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Research Paper

Synergistic Effect of Combination of Phenethyl Isothiocyanate and Sulforaphane or Curcumin and Sulforaphane in the Inhibition of Inflammation

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Purpose. Accumulating evidence from epidemiologic and clinical studies indicates that chronic inflammatory disorders harbor an increased risk of cancer development. Curcumin (CUR) has been strongly linked to the anti-inflammatory effect. On the other hand, isothiocyanates such as sulforaphane (SFN) and phenethyl isothiocyanate (PEITC) are strong phase-II detoxifying/antioxidant enzymes inducer. Therefore it is interesting to see if combination of these drugs can inhibit inflammation with higher combined efficacies.
Methods. We used nitric oxide (NO) assay to assess the synergism of the different combinations of CUR, SFN and PEITC. The inflammatory markers, e.g. iNOS, COX-2, prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF) and interleukin-1 (IL-1) levels were determined using RT-PCR, Western blot and ELISA assays.
Results. We report that combination of PEITC + SFN or CUR + SFN has a synergistic effect in down-regulating inflammation markers like TNF, IL-1, NO, PGE_2. The synergism is probably due to the synergistic induction of phase II/antioxidant enzymes including heme-oxygenase1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO-1).
Conclusions. Our data suggest that CUR + SFN and PEITC + SFN combinations could be more effective than used alone in preventing inflammation and possibly its associated diseases including cancer.

KEY WORDS: combination; inflammation; LPS; phase II genes.

INTRODUCTION

It is now widely accepted that inflammation is a critical component of tumor progression. Many cancers, e.g. epithelial skin cancer and colorectal cancer, arise from chronic inflammation and sites of infection (1,2). Inflammatory reagents such as 12-O-tetradecanoylphorbol 13-acetate (TPA) are required for the formation of chemically-induced mouse skin tumors (2). Similarly, chronic inflammation induced by dextran sodium sulfate (DSS) is required for the azoxymethane (AOM)-induced tumor. All these data and models suggest that inflammation is indispensable for tumor formation and progression (2).

Numerous studies have shown that tumor sites often harbor infiltrated inflammatory cells (3). Chemokines are responsible for directing the local accumulation of inflammatory cells into the infected sites. Thus far, research on inflammation-associated cancer development has focused on cytokines and chemokines as well as their downstream targets (4). Several recent studies have identified nuclear factor-κB (NF-κB) as a key modulator in driving inflammation to cancers (4). NF-κB activation in tumor-associated leukocytes, especially macrophages, contributes towards tumorigenesis by upregulating tumor-promoting proinflammatory proteins (6). One of the key molecules mediating the inflammatory processes in tumor promotion is tumor necrosis factor alpha (TNFα) (5). Recent pre-clinical cancer models have provided critical evidence to support the link between chronic, low level TNFα exposure and the acquisition of pro-malignant phenotype (i.e., increased growth, invasion and metastasis) (5). Inflammatory cytokines including interleukin-1 (IL-1) and 6 (IL-6) (7) also serve as autocrine and paracrine growth factors for several cancers. In addition, overproduction of both nitric oxide (NO) and prostaglandins (PGE_2) has been associated with numerous pathological conditions including chronic inflammation and cancer (8).

Anti-inflammatory therapeutic approaches are now considered to be important in preventing cancer initiation and development (1). Numerous studies have shown that phytochemical constituents prevent tumor formation by up-regulating Nrf2, phase II genes, inducing cell cycle arrest and triggering apoptosis. The anti-inflammatory properties of these constituents are also explored. It has been shown that lipopolysaccharides (LPS) induced nitrite and prostaglandin E_2 (PGE_2) synthesis in Raw 264.7 cells was attenuated by phenethyl isothiocyanate (PEITC) in a concentration-dependent manner and the reduction in both iNOS and COX-2 expression were associated with the inactivation of NF-κB and stabilization of IκB-α (8). Curcumin (CUR) is the main

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constituent of the spice turmeric, used in diet and in traditional medicine across the Indian subcontinent. Anti-inflammatory properties of CUR have been well documented. It has long been known that CUR inhibits induction of nitric oxide synthase (NOS) *in vitro* (LPS-activated macrophages) (10) and in-vivo (11). Several mechanisms have been suggested. CUR binds at submicromolar affinity to the myeloid differentiation protein 2 (MD-2), which is the LPS-binding component of the endotoxin surface receptor complex MD-2/TLR4 (Toll-like receptor 4), targeting MD-2 in the inhibition of response to LPS (9). In addition, CUR inhibits LPS-induced MAPK activation and the translocation of NF-κB p65 (12). These data show that CUR effectively targets the LPS-TLR-MAPK-NF-κB pathway and inhibits inflammatory cytokines expression. Sulforaphane (SFN) is best known as a potent phase II gene/protein inducer. Induction of phase II proteins promotes oxidant scavenging and decreases oxidative stress. The inflammation inhibitory effect of SFN has been proven in cardiovascular system (13) and skin (14). Induction of phase II genes protects the cells from UV-induced oxidative stress and inflammation. This protective effect can be used as a means of reducing cancer incidence.

In this study, we investigate whether the combination of PEITC, SFN and CUR would produce synergistic effect in the inhibition of LPS-induced inflammation in RAW 264.7 cells. We found that PEITC + SFN and CUR + SFN

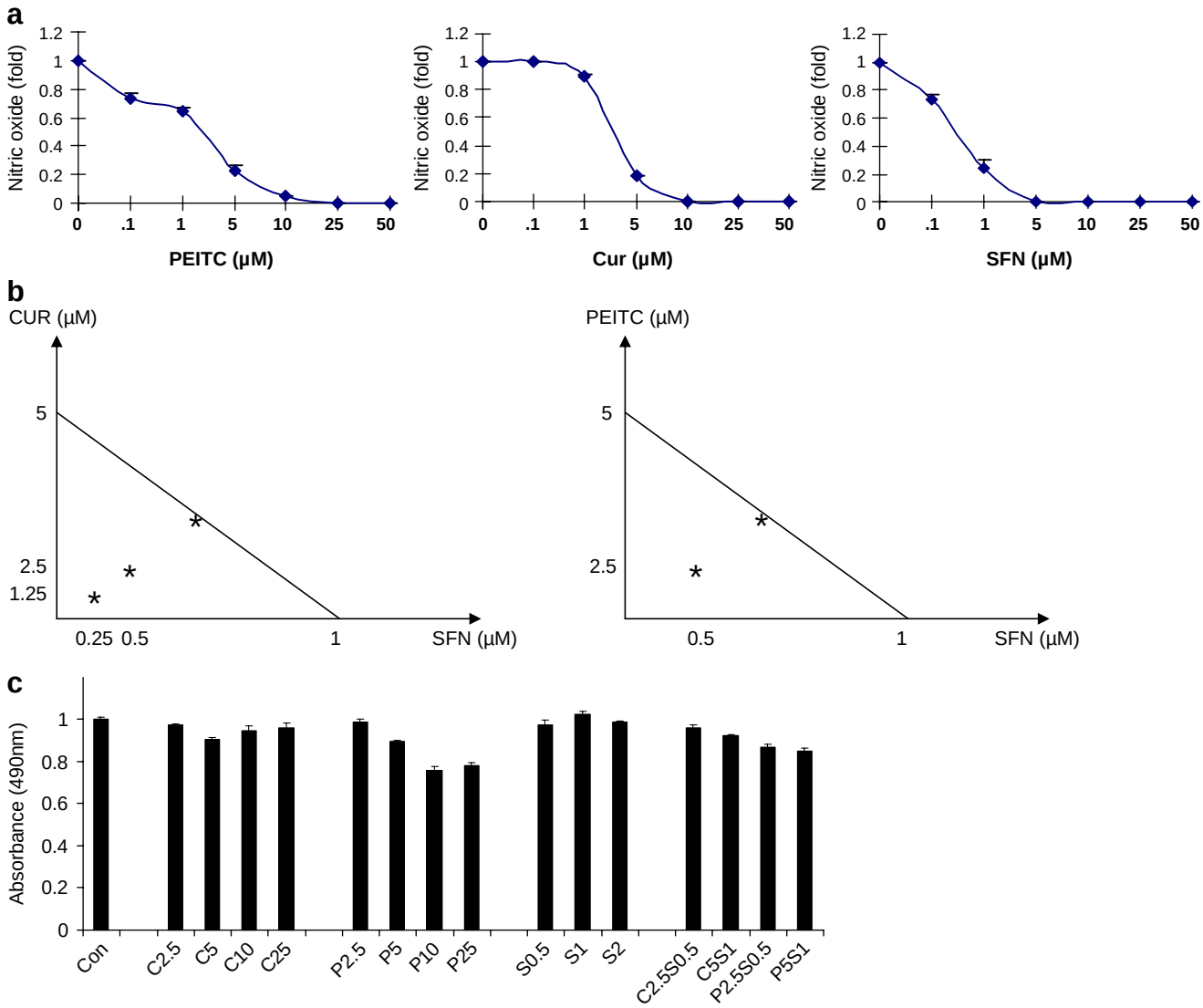

Fig. 1. **A** Dose-dependent inhibition of LPS-induced NO by PEITC, Curcumin and SFN. RAW 264.7 cells were treated with 0.1, 1,5,10, 25, 50 μM PEITC, Curcumin and SFN before LPS treatment. 24 h after treatment, nitric oxide assays were performed on the medium. Data points are representative of three independent experiments. **B** Isobologram analyses of synergy between combinations of CUR + SFN and PEITC + SFN. Several combinations were analyzed for synergy by the method of isobologram analysis as described elsewhere (15,16) and were confirmed as synergistic. Data points are described by concentrations (in μM) as reflected on x- and y-axes respectively, and are representative of three independent experiments. The corresponding combination indices (CI) are smaller than 1, which confirmed the synergy between the combinations. **C** Cytotoxicity of CUR, PEITC, SFN and their combinations. RAW 264.7 cells were seeded on a 96-well plate cultured in 100 μl medium. Each well was treated with compounds and 20 μl MTS/PMS solution was added 24 h after treatment. Cell viability was measured as absorbance at 490 nm. Each data is the average of 3 replicates.

synergistically inhibit LPS-induced inflammation by down-regulating a whole spectrum of inflammatory markers including TNFα, iNOS, NO, COX-2, PGE_2 and IL-1.

MATERIALS AND METHOD

Cell Culture and Reagents

RAW 264.7 cells were obtained from American Type Culture Collection (ATCC). The cells were maintained in Deagle minimum essential medium (DMEM) with 10% fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate, 100 U/mL penicillin and 100 μg streptomycin. Phenethyl isothiocyanate (PEITC), curcumin (CUR) and sulphoraphane (SFN) were obtained from Sigma (St Louis, MO). iNOS, COX-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TNFα, IL-1, PGE_2 ELISA kits were purchased from Calbiochem Technology (San Diego, CA).

Transient Transfection of Nrf2

Dominant negative Nrf2 DNA (Nrf2-M4) construct, encoding Nrf2 peptide (amino acids 401–589) that contains DNA binding domain but lacks transactivation domain, was generated in our laboratory. RAW 264.7 cells were plated into six-well plates and transfected with the GeneJuice (Novagen) and Nrf2-M4 according to the manufacturer's instruction. After transfection, cells were cultured for an additional 24 h and harvested for analysis.

Western Blotting Analysis

RAW 264.7 cells in six-well plates were washed with ice-cold PBS and lysed with 200 μl of whole cell lyses buffer (10 mM Tris–HCl, pH 7.9, 250 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1 mM proteinase inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 100 mM Na3VO4, 5 mM ZnCl2, 2 mM indole acetic acid). The cell lysates were centrifuged at 12,000 g for 10 min at 4°C. The protein concentrations of the supernatants of the whole cell lysate were determined using a Bio-Rad protein assay kit. An equal amount of protein (20 μg) was then resolved on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane using semi-dry transfer system. The membrane was blocked in 5% non-fat milk for 1 h at room temperature and then incubated overnight at 4°C with a primary antibody specifically recognizing iNOS and COX-2. After incubation with the primary antibody, the membrane was washed with TBST (20 mM Tris–HCl, 8 g/L NaCl, 0.1% Tween 20, pH 7.6) three times, then incubated in horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution) for 45 min at room temperature followed by an additional three washes with TBST. Detection was performed using ECL reagents (Bio-Rad).

RT-PCR and PCR

Total RNA from mouse RAW 264.7 cells were isolated by Trizol (Invitrogen, Carlsbad, CA). Total RNA samples were converted to single-stranded cDNA by the Superscript First-Strand Synthesis System III (Invitrogen). The resulting cDNA was amplified by the PCR supermix kit (Invitrogen). PCR conditions are as follows: 94°C for 10 min followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The 5' and 3' primers used for amplifying iNOS were GTGGTGACAAAGCACATTTGG and GGC TGGACTTTTCACTCTGC, COX2 were TCCTCCTG GAACATGGACTC and TGATGGTGGCTGTTTTGGTA, TNF were: ACGGCATGGATCTCAAAGAC and GGTCACTGTCCCAGCATCTT, IL-1 were GAGTGTGG

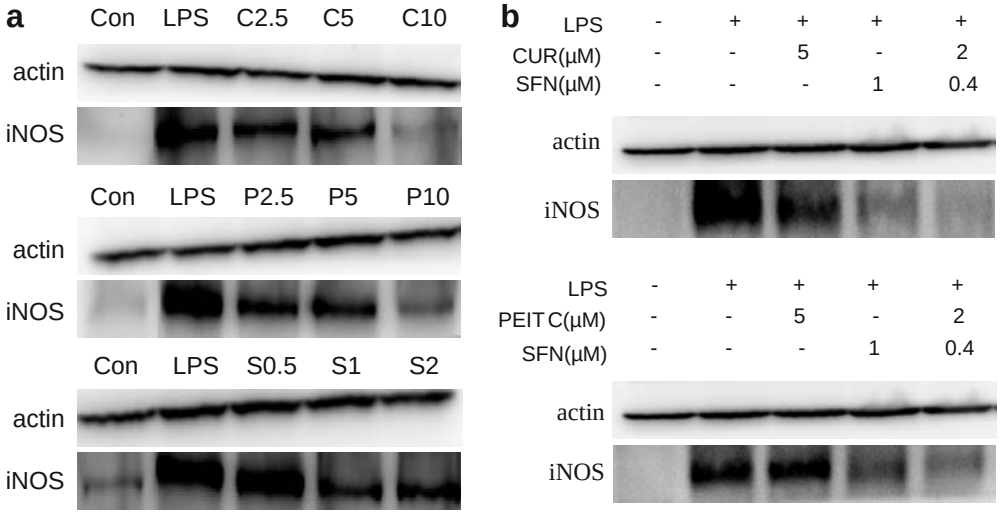

Fig. 2. A Dose-dependent inhibition of LPS-induced iNOS expression by Curcumin, PEITC and SFN. RAW 264.7 cells were pre-treated with PEITC, Curcumin and SFN before LPS treatment. 24 h after treatment, iNOS expression was detected by western blotting. Blots are representative of three independent experiments. **B** iNOS expression with the combinations of CUR + SFN and PEITC + SFN. The proteins were immunoblotted using iNOS antibody as indicated using actin as the control. Blots are representative of three independent experiments.

ATCCCAAGCAAT and CTCAGTGCAGGCTATGG ACCA, HO-1 were AAGAGGCTAAGACCGCCTTC and GTCGTCGTCAGTCAACATGG and NQO1 were CAGA TCCTGGAAGGATGGAA and AAGTTAGTCC CTCGGCCATT. PCR products were resolved on 1% agarose gels and visualized under UV lamps.

Nitric Oxide Assay

Nitric oxides secreted by RAW 264.7 cells were measured by Griess reagent (Promega). A nitric oxide standard curve (0.1M sodium nitrite in water, diluting to 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0 μM) was prepared. 50 μl of the samples to be measured were pipetted into a 96-well plate. Using a multichannel pipette, 50 μl sulfanilamide (1% sulfanilamide in 5% phosphoric acid) solution was added to each sample and the mixture was incubated for 10 min at room temperature protected from light. Following that, 50 μl NED (0.1% N-1-napthylethylenediamine dihydrochloride in water) solution was added to all the wells and the mixture was incubated for 10 min at room temperature protected from light. Purple color started to appear and the absorbance was measured at a wavelength between 520–550 nm.

TNFα, IL-1 and PGE_2 ELISA Assay

TNFα, IL-1 and PGE_2 assays ware performed according to the protocols of manufacturers. For the TNF ELISA assay, 50 μl of incubation buffer was first added to all the wells. Next, 50 μl standard diluent buffer and 50 μl of sample were added to each well. 50 μl biotin conjugate was added afterwards, mixed well and the reaction was incubated for 90 min at room temperature. The wells were then aspirated and washed thoroughly 4 times. 100 μl streptavidin-HRP working solution was then added and incubated for 45 min at room temperature. The reaction was stopped by adding 100 μl stop solution and the reaction was read at 450 nm. IL-1 and PGE_2 ELISA assay was performed similarly according to the standard protocols provided by Calbiochem.

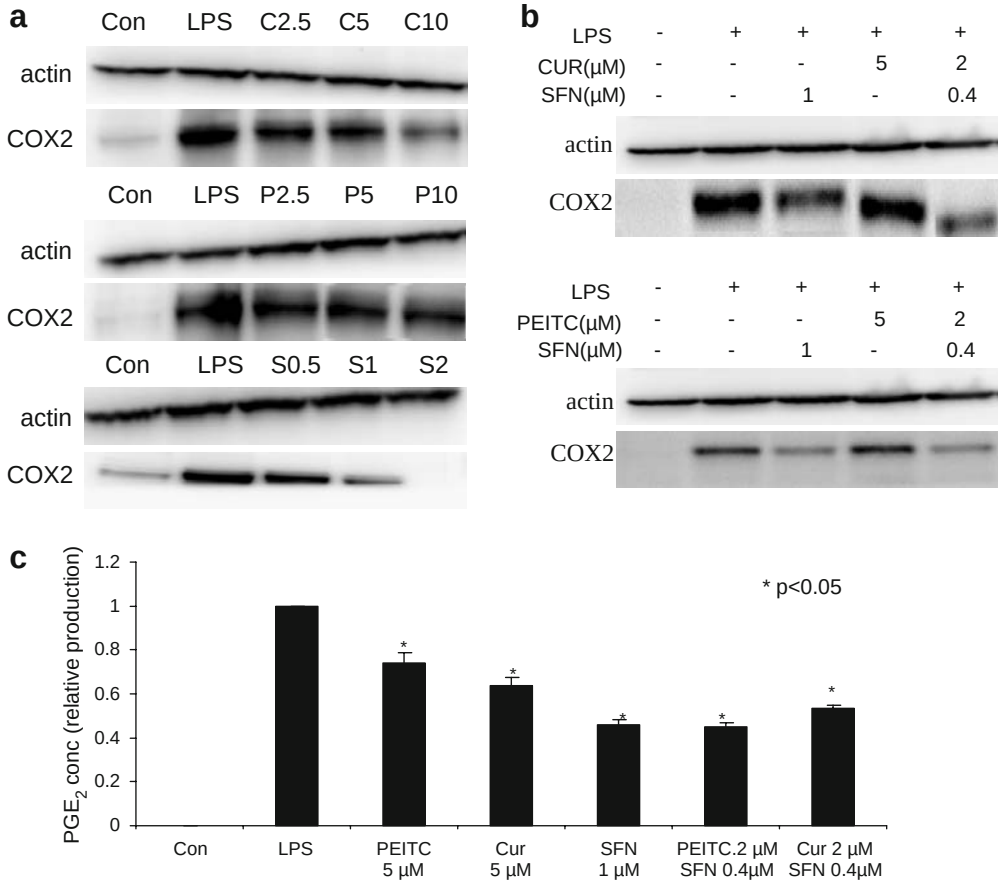

Fig. 3. **A** Dose-dependent inhibition of LPS-induced COX-2 expression by Curcumin, PEITC and SFN. RAW 264.7 cells were pre-treated with PEITC, Curcumin and SFN before LPS treatment. 24 h after treatment, COX-2 expression was detected by Western blotting. Blots are representative of three independent experiments. **B** COX-2 expression with the combinations of CUR + SFN and PEITC + SFN. The proteins were immunoblotted using COX-2 antibody as indicated using actin as the control. Blots are representative of three independent experiments. **C** PGE_2 concentration in the medium with the combinations of CUR + SFN and PEITC + SFN. The experiments were repeated twice with duplicates. Student's *t*-test was used to compare the means between control groups and treated groups $*p<0.05$.

MTS Assay

RAW 264.7 cells were seeded on a 96-well plate cultured in 100 μl medium. Each well was treated with compounds and 20 μl MTS/PMS solution (Promega) was added 24 h after treatment. The plate was incubated for 1 h at 37°C in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was then recorded using an ELISA plate reader.

RESULTS

PEITC + SFN and CUR + SFN Synergistically Inhibit NO Expression of LPS-Stimulated RAW 264.7 Cells

Nitric oxide (NO) assays were performed to determine the IC_{80} of PEITC, CUR and SFN on their inhibition of NO release of RAW 264.7 cells upon LPS (1 μg/mL) stimulation (Fig. 1a). The IC_{80} of PEITC is 5 μM, CUR is 5 μM and SFN is 1 μM, indicating their potency of inhibition of NO expression is: SFN>PEITC = CUR. Isobologram analysis was performed as described previously (15,16). Briefly, 1/2, 1/4, 1/8 of the IC_{80} of PEITC + SFN and CUR + SFN were tested in combination, i.e. (2.5 μM PEITC + 0.5 μM SFN, 1.25 μM PEITC + 0.25 μM SFN, 0.625 μM PEITC + 0.125 μM SFN). We found that at lower doses, combination of PEITC + SFN (1.25 μM/0.25 μM, combination index (CI) <1) or CUR + SFN (0.625 μM/0.125 μM, CI<1) produced better inhibition effect of NO production than the compound was used alone (Fig. 1b). The synergistic effect was not only observed in IC_{80}, but it was also observed in IC_{50} and IC_{90} (CI ranging from 0.2 to 0.8) (data not shown). In addition, using MTS assays, we showed that combination of compounds did not result in higher toxicity (Fig. 1c).

PEITC + SFN and CUR + SFN Synergistically Inhibit iNOS Expression

RAW 264.7 cells were treated with increasing concentration of CUR, PEITC and SFN and LPS-induced iNOS expression was inhibited dose-dependently (Fig. 2a). Next, RAW cells were treated with 5 μM PEITC, 5 μM CUR, 1 μM SFN and in combination (2 μM PEITC + 0.4 μM SFN or 2 μM CUR + 0.4 μM SFN). Consistent with NO assays, SFN alone inhibited the expression of iNOS better than PEITC or CUR. In addition, when combined with PEITC or CUR at low doses, the reduction was even more pronounced than SFN alone (Fig. 2b).

PEITC + SFN and CUR + SFN Synergistically Inhibit COX-2 Expression and PGE_2

Apart from iNOS, another inflammatory gene COX-2 was investigated and similar observation was found. CUR, PEITC and SFN dose-dependently inhibited LPS-induced COX-2 expression (Fig. 3a). CUR (5 μM) or PEITC (5 μM) alone did not cause substantial reduction in COX-2 expression, but CUR (2 μM) + SFN (0.4 μM) strongly inhibited the COX-2 expression up to 50% (Fig. 3b). PEITC (2 μM) in combination with SFN (0.4 μM) showed additive effect in the inhibition rather than synergistic (Fig. 3b). Since there is synergistic effect of CUR + SFN and PEITC + SFN in inhibition of COX-2 expression, we expect the downstream

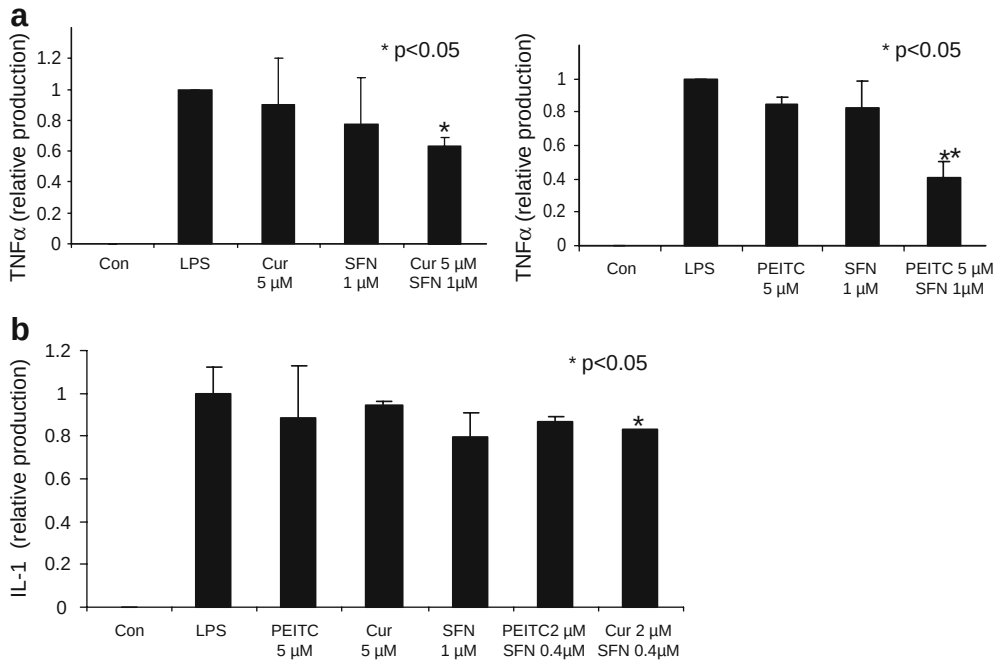

Fig. 4. **A** TNFα expression with the combinations of CUR + SFN and PEITC + SFN. TNFα assays are representative of three independent experiments with replicates. Student's *t*-test was used to compare the means between the control groups and treated groups $*p<0.01$ **B** IL-1 concentration in the medium with the combinations of CUR + SFN and PEITC + SFN. The experiments were repeated twice with duplicates. Student's *t*-test was used to compare the means between control groups and treated groups $*p<0.05$.

inflammatory signaling molecule PGE_2 to be synergistically inhibited as well. Indeed, the PEITC + SFN combination showed synergistic effect while CUR + SFN showed additive effect in downregulation of PGE_2 production (Fig. 3c).

PEITC + SFN and CUR + SFN Synergistically Inhibit TNFα and IL-1

Extensive studies have been conducted and concluded that cytokines like TNFα and IL-1 are the major products of inflammatory cells and contribute to the progression of cancer. We showed that CUR (5 μM) or PEITC (5 μM) alone did not produce any significant effect but CUR (2 μM) + SFN (0.4 μM) and PEITC (2 μM) + SFN (0.4 μM) could suppress TNF release up to 40% and 60% (Fig. 4a). This again confirms the synergistic effect of CUR + SFN in inhibiting inflammation. On the other hand, the individual drug's inhibition effect on IL-1 is limited. Nonetheless, CUR + SFN significantly inhibited IL-1 release to up to 20% (Fig. 4b).

PEITC + SFN and CUR + SFN Do Not Synergistically Inhibit Inflammatory mRNA Expression, but Phase II Genes are Synergistically Induced

Analysis of mRNA expression showed SFN alone inhibited iNOS, COX2, TNF, IL-1 mRNA expression, and combinations did not produce better effect than SFN alone (Fig. 5a). On the other hand, SFN alone did not induce HO-1 and NQO-1 mRNA to a great extent. However, PEITC + SFN and CUR + SFN combinations synergistically induced HO-1 and NQO-1 mRNA (Fig. 5b). Transfection with dominant negative Nrf2 knock-down the HO-1 protein expression by 40% in RAW 264.7 cells (Fig. 5c). Transfection with dominant negative Nrf2 resulted to an increase in iNOS and COX-2 protein expression at 24 h but an increase in their mRNA at 6 h was not observed (Fig. 5c). Applying antioxidant glutathione (GSH) to the cells 6 h after LPS treatment significantly attenuated iNOS protein expression (Fig. 5d).

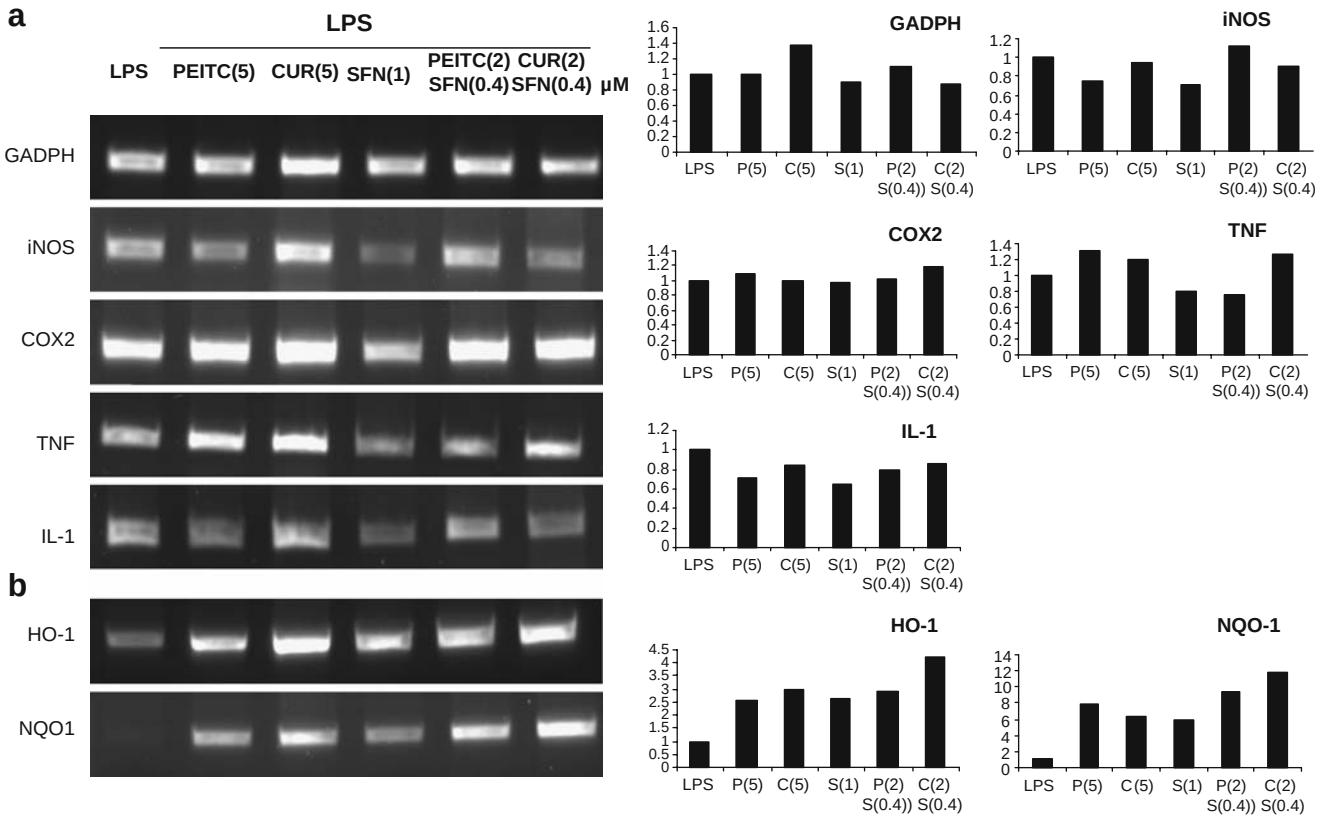

Fig. 5. **A** mRNA expression level of inflammatory genes with the combinations of CUR + SFN and PEITC + SFN. mRNA were extracted 6 h after treatments. GADPH, iNOS, COX-2, TNF and IL-1 mRNA levels were measured. Results are representative of three independent experiments. **B** mRNA expression level of phase II genes with the combinations of CUR + SFN and PEITC + SFN. mRNA were extracted 6 h after treatments and HO-1 and NQO1 mRNA levels were measured. Results are representative of three independent experiments. **C** iNOS, COX-2 mRNA and protein in wild type and dominant negative Nrf2-RAW 264.7 cells. RAW 264.7 cells were treated with empty vector (control), LPS and LPS + dominant negative Nrf2 (dn–Nrf2). iNOS, COX-2, TNF and IL-1 mRNA were extracted at 6 h after treatment. In addition, HO-1, iNOS and COX-2 proteins were extracted at 24 h after LPS treatment. The experiments were repeated twice with duplicates. The relative expression ratio were shown as well. **D** Antioxidant effect in downregulation of iNOS protein. 1 μM glutathione (GSH) was added 6 h after LPS treatment and iNOS protein was blotted 24 h later. The experiments were repeated twice with duplicates.

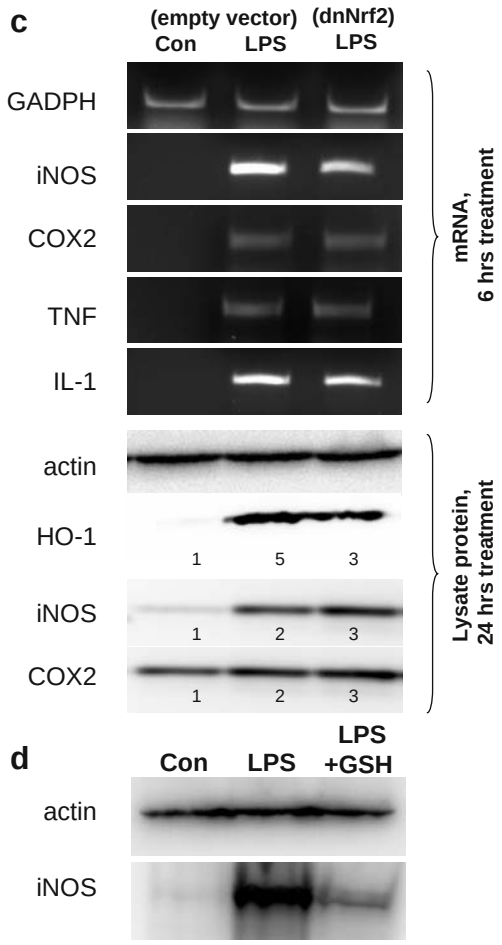

Fig. 5. (continued)

DISCUSSION

Chou *et al*. reported that "The use of multiple drugs may target multiple targets, multiple subpopulations, or multiple diseases simultaneously. The use of multiple drugs with different mechanisms or modes of action may also direct the effect against single target or a disease and treat it more effectively. The possible favorable outcomes for synergism include 1) increasing the efficacy of the therapeutic effect, 2) decreasing the dosage but increasing or maintaining the same efficacy to avoid toxicity, 3) minimizing or slowing down the development of drug resistance, and 4) providing selective synergism against target (or efficacy synergism)" (15). In fact, drug combinations have been widely used and become the choice for treating cancer, immunosuppressant and cardiovascular diseases (15). Our lab has recently showed that there is a synergistic effect of a combination of sulforaphane and (−) Epigallocatechin-3-gallate in HT-29 transfected with AP-1 (16). Our *in vivo* studies showed that SFN and dibenzoylmethane (DBM) combination are potent regimen for chemoprevention of gastrointestinal cancer (17).

Several studies have shown that CUR can activate Nrf2-ARE signaling pathways leading to induction of phase II and antioxidant enzymes such as (glutathione-S transferase) GST and heme-oxygenase1 (HO-1) (18,19). SFN, on the other hand significantly inhibited NF-κB transcriptional activity, nuclear transloction of p65, and gene expression of NF-κB-regulated genes in human prostate PC-3 C4 cells stably transfected with NF-κB-luciferase reporter gene (20).

Our aim in the current study is to determine if the use of multiple drugs with different mechanisms or modes of action can treat inflammation more effectively. Our data clearly shows that CUR + SFN or PEITC + SFN combinations effectively and synergistically inhibited inflammation induced by LPS in RAW 264.7 cells as evidence by the decrease in iNOS, COX-2 protein expression and NO, PGE_2, TNFα and IL-1 production in the medium. Generally, PEITC + SFN or CUR + SFN works better than alone even at a lower dose. The whole spectrum of inflammatory markers was inhibited only when the drugs are administered together. Our data showed that SFN was most effective in inhibiting iNOS protein expression and CUR most effective in inducing Nrf-2 genes such as HO-1 and NQO-1. CUR + SFN showed most synergistic effect (PEITC + SFN to a lesser extent) in reducing iNOS and its related product NO. This combination almost abrogated the iNOS enzyme expression completely in LPS-stimulated RAW 264.7 cells and a very high inhibitory effect of NO release was observed even at a combination of very low doses. This could be very significant in inhibiting inflammation and possibly cancer as NO is the major product in cells that results in oxidative stress and cause damage to lipid, DNA and proteins in the cell.

Cytokines contributes to the microenvironment at the cancer sites. It is believed that TNFα, IL-1, IL-6 act as autocrine and paracrine signals, binding to receptors of cells and trigger the uncontrolled proliferation of the cells (21). Cytokines are one of the reasons why infiltrated macrophages could cause cancer in the infected sites. High level of cytokines is released into the environment, initiates and promotes tumorigenesis (22). Our results showed that the combinations of drugs synergistically inhibited TNFα and IL-1 production in the medium.

To gain further information about the mechanism of synergistic effect of CUR + SFN, we measured the mRNA level of the inflammatory markers and phase II genes. Our results showed that PEITC had a moderate effect on reducing the inflammatory gene transcription and CUR had no effect at all. In contrast, induction of Nrf2 regulated genes HO-1 and NQO1 by SFN was minimal, PEITC had moderate effect and CUR had a very strong induction effect. A few studies have suggested that phase II genes expression is essential in reducing inflammation (23,24). Therefore, it is possible that CUR + SFN inhibit inflammation by two distinct pathways, namely induction of phase II genes and inhibition of inflammatory genes. Surprisingly, CUR + SFN did not synergistically reduce inflammatory genes (TNFα, iNOS, COX-2 mRNA) compared to when they were used alone (Fig. 5a). Rather, the effect seems to be additive. In contrast, the synergistic effect is obvious for phase II genes HO-1 and NQO-1 (Fig. 5b). We next investigated why the combinations reduced iNOS, COX-2 synergistically at the protein level but not at mRNA level. Our results provide a possible mechanism for the synergistic effect shown in protein level. Raw 264.7 cells transfected with dominant-negative Nrf2 knockdown the inducible HO-1 level by LPS. We found that iNOS, COX-2 mRNA at 6 h after LPS treatment was not higher in

HO-1 knock-down RAW 264.7 cells than in the wild type. However at 24 h after LPS treatment, the iNOS and COX-2 protein expression is higher in HO-1 knockdown RAW 264.7 cells (Fig. 5c). This suggests that the expression of HO-1 is crucial in preventing LPS-induced iNOS and COX-2 protein expression. It is suggested that HO-1 can increase the antioxidant status by the formation of the very strong antioxidant bilirubin (25,26) and that a higher cellular antioxidant level can prevent LPS or TNFα induced inflammation (27). In fact, our result showed that addition of glutathione (GSH) 6 h after LPS treatment reduced iNOS and COX-2 protein expression (Fig. 5d). Taken all these together, we conclude that CUR + SFN synergistically up-regulated HO-1, leading to a higher cellular antioxidant capacity and therefore reduced iNOS and COX-2 protein expression and their related inflammatory molecules. In conclusion, our study shows that utilizing two different pathways regulating inflammation, we could achieve a better regimen to target inflammation and its related diseases.

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