Sulforaphane down-regulates COX-2 expression by activating p38 and inhibiting NF-κB-DNA-binding activity in human bladder T24 cells

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Abstract. Cyclooxygenase-2 (COX-2) overexpression has been associated with the grade, prognosis and recurrence of transitional cell carcinoma (TCC) of the bladder. In this study, sulforaphane, a dietary isothiocyanate, down-regulated COX-2 expression in human bladder transitional cancer T24 cells at both transcriptional- and translational levels. Sulforaphane (5-20 μM) induced nuclear translocation of NF-κB and reduced its binding to the COX-2 promoter, a key mechanism for suppressing COX-2 expression by sulforaphane. Moreover, sulforaphane increased expression of p38 and phosphorylated-p38 protein. A specific inhibitor of p38 MAPK, SB202190, was used to further investigate its pivotal role in sulforaphane-mediated down-regulation of COX-2. Exposure of T24 cells to SB202190 1 hour prior to sulforaphane treatment abolished the effect of sulforaphane on COX-2 mRNA down-regulation, but enhanced COX-2 transcription. Furthermore, SB202190 alone induced NF-κB translocation to the nucleus, promoted NF-κB binding to the COX-2 promoter and resulted in up-regulation of COX-2 expression. Taken together, these data suggest that p38 is essential in sulforaphane-mediated COX-2 suppression and provide new insights into the molecular mechanisms of sulforaphane in the chemoprevention of bladder cancer.

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Abbreviations: ChIP, chromatin immunoprecipitation; COX-2, cyclooxygenase-2; ITC, isothiocyanate; MAPK, mitogen-activated protein kinase; SFN, sulforaphane; TCC, transitional cell carcinoma

Key words: isothiocyanate, sulforaphane, bladder cancer, COX-2, NF-κB, MAPKs, chemoprevention

Introduction

Sulforaphane (SFN), a widely-studied isothiocyanate (ITC) derived from cruciferous vegetables, has been shown to possess chemopreventive activities in many *in vivo* and *in vitro* models. The anticarcinogenic effects of SFN are mainly attributed to the activities on up-regulation phase II detoxification enzyme and down-regulation of phase I enzyme expressions (1-3); induction of cell cycle arrest and apoptosis (4,5). Recently, sulforaphane has been shown as a potent inhibitor of histone deacetylase (6-8). Histone deacetylase inhibitors influence gene expression by enhancing acetylation of histones in specific chromatin domains, result in cell cycle arrest and apoptosis, and represent a promising class of antitumor drugs (9). It has recently been shown that sulforaphane possesses bioactivities and effects on transcriptional factors, receptors, and kinases (10-12) that modulate cell cycle and chemopreventive gene expression. Since ingested ITCs are mainly metabolised through the mercapturic acid pathway (13), and sulforaphane metabolites are stored in the bladder for relatively longer period particularly in the bladder epithelium (14). Therefore, the bladder could be an important target organ for dietary ITCs. It has previously been shown that sulforaphane affected cell cycle progression and induced apoptosis in human bladder cancer T24 cells (15). Moreover, epidemiological studies also suggested that consumption of cruciferous vegetables may be more beneficial in decreasing the risk of bladder cancer (16).

Despite extensive research, pathologic and molecular markers for both the prognosis and grade of bladder cancer are lacking (17). Cyclooxygenase-2 (COX-2), the key enzyme in the synthesis of prostaglandin from arachidonic acid (AA), is undetectable in most quiescent cells, but is inducible in the response to cytokines, mitogens, growth factors and tumor promoters. A large body of evidence suggests that COX-2 is overexpressed in human bladder cancer and in chemically-induced rodent bladder cancers (18,19). COX-2 overexpression is closely related to the progression, prognosis and recurrence of transitional cell carcinoma (TCC) of the urinary bladder (20). Based on these findings, targeted inhibition of COX-2 may be a promising approach to prevent bladder cancer

(21). Human COX-2 promoter contains several functionally-important enhancer elements which serve as binding sites for various specific transcription factors (22). COX-2 transcription is regulated through these transcription factors, especially nuclear factor-κB (NF-κB), cyclic AMP response element binding protein (CREB) and C/EBP (23). NF-κB is a ubiquitous and pleiotropic transcription factor closely linked with carcinogenesis by a variety of pathways such as cell cycle progression, apoptosis, invasion, metastasis and inflammation (24). Although regulation of COX-2 expression by p38 mitogen-activated protein kinase (MAPK) has been primarily investigated in inflammation, the relationship between the expression of COX-2 and p38 MAPK in bladder cancer cells remains unclear. In this study, the role of p38 in SFN-mediated down-regulation of COX-2 mRNA and protein was examined using a specific p38 inhibitor, SB202190. In addition, the DNA-binding activities of NF-κB were studied using Chromatin Immunoprecipitation (ChIP) following treatments of T24 cells with SFN and SB202190.

Materials and methods

Reagents. SFN (1-isothiocyanato-4-methylsulphinylbutane) was purchased from LKT laboratory (Alexis Biochemicals, UK). Anti-NF-κB, anti-p38, anti-ß-actin and phosphorylated p38 antibodies, rabbit- and horseradish peroxidase-conjugated (HRP) anti-rabbit and anti-goat antibodies were all purchased from Santa Cruz Biotechnology (Autogen Bioclear, UK). Anti-COX-2 antibody was obtained from Upstate Cell Signaling (UK) and p38 inhibitor SB202190 from Tocris (UK). Nuclear Extraction Kit was purchased from Chemicon® International (UK). Chromatin Immunoprecipitation Kit (EZ ChIP™) was obtained from Upstate Cell Signaling. Protease inhibitor cocktail tablets and phosphatase inhibitor cocktail were from Roche Applied Science (UK) and Sigma (UK), respectively. RPMI-1640 medium was purchased from Invitrogen Corp. (UK). Bradford reagent, dimethyl sulfoxide (DMSO) and phenylmethylsulphonylfluoride (PMSF) were all from Sigma.

Cell culture. Human bladder cancer T24 cells were purchased from the European Collection of Cell Cultures (ECACC) and grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 μg/ml penicillin and 100 U/ml streptomycin, 2 mM L-glutamine. Cells were cultured in a humidified atmosphere (95% air and 5% CO_2) at 37˚C.

Total RNA extraction, RT-PCR and real-time PCR. Total RNA was isolated using a GenElute™ Total Mammalian RNA Kit (Sigma) according to the manufacturer's instructions. RNA concentration and purity were determined by absorbance measurement at 260 and 280 nm using a nanodrop spectrophotometer (Labtech International, UK). Reverse transcriptions (RT) consist of 1 μg total RNA, 1 μl random primer, 0.25 μl Bioscript Polymerase, 1 μl dNTP, 4 μl buffer and 1 μl RNase inhibitor, complemented with PCR water to a total volume 20 μl. The RT reactions were carried out at 70˚C for 10 min and 42˚C for 1 h.

Target mRNA was quantified by real-time PCR (TaqMan®) using an ABI 7500 Sequence Detection System (Applied Biosystems). Forward/reverse primers and the fluorogenic TaqMan probes were designed using ABI Primer Express Software and synthesized by Sigma. Primers/probe sets were homology-searched to ensure that they were specific for COX-2 mRNA transcripts using a NCBI BLAST search. 18S rRNA was used as a housekeeping gene. The probes were labelled with a 5' reporter dye, FAM (6-carboxy-fluoroscein) and 3' quencher dye, TAMRA (6-carboxytetra-methylrhodamine). Real-time PCR was carried out in a 96-well plate (in triplicate) using TaqMan two-step PCR Mastermix Reagent Kit. TaqMan threshold cycle number (C_t) was normalized into relative using the $2^{\Delta\Delta Ct}$ method, $\Delta\Delta C_t = (C_{t,\,Target}-C_{t,\,18S})_{treatment} - (C_{t,\,Target}-C_{t,18S})_{control}$. Standard curves were constructed using 0.5-100 ng of total RNA in triplicate. Primers and probes used in this study are listed in Table I.

Nuclear fraction extraction and Western blot analysis. T24 cells were harvested after the desired treatment with Trypsin-EDTA (T/E) buffer, pelleted by centrifugation and the cell pellets were washed with cold phosphate-buffered saline (PBS). Nuclear- and cytoplasmic extractions were isolated using Chemicon's Nuclear Extraction Kit. Briefly, each pellet was suspended in cell lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF and 1X protease inhibitor cocktail and incubated on ice for 30 min. Thereafter, lysates were centrifuged at 10,000 g for 10 min at 4˚C, and supernatant fractions used for Western blot analysis. The equivalent amounts of protein (30 μg) were mixed with 5X SDS-PAGE loading buffer and dithiothreitol (DTT) reducing agent and subjected to 8-10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred onto PVDF membranes (Bio-Rad) using a semi-dry transfer system. The membranes were blocked with 5% fat-free dry milk in PBS (pH7.4) containing 0.1% Tween-20 for 30 min at room temperature, followed by incubation with primary antibodies in PBS overnight at 4˚C. The membranes were washed three times with PBS containing 0.1% Tween-20, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and washed three times with PBS containing 0.1% Tween-20. The protein of interest was visualized with enhanced chemiluminescent (ECL) system (GE Healthcare, UK) according to the manufacturer's instructions. The membranes were then exposed to CL-Xposure film (Perbio Science, UK).

Chromatin immunoprecipitation (ChIP). T24 cells were plated in a 10-cm dish at the density of $5x10^5$/ml and Chromatin Immunoprecipitation performed using an EZ ChIP kit (Upstate Cell Signaling), as described in the instruction manual. In brief, cells were fixed with 10 ml formaldehyde (37%, Sigma) for 10 min to crosslink proteins to DNA. Cell suspensions were then sonicated and the chromatin sheared to a manageable size (~200-1000 bp, which is small enough to achieve a high degree of resolution required for ChIP analysis). The size of DNA fragment was confirmed by gel electrophoresis. Ten μl (1%) of the supernatant was used as input before immuno-precipation with the following antibodies. The cross-linked protein/DNA complexes were subjected to immunoselection with anti-NF-κB(p65) antibody, positive antibody was anti-acetyl-Histone H3, and negative antibody was normal rabbit

Table I. Primers and probe sequences for COX-2, COX-2 promoter and 18S rRNA.

COX-2	
Forward primer	5'-GAATCATTCACCAGGCAAATTG-3'
Reverse primer	5'-TCTGTACTGCGGGTGGAACA-3'
Probe	5'-TCCTACCACCAGCAACCCTGCCA -3'
COX-2 promoter (25)	
Forward primer	5'-CAATAAATAGGAGTGCCATAAATG-3'
Reverse primer	5'-ACGGATGACTAAAATTCCATCT-3'
Probe	5'-AAGCCTTTCTCCTCCTCTAGTCAT-3'
18S rRNA	
Forward primer	5'-GGCTCATTAAATCAGTTATGGTTCCT-3'
Reverse primer	5'-GTATTAGCTCTAGAATTACCACAGTTATCCA-3'
Probe	5'-TGGTCGCTCGCTCCTCTCCCAC-3'

IgG. Finally, protein/DNA complexes were purified using a spin column. Any DNA sequence cross-linked to the NF-κB(p65) protein would co-precipitate as part of the chromatin complex. Following immunoselection of chromatin fragments and purification of associated DNA, real-time PCR was performed to quantify the DNA sequence associated with NF-κB protein using specific COX-2 promoter probe and primers. C_t values of COX-2 promoter in each group were standardized individually against its corresponding 18S rRNA input. COX-2 promoter percent binding with NF-κB(p65) was presented with $\Delta\Delta C_t$ value from three different experiments (mean ± SD).

Statistical analysis. Results were expressed as mean ± SD (from at least three measurements). Data were analyzed by One-way ANOVA, followed by either Dunnett's t-test for separate comparisons with the control group or Sidak's t-test for multiple-group comparisons. Differences were considered significant at $p<0.05$.

Results

SFN inhibits both COX-2 mRNA and protein in T24 cells. SFN at 5, 10 and 20 μM decreased COX-2 mRNA expression by 28, 69, and 87% after 10-h treatment, and by 18, 53 and 85% after 24 h treatment (Fig. 1A). SFN down-regulated COX-2 at both the mRNA level and protein level; SFN (20 μM) decreased the expression of COX-2 protein by 23, 32 and 43% at 8, 12 and 24 h, respectively (Fig. 1B). This down-regulation of COX-2 protein was dose-dependent after treatment with 5, 10 and 20 μM SFN for 24 h (Fig. 1C).

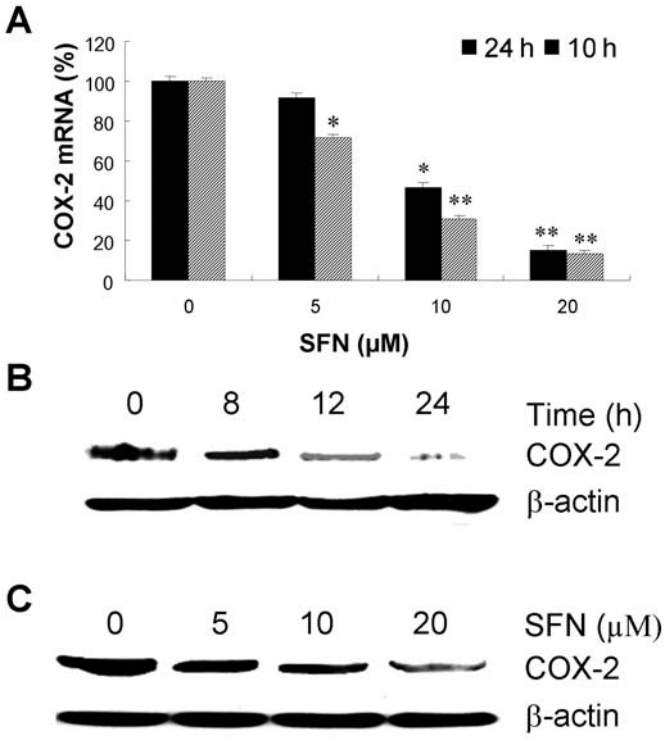

Figure 1. Inhibitory effect of SFN on COX-2 expression. (A) T24 cells were treated with 5, 10 and 20 μM SFN for 10 or 24 h. Data presented were from three different experiments (compared with control group, $^{*}p<0.05$. $^{**}p<0.01$). (B) COX-2 protein was down-regulated by 20 μM SFN after 8, 12 and 24 h. (C) T24 cells were treated with 5, 10 and 20 μM SFN for 24 h and COX-2 was inhibited in a dose-dependent manner.

SFN activates nuclear translocation of NF-κB, but inhibits the NF-κB binding to the COX-2 promoter. Based on these results, a study was undertaken to determine whether SFN could suppress translocation of NF-κB from the cytosol to nucleus and subsequently inhibit the COX-2 mRNA transcription. However, SFN induced translocation of NF-κB to the nucleus in both a dose- and time-dependent manner following 4-24 h of treatment (Fig. 2A and B). To examine whether the NF-κB translocated into the nucleus was bound to the COX-2 promoter, Chromatin Immunoprecipitation (ChIP) in combination with quantitative real-time PCR analysis were performed to detect and relatively quantify the binding of NF-κB to the COX-2 promoter. As shown in

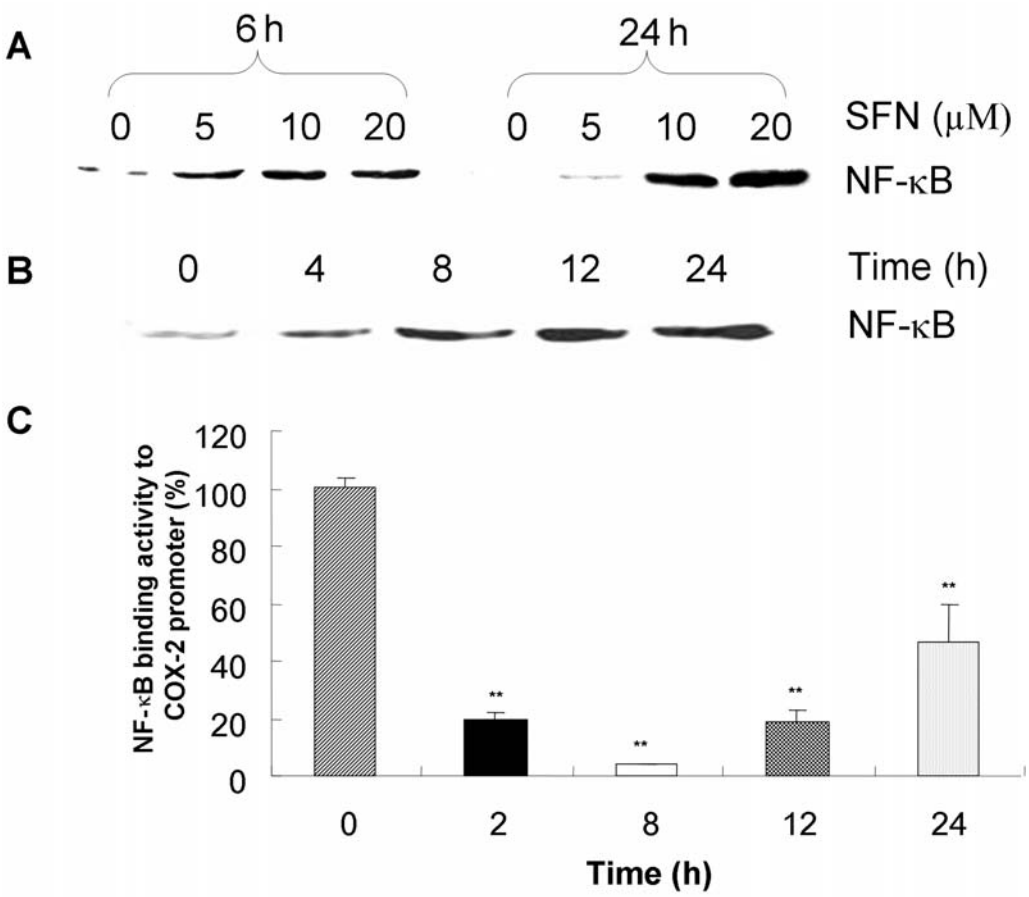

Figure 2. Different effects of SFN on the translocation and binding of NF-κB. (A) The expression of NF-κB induced by SFN for 6 and 24 h. (B) Time course of SFN (20 μM) induced translocation of NF-κB into nucleus. (C) Down-regulation of binding of NF-κB with the COX-2 promoter by SFN (**compared with 0 h, $p<0.01$).

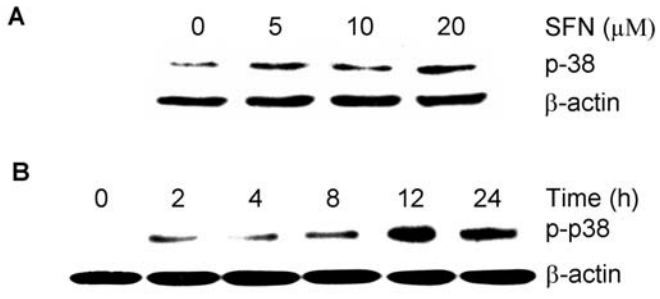

Figure 3. Induction and phosphorylation of p38 protein by SFN treatment. (A) T24 cells were treated with 5, 10 and 20 μM SFN for 24 h. Whole cytosolic fractions were immunoblotted with p38 antibody. (B) T24 cells were treated with 20 μM SFN for the indicated time. The expression of phosphorylated-p38 was analysed in the cytosolic extract.

Fig. 2C, SFN inhibited the binding of NF-κB to the COX-2 promoter in T24 cells after exposure for 8-24 h. The strongest inhibition (50-96%) was observed at 8 h, which explains, at least partly, the significant down-regulation of COX-2 mRNA after exposure to SFN (20 μM for 10 h) (Fig. 1A).

p38 MAPK is required for the inhibition of COX-2 by SFN. In the current study, SFN (5-20 μM) induced p38 expression after treatment for 24 h (Fig. 3A), and SFN significantly upregulated phosphorylated p38 (p-p38) in a time-dependent manner (Fig. 3B). This induction of both total p38 and p-p38 expression by SFN suggests that p38 MAPK plays a crucial role in the chemopreventive effect of SFN in human bladder cancer T24 cells.

To further investigate the potential mechanism of p38 MAPK in this process, SB202190 (10 μM), a widely used and effective suppressor of p38 (26), was pre-incubated with T24 cells 1 h before incubation with or without SFN for 24 h. SB202190 alone induced COX-2 mRNA and protein expression. Moreover, pretreatment with SB202190 totally abolished inhibition of COX-2 mRNA by SFN. At the protein level, SB202190 blocked the inhibitory effects following low doses (5-10 μM) of SFN treatment, but not at the higher dose (20 μM) (Fig. 4B). This discrepancy implies that a post-transcriptional mechanism may be involved in COX-2 expression by SFN. A time-course study with 10 μM SFN showed that SB202190 abolished the inhibition of COX-2 by SFN (10 μM) after 8, 12 and 24 h treatments (Fig. 4C). These data suggest that the total level of p38 in T24 cells contributed to the final expression of COX-2. Taken together, these results provide further confirmation that p38 MAPK plays a pivotal role in the inhibition of COX-2 by SFN.

SB202190 overrides SFN-mediated blockade of NF-κB binding to the COX-2 promoter. To further explore the potential

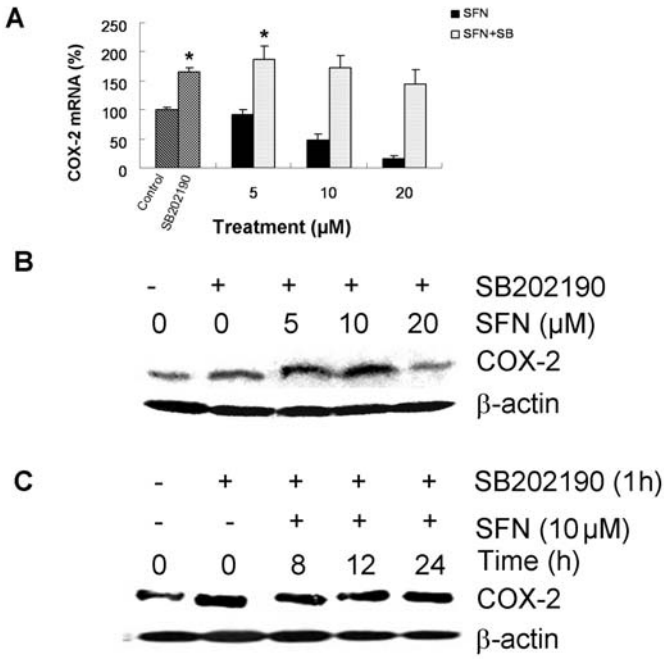

Figure 4. SB202190 abolished the inhibition of COX-2 both in mRNA and protein level. (A and B) Cells were pre-treated with inhibitor SB202190 (10 μM) for 1 h and then treated with 5, 10 and 20 μM SFN (SB202190 group incubated with control medium) for 24 h. *Compared with control, $p<0.05$. (C) Cells were exposed to SB202190 for 1 h, followed by 20 μM SFN for 8, 12 and 24 h. In the SB202190 group, cells were isolated for Western blot analysis after 1 h.

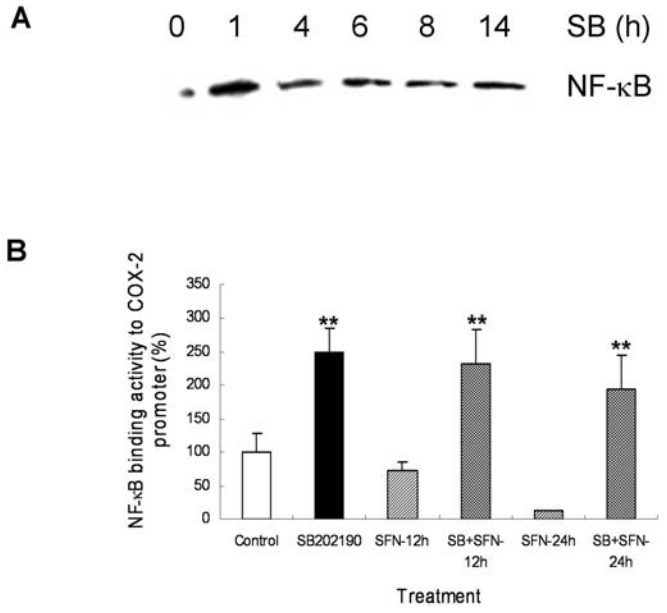

Figure 5. SB202190 induced the translocation and binding of NF-κB. (A) SB202190 (10 μM) induced the translocation of NF-κB. (B) SB202190 reversed the NF-κB DNA binding activity attenuated by SFN treatment for 12 h. T24 cells were exposed to 10 μM SB202190 for 1 h, treated with 10 μM SFN for 12 or 24 h and processed for ChIP. **Compared with control, $p<0.01$.

mechanism by which SB202190 attenuates the inhibitory effect of SFN on COX-2 expression, the effect of SB202190 on the translocation of NF-κB and the binding to the COX-2 promoter were examined. SB202190 (10 μM) significantly increased NF-κB translocation into nucleus after 1-, 4-, 6-, 8- and 14-h incubation. The strongest translocation was observed at 1 h and decreased following longer incubation (Fig. 5A), indicating that the role of SB202190 occurs early. To further elucidate the underlying mechanism of COX-2 induction by SB202190, cells were treated for 12 and 24 h after pre-treatment with SB202190 (10 μM for 1 h) and processed using the ChIP assay. SB202190 alone significantly increased the binding activity (2.5-fold) of NF-κB with the COX-2 promoter in comparison to the control ($p<0.01$) (Fig. 5B). While exposure of cells to SB202190 followed by SFN exposure for 12 and 24 h, binding activities were significantly higher than control (2.3-fold at 12 h and 2.0-fold at 24 h) (Fig. 5B). These results confirmed that SB202190 abolished the inhibitory effect of SFN on COX-2 by increasing NF-κB binding to the COX-2 promoter.

Discussion

For the first time, it has been shown that p38 is essential for the down-regulation of COX-2 expression by SFN in human bladder T24 cells. SFN suppressed both COX-2 mRNA and protein levels by inhibiting NF-κB DNA-binding to the COX-2 promoter. It is already known that COX-2 plays a role in cell proliferation, metastasis and angiogenesis, and these results suggest that targeting COX-2 through p38 MAPK is a novel mechanism for cancer chemoprevention by SFN in human bladder cancer.

It has been demonstrated that SFN at lower doses (5 or 10 μM) inhibited COX-2 and presented that the specific inhibitor of p38 MAPK, SB202190 abolished the suppression of COX-2 by 5 and 10 μM SFN. These findings are important and suggest that sulforaphane is promising for prevention of bladder cancer, since 5 or 10 μM SFN can be readily achievable in the urine following consumption of 100-200 g broccoli sprouts per day (27).

It is well established that NF-κB is closely related with COX-2 mRNA expression (28). These data showed that SFN induced translocation of NF-κB, but reduced binding of NF-κB to the COX-2 promoter in human bladder cancer cells. SFN-mediated translocation of NF-κB to the nucleus and binding to targeting gene can be influenced by many factors (29). Results from Heiss *et al*, suggested that high concentration of SFN was required to prevent NF-κB binding to the COX-2 promoter (30). However, human intervention studies have shown that ITCs stored in the urine can reach more than 100-fold higher than in the plasma (13). Therefore in the present study, high and rapid accumulation of SFN in T24 cells may contribute to the decreased binding activity of NF-κB.

Lower activities of p38 have been reported in human hepatocellular carcinoma (31). This study suggests that the activation of p38 is a crucial and indispensable step involved in the chemopreventive effect of SFN in human bladder cancer, providing evidence of the key role of p38 activators in cancer chemotherapy.

In conclusion, a new pathway linking p38 MAPK with the down-regulation of COX-2 by SFN has been determined in human bladder cancer cells. SFN activated p38 MAPK, then inhibited NF-κB binding to the COX-2 promoter as well as the down-regulation of COX-2 expression. The data presented herein suggest that activators of p38 MAPK may be beneficial in the down-regulation of COX-2 and, in combination with SFN, may provide potential therapeutic strategies for bladder cancer. Taken together, these results indicate that inhibition of COX-2 could be a new target for studies to further examine the chemopreventive and therapeutic effects of SFN in bladder cancer.

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