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RESEARCH PAPER

Impaired transactivation of the human CYP2J2 arachidonic acid epoxygenase gene in HepG2 cells subjected to nitrative stress

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Background and purpose: Human cytochrome P450 2J2 (CYP2J2) generates epoxyfatty acids that modulate cellular apoptosis and proliferation. CYP2J2 regulation has not been intensively studied but induction of the activator protein-1 (AP-1) subunit c-fos mediates CYP2J2 down-regulation in hypoxia, a component of ischaemic injury. Decreased CYP2J2 expression may contribute to tissue injury.
Experimental approach: HepG2 cells were treated with sodium nitroprusside (SNP) to induce nitrative stress, which has been associated with inflammation and infection in liver and other tissues. CYP2J2 protein and mRNA expression were evaluated by immunoblotting and real-time PCR respectively. The role of mitogen-activated protein (MAP) kinases in CYP2J2 dysregulation was assessed using specific inhibitors and dominant negative MAP kinase expression plasmids. CYP2J2-luciferase reporter constructs and electromobility shift assays (EMSAs) were used to identify SNP-regulated regions in the CYP2J2 gene.
Key results: Cytochrome P450 2J2 was down-regulated by SNP while the AP-1 proteins c-jun and c-fos were up-regulated; inhibition of p38 and ERK MAP kinases normalized their expression. The gene elements at –105/–95 and –56/–63 were required for the down-regulation of CYP2J2 induced by nitrative stress.
Conclusions and implications: p38 and ERK MAP kinases transduce stress stimuli that down-regulate CYP2J2. Targeting these kinases may prevent the loss of CYP2J2 and epoxy-fatty acids that protect cells against deleterious stresses.
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Keywords: cytochrome P450 2J2; activator protein-1; gene regulation; epoxyfatty acids; nitrative stress

Abbreviations: AP-1, activator protein-1; bZIP, basic leucine zipper; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EET, epoxyeicosatrienoic acid; EMSA, electromobility shift assay; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MTT, thiazolyl blue tetrazolium bromide; SNP, sodium nitroprusside

Introduction

Human cytochrome P450 (CYP) 2J2 is widely expressed in many tissues, including heart, liver, kidney, lung, pancreas and endothelial cells (Wu *et al.*, 1996; Zeldin *et al.*, 1997a,b; Gaedigk *et al.*, 2006; Yamazaki *et al.*, 2006). CYP2J2 oxidizes arachidonic acid to a series of isomeric epoxyeicosatrienoic acids (EETs). CYP-derived EETs have been shown to influence numerous physiological processes, including modulation of vasoactivity (Fleming, 2001; Roman, 2002) and inflammatory activity (Node *et al.*, 1999) and processes relevant to disease pathology, including cellular proliferation and apoptosis (Chen *et al.*, 2001; Potente *et al.*, 2003). Following exposure to deleterious stimuli, the balance between cell survival and the removal of damaged cells by apoptosis is critical to ongoing tissue viability (Plachta *et al.*, 2003). The pro-survival properties of EETs may be useful for minimization of tissue and cellular injury. Indeed, the application of EETs protected endothelial cells from hypoxic injury (Yang *et al.*, 2001) and enhanced the recovery of CYP2J2-transgenic mouse hearts from ischaemia (Batchu *et al.*, 2009). Understanding the factors that regulate CYP2J2 gene transactivation may be valuable for the development of strategies to maintain the viability of a range of tissues following exposure to injurious stresses.

We have shown previously that CYP2J2 expression is maintained in normoxic HepG2 cells by the basic leucine zipper (bZIP) protein c-jun, which is an important component of the activator protein-1 (AP-1) transcription complex (Marden *et al.*, 2003). In cells that have been cultured in a low oxygen

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environment, the related AP-1 protein c-fos is up-regulated and the resultant c-jun/c-fos heterodimers are unable to support CYP2J2 transactivation. In the present study we tested the hypothesis that nitrative stress, which contributes to inflammation and infection in liver and other tissues (Horiike *et al*., 2005; Kermorvant-Duchemin *et al*., 2005), may also up-regulate c-fos expression and down-regulate CYP2J2 in HepG2 cells.

The range of AP-1-activating stimuli includes hypoxia, UV radiation, growth factors and oxidative and nitrative stress. Mitogen-activated protein kinases (MAP kinases) are a family of serine-threonine kinases that mediate the cellular response to such extracellular stimuli (Treisman, 1996; Bogoyevitch and Court, 2004; Boutros *et al*., 2008). Important members of the MAP kinase family include the p38 MAP kinase, the extracellular signal-regulated MAP kinases (ERKs, or p42/44) and the c-jun N-terminal kinases (JNKs, or p46/54). In response to extracellular stimuli the MAP kinases are phosphorylated and regulate the downstream activities of bZIP transcription factors, including the AP-1 members c-jun and c-fos (Treisman, 1996). Indeed, nitrative stress induced by the nitric oxide donor sodium nitroprusside (SNP) has been shown to activate MAP kinases differently in a cell-type dependent manner (Callsen and Brüne, 1999; Feng *et al*., 1999; Jun *et al*., 1999a,b; Kim *et al*., 2002). The principal finding to emerge from the present study was that SNP down-regulated CYP2J2 gene expression and increased the cellular expression of the AP-1 protein c-fos. From reporter studies in transfected HepG2 cells, this effect was dependent on AP-1-responsive gene elements in the 5′-flank of the gene. Inhibitors of the p38 and ERK MAP kinases selectively decreased the activation of c-fos and prevented the loss of CYP2J2, thus implicating these kinases in the observed actions of nitrative stress in HepG2 cells.

Methods

Liver donors and preparation of microsomal fractions

Studies in human liver microsomes were approved by the ethics committees of the Western Sydney Area Health Service and the University of Sydney, in accordance with the Declaration of Helsinki. Surplus tissue from liver donors and biopsies (n = 11) was obtained through the Queensland and Australian Liver Transplant Programs (Princess Alexandria Hospital, Brisbane, QLD, and Royal Prince Alfred Hospital, Sydney, NSW, Australia respectively). Tissue was perfused with cold Viaspan solution (DuPont, Wilmington, DE, USA) and snap-frozen in liquid nitrogen. Hepatic microsomes were prepared by differential ultracentrifugation (Murray *et al*., 1986).

Cell culture

HepG2 human hepatoma and MCF-7 human breast cancer cell lines (ATCC, Manassas, VA, USA) and the HEK293 human embryonic kidney cell line (a gift from Dr Roland Stocker, Department of Pathology, University of Sydney) were maintained in monolayer culture in a humidified 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin antibiotic mix, 20 mM HEPES and 14.2 mM sodium hydrogen bicarbonate. Cells were passaged twice weekly and used at passage six in all experiments.

Cells were seeded at densities of 6×10^5 per well (HepG2), 8×10^5 per well (MCF-7) and 1.5×10^5 per well (HEK293) in six-well plates or 1×10^5 per well (HepG2) in 24-well plates and incubated for 24 h, followed by replacement of serum-free DMEM for a further 24 h. After this time some cells were treated with SNP (1 mM) in serum-free DMEM media for varying periods. In preliminary studies, concentrations of SNP up to 2 mM elicited ~10% loss of viability whereas 4 mM SNP decreased viability by ~30%. Because the primary aim was to evaluate CYP2J2 dysregulation in cells subjected to moderate stresses 1 mM SNP was selected for further study. Where SNP treatment continued for 72 h, DMEM containing 1 mM SNP was replenished at 24 h intervals and control cells were treated with phosphate-buffered saline (PBS). Following treatments, cells used for protein analysis were trypsinized, washed twice with ice-cold PBS, pelleted by centrifugation (15 800× g, 3 min) at 4°C and resuspended in sample buffer (300 μL; 400 mM Tris-HCl, 3% sodium dodecyl sulphate, 10% 2-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue). The suspension was passed through a 23 guage needle, heated at 100°C for 5 min and then centrifuged for 3 min at 15 800× g; the lysate supernatant was stored at –20°C. In experiments that assessed the role of MAP kinases the chemical inhibitors (SB203580 20 μM, SP600125 50 μM and PD98059 10 μM) were added to cells 2 h prior to the application of SNP; inhibitors were replenished in fresh media at 24 h intervals. In other experiments dominant negative mutant p38 and ERK1 MAP kinase expression plasmids (1 μg DNA) were transfected into HepG2 cells with Effectene reagent. Twenty-four hours later SNP (1 mM) was applied and was replenished in fresh media at 24 h intervals.

MTT and caspase-3 assays

Cell viability was quantified by the reduction of MTT. Cells were seeded into 24-well plates and MTT (0.3 mg·mL^{-1}) was added at intervals and incubated at 37°C for 2 h, followed by removal of supernatant. After addition of dimethylsulphoxide (0.2 mL), plates were shaken (30 min) and 100 μL aliquots were read at 540 nm in a PerkinElmer multilabel reader (Waltham, MA, USA).

Caspase-3 activity was estimated in cells that had been harvested with trypsin/EDTA. Cells were resuspended in 60 μL of serum-free medium, 50 μL aliquots were transferred to 96-well plates and the reconstituted Caspase-Glo3/7 reagent was added (50 μL); luminescence was measured in a PerkinElmer multilabel reader.

RNA extraction and real-time PCR

Total cellular RNA was extracted directly using the TRI-Reagent according to the manufacturer's protocol (Molecular Research Center), quantified spectrophotometrically and its integrity was confirmed by electrophoresis on 1% denaturing agarose gels. DNase A-treated RNA was used in real-time RT-PCR analysis (Quantitest SYBR Green kit; Qiagen) for CYP2J2 (100 ng), c-fos, c-jun and β-actin (300 ng) and

CYP1A1 and CYP2D6 (50 ng) in a Rotor-Gene 6000 thermal cycler (Corbett Life Science, Mortlake, NSW, Australia). The sequences of gene-specific primers were: CYP2J2 (forward 5′-ACGTTAGAGGAACGCATTCAGGA-3′, reverse 5′-CGAAGGT GATGGAGCAAATGAT-3′), c-jun (forward 5′- CCGTTGCTG GACTGGATTAT-3′, reverse 5′- CCCCAAGATCCTGAAACAGA-3′), c-fos (forward 5′- CTTCCTGTTCCCAGCATCAT-3′, reverse 5′- GTACAGGTGACCACCGGAGT-3′), β-actin (forward 5′-GAGCTACGAGCTGCCTGACG-3′, reverse 5′- GTAGTTTCG TGGATGCCACAG-3′), CYP1A1 (forward 5′- TCCAGAGAC AACAGGTAAAACA-3′, reverse 5′-AGGAAGGGCAGAGGAAT GTGAT-3′) and CYP2D6 (forward 5′- GGTGTGACCCATATG ACATC-3′, reverse 5′-CTCCCCGAGGCATGCACG-3′). Primers (0.5 μM each) were used in a final reaction volume of 25 μL. Each run contained control reactions from which RNA was excluded or added after the RT step. Melting curve analysis confirmed the identity of PCR products. Cell treatments were conducted in triplicate using six-well plates and each experiment was performed on at least three separate occasions.

Plasmid DNA standards were constructed for real-time PCR and confirmed by sequencing (ABI Prism BigDye; Sequencing Facility, University of New South Wales, NSW, Australia). Amplicon sizes were: CYP2J2 (133 bp), c-fos (180 bp), c-jun (168 bp) and β-actin (95 bp), which were cloned into the pCR2.1-TOPO vector; standards were included in each run (0.01–1000 fg). CYP1A1 and CYP2D6 mRNA expression was calculated by the $\Delta\Delta C_T$ method and normalized to β-actin (Livak and Schmittgen, 2001), according to the following: relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_T = (\Delta C_T\text{target} - \Delta C_T\beta\text{-actin})_{treated} - (\Delta C_T\text{target} - \Delta C_T\beta\text{-actin})_{untreated}$. PCR conditions were: reverse transcription at 50°C for 30 min, enzyme inactivation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C (CYP2J2, c-jun, β-actin), 58°C (c-fos) or 60°C (CYP1A1, CYP2D6) for 1 min, and elongation at 72°C for 1 min.

Immunoblot analysis

Cell lysate supernatants (20 μg protein) were prepared as described above, electrophoresed on 10% polyacrylamide gels and transferred to Protran nitrocellulose membranes at 100 V for 1 h (25 mM Tris-HCl, 192 mM glycine and 20% methanol). Membranes were incubated for 1 h in 5% non-fat dried milk in Tris-buffered saline (50 mM Tris-HCl pH 7.4, 200 mM NaCl and 0.05% Tween-20), and then for 2 h with primary antibodies (polyclonal anti-rat CYP2J4, 16 $\mu g \cdot mL^{-1}$; anti-c-fos, 0.75 $\mu g \cdot mL^{-1}$ or anti-c-jun, 0.4 $\mu g \cdot mL^{-1}$), or overnight for MAP kinases and phosphorylated MAP kinases (1:1000 dilutions). Membranes were washed in Tris-buffered saline and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (at 1:1000 for CYP2J2, MAP kinases and phosphorylated MAP kinases, or 1:1500 and 1:2000 dilutions for c-fos and c-jun respectively). Immunoreactive protein signals were visualized by ECL and autoradiography and subjected to densitometry (Bio-RAD GS-800 Densitometer and Quantity One software). Membranes were stripped in Tris-buffer containing 100 mM 2-mercaptoethanol and 2% sodium dodecyl sulphate at 70°C and re-probed with anti-mouse α-tubulin antibody (1:2000 dilution) to normalize for protein loading. Western analysis of CYP2J2 expression in human hepatic microsomes was undertaken similarly (30 μg protein).

Transient transfections and luciferase assays

HepG2 cells were seeded at a density of 3×10^4 cells per well in a 96-well plate format for 24 h. Cells were transfected with promoter constructs (0.3 μg per well) and the phRG-TK Renilla expression plasmid (60 ng per well) to control for transfection efficiency using Effectene transfection reagent according to the manufacturer's protocol for 24 h, after which medium was replaced with serum-free DMEM for 24 h. The CYP2J2 Promoter constructs used were p2J2(–152/+98), p2J2(–49/+98) or p2J2(–152/+98; mt –63/–56, mt –106/–96). Luciferase activities were measured with the Dual-Glo System in a Perkin Elmer Multilabel Reader. All transfection experiments were performed on at least three separate occasions.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared from SNP-treated (1 mM, 24 h) and untreated HepG2 cells as described previously (Marden *et al.*, 2003). Nuclear extracts were frozen in liquid nitrogen and stored at –80°C until used in EMSAs.

Complementary oligonucleotides (sequences in Table 1) were annealed, end-labelled with [^{32}P]-dCTP using the

Table 1 Oligonucleotides used as probes or competitors in EMSA analyses

AP-1 consensus[a]	Sense	CTAGTGA**TGAGTCA**GCCGGATC
AP-1 consensus	Antisense	GATCGATCCGGC**TGACTCA**TCA
2J2E-56	Sense	CGGGGCGGGGACCGTCGCCTGCTGGG
2J2E-56[b]	Antisense	CCCAGCAGG**CGACGGTC**CCCGCCCCG
2J2E-106[c]	Sense	**GGGCTGGGAGG**CGGGGCACGGGG
2J2E-106	Antisense	CGTGCCCCGCCTCCCAGCCCGGG
2J2E-106mt[d]	Sense	AAAAAAAAAAGCGGGGCACGGGG
2J2E-106mt	Antisense	CGTGCCCCGCTTTTTTTTTGGG
STAT5 β-casein promoter[e]	Sense	GGA**CTTCTTGGAATT**AAGGGA
STAT5 β-casein promoter	Antisense	GTCCCTT**AATTCCAAGAAG**TCC

[a]The sense probe has a 5′-CTAG overhang and the antisense probe has a 5′-GATC overhang; AP-1 consensus element is in bold type.
[b]AP-1-like binding region on the antisense strand is in bold type.
[c]AP-1-like binding region on the sense strand is in bold type.
[d]Mutagenized nucleotides are underlined and both strands have a 3′*GGG*-overhang.
[e]STAT5 consensus element in the STAT5β-casein promoter is indicated in bold and the STAT5 antisense oligonucleotide has a 5′-*G* overhang.
AP-1, activator protein-1; EMSA, electromobility shift assay.

Megaprime system and purified using ProbeQuant™ G50 micro columns. Nuclear protein (30 μg for the 2J2-105/-95 and 2J2-56/-63 probes or 5 μg for the AP-1 consensus probe) was incubated with 200 fmol of the 2J2-105/-95 and 2J2-56/-63 end-labelled probes or 50 fmol of the AP-1 consensus probe for 20 min at room temperature and 10 min at 4°C in buffer containing 50 mM NaCl, 10 mM Tris/HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol and 1 μg of poly(2′-deoxyinosinic-2′ deoxycytidylic acid). Protein-DNA complexes were electrophoresed on 5% polyacrylamide gels with 2.5% glycerol in TBE buffer (90 mM Tris/HCl, 90 mM boric acid, 2.5 mM EDTA) at 100 V for ~90 min at 4°C. In competition experiments, 200-fold excesses of unlabelled EMSA probes (self) or the non-specific STAT5 element from the β-casein promoter were added to reactions. In supershift experiments rabbit polyclonal anti-c-jun or anti-c-fos antibodies (2 μg) were incubated with control and SNP-treated nuclear fractions for 1 h at 4°C prior to the addition of end-labelled probes. Following electrophoresis, the gels were dried and autoradiographed.

Data analysis and statistical procedures

Results are expressed as means ± SE throughout. After ANOVA analysis the Fisher's PLSD test was used to detect differences between multiple treatment groups. All data used were derived in triplicate in at least three independent experiments.

Drugs, chemical reagents and other materials

SNP, RNase A, thiazolyl blue tetrazolium bromide (MTT), horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs were purchased from Sigma (St. Louis, MO, USA). The p38 and JNK MAP kinase inhibitors SB203580 and SP600125, respectively, were purchased from Alexis Biochemicals (Farmingdale, NY, USA) and the ERK inhibitor PD98059 was from Cayman Chemicals (Ann Arbor, MI, USA). All other chemicals and reagents were of the highest grade commercially available.

The pGL3 basic luciferase vector, Dual-Glo luciferase assay and the phRG-TK Renilla expression plasmid were purchased from Promega (Annandale, NSW, Australia) and the pCR2.1-TOPO vector was obtained from Invitrogen (Mt Waverley, VIC, Australia). The Effectene transfection reagent, Plasmid Midi kit, QIAquick Gel Extraction kit and QuantiTest SYBR Green one-step RT-PCR kit were from Qiagen (Doncaster, VIC, Australia). TRI Reagent for RNA extraction was from Molecular Research Centre (Astral Scientific, Caringbah, NSW, Australia) and the Megaprime™ DNA labelling System and ProbeQuant™ G-50 microcolumns were purchased from Amersham GE Healthcare (Rydalmere, NSW, Australia). [^{32}P]-dCTP was obtained from PerkinElmer (Rowville, VIC, Australia). The dominant negative mutant p38 and ERK1 MAP kinase expression plasmids were generous gifts from Dr Roger Davis (University of Massachusetts Medical School, Worcester, MA, USA). The CYP2J2-luciferase reporter plasmids were as described previously (Marden *et al.*, 2003; Marden and Murray, 2005). Oligonucleotides were synthesized by Geneworks (Hindmarsh, SA, Australia). Enzymes were from Roche Diagnostics (Castle Hill, NSW, Australia) unless otherwise specified.

Reagents for electrophoresis and immunoblotting were from Bio-RAD Laboratories (Gladesville, NSW, Australia), Whatman® Protran nitrocellulose transfer membranes were from Schleicher and Schuell (Basel, Switzerland) and enhanced chemiluminescence (ECL) detection reagents and autoradiography film (Hyperfilm ECL) were purchased from Amersham GE Healthcare. Rabbit anti-(rat CYP2J4) IgG, which is cross-reactive with human CYP2J2 (Hashizume *et al.*, 2002), was generously provided by Dr Qing-Yu Zhang (Wadsworth Centre, New York State Department of Health, Albany, NY, USA). Primary antibodies directed against c-fos (catalogue number sc-7202) and c-jun (sc-45), were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), rabbit antibodies against p38 (sc-9212), ERK (sc-9102) and JNK (sc-9258) MAP kinases, as well as the corresponding phosphorylated forms (phospho-p38, sc-9211; phospho-ERK, sc-9101 and phospho-JNK, sc-9251), were from Cell Signalling Technology (Arundel, QLD, Australia), and mouse anti-α-tubulin antibody (T9026) and horseradish peroxidase-conjugated anti-rabbit (A4914) and anti-mouse antibodies (A6782) were purchased from Sigma-Aldrich. DMEM was obtained from ThermoTrace (Nobel Park, VIC, Australia); Caspase-Glo3/7 reagent was from Promega (Annandale, NSW, Australia); the pCR2.1-TOPO vector was from Invitrogen.

Results

Effect of SNP treatment on viability and gene expression in HepG2 cells

Consistent with previous reports CYP2J2 was readily detected in human hepatic microsomes, although expression varied somewhat between individuals (n = 12, Figure 1A) (Wu *et al.*, 1996; Gaedigk *et al.*, 2006; Yamazaki *et al.*, 2006). Treatment of HepG2 cells with SNP (1 mM) for 24 h produced a small decrease in viability (to 92 ± 1% of control; $P < 0.05$), which is in accord with the findings of Chung *et al.* (2006); a further decrease was observed after 72 h of treatment (to 84 ± 3% of control, $P < 0.001$; Figure 1B). Concurrent administration of 11,12-EET (10 μM) prevented the decline in HepG2 viability elicited by 72 h of SNP treatment ($P < 0.01$ relative to both control and SNP treatment alone); 11,12-EET alone did not influence viability. Caspase-3 activity was significantly increased by SNP treatment (24 h) to 169 ± 4% of control ($P < 0.001$) and this increase was abrogated by 11,12-EET (129 ± 4% of control; $P < 0.001$ relative to control or SNP treatment alone; Figure 1B). The impact of SNP-induced nitrative stress on the expression of the EET-synthase CYP2J2 in HepG2 cells was also investigated. As shown in Figure 1C, treatment of cells for 24 h, but not 1 or 6 h, elicited a significant decrease in CYP2J2 mRNA expression (relative to the housekeeping gene β-actin) to 60 ± 2% of time-matched control ($P < 0.001$); densitometric analysis of western immunoblots was consistent with these findings (Fig. 1D).

Previous studies have established that SNP modulates the expression of the AP-1 subunit proteins c-jun and c-fos in a range of cell types (Callsen and Brüne, 1999; Feng *et al.*, 1999; Jun *et al.*, 1999a,b; Kim *et al.*, 2002); these proteins also regulate CYP2J2 expression in HepG2 cells (Marden *et al.*, 2003). After 6 h of SNP treatment the expression of c-fos mRNA was

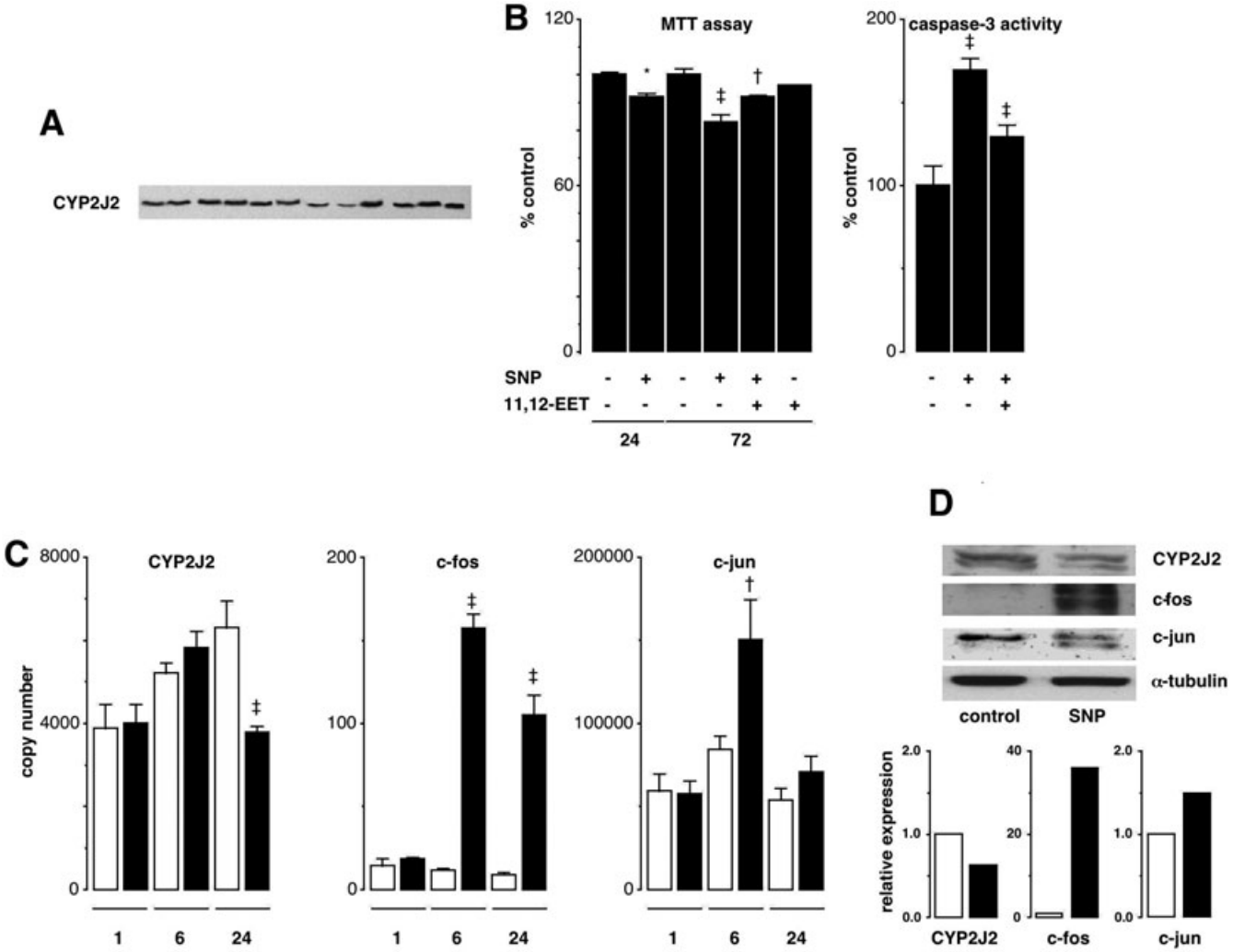

Figure 1 (A) Western immunoblot analysis of CYP2J2 expression in microsomal fractions from 12 individual human livers. (B) Cell viability (MTT assay) and apoptosis (caspase-3 activity) in HepG2 cells after treatment with SNP (1 mM, 24 h) and/or 11,12-EET (10 μM, 2 h prior to SNP). (C) Expression of CYP2J2, c-fos and c-jun mRNAs in HepG2 cells 1, 6 or 24 h after application of SNP (1 mM) (open columns, control; solid columns, SNP). (D) Immunoblot and densitometric analysis showing the down-regulation of CYP2J2 and up-regulation of c-fos and c-jun immunoreactive protein expression in SNP-treated HepG2 cells. A representative of at least two independent experiments is shown. Different from time-matched control: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$. CYP, cytochrome P450; EET, epoxyeicosatrienoic acid; MTT, thiazolyl blue tetrazolium bromide; SNP, sodium nitroprusside.

increased markedly to 13.7 ± 0.7-fold of control ($P < 0.001$; Figure 1C) and remained elevated after 24 h of treatment ($P < 0.001$). In contrast, c-jun mRNA was up-regulated to 1.8 ± 0.3-fold ($P < 0.01$) of time-matched control after 6 h of treatment but this was attenuated by 24 h (Figure 1C). Immunoblot analysis confirmed the increases in expression at the protein level (Figure 1D). Longer term treatment of HepG2 cells with SNP, replenished at 24 h intervals up to 72 h, sustained the decrease in CYP2J2 expression ($P < 0.001$; Figure 2A) as well as the increases in expression of c-fos (Figure 2B) and c-jun mRNAs (Figure 2C).

In comparative studies SNP (1 mM, 24 h) also down regulated CYP2J2 mRNA expression to 54 ± 7% of control in MCF-7 cells ($n = 4$) but HEK293 cells, in which CYP2J2 is also readily detected, were not viable after SNP treatment (not shown). The specificity of the down-regulatory effects of SNP on CYP2J2 expression was assessed in HepG2 cells. Unlike CYP2J2, CYP1A1 mRNA was strongly up-regulated by SNP treatment (1 mM, 24 h) to 4.41 ± 0.83-fold of control but CYP2D6 mRNA was undetectable, either in the presence or absence of nitrative stress (not shown).

Role of MAP kinases in SNP-mediated CYP2J2 down-regulation in Hep G2 cells

Mitogen-activated protein kinases participate in signalling cascades that activate c-jun and c-fos expression in cells. Phospho-p38 and phospho-p42/44 ERK, but not phospho-p46/54 JNK were detected in control HepG2 cell lysates (Figure 3A). SNP (1 mM) stimulated the rapid accumulation of phospho-p38 in cell lysates compared with control, especially by 120 min (Figure 3A). By comparison, phospho-ERK expression was activated weakly over this time frame relative to control and low-level phospho-JNK was detected transiently

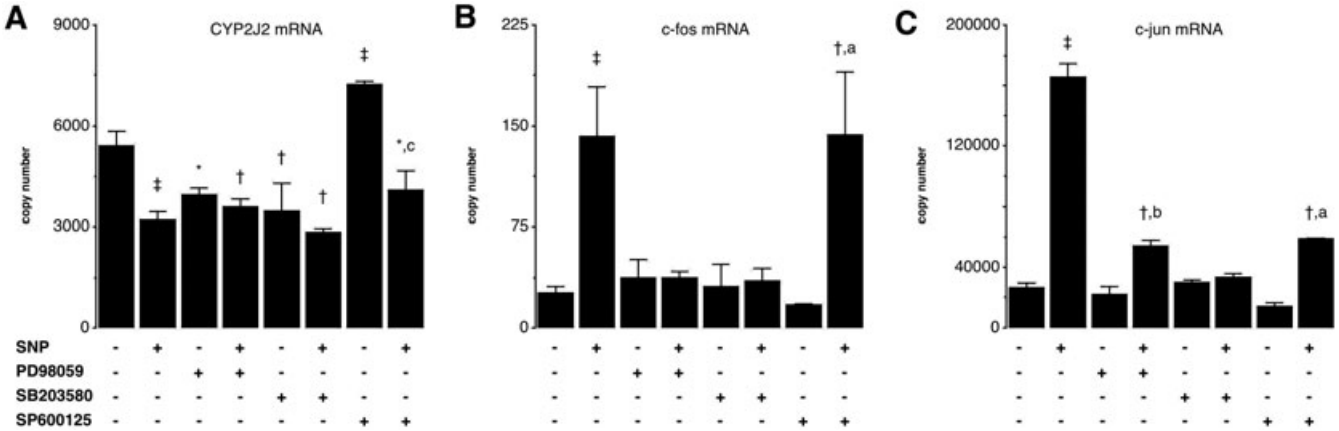

Figure 2 Down-regulation of (A) CYP2J2 mRNA and up-regulation of (B) c-fos mRNA and (C) c-jun mRNA in HepG2 cells that were subjected to nitrative stress induced by SNP (1 mM, at 24 h intervals up to 72 h). MAP kinase inhibitors: PD98059 (10 μM, ERK), SB203580 (20 μM, p38) or SP600125 (50 μM, JNK) were included 2 h prior to SNP treatment and replenished at 24 h intervals. Different from time-matched control: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$. Different from inhibitor alone: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. CYP, cytochrome P450; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; SNP, sodium nitroprusside.

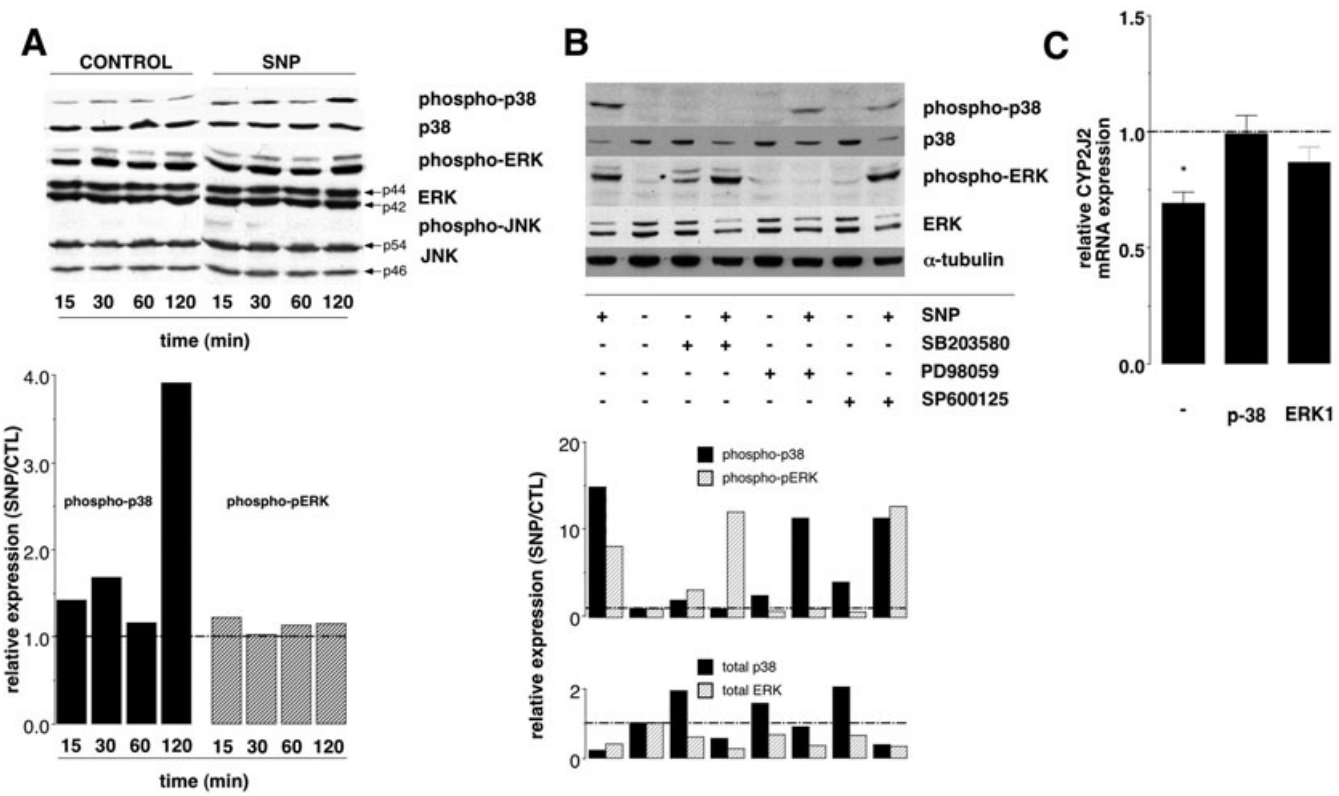

Figure 3 (A) Immunoblots showing phospho-MAP kinase and total MAP kinase expression in HepG2 lysates harvested at intervals (0–120 min) after SNP administration; the lower panel shows findings from densitometric analysis of a representative of three independent experiments. (B) Immunoblots showing phospho-MAP kinase and total MAP kinase expression in lysates from HepG2 cells that had been treated with SNP (1 mM) at 24 h intervals prior to harvest at 72 h. MAP kinase inhibitors: PD98059 (10 μM, ERK), SB203580 (20 μM, p38) or SP600125 (50 μM, JNK) were included 2 h prior to SNP treatment and replenished at 24 h intervals along with SNP. The lower panel shows findings from densitometric analysis of the immunoblots and aligns with the key above; a representative experiment from two independent experiments is shown. (C) Effect of SNP treatment (1 mM) on CYP2J2 mRNA expression in HepG2 cells that had been transiently transfected with dominant negative mutant p38 and ERK MAP kinase expression plasmids (1 μg DNA) 24 h before the first application of SNP; SNP (1 mM) was replenished at 24 h intervals. Different from control: *$P < 0.05$. CYP, cytochrome P450; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; SNP, sodium nitroprusside.

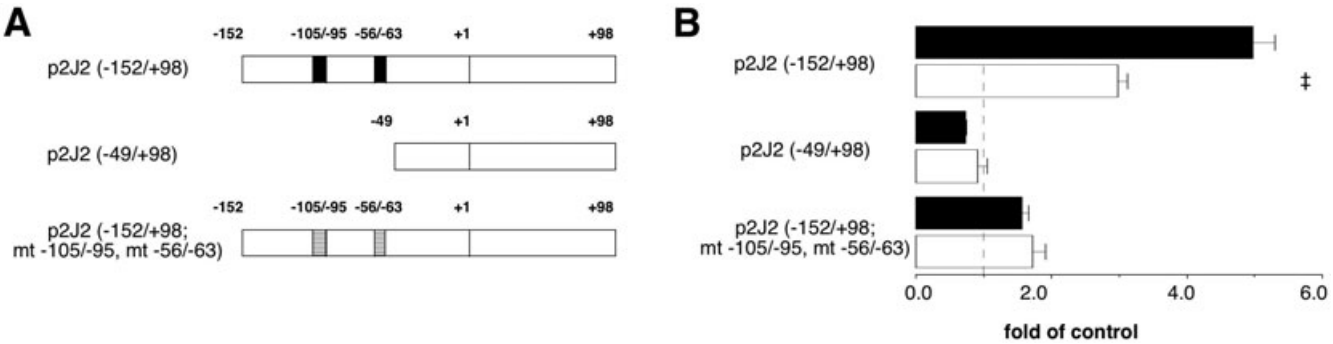

Figure 4 (A) CYP2J2-luciferase reporter constructs showing the intact AP-1-like sites (solid boxes) in the 5′-proximal region [CYP2J2(–152/+98)] that are absent in the CYP2J2(–49/+98) construct and mutagenized (hatched boxes) in the CYP2J2(–152/+98; mt –105/–95, mt –56/–63) construct. (B) Responsiveness of the CYP2J2-luciferase constructs in transfected HepG2 cells to SNP (1 mM, 24 h; open boxes) relative to control (solid boxes). Different from corresponding untreated control: ‡$P < 0.001$. AP-1, activator protein-1; CYP, cytochrome P450.

in lysates soon after SNP treatment. Expression of total immunoreactive MAP kinases was unchanged by SNP treatment over 24 h. The responsiveness of MAP kinases to multiple exposures to SNP at 24 h intervals up to 72 h was retained in the case of phospho-p38 (Figure 3B) and was more pronounced in the case of phospho-ERK compared with short-term treatments; phospho-JNK remained unresponsive. It was apparent from Figure 3B that the SNP-dependent increases in phospho-p38 and phospho-ERK were prevented by coapplication of the p38 and ERK MAP kinase inhibitors respectively. The JNK MAP kinase inhibitor was largely ineffective. From densitometric analysis prolonged nitrative stress for 72 h also elicited decreases in total p38 and ERK MAP kinase expression in HepG2 cells, whereas prolonged treatment with MAP kinase inhibitors in the absence of SNP appeared to increase total p38 but not ERK MAP kinase expression (Figure 3B).

Inhibitors of the p38 and ERK MAP kinases, but not the JNK MAP kinase, prevented the decrease in CYP2J2 mRNA expression mediated by continuous exposure to SNP for 72 h (Figure 2A). c-Fos mRNA up-regulation by SNP was also abrogated by p38 and ERK MAP kinase inhibitors, but not by the JNK inhibitor (Figure 2B), whereas the up-regulation of c-jun by SNP was partially attenuated by each of the inhibitors (Figure 2C). To corroborate the roles of p38 and ERK MAP kinases in CYP2J2 down-regulation HepG2 cells were transfected with dominant negative mutant expression plasmids and then subjected to nitrative stress (SNP 1 mM, applied at 24 h intervals). The SNP-mediated decline in CYP2J2 mRNA expression was prevented by the mutant plasmids (Figure 3C).

Role of AP-1-like promoter elements in SNP-mediated CYP2J2 dysregulation

It has been shown that the regulation of CYP2J2 expression in normoxia and hypoxia by the AP-1 factors c-jun and c-fos is controlled by AP-1-like response elements present in the CYP2J2 5′-flanking region (Marden *et al.*, 2003; Marden and Murray, 2005). The potential role of these elements in mediating the SNP-dependent decline in CYP2J2 expression was tested with CYP2J2-luciferase reporter constructs (Figure 4A). The activity of the wild-type construct p2J2(–152/+98) (4.9 ± 0.3-fold of pGL3 basic) was significantly decreased by SNP treatment to 2.9 ± 0.1-fold of pGL3 basic ($P < 0.001$; Figure 4B). In contrast, the truncated construct p2J2(–49/+98), that lacked the AP-1-like elements, and p2J2(–152/+98; mt –105/–96, mt –56/–63), in which mutagenesis of the elements had occurred, were both unresponsive to SNP (Figure 4B).

SNP treatment increased the binding of nuclear proteins to the CYP2J2 promoter

The binding of proteins to CYP2J2 AP-1-like elements was assessed in nuclear protein fractions from SNP-treated HepG2 cells. The signals produced in EMSAs by double stranded oligonucleotide probes carrying the –105/–95 and –56/–63 elements were intensified in nuclear fractions from SNP-treated HepG2 cells compared with control fractions (SNP versus CTL; Figure 5A,B). Two hundred-fold excess of cold self-oligonucleotides abrogated these signals in SNP-treated nuclear fractions (self; Figure 5A,B), whereas the non-specific competitor corresponding to the STAT5 element from the β-casein promoter (STAT5) was less effective. Antibodies directed against c-jun and c-fos supershifted or block-shifted the binding interactions. In comparative studies the effect of SNP treatment on binding to a double stranded AP-1 consensus oligonucleotide was assessed. As shown in Figure 5C, and similar to the situation with the probes corresponding to the CYP2J2 promoter elements, the AP-1 consensus probe interaction was intensified in nuclear fractions from SNP-treated cells. An excess of the cold AP-1 consensus sequence blocked the interaction in nuclear fractions (self), but the non-specific STAT5 sequence was less effective (STAT5). Antibodies directed against c-jun and c-fos both supershifted and subshifted the binding complexes in SNP-treated nuclear fractions.

Discussion and conclusions

The present findings indicate that exposure to SNP (1 mM) for 24 h decreases CYP2J2 mRNA expression in HepG2 cells and identifies the p38 and ERK MAP kinase isoforms as mediators of this effect; suppression was more pronounced after further administrations of SNP at intervals up to 72 h. A previous

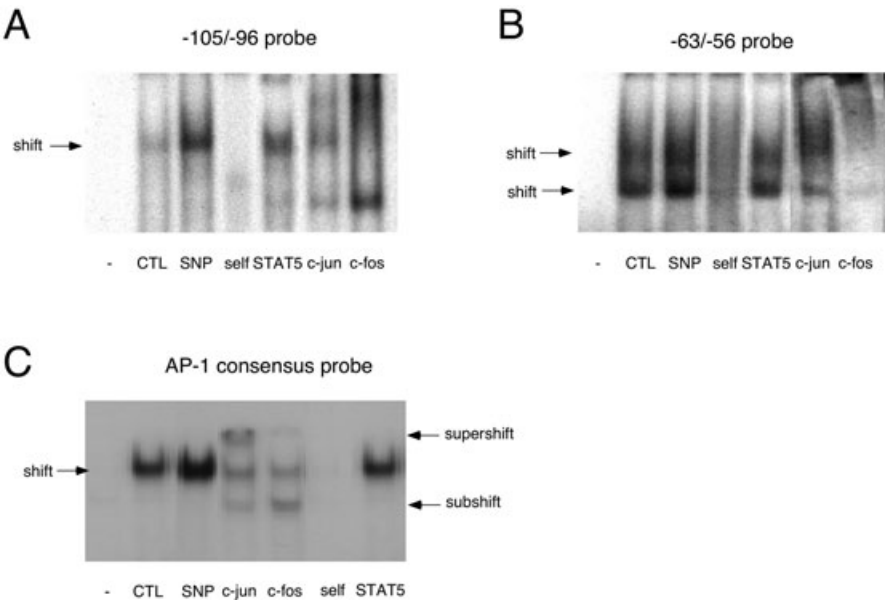

Figure 5 Electromobility shift assay analysis of the binding of double stranded oligonucleotides corresponding to the AP-1-like elements in the CYP2J2 upstream region to nuclear proteins from untreated (CTL) or SNP-treated (SNP) HepG2 cells: (A) CYP2J2-105/-96 element (B) CYP2J2-63/-56 element and (C) the AP-1 consensus element. Gel lanes containing 200-fold excess of the indicated oligonucleotide probe as a cold competitor (self) or the STAT5 element from the β-casein promoter (STAT5) are indicated. Shifted complexes in lanes containing the anti-c-fos or anti-c-jun IgG are also indicated. AP-1, activator protein-1; CYP, cytochrome P450; SNP, sodium nitroprusside.

study found that the AP-1 transcription factor complex is important in the regulation of CYP2J2 expression in response to oxygen availability (Marden *et al.*, 2003). Thus, c-jun predominates in normoxic HepG2 cells whereas hypoxia stimulates the expression of both c-fos and c-jun. In hypoxia the shift from c-jun homodimers, which supported CYP2J2 reporter activity, to c-jun/c-fos heterodimers, which did not, led to the decrease in CYP2J2 expression in cells (Marden *et al.*, 2003). In the present study a similar accumulation of c-fos also occurred in HepG2 cells and was associated with CYP2J2 down-regulation in response to SNP-induced nitrative stress, which resembles pathological stimuli elicited by inflammation and infection (Horiike *et al.*, 2005; Kermorvant-Duchemin *et al.*, 2005).

It has been demonstrated previously that nitric oxide can activate AP-1 (Kim *et al.*, 1997). Similarly, the nitric oxide-releasing agent SNP up-regulated c-fos, junB and, to a lesser extent, c-fos in rat embryo fibroblasts (Pilz *et al.*, 1995), whereas the related agent *S*-nitroso-*N*-acetylpenicillamine increased AP-1 binding activity in cardiomyocytes, as reflected by EMSA analysis (Taimor *et al.*, 2001). The activation of AP-1 expression in HepG2 cells in response to SNP is consistent with these reports.

Previous experiments have indicated that the CYP2J2 gene promoter contains two AP-1-responsive gene elements at –105/–95 and –56/–63 that are positively regulated by c-jun, but not by c-jun/c-fos heterodimers (Marden *et al.*, 2003; Marden and Murray, 2005). SNP decreased the activity of reporter constructs that contained the intact AP-1-like sites, whereas deletion or mutagenesis of the sites abolished this effect of SNP. These findings are in accord with the involvement of the AP-1-responsive elements in the observed decrease in cellular CYP2J2 expression elicited by SNP.

Inhibitors of the p38 and ERK MAP kinases prevented the loss of CYP2J2 expression in response to SNP; in parallel these treatments also prevented the activation of c-fos. In contrast, inhibition of the JNK MAP kinase was without effect, thus indicating that this kinase is not involved in CYP2J2 suppression by nitrative stress. MAP kinases have been implicated previously in cellular responses mediated by nitric oxide, although different isoforms appear to be activated in a cell-type specific manner. Thus, in HL-60 leukaemia cells, SNP rapidly increased p38 phosphorylation within 30 min that was sustained for several hours (Jun *et al.*, 1999b). In contrast, the JNK and ERK MAP kinases were less responsive. The observed phospho-MAP kinase profile was different in RAW264.7 macrophages, with prominent activation of the JNK and p38 isoforms, but not the ERK MAP kinase (Jun *et al.*, 1999a). JNK inhibition prevented nitric oxide-mediated apoptosis in macrophages (Callsen and Brüne, 1999), but inhibitors of ERK and p38 MAP kinases were more effective in rat cardiomyocytes (Kim *et al.*, 2000). Thus, the situation regarding the specificity of MAP kinase activation in SNP-treated cardiomyocytes resembles the present observations in HepG2 cells that have been subjected to nitrative stress.

Nitric oxide is an important biological mediator with diverse actions in physiology and pathology. During cardiac ischaemia and ischaemia-reperfusion injury nitric oxide production is rapidly increased to 10-fold of normal levels (Wang and Zweier, 1996). Nitric oxide and nitric oxide-releasing agents such as SNP have also been found to alter cell viability in a concentration- and cell type-dependent fashion (Pfeilschifter and Huwiler, 1996; von Knethen and Brune, 1997; Callsen and Brüne, 1999; Feng *et al.*, 1999; Jun *et al.*, 1999a,b; Kim *et al.*, 2002). There may be multiple mechanisms by which nitric oxide increases cell death, including

modification of cell macromolecules (Lander *et al.*, 1997), activation of signalling pathways (Callsen and Brüne, 1999; Feng *et al.*, 1999; Jun *et al.*, 1999a,b; Kim *et al.*, 2002) or direct interaction with superoxide, leading to the production of peroxynitrite (Gryglewski *et al.*, 1986). A critical cysteine residue in the cell membrane protein $p21^{ras}$, which is upstream of MAP kinase signalling cascades, has been shown to be S-nitrosylated by nitric oxide (Lander *et al.*, 1997). Thus, the cellular redox status may modulate the regulatory cysteine in $p21^{ras}$ and influence downstream signalling, including MAP kinases and their target genes, such as c-fos.

The decrease in HepG2 cell viability after SNP-induced nitrative stress was prevented by co-administration of exogenous EETs. Similar findings have been made in bovine endothelial cells that were subjected to a low oxygen environment and that were cotreated with 11,12-EET or had been transfected with CYP2J2 cDNA (Yang *et al.*, 2001). Thus, EETs limit endothelial cell injury after exposure to hypoxia and reoxygenation. EETs also attenuated apoptosis in response to a range of deleterious stimuli, including cytotoxic agents and treatment with hydroxygen peroxide (Chen *et al.*, 2001). In the present study 11,12-EET modulated the SNP-mediated increase in caspase-3 activity in HepG2 cells. Phosphorylation of the epidermal growth factor receptor was effected by EETs and led to activation of the survival kinase Akt (Jiang *et al.*, 2005). These anti-apoptotic and proliferative effects of EETs are consistent with the signal to decrease CYP2J2 expression being anti-survival. Indeed, restitution of CYP2J2 or the supply of EETs in the face of deleterious stimuli has been shown to enhance cell survival (Yang *et al.*, 2001) and to enhance cardiac recovery after ischaemia (Batchu *et al.*, 2009). After synthesis EETs may be esterified within cell membranes (Bernstrom *et al.*, 1992; VanRollins *et al.*, 1993). In liver CYP2J2, CYP2C8 and CYP2C9 are alternative EET synthases that influence the profile of stored EETs (Zeldin *et al.*, 1996). Unlike CYPs 2C8 and 2C9, CYP2J2 is active in the formation of 8,9-EET, which is prominent in liver, and the stereochemistry of 14,15-EET extracted from liver is comparable with that produced by CYP2J2 among the alternative EET synthases. Because these EETs have been implicated in physiological processes in several tissues they may also be important in hepatic homeostasis. It will now be of interest to evaluate mechanisms by which EET production could be maintained by stabilizing CYP2J2 expression in cells that have been exposed to injurious stresses.

In summary, the present study found that the CYP2J2 fatty acid epoxygenase was down-regulated by nitrative stress in SNP-treated HepG2 cells. This was mediated by the p38 and ERK MAP kinase isoforms that stimulate the expression of the downstream AP-1 factor c-fos. The present study suggests that CYP2J2 may be important in the prevention of cellular injury by pathological or disease-related stresses; further studies are now warranted to test this possibility.

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Conflict of interest

The authors state no conflict of interest.

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