phosphorylation of Akt (Fig. 6B). CPZ, W7, and LY294002 also

inhibited capsaicin-induced C/EBPβ protein phosphorylation (Fig. 6C). Additionally, W7 and LY294002 reduced the capsaicin-mediated increase in CYP3A4 protein levels, similar to the results obtained for CZP (Fig. 6D). These results indicate that capsaicin-induced TRPV1 activation mediated C/EBPβ downstream signaling.

3.7 Effects of capsaicin on CYP3A1 expression and the metabolism of NIF to DNIF in rats

Young adult male Sprague–Dawley rats exposed to daily doses of 1, 3, and 5 mg/kg/day of capsaicin for 7 days or 1 mg/kg/day of PCN for 3 days showed no differences in body weight between the treated and control groups. There was also no difference in the weight of the liver between rats exposed to PCN and the controls. Immunoblot analysis demonstrated basal protein expression of CYP3A1 (homolog of human CYP3A4) in the liver of adult male Sprague–Dawley rats. Hepatic CYP3A1 protein levels were higher in rats exposed to capsaicin or PCN compared with the control groups (Fig. 7A).

Although the individual pharmacokinetic parameters (C_{max}, T_{max}, AUC, and k_e) of NIF and DNIF in the capsaicin-pretreated groups were not significantly different from those in the control group (data not shown), the ratios of AUC_{DNIF} to AUC_{NIF} were significantly increased with increasing doses of capsaicin (Fig. 7B): the mean metabolism ratio in the control group was approximately 2.7 ± 0.3%, and the repeated pretreatment of capsaicin at the doses of 1 and 3 mg/kg for 7 days increased the mean ratio by 16% ($p = 0.103$) and 35% ($p = 0.005$), respectively.

4 Discussion

The aim of this study was to determine the effect of capsaicin on the expression of CYP3A4 in vitro using human hepatoma HepG2 cells and in vivo using an animal model. We demonstrated that capsaicin significantly induced CYP3A4 expression through C/EBP and hPXR signaling.

hPXR is an orphan nuclear receptor activated at the level of transcription by pregnanes and a number of structurally diverse drugs, including rifampicin and metyrapone. hPXR has been shown to regulate the drug- and corticosteroid-inducible expression of CYP3A4 in hepatocytes, the major expressed CYP responsible for the metabolism of most drugs. In the present study, capsaicin enhanced the binding of hPXR to XREM or ER6. The capsaicin-induced CYP3A4 protein level was also decreased by the treatment of hPXR antagonist. This means hPXR pathway is one of the functional mechanisms of capsaicin-induced CYP3A4 expression.

C/EBPβ (LAP and LIP) are transcription factors involved in the constitutive expression of CYP3A4 genes in the liver [4]. Changes in the LAP:LIP isoform ratio is likely to affect hepatocyte proliferation and differentiation [23, 31]. This ratio increases significantly during liver development, with a transient perinatal peak in LAP expression [23]. Capsaicin induced the protein expression and phosphorylation of LAP and LIP and enhanced the LAP ratio to a greater extent than the LIP ratio in HepG2 cells. The constitutively active C/EBP-specific dominant-negative mutant (AC/EBP) blocked the induction of CYP3A4 mRNA and protein expression by capsaicin in pPXR-transfected HepG2 cells. We found that the upregulation of CYP3A4 by capsaicin was associated with the translational induction of C/EBPβ-LAP, leading to a sharp increase in the LAP:LIP ratio. Recently, it was reported that capsaicin upregulates nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) gene expression mediated through C/EBPβ activation [32]. We also previously reported that capsaicin upregulated C/EBPβ and induced CYP1A1 levels in murine hepatoma cells [12]. These are similar to the pattern of results obtained for CYP3A4 by capsaicin-mediated C/EBPβ activation in HepG2 cells. Our results showed that C/EBPβ activation induced by capsaicin is another important regulatory mechanism of CYP3A4 induction by capsaicin.

The observation of CYP3A4 induction by capsaicin in HepG2 cells seems to be discordant with the previous results by Babbar et al. that suggest no significantly inducible effect of capsaicin for CYP3A4 expression in human normal hepatocytes [33]. In their study, Babbar et al. observed 5, 24, and 36% of increase in CYP3A4 enzymatic activity by 1, 3, and 10 μM capsaicin treatments, respectively. But they could not see the statistical significance for the increase. Their failure to see concretely the induction of CYP3A4 seems to be partially due to small number of human hapatocytes ($n = 3$) in their experiments. Since it has been well known that large inter-individual variation in CYP3A4 regulation including genetic/expressional variation of hPXR, individual difference of CYP3A4 promoter sequences, and genetic/epigenetic factors contributing to differential CYP3A4 expression regulation, use of larger number of human hepatocytes might have reached different conclusion on induction potential of capsaicin on CYP3A4 expression [34].

TRPV1, a known receptor for capsaicin, is also expressed in liver and modulates liver functions [35]. Previous studies reported that liver-derived HepG2 cells express TRPV channels [36, 37]. This receptor activates PI3-K/Akt and CaMKI signal pathways, which leads to activation of C/EBP, one of CYP3A4 expression regulators [29, 38]. In order to determine whether TRPV1 is required for capsaicin-induced CYP3A4 expression, an antagonist for TRPV1 was added together with capsaicin. The capsaicin-induced CYP3A4 expression was significantly decreased, which means TRPV1 contributes to the CYP3A4 induction.

In this study, we used HepG2 cells as model system for studying CYP3A4 expression regulation. Since the expression of hPXR, one of key CYP3A4 regulators, is relatively low in these cells, the induction of CYP3A4 expression through hPXR activation could not be easily observed. But the transient/stable expression of hPXR restores the function of hPXR-mediated signal pathways. Besides hPXR, TRPV1 is

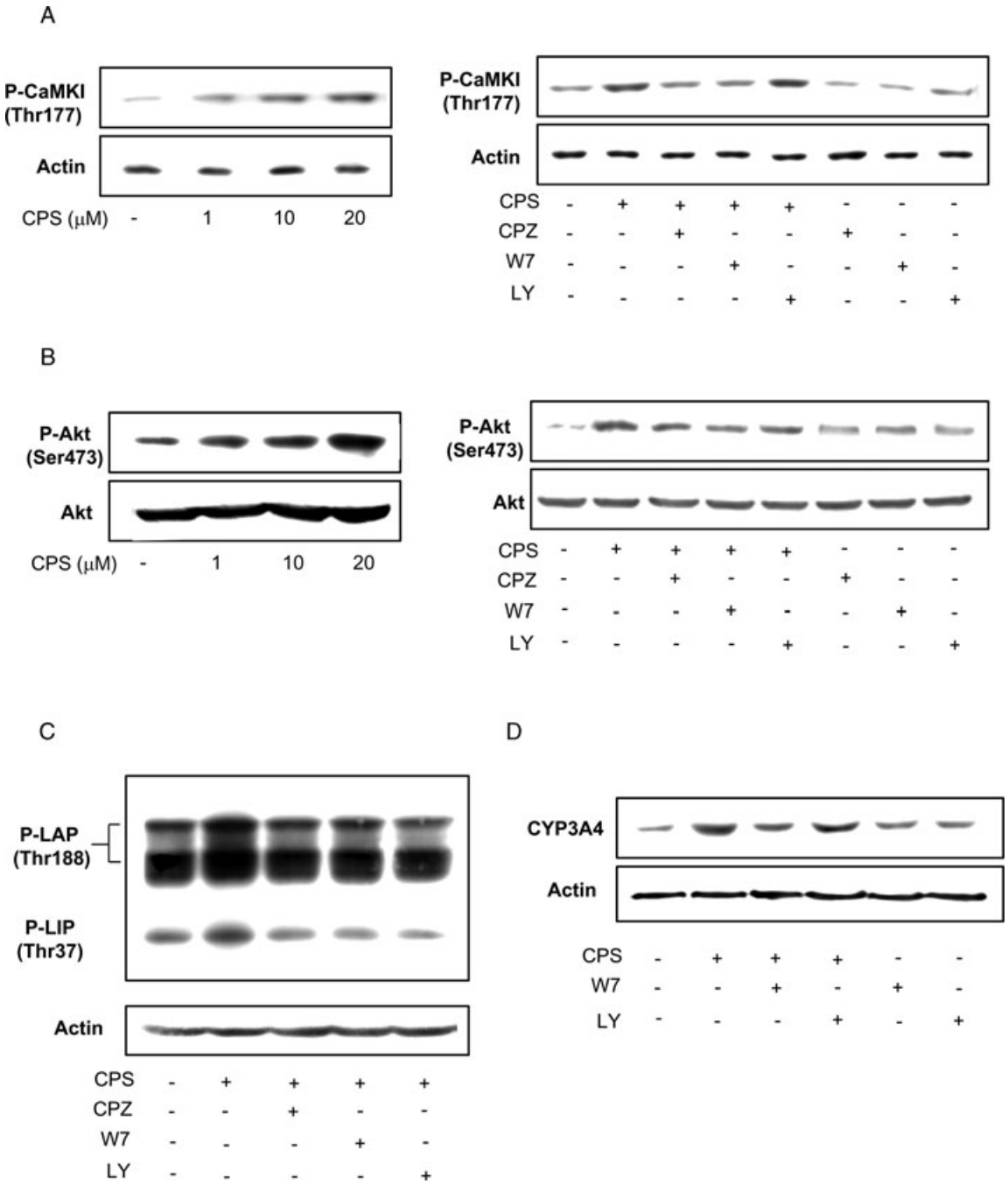

Figure 6. Effects of capsaicin (CPS) on TRPV1 signaling events. (A) Effect of CPS on the activation of the TRPV1 downstream signaling component Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMK). Cells were treated with CPS (1–20 μM) for 5 min. Additionally, cells were pretreated with capsazepine (CPZ; 20 μM), a CaM antagonist (W7; 40 μM), or an Akt inhibitor (LY294002; 10 μM) and then treated with CPS (20 μM) for 30 min. The phosphorylation of CaMKIα at threonine 177 (P-CaMKIα) was then analyzed by Western blotting; actin expression was measured as a loading control. (B) Effects of CPS on the activation of the TRPV1 downstream signaling component Akt. Cells were treated with CPS (1–20 μM) for 10 min. Additionally, cells were pretreated with CPZ (20 μM), W7 (40 μM), or LY294002 (10 μM) for 30 min followed by CPS for 10 min. For Western blotting, the membranes were probed with phospho-Akt-specific antibodies. The phospho-Akt protein levels were compared with total Akt kinase protein. (C) Effect of TRPV1 signaling events on CPS-induced C/EBPβ phosphorylation. Cells were pretreated with CPZ (20 μM), W7 (40 μM), or LY294002 (10 μM) for 30 min followed by CPS (20 μM) for 6 h. The phospho-C/EBPβ protein level was normalized to actin. (D) Effect of CaMK and Akt signaling events on CPS-induced CYP3A4 protein expression. Cells were pretreated with W7 (40 μM) or LY294002 (10 μM) followed by CPS (20 μM) for 24 h. The CYP3A4 protein level was analyzed by the immunoblotting of cell lysates with anti-CYP3A4 antibodies. The CYP3A4 protein level was normalized to actin. Each blot is representative of three independent experiments with similar results.

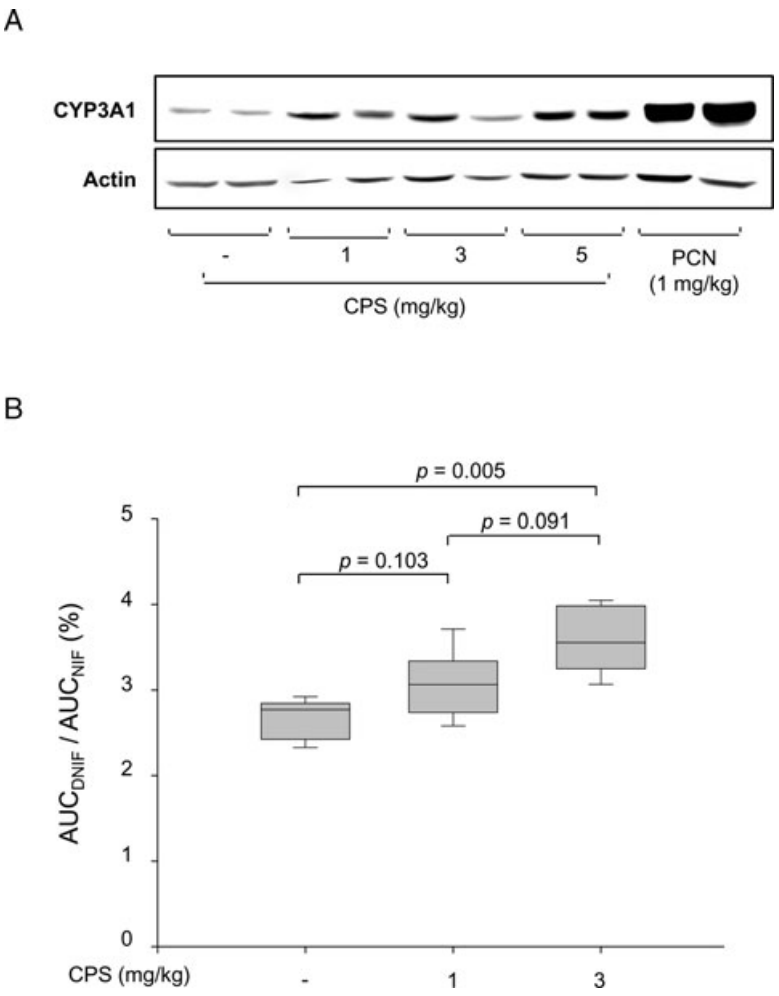

Figure 7. Effects of capsaicin (CPS) on CYP3A1 expression in rats. (A) Effect of CPS on CYP3A1 protein expression in rat liver. CPS was orally administered at 1, 3, and 5 mg/kg/day for 7 days (ethanol:saline = 1:5) or 1 mg/kg/day of pregnenolone 16α-carbonitrile (PCN) was intraperitoneally injected (in olive oil) for 3 days to the treatment groups. The rats were sacrificed and liver microsomes obtained. Immunoblots of 2 μg of hepatic microsomal fractions from rats treated with CPS or PCN are presented. The CYP3A1 protein level was analyzed by immunoblotting of liver microsomes with anti-hCYP3A1 antibodies. The CYP3A1 protein level was normalized to actin. Each blot is representative of three independent experiments. (B) Effect of CPS on the CYP3A1 pharmacokinetics of nifedipine in rats. CPS was orally administered at 1 and 3 mg/kg/day for 7 consecutive days to the treatment groups. On day 8, all three groups were gavaged with 30 mg/kg of nifedipine (NIF). Heparinized blood was serially taken from the subclavicular vein and plasma obtained. The plasma concentrations of NIF and dehydronifedipine (DNIF) were quantified. The analytical data were processed with Analyst software (version 1.4.1; Applied Biosystems). Ratios of the area under the plasma concentration-time curves (*AUC*) for DNIF (AUC_{DNIF}) to that for NIF (AUC_{NIF}) were calculated to measure the effect of CPS. A statistical analysis of the AUC_{DNIF} to AUC_{NIF} ratios was performed using one-way analysis of variance followed by the Tukey test (SPSS 10.0; SPSS Inc., Chicago, IL). $p < 0.05$ was considered to be statistically significant.

also a receptor for many kinds of xenobiotics and is expressed in HepG2 cells. Therefore, if the HepG2 cells are engineered to express hPXR enough to function as signal molecule, these cells will be used as a good model system for studying gene expression regulation by various xenobiotics for drug interaction studies. In spite of these advantages of HepG2 as an alternative model system, it does not mean that all of signal pathways involved in CYP3A4 regulation are intact and working in HepG2 cells like human hepatocytes. Therefore, additional confirmatory studies using human hepatocytes will help to prove our model of capsaicin-induced CYP3A4 upregulation.

The pharmacokinetic study of nifedipine, a representative CYP3A probe drug, supports the hypothesis that capsaicin induces CYP3A expression *in vitro*. Although there was no statistical significance in AUCs of both nifedipine and its metabolite, dehydronifedipine by the treatment of capsaicin due to inter-individual variation, the metabolic ratio was significantly increased, indicating that capsaicin may enhance the metabolism of nifedipine by CYP3A1 induction. One may also think about the contribution of pulmonary metabolism to the elimination of nifedipine as published by Aoki et al. [39]. However, in contrast to the other CYP3A substrates such as lidocaine and midazolam, the pulmonary intrinsic clearance of nifedipine was only 0.15% of its hepatic value, so that the pulmonary metabolism could be negligible.

A recent study reported that capsaicin induced the expression of P-glycoprotein (P-gp) and multi-drug resistance gene (MDR1) in Caco-2 cells [40,41]. The dose of capsaicin in a typical Indian or Thai diet was reported to be about 128 μg/kg of human body weight [42]. Taking the gastric fluid volume to be 1–3 L for an adult human with a body weight of 60 kg, the concentration of capsaicin in the gastric fluid would be equivalent to 8–25 μM. It is therefore possible for dietary capsaicin to affect the bioavailability of co-administered drugs by interfering with the functioning of both CYP3A4 in the liver, and drug transports in gastrointestinal tracts. It is also likely that the dose of capsaicin will be higher in households that regularly consume a large amount of hot chili; whether this will lead to upregulated liver CYP3A4 expression remains to be verified. Until the clinical significance of these findings is established, caution is advised for patients who are likely to co-consume capsaicin with drugs of narrow therapeutic index whose bioavailability is strongly influenced by CYP3A4

efflux activity. Our study also supports the concept that capsaicin can change the expression of CYP3A4, affecting drug metabolism and altering the therapeutic and toxicologic responses to drugs, which may in turn lead to adverse drug interactions.

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