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Sulforaphane induces CYP1A1 mRNA, protein, and catalytic activity levels via an AhR-dependent pathway in murine hepatoma Hepa 1c1c7 and human HepG2 cells

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ABSTRACT

Recent reports have proposed that some naturally occurring phytochemicals can function as anticancer agents mainly through inducing phase II drug detoxification enzymes. Of these phytochemicals, isothiocyanates sulforaphane (SUL), present in broccoli, is by far the most extensively studied. In spite of its positive effect on phase II drug metabolizing enzymes, its effect on the phase I bioactivating enzyme cytochrome P450 1a1 (Cyp1a1) is still a matter of debate. As a first step to investigate this effect, Hepa 1c1c7 and HepG2 cells were treated with various concentration of SUL. Our results showed that SUL-induced CYP1A1 mRNA in a dose- and time-dependent manner. Furthermore, this induction was further reflected on the protein and catalytic activity levels. Investigating the effect of SUL at the transcriptional level revealed that SUL increases the Cyp1a1 mRNA as early as 1h. The RNA polymerase inhibitor actinomycin D (Act-D) completely abolished the SUL-induced Cyp1a1 mRNA. Furthermore, SUL successfully activated AhR transformation and its subsequent binding to the XRE. At the post-transcriptional level, SUL did not affect the levels of existing Cyp1a1 mRNA transcripts. This is the first demonstration that the broccoli-derived SUL can directly induce *Cyp1a1* gene expression in an AhR-dependent manner and represents a novel mechanism by which SUL induces this enzyme.

1. Introduction

The aryl hydrocarbon receptor (AhR) is a member of basic-helix–loop–helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription proteins. Inactive AhR resides in the cytoplasm bound to two 90-kDa heat-shock proteins (HSP90), the 23-kDa heat shock protein (p23), and hepatitis B virus X-associated protein 2 (XAP2). Upon ligand binding, the AhR-ligand complex dissociates from the cytoplasmic complex and translocates to the nucleus where it associates with the aryl hydrocarbon nuclear translocator (ARNT) [1]. The whole complex then acts as a transcription factor that binds to a specific DNA recognition sequence, termed the xenobiotic responsive element (XRE), located in the promoter region of a number of AhR-regulated genes. Among the AhR-regulated genes, CYP1A1 has received considerable attention since it is highly induced by a broad range of AhR ligands such as polycyclic and halogenated aromatic hydrocarbons (PAHs and HAHs) via the AhR-XRE transcription pathway [2]. Moreover, CYP1A1 is capable of producing polar, toxic, or even carcinogenic metabolites from these AhR ligands [3].

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Abbreviations: Act-D, actinomycin D; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; Cyp1a1 and CYP1A1, cytochrome P450 1a1 and 1A1; DMSO, dimethylsulfoxide; DMBA, 7,12-dimethylbenz[*a*]anthracene; EMSA, electrophoretic mobility shift assay; EROD, 7-ethoxyresorufin *O*-deethylase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacaetylase; Hepa1c1c7, murine hepatoma Hepa1c1c7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HO-1, heme oxygenase-1; PAGE, polyacrylamide gel electrophoresis; PAH, polycyclic aromatic hydrocarbon; SUL, isothiocyanate sulforaphane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

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Diet plays a pivotal role in determining the incidence of various cancers. Research conducted on the effect of diet and the increased incidence of cancer have demonstrated that high fibre, low fat diets accompanied by high consumption of fruits and vegetables may protect against development of many cancers [4]. For example, consumption of cruciferous vegetables has been associated with protection against various types of cancers such as, breast and colon cancers [5,6].

Sulforaphane (SUL) is an isothiocyanate found in cruciferous vegetables, with particularly high levels detected in broccoli and broccoli sprouts [7]. Over a decade ago, this phytochemical was identified as a likely chemopreventive agent based on its ability to induce phase II detoxification enzymes [7]. Once disrupted, the plant cell containing the physically segregated enzyme myrosinase (thioglucoside glucohydrolase; EC 3.2.3.1) will hydrolyze the co-existing glucosinolates (β-thioglucoside *N*-hydroxysulfates) liberating free isothiocyanates, such as SUL [7].

Recent reports have proposed that some naturally occurring phytochemicals can function as anticancer agents mainly through inducing phase II drug detoxification enzymes such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and glutathione-*S*-transferase (GST) [7,8]. Of these phytochemicals, SUL is by far the most extensively studied [9]. Aside from its chemopreventive effect via the induction of phase II detoxification enzymes, various studies have shown that SUL may also act through epigenetic mechanism [10]. SUL has been shown to inhibit histone deacetylase activity (HDAC) in human colon and prostate cancers [10]. The working hypothesis for using SUL as HDAC inhibitor is that DNA/chromatin interactions are kept in a constrained state in the presence of HDAC/co-repressor complexes, but HDAC inhibitors enable histone acetyltransferase/co-activator (HAT/CoA) complexes to transfer acetyl groups to lysine in histones, thus loosening the interactions with DNA and facilitating transcription factor access and gene activation [10]. Recently, it has been demonstrated that SUL can act as a direct inducer of human β-defensin-2 (HBD-2), an antimicrobial peptide that can be induced by HDAC inhibitors, in colonocytes suggesting a more direct role of SUL in the treatment of colonic Crohn's disease [11].

In spite of its positive effect on phase II drug detoxification enzymes, its effect on the phase I bioactivating enzyme cytochrome P450 1a1 (Cyp1a1) is still a matter of debate. Previous studies have demonstrated that SUL is capable of inducing CYP1A1 mRNA in primary human hepatocytes [12]. Furthermore, SUL precursor, glucoraphanin, has been shown to significantly increase the pulmonary CYP1A protein and catalytic activity levels [13]. On the other hand, several studies have demonstrated that activation of the AhR is the first step in a series of molecular events leading to the induction of CYP1A1. Therefore, the expression level of CYP1A1 is considered to be a useful biomarker of exposure to AhR ligands [14]. Furthermore, a well established link between induction of CYP1A1 and cancer has been reported [15].

The aims of this work were to examine the effect of SUL on constitutive expression levels of Cyp1a1 and to investigate the mechanisms by which SUL modulates the expression of Cyp1a1.

2. Materials and methods

2.1. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 7-ethoxyresorufin, fluorescamine, isothiocyanate sulforaphane (SUL), anti-goat IgG peroxidase secondary antibody, 7,12-dimethylbenz[*a*]anthracene (DMBA), and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent was purchased from Invitrogen (San Diego, CA). High-Capacity cDNA Reverse Transcription Kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Actinomycin-D (Act-D) was purchased from Calbiochem (San Diego, CA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1 goat polyclonal primary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [γ^{32}P]ATP was supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, ON).

2.2. Animals and ethics

All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Hartley guinea pigs weighing 250–300 g were obtained from Charles River Canada (St. Constant, QC, Canada). All animals were exposed to 12 h of light and 12 h of dark daily and given free access to food and water.

2.3. Cell culture

Murine hepatoma Hepa 1c1c7 and human hepatoma HepG2 cell lines, ATCC number CRL-2026 and HB-8065, respectively (Manassas, VA), were maintained in Dulbecco's modified Eagle's medium (DMEM), without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM l-glutamine, 50 μg/ml amikacin, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

2.4. Chemical treatments

Cells were treated in serum free medium with various concentrations of SUL (1–20 μM) as described in figure legends. SUL was dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

2.5. Effect of SUL on cell viability

The effect of SUL on cell viability was determined using the MTT assay as described previously [16]. MTT assay

measures the conversion of MTT to formazan in living cells via mitochondrial enzymes of viable cells. In brief, Hepa 1c1c7 cells were seeded into 96-well microtiter cell culture plates and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. Cells were treated with various concentrations of SUL (1–40 μM). After 24 h incubation, the medium was removed and replaced with cell culture medium containing 1.2 mM MTT dissolved in phosphate buffered saline (PBS) (pH 7.4). After 2 h of incubation, the formed crystals were dissolved in isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using the Bio-Tek EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT).

2.6. RNA extraction and quantitative real-time PCR of CYP1A1, and HO-1

After incubation with the test compound for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen), and quantified by measuring the absorbance at 260 nm. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1.0 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit with random primers. Real-time PCR reactions were performed on an ABI 7500 real-time PCR system (Applied Biosystems), using SYBR® Green PCR Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 10 min at 95 °C, and 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Primers and probes for mouse Cyp1a1 were: Forward primer 5′-GGT TAA CCA TGA CCG GGA ACT-3′, reverse primer 5′-TGC CCA AAC CAA AGA GAG TGA-3′. Heme oxygenase-1 (HO-1): forward primer 5′-GTG ATG GAG CGT CCA CAG C-3′, reverse primer 5′-TGG TGG CCT CCT TCA AGG-3′, and for β-actin: forward primer 5′-TAT TGG CAA CGA GCG GTT CC-3′, reverse primer 5′-GGC ATA GAG GTC TTT ACG GAT GTC-3′. Human CYP1A1: forward primer 5′-CGG CCC CGG CTC TCT-3′, reverse primer 5′-CGG AAG GTC TCC AGG ATG AA-3′, and human β-actin: forward primer 5′-CTG GCA CCC AGC ACA ATG-3′, reverse primer 5′-GCC GAT CCA CAC GGA GTA CT-3′ were purchased from Integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of CYP1A1 (target gene) between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: Fold change = $2^{\Delta(\Delta C_t)}$, where $\Delta C_t = C_{t(\text{target})} - C_{t\beta\text{-actin}}$ and $\Delta(\Delta C_t) = \Delta C_{t(\text{treated})} - \Delta C_{t(\text{untreated})}$.

2.7. Protein extraction and Western blot analysis

Twenty-four hours after incubation with the test compound, cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml of protease inhibitor cocktail. The cell homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000*g* for 10 min at 4 °C. Proteins (25 μg) were resolved by denaturing electrophoresis, as described previously [17]. Briefly, the cell homogenates were dissolved in 1× sample buffer, boiled for 5 min, separated by 10% SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4 °C in blocking buffer containing 5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween 20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris–base). After blocking, the blots were incubated with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at room temperature, or primary polyclonal goat anti-mouse Gapdh antibody for overnight at 4 °C in TBS containing 0.05% (v/v) Tween 20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody for Cyp1a1 and Gapdh was carried out in blocking buffer for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (Amersham, Arlington Heights, IL). The intensity of Cyp1a1 protein bands was quantified, relative to the signals obtained for Gapdh protein, using ImageJ software.

2.8. Determination of CYP1A1 enzymatic activity

CYP1A1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity was performed on intact, living cells using 7-ethoxyresorufin as a substrate, as previously described [17]. Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay [18].

2.9. Electrophoretic mobility shift assay (EMSA)

For preparation of guinea pig hepatic cytosol, freshly excised livers, from male Hartley guinea pigs (250–300 g), were homogenized in ice-cold HEGD buffer (25 mM HEPES, 5 mM EDTA, 10% glycerol, pH 7.4) using three passes with a Teflon-glass homogenizer. The resulting homogenate was centrifuged at 9000 *g* for 20 min at 4 °C followed by centrifugation of the supernatant at 100,000*g* for 60 min at 4 °C. Aliquots of guinea pig liver cytosol (100,000 *g* supernatant) were stored at −80° until use.

Hepatic cytosol of untreated guinea pig was incubated with the test compounds for a final concentration of 20 nM TCDD, and 5 μM SUL for 2 h at 20 °C. Protein concentrations for the cytosolic extracts were determined using the method of Lowry [19]. To visualize the ability of SUL to induce the transformation and subsequent DNA binding of the AhR, a complementary pair of synthetic oligonucleotides containing the sequence 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ and 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′, corresponding to the XRE binding site, were synthesized and radiolabeled with [γ^{32}P]ATP as previously described [20] and used as a DNA probe in all experiments. Binding reactions using aliquots of 120 μg cytosolic, and excess radiolabeled oligonucleotides were allowed to proceed for 15 min at 20 °C in a buffer containing 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 25 mM HEPES, 400 ng poly(dI–dC), and 0.4 mM KCl. To determine the

specificity of binding to the oligonucleotide, a 100-fold M excess of unlabeled XRE probe was added to the binding reaction prior to addition of the γ^{32}P-labeled probe. Protein–DNA complexes were separated under non-denaturing conditions on a 4% polyacrylamide gel using 1× TBE (90 mM of Tris borate, 90 mM of boric acid, 4 mM of EDTA) as a running buffer. The gels were dried and the protein–DNA complexes were visualized by autoradiography.

2.10. Cyp1a1 mRNA stability

The half-life of Cyp1a1 mRNA was analyzed by an Act-D-chase assay. Cells were pre-treated with 1 μM DMBA for 6 h. Cells were then washed and incubated with 5 μg/ml Act-D, to inhibit further RNA synthesis, immediately before treatment with (5 μM) SUL. Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with SUL. Real-time PCR reactions were performed using SYBR® Green PCR Master Mix (Applied Biosystems). The fold change in the level of Cyp1a1 (target gene) between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: Fold change = $22^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_{t(\text{target})} - C_{t(\beta\text{-actin})}$ and $\Delta(\Delta C_t) = \Delta C_{t(\text{treated})} - \Delta C_{t(\text{untreated})}$.

2.11. Statistical analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, Inc., CA). A one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess statistical significance. The differences were considered significant when $p < 0.05$.

3. Results

3.1. Effect of SUL on cell viability

To determine the maximum non-toxic concentrations of SUL to be utilized in the current study, Hepa 1c1c7 cells were exposed for 24 h to increasing concentrations of SUL (1–40 μM). The MTT assay showed that the concentrations ranging from 1 to 10 μM did not affect cell viability. On the other hand the concentrations of 20 and 40 μM decreased cell viability by 20% and 40%, respectively (Fig. 1). Based on these findings all subsequent studies were conducted at concentrations of SUL ranging from 1 to 10 μM.

3.2. Concentration and time-dependent effect of SUL on constitutive Cyp1a1 mRNA in Hepa 1c1c7 cells

To examine whether SUL is able to induce the expression of Cyp1a1 mRNA, Hepa 1c1c7 were treated with various concentrations of SUL (Fig. 2A). Thereafter, Cyp1a1 mRNA was assessed using real-time PCR. Our results showed that SUL increased Cyp1a1 mRNA dose-dependently starting from the lowest concentration tested (1 μM) to reach 1.5-fold, and reaching a maximum induction at the highest concentration tested (10 μM) to reach 2.5-fold.

To better understand the kinetics of Cyp1a1 mRNA in response to SUL, Cyp1a1 mRNA levels were measured at different time points after treating Hepa 1c1c7 cells with 5 μM SUL. Fig. 2B shows that SUL-induced Cyp1a1 mRNA in a time-dependent manner. SUL treatment caused a maximal induction of the Cyp1a1 mRNA (~3-fold) at 6 h. However, a 1.5-fold induction occurred as early as 1 h (Fig. 2B) suggesting the involvement of transcription mechanism.

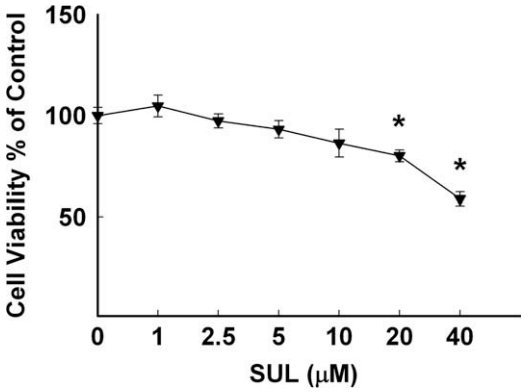

Fig. 1. Effect of SUL on cell viability. Hepa 1c1c7 cells were treated for 24 h with SUL (1, 2.5, 5, 10, 20, and 40 μM). Cell cytotoxicity was determined using MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE ($n = 8$). (*) $P < 0.05$, compared to control (concentration = 0 μM).

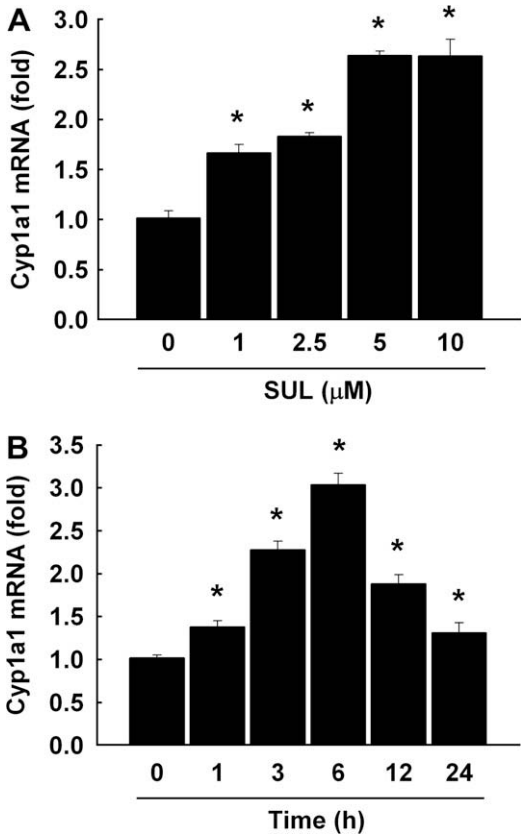

Fig. 2. Effect of SUL on Cyp1a1 mRNA using real-time PCR in Hepa 1c1c7 cells. (A) Hepa 1c1c7 cells were treated for 6 h with increasing concentrations of SUL (1–10 μM). (B) Hepa 1c1c7 cells were treated with 5 μM SUL at different time points. First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa 1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (*) $P < 0.05$, compared to control (concentration = 0 μM) or (time = 0 h).

3.3. Concentration-dependent effect of SUL on Cyp1a1 protein and catalytic activity in Hepa 1c1c7 cells

To further examine whether the induction of Cyp1a1 mRNA in response to SUL treatment is translated into functional protein and enzyme catalytic activity, Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of SUL, and the Cyp1a1 protein and catalytic activity levels were determined using Western blot analysis and EROD assay, respectively. Fig. 3A shows that SUL induces the Cyp1a1 protein in a concentration-dependent manner with a maximum induction at 10 μM. Furthermore, SUL induced the Cyp1a1 EROD activity with a maximum induction at the concentration of 2.5 μM. However, at higher concentrations the activity seemed to reach plateau at 2.5 μM, thereafter started to descend at 5 and 10 μM (Fig. 3B).

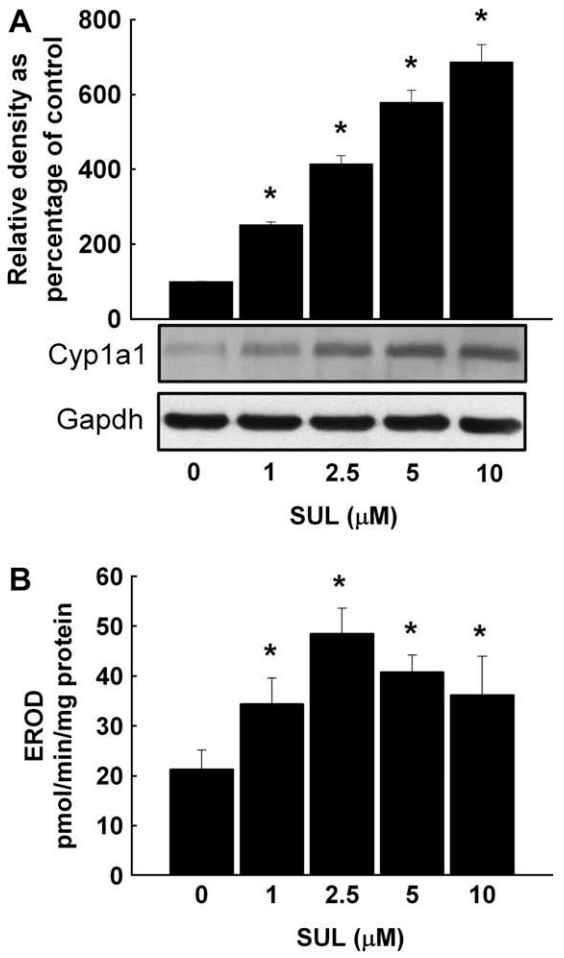

Fig. 3. Effect of SUL on constitutive Cyp1a1 protein and catalytic activity in Hepa 1c1c7 cells. (A) Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of SUL (1–10 μM). Protein (25 μg) was separated on a 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary Cyp1a1 antibody for 2 h at 4 °C, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as loading control. One of three representative experiments is shown. (*) $P < 0.05$, compared to control (concentration = 0 μM). (B) EROD activity was measured in intact living cells treated with increasing concentrations of SUL (1–10 μM) for 24 h. Cyp1a1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 8$). (*) $P < 0.05$, compared to control (concentration = 0 μM).

3.4. Concentration-dependent effect of SUL on CYP1A1 mRNA and catalytic activity in HepG2 cells

To further confirm that the effect of SUL on *CYP1A1* gene expression is not species-specific, we examined the effect of SUL on CYP1A1 mRNA and catalytic activity levels using human hepatoma HepG2 cells. Similar to the data obtained with Hepa 1c1c7 cells, CYP1A1 mRNA was increased in concentration-dependent manner in response to SUL in HepG2 cells. SUL at the concentration of 1 μM increased CYP1A1 mRNA by ~2-fold, while the highest concentration tested, 10 μM, caused a 3.5-fold induction in CYP1A1 mRNA levels (Fig. 4A). Furthermore, SUL increased the CYP1A1-dependent EROD activity initially at the concentration of 1 μM and reached the maximum at the concentration of 2.5 μM (Fig. 4B). Of interest, further increase in SUL concentrations caused a decrease in the induction of CYP1A1 catalytic activity at the concentrations of 5 and 10 μM.

3.5. Transcriptional induction of Cyp1a1 by SUL

In order to understand the mechanism by which SUL induces the Cyp1a1 mRNA, and to examine whether the increase in Cyp1a1 expression was a result of *de novo* RNA synthesis or a post-transcriptional effect, Hepa 1c1c7 cells were pretreated with the transcriptional inhibitor, acti-

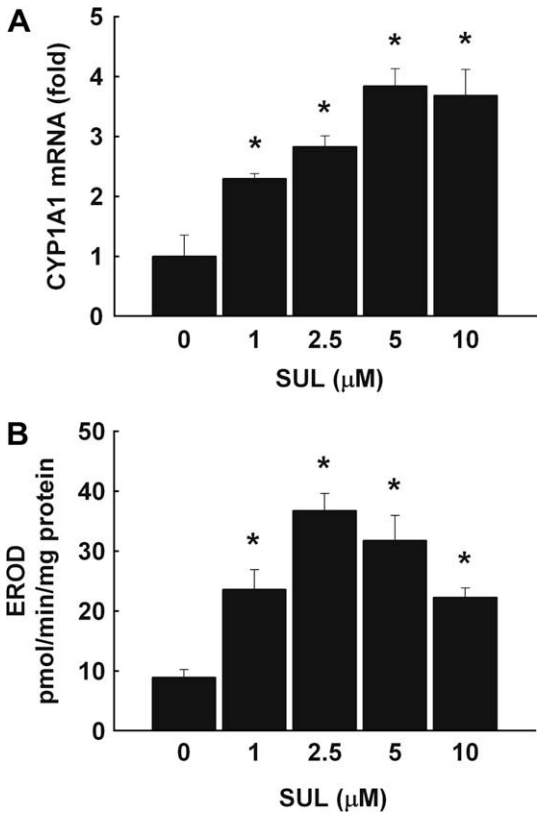

Fig. 4. Effect of SUL on constitutive CYP1A1 mRNA and catalytic activity levels in HepG2 cells. (A) HepG2 cells were treated for 6 h with increasing concentrations of SUL (1–10 μM). First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (*) $P < 0.05$, compared to control (concentration = 0 μM). (B) EROD activity was measured in intact living cells treated with increasing concentrations of SUL (1–10 μM) for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 8$). (*) $P < 0.05$, compared to control (concentration = 0 μM).

nomycin D (Act-D) prior to the addition of SUL. If SUL increases the Cyp1a1 mRNA levels through increasing its stability we would expect no effect for Act-D on Cyp1a1 mRNA levels in response to SUL treatment. Our results demonstrated that pretreatment of the cells with Act-D completely abolished the induction of Cyp1a1 mRNA in response to SUL (Fig. 5A), implying a requirement of *de novo* RNA synthesis.

In an effort to determine the ability of SUL to activate the cytosolic AhR and the subsequent DNA-binding, EMSA was performed on untreated guinea pig hepatic cytosol incubated with vehicle, SUL or TCDD as a positive control *in vitro*. Fig. 5B shows that SUL induced the AhR/ARNT/XRE complex formation, as determined by the shifted band, compared to that of TCDD. Collectively, our data indicate that SUL-induced Cyp1a1 expression through a transcriptional mechanism.

3.6. Post-transcriptional modification of Cyp1a1 mRNA by SUL

The level of subcellular mRNA transcripts is a function of its synthesis, processing, and degradation rates. To further investigate if the observed increase in Cyp1a1 mRNA by SUL could be attributed to a post-transcriptional stabilization of the mRNA, Act-D-chase experiments assessing the half-life of Cyp1a1 mRNA in the presence and absence of SUL were performed. As shown in Fig. 6, Cyp1a1 mRNA decayed rapidly with an apparent half-life of 2.76 ± 0.19 h. In addition, SUL did not significantly alter the half-life of Cyp1a1 mRNA, indicating that the increase of Cyp1a1 mRNA transcripts in response to SUL was not due to a post-transcriptional stabilization of the mRNA. These results clearly show that SUL induction was through a transcriptional mechanism.

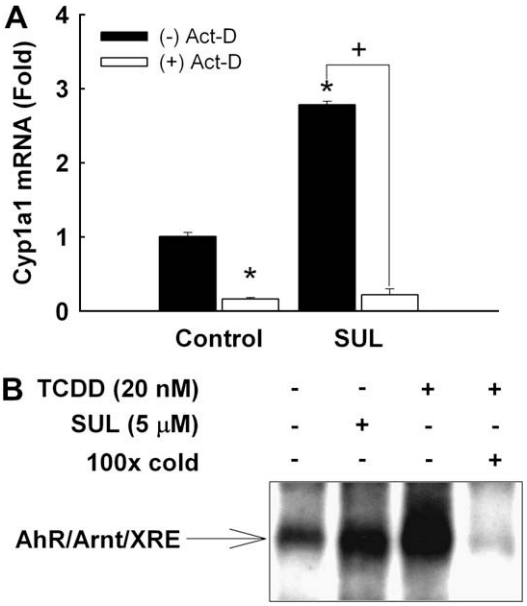

Fig. 5. Transcriptional induction of Cyp1a1 by SUL. (A) Effect of the RNA polymerase inhibitor, actinomycin-D (Act-D) on SUL-mediated induction of Cyp1a1 in Hepa 1c1c7 cells. Cells were pretreated with Act-D (5 μg/ml) 30 min before exposure to SUL (5 μM) for another 6 h. First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (*) $P < 0.05$, compared to control (concentration = 0 μM); (+) $P < 0.05$, compared to SUL (concentration = 5 μM). (B) Untreated guinea pig cytosolic extracts were incubated for 2 h with vehicle, SUL (5 μM) or, TCDD (20 nM). The cytosolic extracts were mixed with [γ^{32}P]-labeled XRE, and the formation of AhR/ARNT/XRE complexes was analyzed by EMSA. The specificity of binding was determined by incubating the protein treated with TCDD with 100-fold molar excess of cold XRE. The arrow indicates the specific shift representing the AhR/ARNT/XRE complex. This pattern of AhR activation was observed in three separate experiments, and only one is shown.

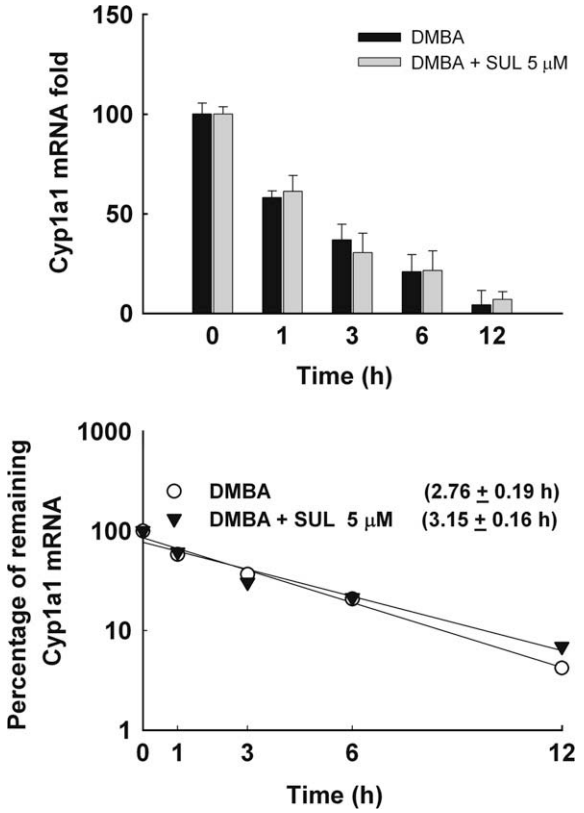

Fig. 6. Effect of SUL on Cyp1a1 mRNA half-life using real-time PCR. After cells were induced with (1 μM) DMBA for 6 h, 5 μg/ml of Act-D was added to inhibit further RNA synthesis, immediately before treatment with (5 μM) SUL. Total RNA was extracted at 0, 1, 3, 6 and 12 h after incubation with SUL and subjected to real-time PCR. mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geqslant 0.85$) to a semilog plot of mRNA amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $N = 3$). (*) $P < 0.05$ compared to DMBA alone.

3.7. Effect of SUL on HO-1 mRNA

In an attempt to explain the reduced induction of Cyp1a1 catalytic activity in response to SUL at higher concentration tested, we sought to determine its effect on HO-1, a rate limiting enzyme of heme degradation. For this reason, Hepa 1c1c7 cells were treated with increasing concentration of SUL (1–10 μM) (Fig. 7). Thereafter, HO-1 mRNA was assessed using real-time PCR. Our results showed that SUL significantly increased the HO-1 mRNA in a concentration-dependent manner. Initially, SUL at the concentration of 1 μM increased HO-1 mRNA by 5-fold, while the highest concentration tested 10 μM increased HO-1 mRNA by 9-fold (Fig. 7).

4. Discussion

The present work provides the first demonstration that SUL modulates *Cyp1a1* gene expression at the transcriptional level.

AhR ligands can be classified according to their origin into synthetic and natural [2]. The majority of the AhR ligands identified up to date, fall in the first class which includes planar, hydrophobic HAHs and PAHs [2]. Further-

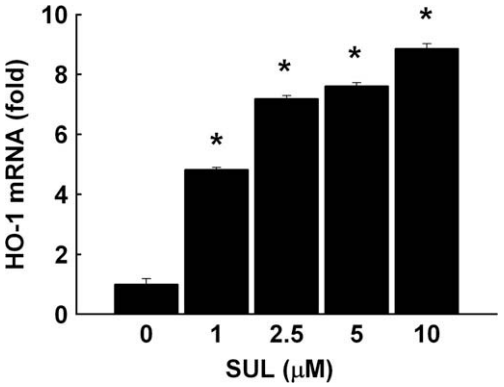

Fig. 7. Effect of SUL on HO-1 mRNA. Hepa 1c1c7 cells were treated for 6 h with increasing concentrations of SUL (1–10 μM). First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa 1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (*) $P < 0.05$, compared to control (concentration = 0 μM).

more, there has been another classification for the AhR ligands based on their planar configuration into "classical" and "non-classical" ligands [2]. Non-classical AhR ligands have been previously defined to be those ligands whose structures and physiochemical properties significantly differ from those of PAHs and HAHs [21,22]. The majority of these non-classical AhR ligands has low affinity to the AhR and is relatively weak inducers of Cyp1a1, compared to TCDD. Thus, a wide range of structural diversity in AhR ligands indicates that a greater spectrum of chemicals can interact with and activate this receptor than previously thought [2].

The importance of the toxicological sequences of AhR ligands arises from the ability of these ligands, typified by PAH, to induce a battery of genes, in hepatic and extra-hepatic tissues, through activating the AhR [23]. In addition to their ability to activate the AhR, these ligands, with the exception of TCDD which is poorly metabolized, are also substrates for phase I AhR-regulated enzymes such as CYP1A1 [23]. Such an interaction would result in metabolizing these ligands and thus the formation of diol-epoxides capable of forming covalent adducts when these genotoxic metabolites interact with guanines in critical genes initiating tumorigenesis in addition to other toxicological consequences [23].

Epidemiological data have demonstrated that consumption of fruits and vegetables on a regular basis is accompanied by a significant decrease in cancer incidences [24,25]. For example, 3–5 servings of crucifers, to which broccoli belongs, has been shown to decrease the risk of prostate cancer by up to 40% [26]. The effect of these crucifers on cancer has been attributed to a class of secondary metabolites termed glucosinolates [26]. Once hydrolyzed glucosinolates give rise to bioactive isothiocyanates [8,27]. The types and concentrations of these glucosinolates vary among members of the cruciferous family [28]. Broccoli in particular is rich in glucoraphanin, which upon hydrolysis releases SUL [28].

Pharmacokinetic studies on humans have shown that a single dose of isothiocyanates (200 μM), assuming 77.2% to be SUL, was rapidly absorbed with a maximum plasma concentration of 2.2 μM/L at 1 h after ingestion, and declined with a half-life of ~1.8 h [29]. In another study where SUL (200 μM) was administered to eight breast cancer female patients, the mean plasma concentration of SUL was 0.92 ± 0.72 μM after ~90 min of administration, confirming the findings of the first study [30]. These pharmacokinetic parameters were also evaluated in rats in which SUL was detectable in the plasma 1 h after dosing (50 μM) p.o., and reaching a peak concentration of 20 μM at 4 h after dosing, and declined with a half-life of 2.2 h [31]. Therefore, the concentrations used in the current study are of great relevance to those of humans.

In the current study, we hypothesize that SUL mediates the induction of CYP1A1 in Hepa 1c1c7 and HepG2 cells. Thus the objectives of the current study were to examine the effect of SUL on the constitutive expression levels of *CYP1A1*, and to explore the underlying mechanisms involved in the modulation of *CYP1A1* gene expression by SUL.

The regulation of *CYP1A1* gene expression involves activation of a cytosolic transcriptional factor, AhR, as the first step in a series of molecular events promoting *CYP1A1* transcription and translation processes [32]. We firstly examined the effect of different concentrations of SUL on CYP1A1 mRNA. Our results demonstrated that SUL increased the CYP1A1 mRNA in a dose-dependent manner. In agreement with our results, it has been previously shown that SUL significantly increased the CYP1A1 mRNA dose-dependently in primary human hepatocytes [12]. To further enhance our understanding on how SUL mediates the induction of Cyp1a1 mRNA, we performed the time-dependent study. Surprisingly, we found that SUL started to induce Cyp1a1 mRNA as early as 1 h. This result suggests a transcriptional activation of *Cyp1a1* gene expression in response to SUL.

To examine, whether the induction of Cyp1a1 mRNA is further translated to protein and catalytic activity levels, we measured Cyp1a1 protein and catalytic activity using Western blot analysis, and EROD activity, respectively. In agreement with our mRNA results, Cyp1a1 protein levels were increased dose-dependently in response to SUL. On the other hand, CYP1A1 catalytic activity levels in both Hepa 1c1c7 and HepG2 cells increased at the low concentrations of SUL (1 and 2.5 μM). However, at the higher concentrations, there was an unexpected decline in the induction of CYP1A1 catalytic activity suggesting post-translational modification to the CYP1A1 protein in response to SUL. In agreement with our results, it has been previously shown that glucoraphanin, precursor of SUL, significantly increases CYP1A1 mRNA and catalytic activity levels in rat livers [33]. In contrast to our results it has been previously reported that treatment of primary rat hepatocytes with SUL at the concentration of (5–25 μM) causes a significant decrease in the induction of CYP1A1 catalytic activity [34]. This course of inhibition was not CYP1A1-specific, as CYP1A2 and CYP3A4 catalytic activities in primary human hepatocytes were also decreased in response to SUL treatment [34]. This effect of SUL on CYPs could be

attributed to its possible post-translational effect and subsequently its effect on CYPs activities.

The ability of SUL to increase *CYP1A1* gene expression prompted us to investigate the effect of SUL on the transcriptional levels. For this reason we performed two distinct experiments. We firstly used the transcription inhibitor Act-D. Our results showed that Act-D completely abolished the induction of Cyp1a1 mRNA in response to SUL, implying that SUL increases the *de novo* Cyp1a1 mRNA synthesis. Secondly, we performed EMSA using untreated guinea pig hepatic cytosol. Because of low efficiency of transformation of the mouse AhR, due to the extreme resistance of HSP90 to dissociate from the mouse AhR, while the greatest degree of transformation of the guinea pig AhR in response to AhR ligand [35], we used guinea pig cytosol as a model. Therefore, freshly isolated hepatic cytosol of untreated guinea pig was incubated with SUL 5 μM or TCDD 20 nM as positive control. Our results showed that SUL was able to induce the AhR transformation and its subsequent binding to XRE in untreated guinea pig hepatic cytosol in a manner similar to that obtained with TCDD, further confirming a transcriptional mechanism.

At the post-transcriptional level, our results showed that the Cyp1a1 mRNA induced by DMBA is short-lived, with an estimated half-life of 2.76 ± 0.19 h. Our results are in agreement with previous reports which showed that the half-life of Cyp1a1 mRNA induced by TCDD in Hepa 1c1c7 cells ranges from 3–4.5 h [36–38]. On the other hand, SUL did not significantly alter the stability of Cyp1a1 mRNA, suggesting that post-transcriptional mechanism is not involved in the modulation of Cyp1a1 mRNA by SUL.

The discrepancy between the effect of SUL on CYP1A1 mRNA and protein levels, and its effect on the CYP1A1 catalytic activity level prompted us to investigate the effect of SUL on HO-1 mRNA, a rate limiting enzyme of heme degradation. CYP1A1 is a heme containing protein, and the lost of which will result in the formation of a hollow functionless protein. SUL increased HO-1 mRNA in a concentration-dependent manner, which in turn might have participated in the formation of a hollow CYP1A1 protein.

Previous studies have demonstrated that oltipraz, a promising cancer chemopreventive agent, exerts similar effect on HepG2 cells [39]. At first oltipraz was recognized as a monofunctional inducer that selectively induces phase II detoxifying enzymes through activating the antioxidant responsive element (ARE). However, recent studies have demonstrated the capability of oltipraz to bind to increase the AhR/ARNT/XRE complex formation as evident by EMSA using nuclear extracts of HepG2 cells [39]. Furthermore, it was shown that oltipraz was able to induce the CYP1A1 mRNA levels in the rat hepatoma H4IIE cell line [39]. We herein provide the first evidence that SUL behavior is similar to that of oltipraz. In the current study, we demonstrated that, although SUL is a chemopreventive agent that was thought to exert its effect solely through inducing the phase II detoxifying enzymes, it also increases the CYP1A1 gene expression in both Hepa 1c1c7 and HepG2 cells.

CYP1A1 contains multiple copies of XRE but not ARE, and the activation of which will help in transforming some types of carcinogens into nontoxic metabolites. In contrast, it could also generate more electrophilic metabolites, which in turn will act as a second messenger to activate phase II detoxifying enzymes. It is also a possibility that the activation of *CYP1A1* gene expression would result in the formation of carcinogens from their pre-carcinogen precursors, and thus compromising SUL chemopreventive effect. This effect could be of great importance if SUL clinical trials prove to be unsuccessful. Finally, our data suggest that a simple classification of monofunctional and bifunctional inducers might be an incomplete classification, and further suggesting critical evaluation, especially, for phase II monofunctional inducers.

Conflict of interest statement

There are no conflicts of interest.

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