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RESEARCH PAPER

CCAAT/ enhancer-binding protein β activation by capsaicin contributes to the regulation of CYP1A1 expression, mediated by the aryl hydrocarbon receptor

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BACKGROUND AND PURPOSE
Capsaicin, a constituent of peppers, has been linked to the suppression of tumorigenesis and carcinogenesis. The influence of capsaicin on cytochrome P450 (CYP) 1A1, which is involved in metabolism of carcinogens, and the underlying mechanisms remain unclear. Here, we examined the effect of capsaicin on CYP1A1 expression in mouse hepatoma cells.

EXPERIMENTAL APPROACH
Murine hepatoma Hepa-1c1c7 cells were incubated with capsaicin and/or 3-methylcholanthrene (3-MC). Effects of capsaicin on CYP1A1 levels were determined by analysing mRNA expression, transcription activity and protein expression. Regulation of CYP1A1 was investigated by determining transcriptional factor expression, activation and binding activity with cotreatment with target signal antagonists.

KEY RESULTS
Capsaicin alone slightly induced CYP1A1 activity, mRNA expression, protein level and promoter activity. Treatment with transient receptor potential vanilloid type-1 receptor (TRPV1) or aryl hydrocarbon receptor (AhR) antagonist decreased induction of CYP1A1 expression by capsaicin. Additionally, capsaicin significantly inhibited 3-MC-induced CYP1A1 mRNA and protein level and xenobiotic response element–luciferase activity. Capsaicin also inhibited 3-MC-induced AhR transactivation and nuclear localization of AhRs. Moreover, capsaicin increased Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMK) and CCAAT/ enhancer-binding protein β (C/EBPβ) activation, downstream of TRPV1 receptors. Capsaicin-induced C/EBPβ activation inhibited induction of CYP1A1 mRNA and protein by 3-MC.

CONCLUSIONS AND IMPLICATIONS
Capsaicin alone weakly induced CYP1A1 expression, and 3-MC-induced CYP1A1 levels were suppressed by capsaicin. Activation of C/EBPβ and inhibition of 3-MC-induced AhR transactivation by capsaicin contributed to the suppression of CYP1A1 expression. Capsaicin has a potential chemopreventive effect through inhibiting induction of CYP1A1 by poly aryl hydrocarbons.

Abbreviations
3-MC, 3-methylcholanthrene; AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator protein; C/EBPβ, CCAAT/enhancer-binding proteinβ; CaMK, Ca^{2+}/calmodulin (CaM)-dependent protein kinase; CPZ, capsazepine; PAH, poly aryl hydrocarbon; TRPV1, transient receptor potential vanilloid type-1 receptor; XRE, xenobiotic response element

Introduction

Cytochrome P450s (CYP) are a superfamily of haem-containing monooxygenase enzymes that metabolize foreign compounds, such as drugs and environmental chemicals, and endogenous substrates, such as steroids and fatty acids (Nelson *et al.*, 1996). The transcriptional induction of the CYP1A1 gene is the most studied cellular response to poly aryl hydrocarbons (PAHs). This gene encodes the CYP1A1 enzyme (nomenclature follows Alexander *et al.*, 2009), which catalyses the oxidative catabolism of PAHs and generates genotoxic metabolites that can enter the nucleus and bind to specific DNA residues, leading to mutagenesis (Dipple, 1994).

The aryl hydrocarbon receptor (AhR) binds to environmental contaminants, such as the PAHs 3-methylcholanthrene (3-MC) (Thomas *et al.*, 1983), benzo[a]pyrene (Conney, 1986), 7,12-dimethylbenz[a]anthracene (Kleiner *et al.*, 2002) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Whitlock, 1987). 3-MC, a potent environmental contaminant and potent carcinogen, triggers the induction of CYP1A1 proteins via an intracellular AhR activation (Poland and Knutson, 1982). AhR binds to xenobiotics, such as 3-MC, with extremely high affinity, and subsequently, the complex is translocated to the nucleus where the AhR–ligand complex binds the AhR nuclear translocator (ARNT) protein (McGuire *et al.*, 1994; Heid *et al.*, 2000). In the nucleus, the ligand-bound AhR/ARNT heterodimer binds to xenobiotic-responsive element (XRE) sequences, which are enhancer DNA elements present in the 5′-flanking region of target genes, such as CYP1A1.

Additionally, the AhR that binds to the XRE in the gene promoter is related to a member of the CCAAT/ enhancer-binding protein (C/EBP) family of transcription factors (Cho and Kim, 2003; Shin *et al.*, 2005). C/EBP is known to play important roles in regulating the expression of multiple hepatocyte-specific genes (Lekstrom-Himes and Xanthopoulos, 1998), and it helps to control hepatocyte progression through the cell cycle (Timchenko *et al.*, 1996; Cho and Kim, 2003). Thus, the C/EBPs are likely to be important targets for regulation during liver regeneration. The AhR/ARNT complex is related to the C/EBPβ transcription factors (Cho and Kim, 2003; Shin *et al.*, 2005). Thus, C/EBPβ showed overlapping DNA binding specificity to that of the AhR (Carrier *et al.*, 1994). Capsaicin up-regulated the gene encoding C/EBP homologous protein (CHOP) in human prostate epithelial cells (Sanchez *et al.*, 2008). Oltipraz or flavonoid compounds, including PD98059, promote nuclear translocation and activation of C/EBPβ, which leads to the inhibition of CYP1A1 expression via binding of activated C/EBPβ to the C/EBP binding site present in the gene promoter (Cho and Kim, 2003; Shin *et al.*, 2005). The phosphoinositide 3-kinase (PI3-kinase) /Akt pathway controls nuclear translocation of C/EBPβ (Venugopal and Jaiswal, 1998; Kang *et al.*, 2003).

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a major pungent constituent of a variety of capsicum fruits, such as hot peppers, and its content in hot red peppers ranges from 0.1% to 1.0% (Gonzalez, 1990). Capsaicin stimulates the transient receptor potential vanilloid type-1 receptor (TRPV1), located primarily on polymodal C-fibres, and initiates a complex cascade of events, including neuronal excitation and the release of pro-inflammatory mediators, desensitization of the receptor and neuronal toxicity (Lee *et al.*, 1991; Szallasi and Blumberg, 1991; Caterina *et al.*, 1997; Salazar *et al.*, 2009). Previous studies on the metabolism of capsaicin by P450 enzymes have demonstrated the formation of multiple products arising from aromatic and alkyl hydroxylation (Surh *et al.*, 1995; Surh and Lee, 1995; Reilly and Yost, 2006). Capsaicin is converted to a highly reactive phenoxy radical intermediate by CYP2E1, and such reactive species exert chemoprotective activity against some chemical carcinogens and mutagens (Surh *et al.*, 1995).

However, the influence of capsaicin on CYP1A1 expression in hepatocytes and the relationship between the capsaicin inhibitory effect and C/EBP expression remain unclear. We have therefore investigated the effect of capsaicin on CYP1A1 expression in Hepa-1c1c7 cells. Our results demonstrated that capsaicin can regulate the expression of CYP1A1 through AhR- and C/EBPβ-dependent mechanisms.

Methods

Cell culture and treatment

The mouse hepatoma cell line, Hepa-1c1c7, was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in α-minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37°C. Capsaicin and 3-MC were dissolved in ethanol or dimethylsulfoxide (DMSO), respectively, and stock solutions were added directly to the culture media. The control cells were treated with only DMSO, and the final DMSO concentration was kept below 0.2%.

Assay for cytotoxicity

Cell cytotoxicity was examined using a WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)–based assay kit according to the manufacturer's instructions. Briefly, Hepa-1c1c7 cells (5×10^5 cells per well) in 10% FBS–α-MEM were seeded into 96-well plates. Capsaicin (10–50 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., Beverly, MA, USA). Capsaicin did not interfere at this wavelength.

Ethoxyresorufin-O-deethylase (EROD) activity assay

Cells were incubated with 3-MC (1 μM) and/or capsaicin (10–50 μM) for 18 h. After incubation, the medium was removed, and the wells were washed twice with fresh medium. EROD activity was measured in intact cells grown in 48-well plates, as described previously (Ciolino *et al.*, 1998). The fluorescence intensity was measured after 30 min using a FL600 plate reader (Biotek, Winooski, VT, USA), with excitation at 530 nm and emission at 590 nm. A standard curve was generated using resorufin.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from treated cells using RNAiso Reagent (Takara, Tokyo, Japan) according to the manufactur-

er's protocol. The following primers were used: mouse N-terminal region of TRPV1 forward, 5′-CTATGATCGCAGG AGCATCTTCGA-3′; reverse, 5′-GAACTTCACAATGGCCAGCT GGTT-3′ (466 bp); mouse C-terminal region of TRPV1 forward, 5′-TGCCTGTGGAGTCCCCACCACACAA-3′; reverse, 5′-AGGGAGAAGCTCAGGGTGCGCTTGA-3′ (511 bp) and the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5′-CCCTTCATTGACCTCAACTA-3′; mouse GAPDH reverse, 5′-CCAAAGTTGTCATGGATGAC-3′ (397 bp). PCR was carried out for 35 cycles using the following conditions: denaturation at 98°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 40 s. The band intensities of the amplified DNAs were visualized using the SYBR® Safe DNA gel stain kit.

RNA preparation and mRNA analysis by real-time quantitative PCR

Cells were incubated with 3-MC (1 μM) and/or capsaicin (10–50 μM) for 6 h. Total RNA from the treated cells was prepared with the RNAiso reagent (Takara), according to the manufacturer's protocol. For the detection of CYP1A1, total RNA was extracted after stimulation and treatment. Product formation was monitored continuously during PCR using Sequence Detection System software (ver. 1.7; Applied Biosystems, Foster City, CA, USA). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR®). The expression levels of CYP1A1 in the exposed cells were compared with those in control cells at each time point using the comparative cycle threshold (Ct)-method (Johnson *et al.*, 2000). The following primer sequences were used: CYP1A1 forward, 5′-GGTTAACCATGACCGGGAACT-3′; CYP1A1 reverse, 5′-TGCCCAAACCAAAGAGAGTGA-3′; β-actin forward: 5′-TGGCACCCAGCACAATGAA-3′, β-actin reverse: 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of β-actin, a housekeeping gene.

Luciferase (Luc) and β-galactosidase (β-gal) assays

Cells were transfected with 1 μg of pGL3-CYP1A1-Luc, pXRE-Luc, pC/EBP-Luc, AC/EBP plasmid and/or 0.2 μg of pCMV-β-gal per well using Lipofectamine™ 2000. Plasmid pGL3-CYP1A1-Luc was created by inserting the mouse CYP1A1 upstream regulatory region (–1395 to +7) PCR product into the pGL3 basic vector (Kim *et al.*, 2008). XRE-driven luciferase reporter plasmid (–1306 to –824 of the murine CYP1A1; Jeong and Kim, 2002) containing four XREs was used to examine the specific activation of XRE. pC/EBP-Luc was purchased from Stratagene (La Jolla, CA, USA). AC/EBP expression vector (gift from Dr Keon Wook Kang, Chosun University, South Korea), encoding a mutant of the C/EBP region. At 4 h after transfection, fresh medium containing 10% FBS was added to the cells. Cells were incubated with 3-MC (1 μM) and/or capsaicin (10–50 μM) for 18 h and lysed. The lysed cell preparations were then centrifuged (14 240 × *g*, 10 min), and the supernatant was assayed for both luciferase and β-galactosidase activity. Luciferase activity was measured using the luciferase assay system (Promega, Madison, WI, USA) with a luminometer, according to the manufacturers' instructions. The β-galactosidase assay was carried out 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 *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.

Western blotting

Cell lysates were prepared after capsaicin or 3-MC treatment and resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were probed with the appropriate primary antibodies, followed by incubation with horseradish peroxidase–conjugated secondary antibody. The blots were visualized using an ECL Western blot kit (Animal Genetics Inc., Suwon, Kyonggi-do, Korea), according to the manufacturer's protocol.

Electrophoretic mobility shift analysis

Nuclear extracts were prepared, and electrophoretic mobility shift analyses were performed, as described previously (Jeong *et al.*, 1997; Han *et al.*, 2007). Cells were incubated with capsaicin or 3-MC for 3 h. Briefly, the XRE oligonucleotide probe (5′-GGAGTTGCGTGAGAAGAGCC-3′) and C/EBP oligonucleotide probe (5′-ACCGGGCTTACGCAATTTTTTTAAGG-3′) were labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The binding reaction was carried out in a total volume of 25 μL in buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 4% glycerol, 0.1 μg·mL^{-1} sonicated salmon sperm DNA, 10 μg of nuclear extracts and 100 000 cpm of the labelled probe. A 200-fold excess of unlabeled oligonucleotide (competitor) was added, where appropriate. After 15 min incubation at room temperature, 2 μL of 0.1% bromophenol blue was added, and the samples were separated by electrophoresis on a 5% non-denaturing polyacrylamide gel at 120 V for 2 h. Finally, the gel was dried and exposed to X-ray film. For the supershift assay, the C/EBPβ antibody (1 μg) was added to the reaction mixture and additionally incubated for 1 h.

Immunocytochemistry

Cells grown on poly-L-lysine-coated coverslips were treated with 50 μM of capsaicin and/or 3-MC for 3 h. Next, the cells were washed with PBS and fixed with 2% (w/v) paraformaldehyde. After permeabilization, coverslips were blocked with 1% BSA and then incubated with anti-AhR polyclonal or C/EBPβ antibodies (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h. After washing in PBS, the cells were incubated with secondary antibody (1:500, Alexa Fluor 488–conjugated donkey anti-goat antibody; Molecular Probes, Eugene, OR, USA). The sections were counterstained with 1 μg·mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. Cells were then mounted with mounting medium (Dako, Hamburg, Germany) and analysed by confocal laser scanning microscopy (Zeiss, Jena, Germany).

Statistical analysis

All experiments were repeated at least three times, and 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 treatment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for *P*-values of <0.01.

Materials

All chemicals and cell culture materials were obtained from the following sources: capsaicin (purity, >99.8%), 3-MC, capsazepine (CPZ) and DAPI from Sigma Chemical Co. (St. Louis, MO, USA); 7-ethoxyresorufin from Pierce Chemical Co. (Rockford, IL, USA); the WST-1 assay kit from Roche Co. (London, UK); Lipofectamine™ 2000, SYBR® Safe DNA Gel Stain kit and α-MEM from Invitrogen Co. (Carlsbad, CA, USA); FBS, penicillin–streptomycin solution and trypsin from Life Technologies, Inc. (Carlsbad, CA, USA); pCMV-β-gal from Clontech (Palo Alto, CA, USA); a protein assay kit from Bio-Rad Laboratories, Inc. (Hercules, CA, USA); antibodies [AhR (N-19), CYP1A1, C/EBPβ (C-19), phospho-CaMKIα (Thr177) and β-actin (C4)] from Santa Cruz Biotechnology, Inc.; anti-Akt/phospho-Akt (Ser473), phospho-C/EBPβ (Thr188/Thr37) (C-19), and secondary antibodies (HRP-linked anti-rabbit and anti-mouse IgG) from Cell Signaling Technology (Danvers, MA, USA); 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH-223191; 10 μM), CaMK inhibitor W7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; 40 μM] and LY294002 (10 μM) from Calbiochem (La Jolla, CA, USA); and ECL chemiluminescence system and PVDF membrane from Amersham Pharmacia Biotech (Uppsala, Sweden). PCR oligonucleotide primers were custom- synthesized by Bioneer Co. (Daejeon, Chungcheongnam-do, Korea). All chemicals were of the highest grade commercially available.

Results

Capsaicin regulates CYP1A1 protein, mRNA expression and promoter activity

To determine the optimal concentrations for use in our studies, the potential cytotoxicity of capsaicin was tested in Hepa-1c1c7 cells. The chemical structure of capsaicin is illustrated in Figure 1A. Figure 1B shows that 1, 10, 25 and 50 μM capsaicin did not affect cell viability; however, 100 μM, the highest concentration tested, caused a 51% decrease in cell viability in Hepa-1c1c7 cells. Thus, all subsequent studies were conducted using 1–50 μM capsaicin.

To examine the effect of capsaicin on CYP1A1, we tested CYP1A1 activity in capsaicin-treated Hepa-1c1c7 cells; 3-MC was used as a positive control in these experiments. CYP1A1 enzyme activity was measured by the EROD activity assay. When cells were treated with 1 μM 3-MC for 18 h, there was a significant increase in EROD activity (Figure 1C). Capsaicin alone slightly increased EROD activity, and 3-MC-inducible EROD activity was reduced markedly by capsaicin, in a dose-dependent manner (Figure 1C). Next, we tested the effects of capsaicin on CYP1A1 gene expression in a real-time PCR assay. Capsaicin alone induced CYP1A1 mRNA expression (Figure 1D). Additionally, capsaicin suppressed the 3-MC-induced CYP1A1 mRNA level in Hepa-1c1c7 cells (Figure 1D).

We next transfected Hepa-1c1c7 cells with the CYP1A1-Luc reporter construct. Treatment with capsaicin alone slightly enhanced CYP1A1 luciferase activity in Hepa-1c1c7 cells (Figure 1E). Capsaicin reduced CYP1A1 luciferase activity markedly in 3-MC-treated cells (Figure 1E). To confirm the effect of capsaicin, we also measured the protein expression of CYP1A1 by immunoblot assay. Capsaicin alone slightly induced CYP1A1 protein levels, similar to the pattern of results obtained for gene expression, enzyme and promoter activity (Figure 1F). However, 3-MC-inducible CYP1A1 protein levels were suppressed markedly upon treatment with 3-MC and capsaicin (Figure 1F). Thus, capsaicin could regulate the transcriptional activation of CYP1A1 in Hepa-1c1c7 cells.

Capsaicin regulates AhR activation in Hepa-1c1c7 cells

To demonstrate that capsaicin regulates the expression of CYP1A1 by XRE activation, we performed a XRE reporter assay. Treatment with capsaicin alone slightly enhanced XRE reporter activity in a dose-dependent manner (Figure 2A). However, capsaicin significantly suppressed 3-MC-induced XRE reporter activity in Hepa-1c1c7 cells (Figure 2A). Additionally, we investigated the XRE and AhR binding ability with capsaicin treatment using an electrophoretic mobility shift analysis (EMSA). We used a typical consensus XRE sequence from the CYP1A1 gene, along with nuclear extracts from Hepa-1c1c7 cells. As shown in Figure 2B, capsaicin and 3-MC increased the binding of AhR to the typical consensus XRE sequence from the CYP1A1 promoter. Capsaicin inhibited the 3-MC-induced binding of AhR to the XRE sequence from the CYP1A1 promoter (Figure 2B). The specificity of this interaction was confirmed using a 200-fold excess of unlabelled XRE oligonucleotide to compete with the 3-MC-induced binding of AhR to ^{32}P-XRE. These results indicated that capsaicin could regulate the AhR that binds to the XRE in the CYP1A1 gene. Using fluorescence microscopy, we examined whether capsaicin regulated the nuclear translocation of AhR in Hepa-1c1c7 cells. In control cells, AhR protein was localized in the cytoplasm; however, after capsaicin and 3-MC treatment, AhR was found almost exclusively in the nucleus (Figure 2C). Capsaicin inhibited the 3-MC-induced nuclear translocation of AhR (Figure 2C). Thus, capsaicin can regulate AhR, and the inhibition of CYP1A1 expression by capsaicin occurred during inhibition of AhR transactivation.

Role of AhR and TRPV1 in capsaicin-mediated up-regulation of CYP1A1

To determine whether capsaicin-mediated down- and up-regulation of CYP1A1 expression was correlated with AhR activation and a TRPV1 receptor signalling pathway, we examined the effect of AhR inhibitor and a TRPV1 receptor antagonist. The AhR inhibitor, CH-223191 (CH), blocked the binding of TCDD to AhR and inhibited TCDD-mediated nuclear translocation and DNA binding of AhR (Kim *et al.*, 2006). Treatment with CH reduced the capsaicin-mediated increase in enzyme activity and protein level of CYP1A1 (Figure 3A–B). As capsaicin exerts its effects by binding to TRPV1 receptors (Yang *et al.*, 2010; Ziglioli *et al.*, 2009), we determined whether Hepa-1c1c7 cells expressed the TRPV1

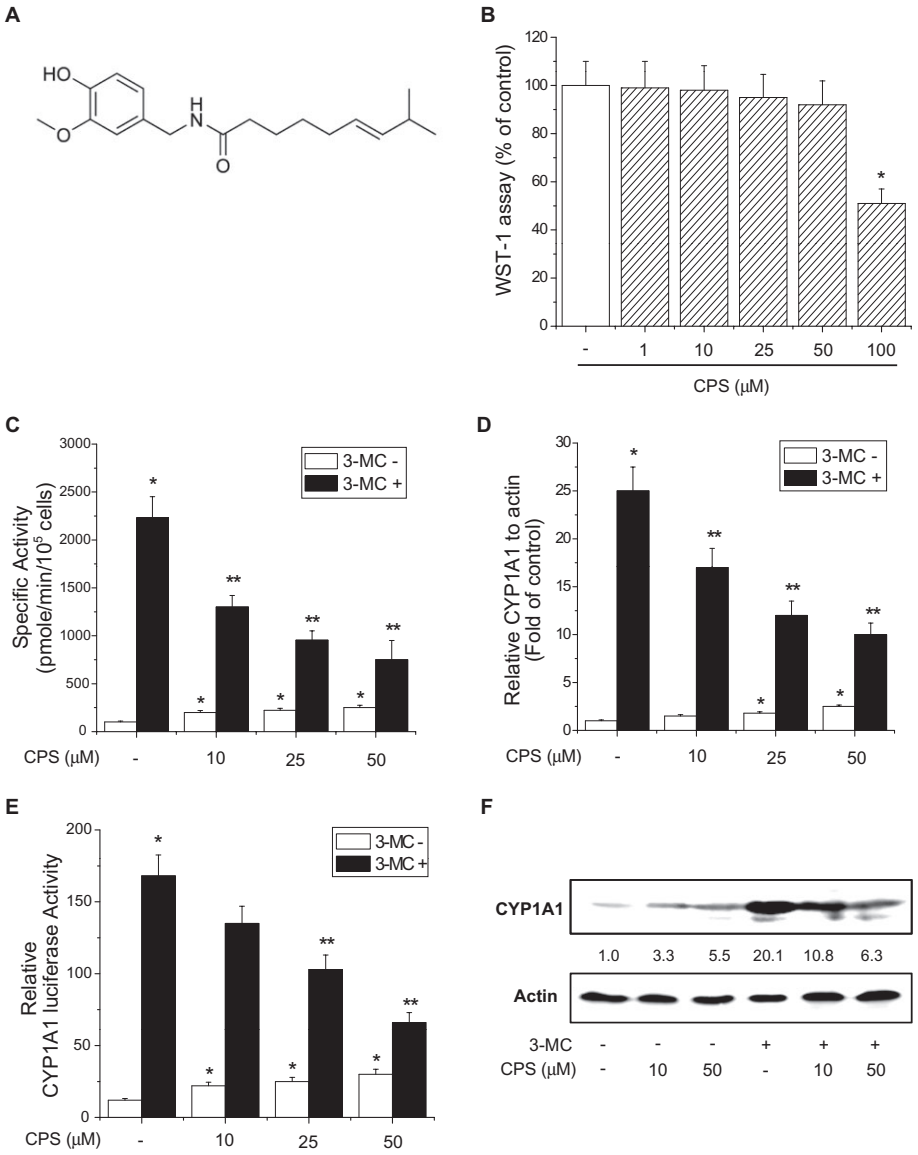

Figure 1

Effect of capsaicin on CYP1A1 expression. (A) Structure of capsaicin. (B) Hepa-1c1c7 cells were seeded in 96-well plates and treated with various concentrations of capsaicin (CPS; 1–100 μM) for 24 h. Cell viability was assessed using the WST-1 assay for the rapid detection of cytotoxicity. *$P < 0.01$, significantly different from control. (C) Effects of capsaicin on EROD activity. EROD activity was measured in cells treated with capsaicin (CPS; 10–50 μM) and/or 3-MC for 18 h. *P and **$P < 0.01$, significantly different from the control and 3-MC, respectively. (D) Effect of capsaicin on CYP1A1 mRNA expression. Cells were incubated with capsaicin (CPS; 10–50 μM) and/or 3-MC (1 μM) for 6 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1A1 mRNA expression, relative to actin expression. CYP1A1 mRNA expression was compared between treated and untreated cells at each time point. *P and **$P < 0.01$, significantly different from control and 3-MC respectively. (E) Effect of capsaicin on CYP1A1 promoter activity. Cells were transfected with CYP1A1-Luc and cultured with capsaicin (CPS) and/or 3-MC for 24 h, harvested and assayed for luciferase activity. *P and **$P < 0.01$, significantly different from the control and 3-MC respectively. (F) Effect of capsaicin on CYP1A1 protein expression. Cells were incubated with capsaicin (CPS; 10 and 50 μM) or 3-MC (1 μM) for 24 h. The CYP1A1 protein level was analysed by immunoblots of cell lysates probed with anti-hCYP1A1 antibody. The CYP1A1 protein level was normalized to that of actin. Each blot is representative of three independent experiments. The densitometry data presented below the bands are the fold-change compared with the control after normalization with the respective loading control value. EROD, ethoxyresorufin-O-deethylase; PCR, polymerase chain reaction.

receptor gene and found that Hepa-1c1c7 cells did express mRNA for TRPV1 receptors (Figure 3C). CPZ, a synthetic competitive antagonist of capsaicin (Bevan *et al.*, 1992), is a TRPV1 receptor antagonist capable of blocking the activation induced by capsaicin, heat, and protons (Tominaga *et al.*, 1998). CPZ reduced the capsaicin-mediated increase in enzyme activity and protein level of CYP1A1 in Hepa-1c1c7 cells (Figure 3D–E). Thus, the induction of CYP1A1 expression by capsaicin may involve TRPV1 receptor– and AhR-dependent signalling pathways.

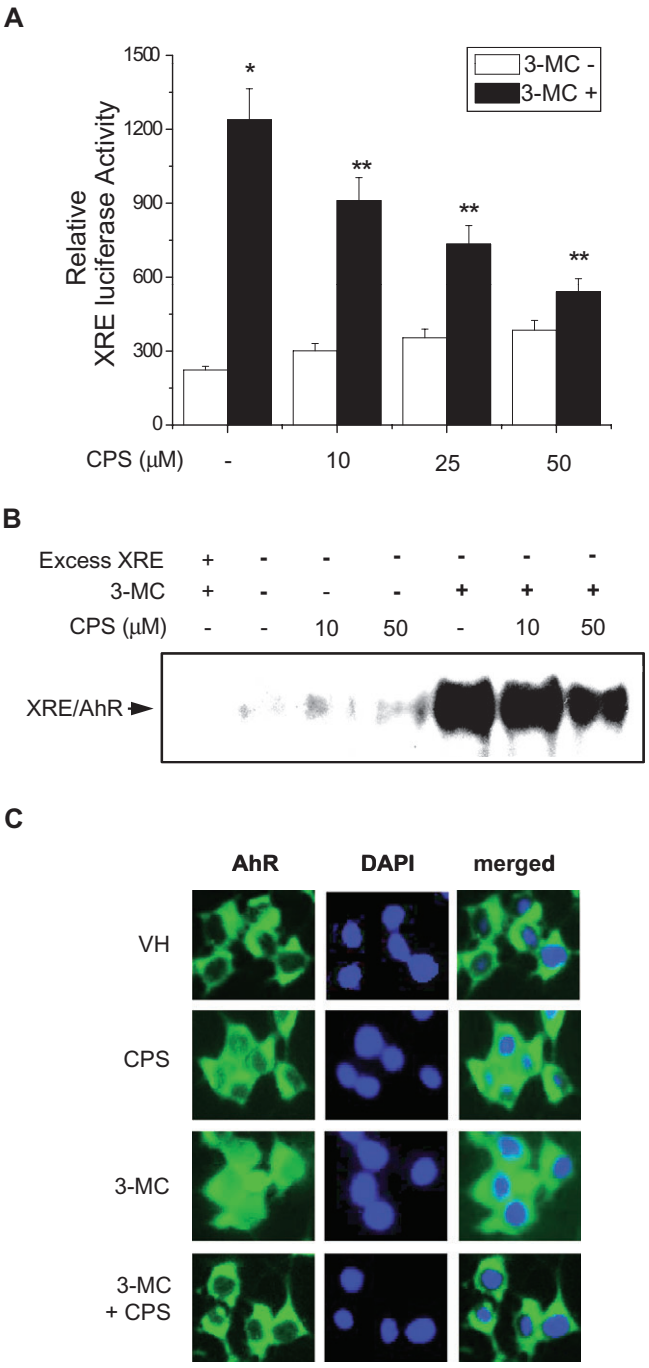

Figure 2

Effects of capsaicin on XRE and AhR binding. (A) Effects of capsaicin on XRE promoter activity. Cells were transfected with the XRE-Luc construct and then treated with capsaicin (CPS; 10–50 μM) and/or 3-MC (1 μM) for 18 h. Cells were then harvested and assayed for luciferase activity. * and **$P < 0.01$, significantly different from the control and 3-MC, respectively. (B) Effects of capsaicin on XRE-AhR binding activity. The cells were treated with capsaicin (CPS; 10 and 50 μM) or 3-MC (1 μM) for 3 h. XRE-AhR binding activity was measured using an electrophoretic mobility shift assay. The arrow indicates the AhR-XRE complex or supershift. Excess XRE: 200-fold excess of non-labelled XRE. (C) Effects of capsaicin on AhR nuclear translocation. The cells were treated with capsaicin (CPS; 50 μM) and/or 3-MC for 3 h. Cells were fixed and subjected to immunocytochemistry using an AhR antibody (left column), and the images were superimposed with DAPI-stained images (middle column); merged images (right column). This figure is representative of three independent experiments. XRE, xenobiotic response element; AhR, aryl hydrocarbon receptor; Luc, luciferase ; 3-MC, 3-methylcholanthrene; DAPI, 4′,6-diamidino-2-phenylindole.

Upstream signal pathway in capsaicin-mediated up-regulation of CYP1A1

We next investigated changes in signalling pathways downstream of TRPV1 receptors, by capsaicin. Capsaicin concentration-dependently enhanced phosphorylation of Ca^{2+}/calmodulin (CaM)–dependent protein kinase I (Thr177) (CaMKI), a downstream component of the TRPV1 receptor signalling pathway (Figure 4A). The TRPV1 receptor antagonist, CPZ, reduced capsaicin-induced CaMKI (Thr177) phosphorylation (Figure 4A). However, the AhR inhibitor CH did not affect capsaicin-induced CaMKI (Thr177) phosphorylation (Figure 4A). TRPV1 receptors mediate intracellular calcium release activity and calcium regulates the PI3-kinase /Akt pathway (El Andaloussi-Lilja *et al.*, 2009; Nabissi *et al.*, 2010). Capsaicin concentration-dependently induced phosphorylation of Akt (Figure 4B). Furthermore, the TRPV1 receptor antagonist CPZ or the CaM antagonist W7 inhibited capsaicin-induced phosphorylation of Akt (Figure 4B). W7 or the PI-3 K inhibitor LY294002 reduced the capsaicin-mediated increase in CYP1A1 gene expression and protein level (Figure 4C, D). These results indicate that capsaicin-induced CYP1A1 expression may be regulated by CaM and Akt activation–dependent signalling events.

Capsaicin induces C/EBPβ activation in Hepa-1c1c7 cells

The activated PI3-kinase/Akt pathway regulates C/EBPβ expression and phosphorylation (Venugopal and Jaiswal, 1998; Kang *et al.*, 2003). A major truncated form having a molecular weight close to that of the 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) (Descombes and Schibler, 1991). We further examined the effect of C/EBPβ expression and phosphorylation by capsaicin. Capsaicin significantly induced LAP and LIP C/EBPβ protein expression and phosphorylation (Figure 5A, B). However, 3-MC did not change expression and phosphorylation of C/EBPβ (data not shown). The β-actin control was expressed constitutively and was unaffected by capsaicin treatment. To clarify the mechanism of C/EBPβ protein expression, we transfected Hepa-1c1c7 cells with C/EBP-Luc reporter constructs. Capsaicin increased the C/EBP luciferase activity in a dose-dependent manner (Figure 5C). Thus, capsaicin may regulate the transcriptional activation of C/EBP in Hepa-1c1c7 cells. Next, to demonstrate that capsaicin induced C/EBP transactivation, we performed an EMSA assay. We used a typical consensus C/EBP sequence, along with nuclear

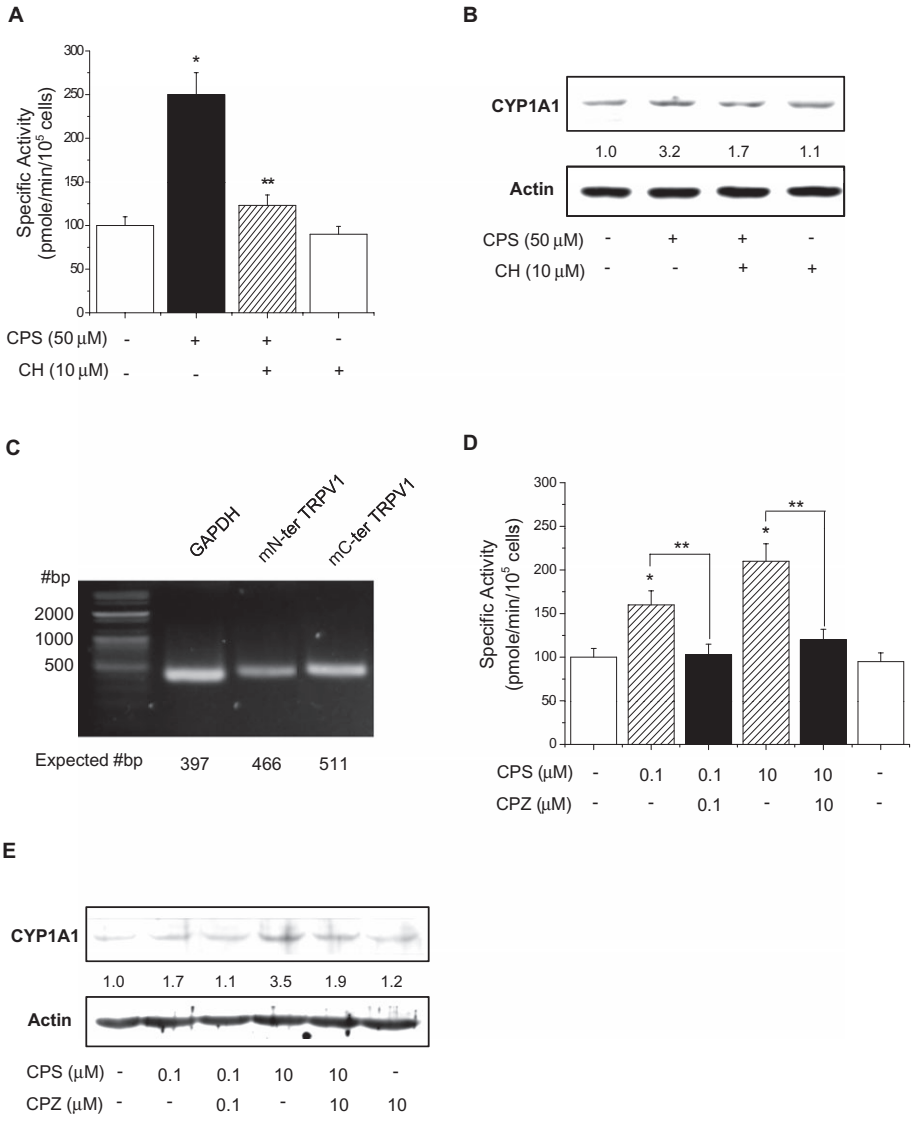

Figure 3

The role of AhR and TRPV1 in capsaicin-mediated CYP1A1 expression. (A) Effect of CH-223191 (CH) on capsaicin-induced CYP1A1 enzyme activity. Cells were pretreated with CH (10 μM) for 30 min and then incubated with 50 μM of capsaicin (CPS) for 18 h. * and **$P < 0.01$, significantly different from the control and capsaicin respectively. (B) Effect of CH on capsaicin-induced CYP1A1 protein expression. Cells were preincubated with CH (10 μM) and then treated with capsaicin (CPS; 50 μM) for 24 h. (C) Effect of capsazepine (CPZ) on capsaicin-induced CYP1A1 enzyme activity. Cells were pretreated with CPZ (0.1 or 10 μM) for 30 min and then incubated with capsaicin (0.1 or 10 μM) for 18 h. *P and **$P < 0.01$, significantly different from the control and capsaicin respectively. (D) Effect of capsazepine on capsaicin-induced CYP1A1 protein expression. Cells were pre-incubated with capsazepine (CPZ; 0.1 or 10 μM) and then treated with capsaicin (CPS; 0.1 or 10 μM) for 24 h. CYP1A1 protein was analysed by immunoblots of cell lysates probed with anti-CYP1A1 antibody. Levels of CYP1A1 protein were normalized to that of actin. Each blot in this figure is representative of three independent experiments with similar results. The densitometry data presented below the bands are the fold-change compared with actin after normalization with the respective loading control value. TRPV, transient receptor potential vanilloid type-1 receptor; AhR, aryl hydrocarbon receptor.

extracts from Hepa-1c1c7 cells. Capsaicin enhanced the binding of C/EBP to the typical consensus C/EBP response sequence (Figure 5D). Supershift analysis, with a highly specific antibody directed against C/EBPβ, indicated that the C/EBP binding complex contained C/EBPβ (Figure 5D). The specificity of this interaction was confirmed by showing that a 200-fold excess of unlabelled C/EBP oligonucleotide competed with the binding of C/EBPβ to ^{32}P-C/EBP. Additionally, we examined whether capsaicin regulated the nuclear translocation of C/EBPβ in Hepa-1c1c7 cells. Capsaicin increased the nuclear translocation of C/EBPβ (Figure 5E). Additionally, the TRPV1 receptor antagonist CPZ, the CaM antagonist W7 and the inhibitor of PI3-kinase/Akt LY294002 all inhibited capsaicin-induced C/EBPβ protein phosphorylation (Figure 5F). CPZ, W7 or LY294002, used alone, did not affect C/EBPβ protein phosphorylation (Figure 5F). Therefore,

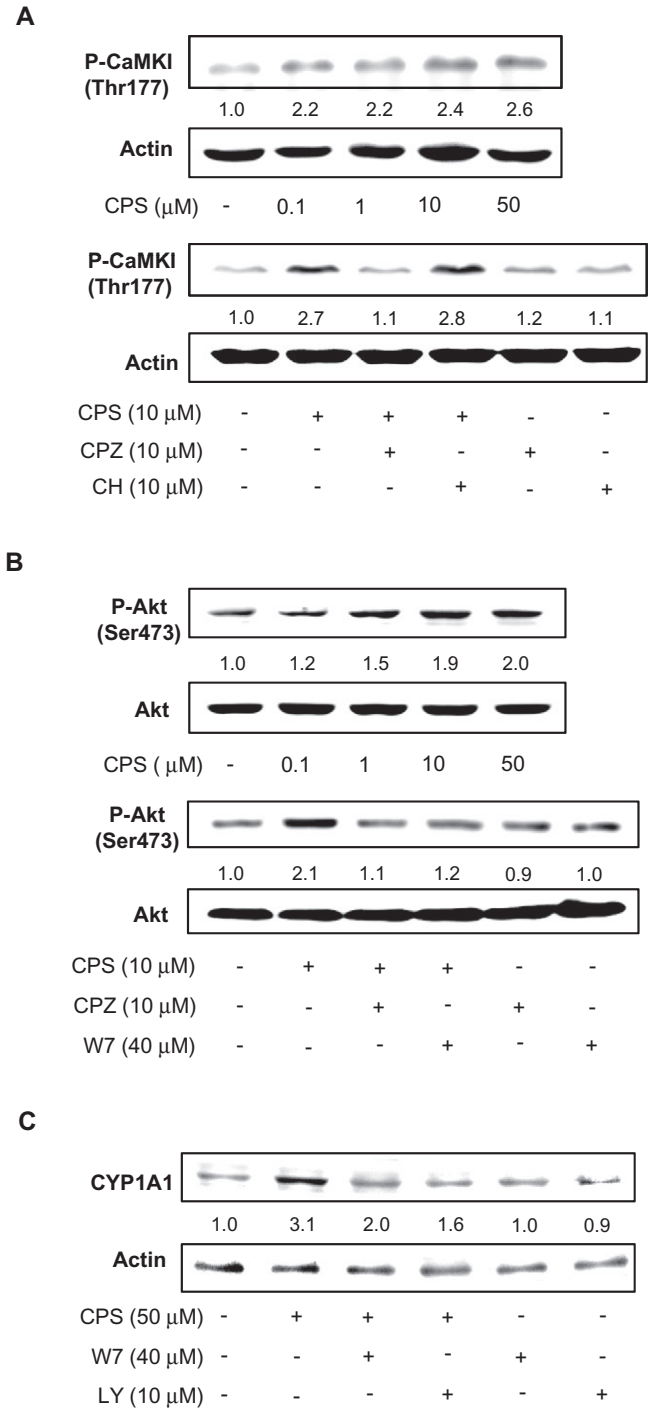

Figure 4

Effects of capsaicin on TRPV1 receptor signalling events. (A) Effects of capsaicin on activation of Ca^{2+}/calmodulin (CaM)–dependent protein kinase (CaMKI), a signalling component, downstream of TRPV1 receptor activation. Cells were treated with capsaicin (CPS; 0.1–50 μM) for 30 min. In addition, cells were pretreated with capsazepine (CPZ; 0.1 or 10 μM) and then treated with capsaicin (0.1 or 10 μM) for 30 min. Phosphorylated CaMKIα at threonine-177 (P-CaMKIα) was then analysed by Western blotting; actin expression was measured as a loading control. (B) Effects of capsaicin on activation of Akt, a signalling component, downstream of TRPV1 receptor activation. Cells were treated with capsaicin (0.1–50 μM) for 10 min. Cells were pretreated with capsazepine (CPZ; 10 μM) or W7 (40 μM) for 30 min and treated with capsaicin for 10 min. The Western blotting membranes were probed with phospho-Akt specific antibody. The phospho-Akt protein levels are compared with total Akt kinase protein. (C) Effect of W7 and LY294002 (LY) on capsaicin-induced CYP1A1 protein expression. Cells were pretreated with W7 (40 μM) or LY (10 μM) and then treated capsaicin (50 μM) for 24 h. CYP1A1 protein was analysed by immunoblots of cell lysates probed with anti-CYP1A1 antibody. The CYP1A1 protein level was normalized to that of actin. Each blot in this figure is representative of three independent experiments with similar results. The densitometry data presented below the bands are the fold-change compared with the control after normalization with the respective loading control value. TRPV1, transient receptor potential vanilloid type-1 receptor.

capsaicin may induce C/EBPβ activation through signalling pathways downstream of TRPV1 receptors.

C/EBPβ activation is required for capsaicin-mediated down-regulation of CYP1A1

Recently, it has been reported that the C/EBPβ is involved in the formation of the transcription complexes binding to the XRE (Cho and Kim, 2003; Shin *et al.*, 2005). To correlate the activation of C/EBP with the inhibition of 3-MC-mediated CYP1A1 level by capsaicin, a constitutively active C/EBP-specific dominant-negative mutant (AC/EBP) was expressed in cells. Expression of AC/EBP abolished the ability of capsaicin to suppress C/EBPβ protein expression (Figure 6A). The expression of AC/EBP alone did not affect the C/EBP protein level, and the control actin protein was unaffected by AC/EBP overexpression. The expression of AC/EBP also blocked the ability of capsaicin to suppress the 3-MC induction of CYP1A1 enzyme activity, mRNA and protein levels (Figure 6B–E). Thus, 3-MC-induced CYP1A1 activation was blocked by capsaicin-mediated C/EBPβ transcriptional activation.

Additionally, the capsaicin-inducible expression of CYP1A1 protein was abolished by the expression of AC/EBP (Figure 6F). This result showed a pattern similar to that of CYP1A1 gene expression, enzyme and promoter activity (data not shown). Therefore, capsaicin-inducible CYP1A1 expression may regulate C/EBPβ activation by itself.

Discussion

The aim of this study was to determine the effect of capsaicin on CYP1A1 expression in mouse hepatoma Hepa-1c1c7 cells. We demonstrated that capsaicin alone was a weak inducer of CYP1A1 expression, and the induction of CYP1A1 by 3-MC was moderately inhibited by capsaicin. Our findings suggest that this effect of capsaicin was mediated through C/EBP and AhR signalling pathways.

Treatment of capsaicin increased CYP1A1 enzyme activity, gene expression and protein levels in Hepa-1c1c7 cells. Capsaicin also induced XRE luciferase activity and AhR/XRE

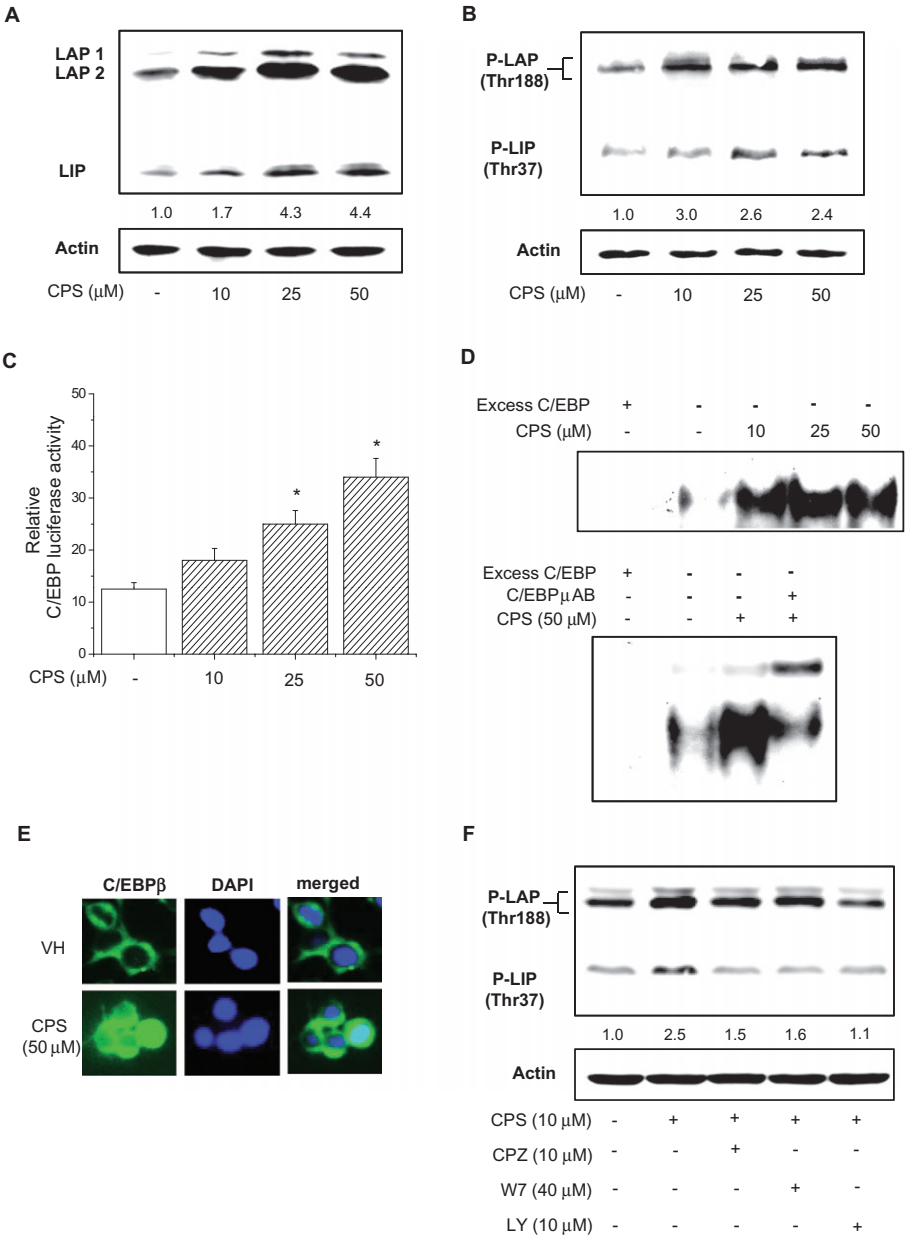

Figure 5

Effect of capsaicin on C/EBP expression. (A) Effect of capsaicin on C/EBPβ protein expression. Cells were cultured with capsaicin (CPS; 10, 25 and 50 μM) for 24 h. Levels of C/EBPβ protein (as LAP and LIP components) level was analysed by immunoblots of cell lysates probed with anti-C/EBPβ antibody. The C/EBPβ protein level was normalized to that of actin. (B) Effect of capsaicin on C/EBPβ activation. Cells were cultured with capsaicin (CPS; 10, 25, and 50 μM) for 6 h. Phospho-C/EBPβ (P-LAP, P-LIP) was analysed by immunoblots of cell lysates probed with anti-phospho-C/EBPβ (Thr188/37) antibody. The phospho-C/EBPβ protein was normalized to that of actin. (C) Effect of capsaicin on C/EBP promoter activity. Cells were transfected with CYP1A1-Luc and then incubated with capsaicin (CPS; 10, 25 and 50 μM) for 24 h, harvested and assayed for luciferase activity. *$P < 0.01$, significantly different from the control. (D) Effect of capsaicin on protein binding to the CYP1A1 C/EBP binding site. Nuclear extracts were prepared from cells treated with capsaicin (CPS; 10, 25 and 50 μM) for 3 h. For immuno-inhibition, the nuclear extract obtained from cells treated with 50 μM of capsaicin for 3 h was incubated with an anti-C/EBPβ antibody for 1 h. The immuno-depleted extract was mixed with labelled probe. Competition experiments using a 200-fold excess C/EBP binding oligonucleotide confirmed the specificity of C/EBP DNA binding. (E) Effects of capsaicin on C/EBPβ nuclear translocation. The cells were treated with capsaicin (CPS; 50 μM) for 3 h. Cells were fixed and subjected to immunocytochemistry using a C/EBPβ antibody (left column), and the images were superimposed with DAPI-stained images (middle column); merged images (right column). This figure is representative of three independent experiments. (F) Effect of capsazepine (CPZ), W7 or LY294002 (LY) on capsaicin-induced C/EBPβ phosphorylation. Cells were pretreated with CPZ (10 μM), W7 (40 μM) or LY (10 μM) for 30 min and then incubated with capsaicin (CPS; 10 μM) for 6 h. The phospho-C/EBPβ protein level was normalized to that of actin. Each blot in this figure is representative of three independent experiments with similar results. The densitometry data presented below the bands are the fold-change compared with the control after normalization with the respective loading control value. C/EBP, CCAAT/ enhancer-binding protein; Luc, luciferase; DAPI, 4′,6-diamidino-2-phenylindole.

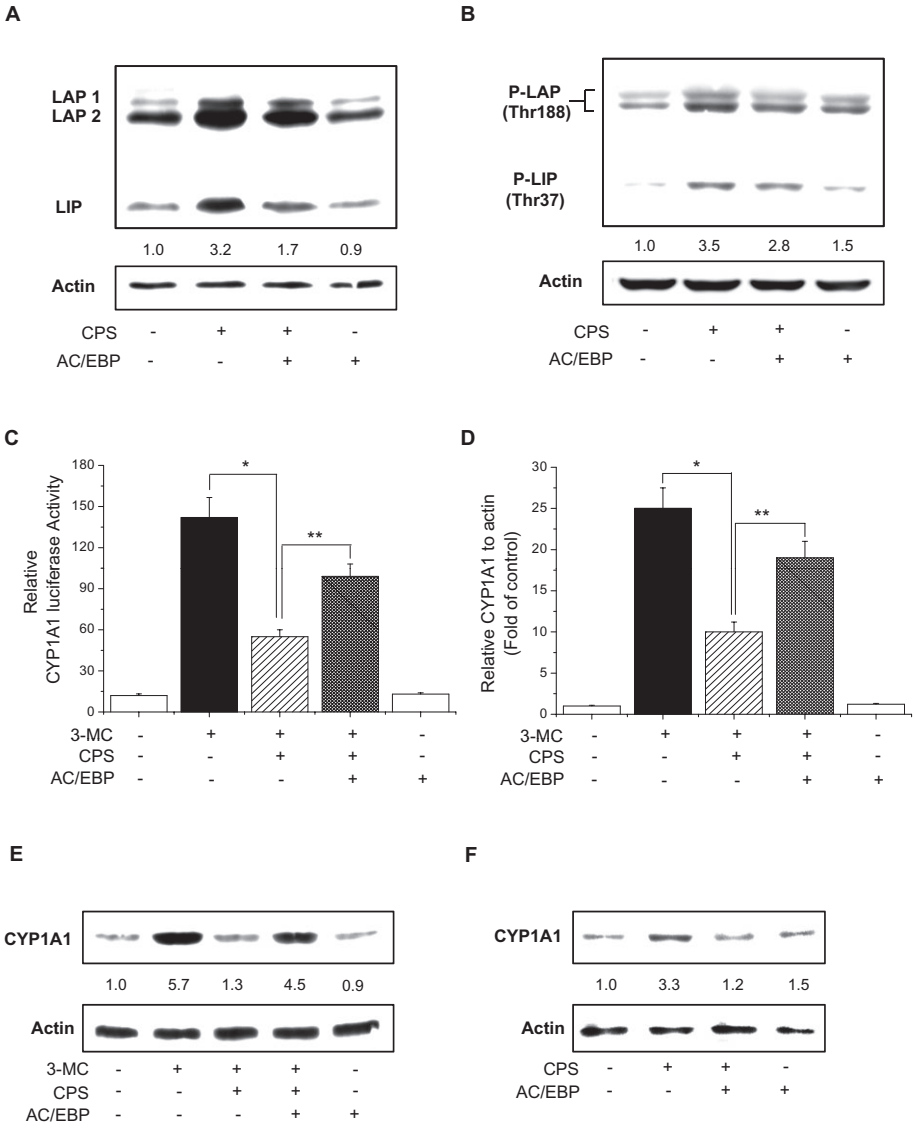

Figure 6

The role of C/EBP in capsaicin-mediated CYP1A1 expression. (A) Effect of AC/EBP on capsaicin-induced C/EBPβ protein expression. Cells were transfected with AC/EBP vector, a mutation plasmid of the C/EBP region, for 24 h and then incubated with 50 μM of capsaicin (CPS) for 24 h. C/EBPβ protein (as LAP, LIP components) was analysed by immunoblots of cell lysates probed with anti-C/EBPβ antibody. The C/EBPβ protein level was normalized to that of actin. Each blot is representative of three independent experiments. (B) Effect of AC/EBP on capsaicin-reduced EROD activity. EROD activity was measured in cells transfected with AC/EBP vector and then treated with capsaicin (CPS; 50 μM) and/or 3-MC (1 μM) for 18 h. * and ** $P < 0.01$, significantly different from 3-MC and 3-MC + capsaicin respectively. (C) Effect of AC/EBP on capsaicin-suppressed CYP1A1 promoter activity. Cells were cotransfected with CYP1A1-Luc and AC/EBP for 4 h and then cultured with capsaicin (CPS) and/or 3-MC for 24 h. Cells were harvested and assayed for luciferase activity. * P and ** $P < 0.01$, significantly different 3-MC and 3-MC + capsaicin respectively. (D) Effect of AC/EBP on capsaicin-mediated CYP1A1 mRNA expression. Cells were transfected with AC/EBP for 24 h and then incubated with capsaicin (CPS; 50 μM) and/or 3-MC (1 μM) for 6 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1A1 mRNA expression relative to actin expression. CYP1A1 mRNA expression was compared between treated and untreated cells at each time point. * and ** $P < 0.01$, significantly different 3-MC and 3-MC + capsaicin, respectively, by the Newman–Keuls test. (E) Effect of AC/EBP on capsaicin-mediated CYP1A1 protein expression. Cells were transfected with AC/EBP for 24 h and then incubated with capsaicin (CPS; 50 μM) and/or 3-MC (1 μM) for 24 h. (F) Effect of AC/EBP on capsaicin-inducible CYP1A1 protein expression. Cells were transfected with AC/EBP for 24 h and then incubated with capsaicin (CPS; 50 μM) for 24 h. The CYP1A1 protein level was analysed by immunoblots of cell lysates probed with anti-hCYP1A1 antibody. The CYP1A1 protein level was normalized to that of actin. Each blot is representative of three independent experiments. C/EBP, CCAAT/ enhancer-binding protein; 3-MC, 3-methylcholanthrene; Luc, luciferase; EROD, ethoxyresorufin-O-deethylase.

binding ability. In the present study, we further revealed that induction of CYP1A1 was regulated via TRPV1 receptors as well as AhR, in the presence of capsaicin. The TRPV1 receptor–dependent induction of CYP1A1 by capsaicin implies a pathway of CYP1A1 induction, distinct from that via AhR. TRPV1 receptors are selectively activated by capsaicin, the primary pain-producing chemical in hot peppers, and a variety of exogenous and endogenous respiratory toxicants (Van Der Stelt and Di Marzo, 2004; Thomas *et al.*, 2007). TRPV1 receptor activation has been reported to induce CYP1A1 activity (Dai *et al.*, 2008; Han *et al.*, 2009). Treatment with CPZ, a synthetic competitive antagonist of TRPV1 receptors, suppressed capsaicin-inducible CYP1A1 gene expression, enzyme, promoter activity and protein level in Hepa-1c1c7 cells. Capsaicin also enhanced phosphorylation of CaMKI and Akt, downstream components of the TRPV1 receptor signalling pathway. Thus, the TRPV1 receptor signalling pathway could participate in capsaicin-mediated CYP1A1 expression. CYP1A1 catalyses the mono-oxidation of carcinogens, creating reactive epoxides that can bind DNA and cause mutations (Nebert and Dalton, 2006), while CYP1A1 activation also provides better substrates than the carcinogens, including 3-MC, for phase II enzyme conjugation, thus facilitating detoxification (Ciolino *et al.*, 2006; Han *et al.*, 2009). For example, rutaecarpine from *Evodia* fruit induces CYP1A1 expression (Han *et al.*, 2009), and it also induces expression of phase 2 enzymes, including NADPH:quinone reductase (Ahn *et al.*, 2008). Therefore, capsaicin-mediated CYP1A1 induction may be a key step in detoxification and chemoprevention through AhR and TRPV1 receptor activation.

The 3-MC/AhR/ARNT complex translocates to the nucleus and the complex binds to XRE sequences (McGuire *et al.*, 1994; Heid *et al.*, 2000). 3-MC-induced CYP1A1 enzyme activity, gene expression, and protein levels were decreased by capsaicin. In addition, capsaicin suppressed the 3-MC-induced XRE reporter activity, AhR/XRE binding ability and nuclear translocation of AhR. Capsaicin-mediated antagonism of CYP1A1 appears to result from 3-MC-induced recruitment of XRE, and this may reduce reactive epoxides that cause mutations. This novel mechanism of antagonism may form the basis for the chemopreventive effects of capsaicin. Phytochemicals show some apparent differences in the AhR agonist or antagonist activities of individuals (Ciolino *et al.*, 1999). However, previous studies have demonstrated that many of phytochemicals exhibit weak AhR agonist and/or partial antagonist activities (Ciolino *et al.*, 1998; Ashida *et al.*, 2000; Quadri *et al.*, 2000; Chun *et al.*, 2001; Zhang *et al.*, 2003). Capsaicin may prove to be an effective chemopreventive agent, and it was also shown to be an agonist/antagonist of the AhR.

TRPV1 receptors mediate calcium release activity, and the raised intracellular calcium then activates the PI3-kinase/Akt pathway (El Andaloussi-Lilja *et al.*, 2009; Nabissi *et al.*, 2010). The activated PI3-kinase/Akt pathway regulates C/EBP expression and phosphorylation (Venugopal and Jaiswal, 1998; Kang *et al.*, 2003). C/EBPs are known to play important roles in regulating the expression of multiple hepatocyte-specific genes (Diehl, 1998; Lekstrom-Himes and Xanthopoulos, 1998). These transcription factors also help to control hepatocyte progression through the cell cycle (Hendricks-Taylor and Darlington, 1995; Timchenko *et al.*, 1996). Thus, the C/EBPs are likely to be important targets for regulation during liver regeneration. A previous study reported that capsaicin up-regulated the gene encoding CHOP in human prostate epithelial cells (Sanchez *et al.*, 2008). Oltipraz or flavonoid compounds, including PD98059, promote nuclear

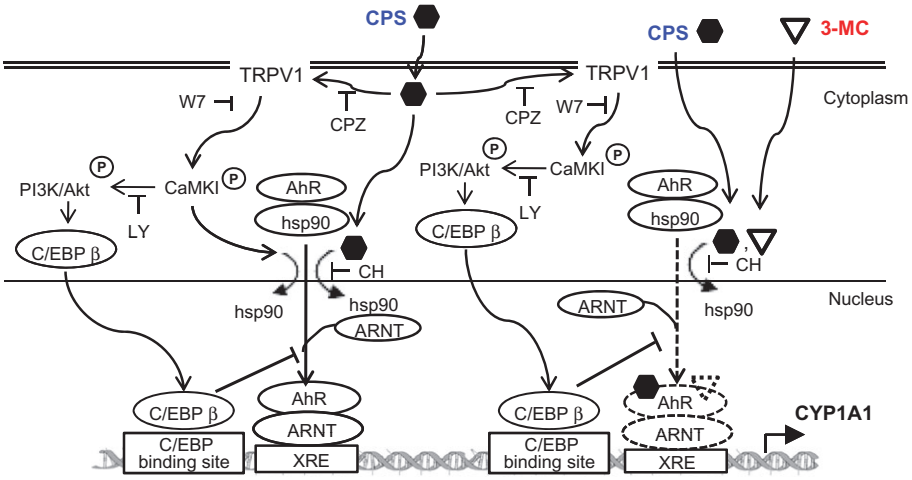

Figure 7

Diagram of the regulation of CYP1A1 by capsaicin in Hepa-1c1c7 cells. Capsaicin (CPS) alone weakly enhanced AhR activation. Additionally, capsaicin activates the TRPV1 receptor and then induces raised intracellular calcium and thus activates the PI3-kinase/Akt signalling pathway. PI3-kinase/Akt signalling induces C/EBPβ protein expression and activation. PAHs such as 3-MC and capsaicin bind to AhR and are then degraded by hsp90. The PAHs/AhR complex binds ARNT and this complex is then translocated to the nucleus. The translocated capsaicin or PAHs and AhR/ARNT complex binds to the XRE site and thus CYP1A1 expression is increased. C/EBPβ activated by capsaicin prevents the PAHs/AhR/ARNT complex from binding to the XRE. C/EBP, CCAAT/ enhancer-binding protein; PAH, poly aryl hydrocarbon; TRPV1, transient receptor potential vanilloid type-1 receptor; 3-MC, 3-methylcholanthrene; AhR, aryl hydrocarbon receptor ; XRE, xenobiotic response element.

translocation and activation of C/EBPβ, which leads to the inhibition of CYP1A1 expression via binding of activated C/EBPβ to the C/EBP binding site present in the gene promoter (Cho and Kim, 2003; Shin *et al*., 2005).

We found that capsaicin increased the C/EBP luciferase activity and transcription factor binding ability. The C/EBPβ protein is composed of LAP and LIP components in liver cells (Descombes and Schibler, 1991) and, in our work, capsaicin significantly induced LAP and LIP C/EBPβ protein expression and phosphorylation. C/EBP-specific dominant-negative mutant (AC/EBP) overexpression blocked capsaicin-induced CYP1A1 protein expression, enzyme activity and mRNA expression. Thus, capsaicin induced the transcriptional activation of C/EBPβ and activated C/EBPβ played a critical role in capsaicin-mediated inhibition of CYP1Al expression.

Many natural compounds that modulate the metabolism of carcinogens can be both inhibitors and stimulators of the activities of CYP1A1 enzymes (Hwang *et al*., 2008). CYP1A1 catalyses the oxidative catabolism of PAHs, and PAH-induced CYP1A1 expression can lead to formation of carcinogenic metabolites that enter the nucleus and bind to specific DNA residues, leading to mutagenesis and carcinogenesis (Dipple, 1994). It would be expected that the suppression of CYP1A1 induction would block the activation of certain xenobiotics to carcinogenic metabolites. Capsaicin could induce this potential cancer chemoprevention effect through C/EBPβ-mediated inhibition of CYP1A1 expression by capsaicin. Taken together, our results reveal that capsaicin alone enhanced AhR-mediated CYP1A1 expression and it induced C/EBPβ activation, mediated by the TRPV1 receptor and Akt signalling pathways (Figure 7). Additionally, capsaicin suppressed PAH-induced CYP1A1 expression, and this capsaicin-reduced CYP1A1 expression was mediated by capsaicin-induced C/EBPβ activation (Figure 7). Our results suggest capsaicin is a potential chemopreventive agent that reduces 3-MC-induced CYP1A1 levels via activation of C/EBPβ and blocking of the AhR signalling pathway.

Conflict of interest

None.

Acknowledgements

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