

Nuclear Factor κ B Is a Molecular Target for Sulforaphane-mediated Anti-inflammatory Mechanisms*

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Elke Heiss, Christian Herhaus, Karin Klimo, Helmut Bartsch, and Clarissa Gerhäuser‡

From the Deutsches Krebsforschungszentrum Heidelberg, Division of Toxicology and Cancer Risk Factors, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Sulforaphane (SFN), an aliphatic isothiocyanate, is a known cancer chemopreventive agent. Aiming to investigate anti-inflammatory mechanisms of SFN, we here report a potent decrease in lipopolysaccharide (LPS)-induced secretion of pro-inflammatory and pro-carcinogenic signaling factors in cultured Raw 264.7 macrophages after SFN treatment, i.e. NO, prostaglandin E₂, and tumor necrosis factor α . SFN did not directly interact with NO, nor did it inhibit inducible nitric-oxide synthase enzymatic activity. Western blot analyses revealed time- and dose-dependent reduction of LPS-induced inducible nitric-oxide synthase as well as Cox-2 protein expression, which was suppressed at the transcriptional level. To reveal the target of SFN beyond its anti-inflammatory action, we performed electrophoretic mobility shift assay analyses of transcription factor-DNA binding. Consequently, nuclear factor κ B (NF- κ B), a pivotal transcription factor in LPS-stimulated pro-inflammatory response, was identified as the key mediator. SFN selectively reduced DNA binding of NF- κ B without interfering with LPS-induced degradation of the inhibitor of NF- κ B nor with nuclear translocation of NF- κ B. Because SFN can interact with thiol groups by dithiocarbamate formation, it may impair the redox-sensitive DNA binding and reactivation of NF- κ B. Sulforaphane could either directly inactivate NF- κ B subunits by binding to essential Cys residues or interact with glutathione or other redox regulators like thioredoxin and Ref-1 relevant for NF- κ B function. Our data provide novel evidence that anti-inflammatory mechanisms contribute to sulforaphane-mediated cancer chemoprevention.

butane: CH₃S(O)(CH₂)₄-N=C=S) is a naturally occurring cancer chemopreventive agent found as a precursor glucosinolate in cruciferous vegetables like broccoli (1). Chemoprevention involves preventing, delaying, or reversing carcinogenesis by intervention with nontoxic compounds, synthetic chemicals, or natural compounds before malignancy (2). With this respect, SFN has been shown to prevent 7,12-dimethylbenz[*a*]anthracene-induced preneoplastic lesions in mouse mammary glands (3) and rat mammary tumorigenesis (4). These effects were initially attributed to modulation of carcinogen metabolism by monofunctional induction of phase II detoxication enzymes and glutathione (GSH) levels (3) and by inhibition of human and rat cytochromes P-450 and benzo[*a*]pyrene-DNA binding (summarized in Ref. 5). Recently, additional mechanisms indicative of prevention of carcinogenesis at various stages have been reported, including inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase activity, induction of cell differentiation, initiation of cell cycle arrest, and apoptosis in human colon cancer cells (6, 7). Subsequently, SFN was demonstrated to inhibit azoxymethane-induced aberrant crypt foci in rat colon as a further indication for its potential to prevent colon cancer (8).

So far, no data were available regarding anti-inflammatory effects of SFN, although chronic inflammation and carcinogenesis are thought to be mechanistically linked (9). Chronic inflammation and infections lead to the up-regulation of a series of enzymes and signaling proteins in affected tissues and cells. These pro-inflammatory enzymes, including the inducible forms of nitric-oxide synthase (iNOS) and cyclooxygenase (Cox-2), responsible for the elevated levels of NO and prostaglandins (PGs), respectively, are known to be involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson's and Alzheimer's disease, and colon cancer (10–14). The constitutive epithelial and neuronal forms of nitric-oxide synthase contribute relatively little to either inflammation or carcinogenesis. On the other hand, iNOS plays an important role in the inflammatory response of tissues to injury and infectious agents. Although iNOS provides a benefit to the organism in terms of immune surveillance, aberrant or overproduction of NO has been implicated in the pathogenesis of cancer *via* reactive nitrogen oxide species-mediated reactions like nitrosative deamination of DNA bases, lipid peroxidation, and DNA strand breaks (15, 16). Elevated levels in the expression of the inducible Cox-2 have been detected in various tumor types and may account for excessive PG production (17). In addition to their role as pro-inflammatory mediators, PGs were demonstrated to suppress immune functions, to inhibit apoptosis, to enhance proliferation, and to increase the invasiveness of cancer cells (18–20). Consequently, inhibition of expression and enzymatic activity of Cox-2 and down-regulation of PG levels is regarded as a rational and feasible strategy in cancer chemoprevention with first positive results in human trials

Sulforaphane (SFN)¹ (1-isothiocyanato-(4*R*)-(methylsulfinyl)

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‡ To whom requests for reprints should be addressed: DKFZ Heidelberg, C0202 Chemoprevention, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. Tel.: 49-6221-42-33-06; Fax: 49-6221-42-33-59; E-mail: c.gerhauser@dkfz.de.

¹ The abbreviations used are: SFN, sulforaphane; AP-1, activator protein 1; Cox, cyclooxygenase; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehydephosphate dehydrogenase; GSH, glutathione; I κ B, inhibitor of NF- κ B; iNOS, inducible nitric-oxide synthase; NF- κ B, nuclear factor κ B; LPS, lipopolysaccharide; PG, prostaglandin; Ref-1, redox factor-1; TNF- α , tumor necrosis factor α ; TRX, thioredoxin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RT, reverse transcriptase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; IL, interleukin; ODC, ornithine decarboxylase; C/EBP, CCAAT/enhancer-binding protein.

(21). TNF- α and other inflammatory cytokines were shown to stimulate tumor promotion and progression of initiated cells as well as of preneoplastic lesions (22). Recently, Fujiki *et al.* presented evidence that tumor promotion in TNF- α (-/-) knock-out mice was significantly suppressed in comparison with TNF- α (+/+) mice (23). Thus, TNF- α can be considered as an endogenous tumor promoter and a central mediator in cancer development.

For our studies, we have used the murine macrophage cell line Raw 264.7, which can be stimulated with bacterial lipopolysaccharides (LPS) to mimic a state of infection and inflammation. In this report, we summarize novel anti-inflammatory mechanisms mediated by SFN based on the inhibition of LPS-mediated induction of iNOS, Cox-2, and TNF- α . We have analyzed the mechanism of the observed inhibition of iNOS induction by RT-PCR and EMSA analyses of transcription factor-DNA binding and have identified nuclear factor κ B (NF- κ B) as an important target. These anti-inflammatory properties of SFN may contribute to its chemopreventive potential.

EXPERIMENTAL PROCEDURES

Chemicals—All cell culture media and supplements were obtained from Life Technologies, Inc. Fetal bovine serum was from Greiner Labortechnik GmbH (Frickenhausen, Germany). Primary antibodies for iNOS (sc-650), Cox-2 (sc-1747), p65 (sc-109A), p50 (sc-114X), I κ B- α (sc-371), I κ B- β (sc-945), and horseradish peroxidase-conjugated secondary antibodies (sc-2004 and sc-2020) and consensus oligonucleotides for NF- κ B (sc-2505), AP-1 (sc-2501), and C/EBP β (sc-2525) were obtained from Santa Cruz (Heidelberg, Germany). Alexa 488-coupled fluorescent anti-rabbit antibody used for immunocytochemistry and SIN-1 were obtained from Molecular Probes (Möbitec, Göttingen, Germany). Vectashield was from Alexis (Grünberg, Germany). γ -³²P-labeled ATP was purchased from ICN (Costa Mesa, CA). BioTrak TNF- α enzyme-linked immunosorbent assay kit was obtained from Amersham Pharmacia Biotech. A prostaglandin screen colorimetric kit was purchased from Cayman Chemical Company (Ann Arbor, MI). RNAClean used for RNA extraction was from Hybaid AGS (Heidelberg, Germany), and an Advantage RT for PCR kit and Advantage cDNA and Amplimer kits for iNOS and GAPDH were purchased from CLONTECH (Heidelberg, Germany). All materials, equipment, and biotinylated marker proteins for gel electrophoresis were from Bio-Rad. All other chemicals were obtained from Sigma.

Synthesis of SFN—SFN was synthesized following the method of Kim and Yi (24). 300 mg (2.22 mmol) of (\pm)-1-amino-4-(methylsulfinyl)butane, obtained according to Ref. 25 in 98% yield, were dissolved in 10 ml of CH₂Cl₂, and 515 mg (2.22 mmol) of 1,1'-thiocarbonyldi-2(1H)-pyridone were added at room temperature. The reaction mixture was stirred for 1 h and then washed with saturated aqueous NaHCO₃ solution. The aqueous phase was extracted with 3 \times 30 ml of CH₂Cl₂. The organic fractions were combined and dried over Na₂CO₃, and the solvent was removed *in vacuo*. Distillation yielded 250 mg (64%) of a pale yellow oil. For ¹H nuclear magnetic resonance (in CDCl₃), δ : 1.83–2.02 (m, 4H), 2.59–2.8 (m, 2H), 2.61 (s, ³H), 3.62 (t, J = 6.0 Hz, 2H) (Bruker AC-250-Spectrometer). For ¹³C NMR (CDCl₃), δ : 20.0, 28.9, 38.6, 44.5, 53.4 (Bruker AC-250-Spectrometer). Formula: C₆H₁₁NOS₂. Analysis: Calculated C 40.65, H 6.25, N 7.90, S 36.17; Found: C 40.77, H 6.50, N 7.96, S 36.39 (Carlo Erba EA 1108-Elemental Analyzer).

Cell Culture—Raw 264.7 murine macrophages were obtained from the Deutsches Krebsforschungszentrum (Heidelberg, Germany) and maintained in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, and 250 ng/ml amphotericin B, supplemented with 10% fetal bovine serum under endotoxin-free conditions at 37 °C in a 5% CO₂ atmosphere. Unless otherwise indicated, the cells were preincubated in Dulbecco's modified Eagle's medium containing fetal bovine serum for 24 h. Then the medium was replaced by serum-free Dulbecco's modified Eagle's medium, and LPSs (from *Escherichia coli*, serotype O111:B4) were added at a final concentration of 500 ng/ml.

Inhibition of LPS-mediated iNOS Induction, PGE₂ Production, and TNF- α Secretion—Raw macrophages were plated at a density of 2 \times 10⁵ cells/well in 96-well plates and incubated overnight. The cells were treated with test compounds and LPS for 24 h. NO production was determined *via* quantitation of nitrite levels in cell culture supernatants according to the Griess reaction and compared with a nitrite standard curve (26). The amount of secreted PGE₂ was determined in

1:2000 diluted cell culture supernatants employing a prostaglandin screen colorimetric assay kit (Cayman) according to the manufacturer's protocol. The amount of secreted TNF- α was measured in 1:10 diluted cell culture supernatants using the BioTrak TNF- α mouse enzyme-linked immunosorbent assay system (Amersham Pharmacia Biotech). Effects on cell growth were estimated by sulforhodamin B staining (27). IC₅₀ values (half-maximal inhibitory concentration) of LPS-induced nitrite production, PGE₂, or TNF- α secretion were generated from the results of eight serial 2-fold dilutions tested in duplicate.

Inhibition of LPS-induced iNOS Enzyme Activity—Raw macrophages were grown in 75-cm² tissue culture flasks to 60–70% confluence, and iNOS expression was induced by treatment with LPS for 12 h. The cells were washed twice with PBS, harvested by scraping, plated into 96-well plates (2 \times 10⁵ cells/well), and incubated in the presence or absence of SFN or *N*-monomethyl-L-arginine, a known inhibitor of NOS enzyme activity, for another 12 h without further stimulation by LPS. Cell viability was measured by the MTT assay (28). In addition, iNOS enzymatic activity was measured in cell lysates essentially as described by Vodovotz *et al.* (29).

Western Blot Analyses—Raw macrophages were plated in 60-mm tissue culture dishes (2.5 \times 10⁶ cells/5 ml) and treated as indicated in the figure legends. The cells were washed with PBS and lysed with boiling 2 \times standard lysis buffer. Protein was determined using the BCA method (30) after precipitation with cold 10% TCA. Total protein (15–40 μ g/lane) was electrophoresed on a 7% (iNOS, Cox-2), 10% (p50 and p65), or 12% (I κ B) reducing SDS-polyacrylamide gel under standard conditions and electroblotted to polyvinylidene difluoride membranes in 20% methanol, 25 mM Tris, and 192 mM glycine. Equal protein loading per lane was ensured by staining either a duplicate gel before blotting or the membrane after blotting. The membranes were blocked with 1% nonfat milk in Tris-buffered saline (10 mM Tris, pH 7.4, 100 mM NaCl) containing 0.01% Tween 20 overnight at 4 °C and incubated with primary antibody (1:400 dilution for I κ B- α and I κ B- β and 1:2500 dilution for Cox-2, iNOS, p50, and p65 in 1% nonfat milk in Tris-buffered saline) for 1 h at 37 °C or overnight at 4 °C. After thorough washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (1:2500) and streptavidin-horseradish peroxidase (1:2500) for 30 min at 37 °C. The membranes were developed using a chemiluminescence system. For quantitation of protein expression, densitometric scans of the obtained autoradiographs were analyzed using the Lucia G software (Nikon, Düsseldorf, Germany).

RT-PCR—Total RNA from 5 \times 10⁶ Raw macrophages (treated as indicated) was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method using RNAClean. 1 μ g of RNA was transcribed into cDNA using the Advantage RT for PCR Kit (CLONTECH). cDNAs of iNOS and GAPDH, respectively, were amplified with the Advantage cDNA and Amplimer Kits (CLONTECH) for 35 cycles of 45 s at 94 °C, 45 s at 65 °C, and 2 min at 72 °C followed by an extension at 72 °C for 7 min. PCR products were separated on 1.8% agarose gels and visualized by ethidium-bromide staining.

Northern Blot Analysis of ODC mRNA Expression—Raw cells were grown in 60-mm cell culture dishes (2.5 \times 10⁶ cells/5 ml) and treated with LPS and SFN as indicated. Total RNA was isolated with RNAClean (Hybaid-AGS). RNA samples (20 μ g/lane) were separated by electrophoresis on a 1.2% agarose gel containing 6.7% formaldehyde according to Sambrook *et al.* (31) and vacuum-blotted to a nylon membrane (Zeta Probe GT, Bio-Rad) in 20 \times SSC. The membrane was baked at 80 °C for 1 h and prehybridized in 0.25 M Na₂HPO₄, pH 7.2, containing 7% SDS. Hybridization was performed at 65 °C for 18 h with an ODC c-DNA probe labeled with [α -³²P]dCTP using a Random Primer labeling kit (Stratagene). After thorough washing with washing solution (20 mM Na₂HPO₄, pH 7.2, 5–1% SDS depending on stringency), the membrane was exposed to x-ray film.

Electrophoretic Mobility Shift Assay—Raw macrophages were plated in 60-mm dishes (2.5 \times 10⁶ cells/5 ml). The cells were treated with SFN at the indicated concentrations and stimulated with LPS for 45 min (NF- κ B and AP-1) or up to 4 h (CEBP β), washed once with PBS, scraped into 0.5 ml cold PBS, and pelleted by centrifugation. Cytosolic and nuclear protein fractions were extracted as described previously (32). Binding reactions were performed at 37 °C for 15 min in 20 μ l of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5 or 1 μ g of poly(dI-dC), 1 mM dithiothreitol, and 30,000 cpm ³²P-labeled oligonucleotide probes for NF- κ B, AP-1, and C/EBP β . DNA-protein complexes were separated from unbound DNA probe on native 6% polyacrylamide gels at 75V in 0.5 \times TBE buffer. The gels were vacuum-dried for 1 h at 80 °C and exposed to x-ray film at -80 °C for 4 to 24 h. For *in vitro* binding studies, nuclear protein from LPS-stimulated Raw 264.7 macrophages was incubated with SFN or

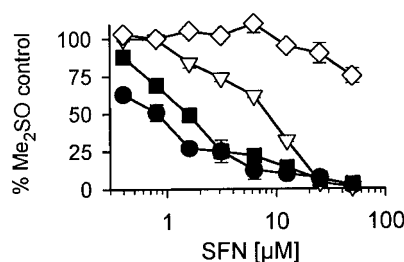


FIG. 1. **Inhibition of LPS-induced generation of NO, PGE₂, and TNF- α .** Raw macrophages were treated with SFN or Me₂SO and stimulated with LPS for 24 h. Nitrite, as a measure of NO production (●), secreted PGE₂ (■), and TNF- α (▽), was determined in cell culture supernatants (levels in unstimulated controls: 0.45 \pm 0.17 nmol nitrite/ml, n = 5; 0.25 \pm 0.1 ng PGE₂/ml, n = 2; <0.5 ng TNF- α /ml, n = 2; after LPS stimulation: 29.1 \pm 4.1 nmol nitrite/ml, n = 5; 5.9 \pm 0.5 ng PGE₂/ml, n = 2; 18.2 \pm 3.0 ng TNF- α /ml, n = 2). Effects of SFN on cell growth were measured by SRB staining (◇).

SFN and mercaptoethanol, respectively, for 75 min at room temperature before the labeled probe was added, and binding reaction and electrophoresis were conducted as described above.

Immunofluorescent Detection of NF- κ B Localization—Raw macrophages were grown on glass coverslips overnight. Cells were treated with Me₂SO alone, Me₂SO and LPS (500 ng/ml), or 20 μ M SFN and LPS (500 ng/ml), respectively, for 45 min. The cells were fixed with ice-cold acetone for 2 min, rinsed in PBS, and permeabilized with 0.4% Triton X-100 in PBS. Unspecific binding sites were blocked with 5% bovine serum albumin in PBS. After incubation with anti-p65 and anti-p50, respectively, overnight at 4 °C and washing with PBS, the Alexa 488-conjugated secondary antibody was applied for 1 h at room temperature. 4',6-Diamidino-2-phenylindole dihydrochloride-stained and Vectashield-mounted cells were analyzed under a Zeiss fluorescence microscope. Digital images were acquired with an AxioCam color digital camera and processed with the Axiovision Rel. 2.05 software package (Carl Zeiss, Göttingen, Germany).

Determination of GSH Levels—Raw macrophages were seeded in 96-well plates (1 \times 10⁵ cells/well). SFN (final concentration, 0.4–50 μ M) was added 1–24 h prior to determination of total GSH and protein levels. The plates were washed three times with PBS and stored at –80 °C until analyzed. GSH was measured essentially as described previously (3) and normalized to protein concentrations determined using the BCA method (30).

RESULTS

The aim of this study was to analyze potential anti-inflammatory properties of SFN and to elucidate the underlying mechanisms of action. We used the murine macrophage cell line Raw 264.7, which releases NO, PGs, and pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, and IL-12 upon stimulation with LPS, thus providing a suitable model for studying inflammatory response in cultured cells.

Inhibition of LPS-mediated NO Generation, PGE₂ Production, and TNF- α Secretion—Treatment of Raw macrophages with SFN caused a dose-dependent inhibition of LPS-induced NO generation (measured *via* nitrite levels in cell culture supernatants by the Griess reaction), PGE₂ production and TNF- α secretion with apparent IC₅₀ values of 0.7, 1.4, and 7.8 μ M, respectively (Fig. 1). The inhibition of these processes was not due to reduction of cell viability. To exclude direct NO-scavenging potential of SFN or interference with the Griess reaction, SFN was incubated for 3 h at room temperature with the NO-donor SIN-1 (7.5 mM in H₂O), which spontaneously releases NO under slightly alkaline conditions *in vitro*. In a concentration range of 0.02–2.5 mM, nitrite levels of SFN-treated samples were similar to that of the Me₂SO solvent control (Fig. 2).

iNOS Enzyme Activity—Focussing on the effect of SFN on NO production, we analyzed whether the inhibitory effect of SFN was due to inhibition of iNOS enzymatic activity. SFN at concentrations of 25 and 50 μ M reduced NO levels in a cellular

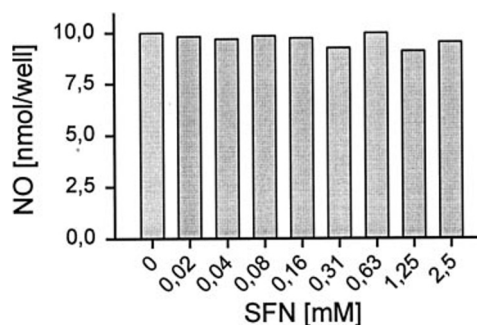


FIG. 2. **Determination of NO scavenging activity of SFN.** SFN in a concentration range of 0–2.5 mM was incubated for 3 h at room temperature with 7.5 mM of the NO donor SIN-1 in H₂O. The nitrite levels were determined *via* the Griess reaction.

assay by 20 and 25% in comparison with the control; however, lowered nitrite levels were attributed to a concomitant reduction in cell viability rather than enzyme inhibition (Fig. 3, *left panel*). In contrast, *N*-monomethyl-L-arginine, an enzyme substrate analogue, inhibited iNOS enzyme activity by a maximum of 60% in a concentration range of 0.8–100 μ M without any signs of toxicity (Fig. 3, *right panel*). These findings were confirmed in a cell-free assay using lysates of LPS-stimulated Raw macrophages as a source of iNOS and L-arginine as a substrate (specific activity, 8 nmol nitrite/mg/h). Nitrite levels were reduced 8.8% by 20 μ M SFN and 46.2% by 100 μ M *N*-monomethyl-L-arginine, respectively.

Influence on Protein and mRNA Expression—A potential inhibitory influence of SFN on LPS-stimulated protein expression was measured by Western blot analyses. SFN suppressed LPS-mediated up-regulation of iNOS protein in a time-dependent manner (Fig. 4A). In the Me₂SO control, iNOS levels were detectable as early as 6 h after LPS addition, whereas 5 μ M SFN completely inhibited iNOS induction at all time points. Similar results were obtained when LPS-mediated Cox-2 expression was measured. Dose-dependent effects of SFN were analyzed 12 h post LPS stimulation (Fig. 4C). In good correlation with the reduction of NO release, 0.8 and 1.5 μ M SFN significantly lowered iNOS protein levels by 27 and 61% in comparison with the control. LPS-induced Cox-2 expression was also inhibited in a dose-dependent manner, whereas TPA-mediated Cox-2 expression in Raw macrophages was not affected by SFN (Fig. 4E). When 5 μ M SFN was added at different time points *after* LPS stimulation, inhibitory effects on iNOS and Cox-2 expression and NO production were no longer detectable when the time interval between LPS stimulation and SFN addition exceeded 4 h (Fig. 5). We concluded that SFN targets an early event in the signal transduction pathway between LPS stimulation and protein induction, presumably at the transcriptional level, and analyzed the effects of SFN on iNOS mRNA expression by RT-PCR (Fig. 4, B and D). In close correlation with the results obtained at the protein level, SFN potently down-regulated iNOS mRNA expression in a time- and dose-dependent manner.

Apart from induction of pro-inflammatory proteins, LPS treatment of Raw macrophages has previously been shown to induce levels of ornithine decarboxylase (ODC) (33). ODC as a key enzyme in polyamine synthesis is overexpressed in many tumor types and accounts for enhanced tumor promotion. By Northern blot analyses, we could demonstrate a maximum of ODC mRNA expression in Raw macrophages 5 h after LPS treatment (Fig. 6A). SFN in a concentration range of 0.4–25 μ M was not able to suppress the observed LPS-mediated ODC mRNA induction (Fig. 6B).

Inhibition of NF- κ B DNA Binding Activity *in Vivo*—To further investigate the mechanism of SFN-mediated inhibition of

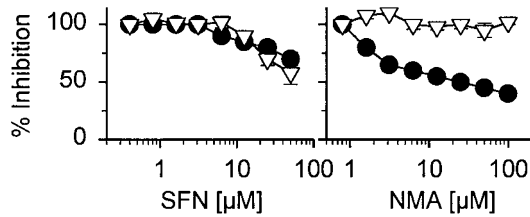


FIG. 3. **Determination of iNOS enzyme activity.** Raw macrophages were stimulated with LPS for 12 h. Then Me₂SO, SFN, or *N*-monomethyl-L-arginine (*NMA*) were added in LPS-free medium. After further incubation for 12 h, the nitrite levels (●) were determined in cell culture supernatants, and inhibitory potential was calculated in comparison with the Me₂SO control (25.8 ± 2.3 nmol nitrite/ml; *n* = 3). Cell viability was measured by the MTT assay (▽).

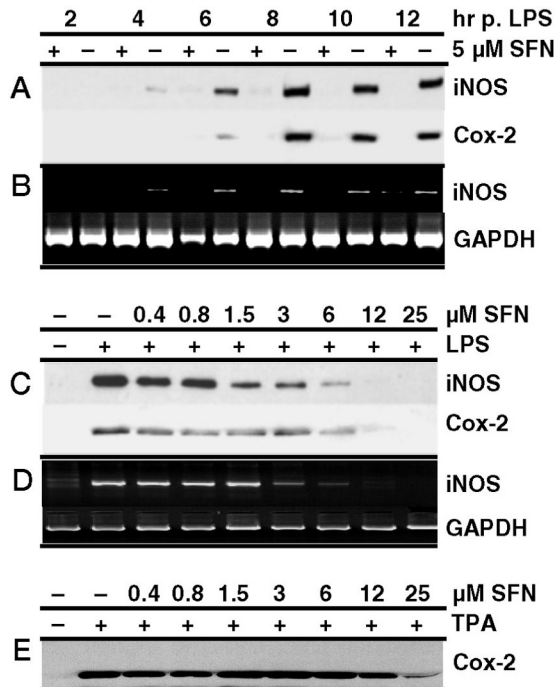


FIG. 4. **Influence on time- and dose-dependent inhibition of iNOS and Cox-2 protein and iNOS mRNA expression.** A and B, time course. Raw macrophages were treated with 5 μM SFN (+) or Me₂SO (-) and stimulated with LPS for 2–12 h as indicated. C and D, dose response. Raw macrophages were treated with Me₂SO (-) or 0.4 to 25 μM SFN, respectively, as indicated and were stimulated with LPS (+) for 12 h. Western blot analyses of iNOS (upper panel) and Cox-2 (lower panel) protein expression (A and C), RT-PCR amplification of iNOS (upper panel) and GAPDH (lower panel) mRNA (B and D). E, Cox-2 induction by TPA. Raw macrophages were treated with Me₂SO (-) or 0.4–25 μM SFN, respectively, and stimulated with 60 ng/ml TPA (+) for 12 h. Whole cell lysates were subjected to Western blot analysis of Cox-2 expression.

iNOS transcription, we focused on transcription factors known to transactivate iNOS, Cox-2, and TNF-α. EMSA analyses demonstrated a selective reduction of NF-κB DNA binding in nuclear extracts obtained from LPS-stimulated Raw macrophages treated with 10 and 20 μM SFN, whereas DNA binding of transcription factors AP-1 and C/EBPβ was not influenced (Fig. 7).

Degradation of IκB—In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor IκB, which upon LPS stimulation is phosphorylated by IκB kinase, ubiquitinated, and rapidly degraded *via* the 26 S proteasome, thus releasing NF-κB (34, 35). SFN (10 μM) had no influence on LPS-induced degradation of IκB-α and IκB-β, constituting the IκB complex. 30 min post LPS-exposure, IκB-α was not detectable in protein lysates of either treated or untreated macrophages. Rather,

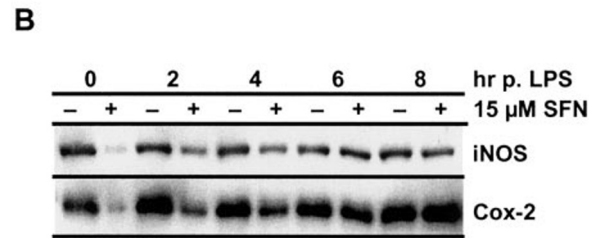
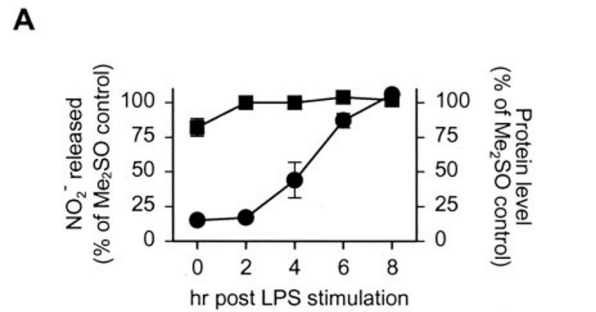


FIG. 5. **Effect of SFN addition at different time points after LPS stimulation.** Raw macrophages were stimulated with LPS (500 ng/ml). SFN (5 μM) was added simultaneously or at time points up to 8 h after LPS exposure as indicated. A, nitrite as a measure of NO production (●) was determined in cell culture supernatants. Effects of SFN on cell growth were measured by SRB staining (■). B, iNOS and Cox-2 protein expression was analyzed by Western blotting.

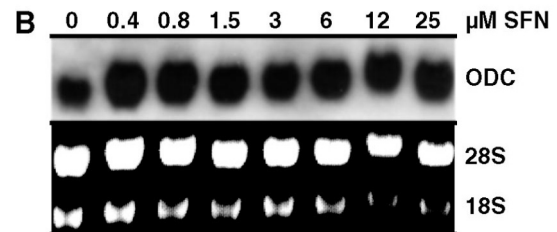
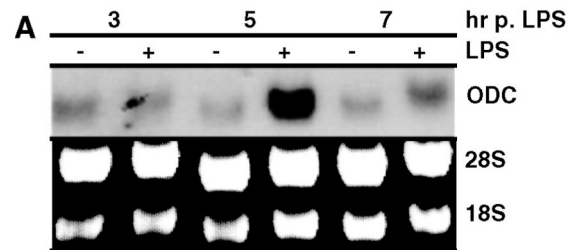


FIG. 6. **Influence on ODC mRNA induction.** A, Northern blot hybridization of RNA samples from cultured Raw macrophages treated with LPS for 3 to 7 h as indicated using an ODC cDNA probe (upper panel). Lower panel, ethidium bromide-stained gel. B, Northern blot hybridization of RNA samples from Raw macrophages using an ODC probe (upper panel). Lower panel, ethidium bromide-stained gel. The cells were treated with LPS (500 ng/ml) and varying concentrations of SFN as indicated for 5 h.

SFN retarded *de novo* synthesis of IκB-α, which reappeared 180 min after LPS stimulation in control samples (Fig. 8A). Because IκB-α transcription is κB-dependent (36), these findings were consistent with SFN-mediated inhibition of NF-κB DNA binding. IκB-β degradation was observed in both SFN-treated and Me₂SO control cells 360 min after LPS stimulation (Fig. 8B).

Nuclear Translocation of NF-κB—The next step to investigate was whether SFN prevented the most prominent subunits of NF-κB, p50 and p65, from translocation to the nucleus after release from IκB. Immunocytochemical detection of p65 re-

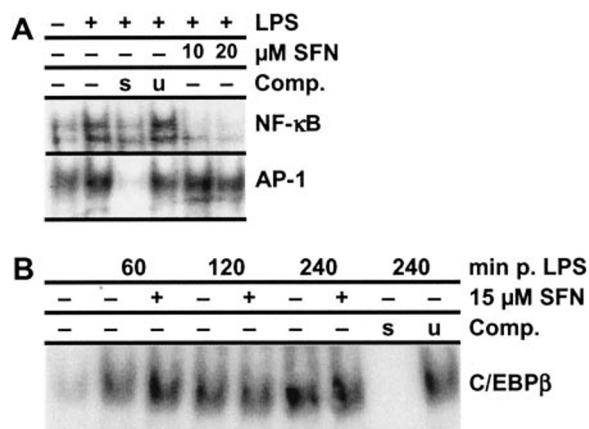


FIG. 7. Electrophoretic mobility shift assay of NF-κB, AP-1, and C/EBPβ DNA-binding capacity. A, raw macrophages were treated with Me₂SO (-) or SFN (as indicated) and stimulated with LPS (+) for 45 min before nuclear protein was isolated. DNA binding was analyzed using specific ³²P-labeled oligonucleotide probes for NF-κB (upper panel) or AP-1 (lower panel). B, cells were treated with Me₂SO (-) or 15 μM SFN (+) and stimulated with LPS for 0–240 min before nuclear protein was isolated and C/EBPβ DNA binding was analyzed. Specificity was demonstrated by co-incubation with a 25-fold excess of unlabeled specific (lane s) or unlabeled probe (lane u; AP-1 for NF-κB, NF-κB for AP-1 and C/EBPβ) for competition (Comp.).

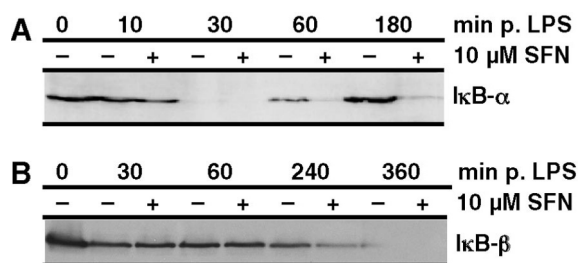


FIG. 8. IκB degradation. Raw macrophages were treated with Me₂SO (-) or 10 μM SFN (+) and stimulated with LPS for the indicated periods of time. Total protein was subjected to Western blot analyses using anti-IκB-α antibody (A) and anti-IκB-β antibody (B).

vealed similar nuclear staining in SFN-treated and control cells after LPS stimulation (Fig. 9A). p50 was localized in cytosol and nuclei of unstimulated cells and was not influenced by LPS stimulation or SFN treatment (Fig. 9B). Western blot analyses with nuclear fractions of correspondingly treated cells and p50- and p65-specific antibodies showed consistent results (Fig. 10).

Inhibition of NF-κB Binding by Exogenous SFN—In addition to preventing NF-κB binding to its consensus sequence in intact cells, SFN exogenously added to nuclear protein was capable of reducing the formation of the DNA-protein complex in a dose-dependent manner (Fig. 11). This inhibitory effect was preventable by preincubation of SFN with an excess of mercaptoethanol, suggesting a thiol-dependent modification of NF-κB subunits by SFN. Identical results were obtained when cytosolic proteins (4–5 μg) of untreated cells, providing the NF-κB-IκB complex, were preincubated in aqueous 0.8% deoxycholate solution containing 1.1% Igepal for 10 min on ice to release active NF-κB dissociated from its inhibitor (data not shown).

Effect of Cellular Glutathione Levels on LPS-induced Nitrite Levels—To further investigate a potential thiol dependence of NF-κB inhibition, we were interested whether changes in cellular GSH levels after pretreatment with SFN for different time periods would modulate SFN-mediated inhibition of iNOS induction. Total GSH levels initially dropped to 43% of control values (75.5 nmol/mg protein) after 4 h of pretreatment with 12.5 μM SFN (Fig. 12A). Then the GSH-depleting effect was

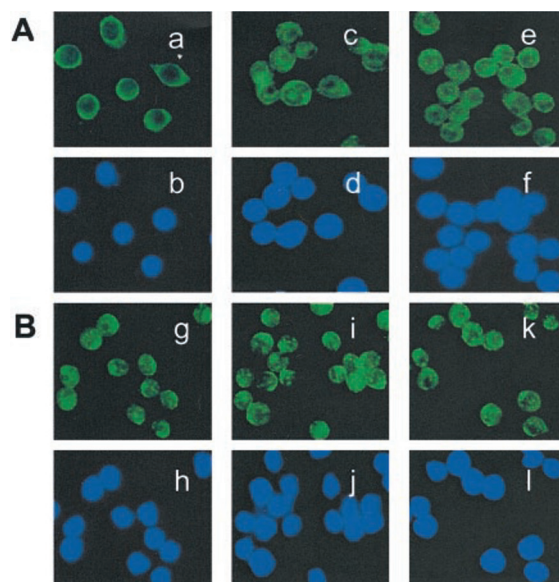


FIG. 9. Immunocytochemical analysis of nuclear translocation of NF-κB. Raw macrophages were grown on coverslips, treated with Me₂SO (panels a–d and g–j) or 20 μM SFN (panels e, f, k, and l), stimulated with LPS for 45 min (panels c–f and i–l), fixed, and subjected to immunocytochemical analysis with anti-p65 (A) or anti-p50 (B) antibody, respectively, and Alexa 488-conjugated secondary antibody (upper panels). Nuclei were stained with DAPI (lower panels). Magnification, 400×.

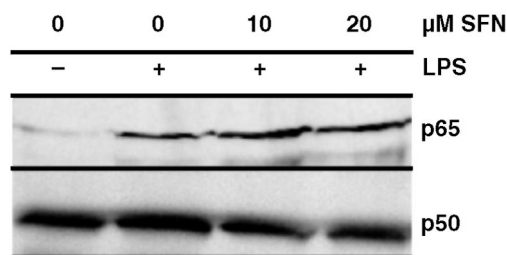


FIG. 10. Western blot analyses of nuclear translocation of NF-κB. Raw macrophages were treated with LPS (500 ng/ml) (+) and 10 or 20 μM SFN, respectively, for 45 min before nuclear protein fractions were subjected to Western blot analyses of p65 (upper panel) and p50 (lower panel) proteins.

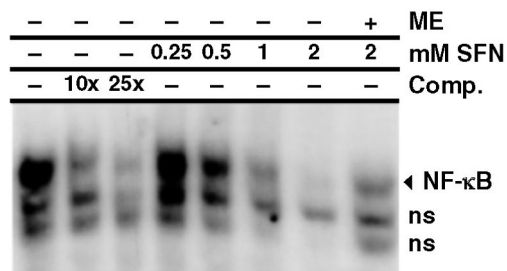


FIG. 11. Inhibition of NF-κB DNA binding by exogenous SFN. Nuclear protein from LPS-stimulated cells was incubated for 75 min with 0-, 10-, or 25-fold excess of specific competitor, 0.25–2.0 mM exogenous SFN as indicated, or 2.0 mM SFN preincubated with 142 mM mercaptoethanol (ME) for 15 min, respectively. The arrow indicates the position of the NF-κB (p50/p65) band. ns, nonspecific binding.

reversed by induction of GSH, resulting in 2-fold elevated levels after 24 h (149.5 nmol/mg protein). When LPS was added under high GSH conditions, the observed IC₅₀ value for SFN-mediated inhibition of NO release was doubled. To relate dose-dependent modulation of GSH levels to the inhibition of NO production, we plotted the values of the area under the (induction or inhibition) curve at each time point against each other and found a significant correlation ($r^2 = 0.96$) (Fig. 12B).

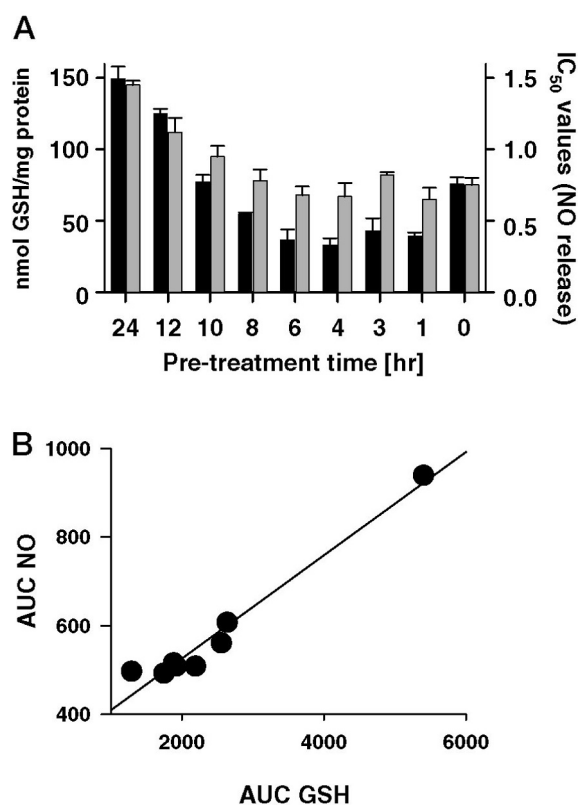


FIG. 12. Effect of cellular GSH levels on LPS-induced nitrite levels. A, Raw macrophages were seeded in three 96-well plates and incubated for 1–24 h with SFN in a concentration range of 0.4–50 μM before total GSH and protein levels were determined at time 0 (total GSH levels in untreated cells at time 0: 76 ± 5 nmol/mg protein, $n = 3$; *black bars*, GSH levels after treatment with 12.5 μM SFN). At time 0, SFN-treated cells were stimulated with LPS for another 24 h, and the IC_{50} values for inhibition of NO release were determined (*gray bars*). B, for each time point, GSH and NO levels were plotted against SFN concentrations. The unprocessed area under the curve (AUC) values for GSH and NO, respectively, were obtained from the area x_{\min} to x_{\max} function in the TableCurve™ XY table status window (Jandel Scientific). For each time point, these values were plotted against each other, and a correlation coefficient of $r^2 = 0.96$ was obtained.

DISCUSSION

The present report provides the first evidence that anti-inflammatory mechanisms could contribute to SFN-mediated cancer chemoprevention. We have demonstrated that SFN down-regulates LPS-mediated induction of iNOS and Cox-2 expression and TNF- α secretion in cultured Raw macrophages. iNOS (and presumably also Cox-2 and TNF- α) induction was repressed at the transcriptional level, and we focused our research on elucidating the underlying molecular mechanisms.

Treatment of Raw macrophages with endotoxins like LPS results in the coordinate activation of a series of signaling mediators, including NF- κB , cAMP, the cJun/cFos heterodimer AP-1, or nuclear factor interleukin-6 (NF-IL6, also known as C/EBP β), transactivating the transcription of LPS-responsive genes after binding to respective binding sites or enhancer elements. As a first approach to identify a target for SFN action, we compared known *cis*-acting elements in the promoter/enhancer region of iNOS (37–39), Cox-2 (40, 41), and TNF- α (literature cited in Ref. 42), assuming that a common LPS-activated nuclear factor might be affected by SFN. We identified binding sites for the NF- κB and C/EBP β families of transcription factors in the promoter regions of all three genes. In macrophages, NF- κB is known to play a critical role in the regulation of genes involved in immune response and to coordinate the expression of pro-inflammatory proteins including

iNOS, Cox-2, and TNF- α (36, 43). C/EBP was initially identified as an IL-1-induced transcription factor (44) and was recently shown to be involved in soluble phospholipase A₂-receptor-dependent Cox-2 up-regulation in NIH3T3 cells (45). Both transcription factors have been reported to interact in the transcriptional regulation of genes (46). EMSA analyses revealed that DNA binding of NF- κB was inhibited by SFN (Fig. 7A), whereas C/EBP β DNA binding was not affected and therefore not further investigated (Fig. 7B). LPS treatment of Raw macrophages has been shown to result in cAMP/cAMP-responsive element-mediated induction of ODC. We excluded a possible interference of SFN with cAMP/cAMP-responsive element-dependent transcription because SFN did not inhibit ODC mRNA induction. This is consistent with our findings that SFN suppresses LPS-mediated gene expression mainly via inhibition of NF- κB activity. The ODC promoter does not contain any known NF- κB binding site, and ODC transcription is therefore not affected by SFN.

To gain more information on the selectivity of SFN for NF- κB , we stimulated Raw macrophages with the tumor promoter TPA. TPA is a known activator of protein kinase C and was shown to up-regulate Cox-2 protein levels in Raw macrophages mainly through AP-1 transactivation (47), without concomitant induction of iNOS. This was ascribed to a lack of Raw macrophages in protein kinase C ϵ essential for activation of NF- κB (48). Under these conditions, SFN did not inhibit Cox-2 protein expression (Fig. 4E), indicating that SFN would not interfere with AP-1 transactivating activity. These findings were confirmed by EMSA analyses (Fig. 7A). Consistently, SFN was reported to enhance AP-1-supported expression of phase II detoxication enzymes by increasing extracellular signal-regulated kinase 2 phosphorylation and kinase activity in hepatoma cells (49). Interestingly, the triterpene ursolic acid has been demonstrated recently to suppress Cox-2 transcription in human mammary epithelial cells by inhibition of TPA-mediated induction of protein kinase C, extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases (50).

Having established NF- κB as a target of SFN, we focused on the mechanism of inactivation. Other than agents like theaflavin, (-)-epigallocatechin gallate, or resveratrol, which have been shown to impair I κB degradation (51–53), SFN was inactive in this respect. Rather, in SFN-treated cells, activated NF- κB translocated to the nucleus, but DNA binding was impaired. We noticed that concentrations of SFN required to prevent activated NF- κB from binding to its consensus sequence were essentially higher than those required to inhibit LPS-mediated iNOS induction (*i.e.* 10–20 μM when added prior to LPS stimulation or 1–2 mM when added exogenously to activated NF- κB). This might partially be due to different incubation periods (45 min for NF- κB activation *versus* 12–24 h in iNOS induction experiments). Also, SFN has been shown recently to accumulate in murine hepatoma cells treated with 5 μM SFN up to a concentration of 900 μM within 30 min (54). This intracellular accumulation was attributed to a reversible binding of SFN to GSH by dithiocarbamate formation. Therefore, intracellular concentrations of 1 mM in our system are feasible but have to be experimentally confirmed.

Inhibition of NF- κB DNA binding was preventable by preincubation of SFN with an excess of mercaptoethanol, suggesting a direct, reversible and thiol-dependent modification of NF- κB subunits or relevant co-factors by SFN. This prompted us to investigate how SFN-mediated effects on intracellular thiol levels (using GSH as a marker) might influence anti-inflammatory activities. In Raw macrophages, preincubation with SFN for various time periods initially resulted in GSH deple-

tion, which was reversed by an autoregulatory feedback induction of GSH with 2-fold elevated levels after 24 h (Fig. 12A). LPS treatment under these conditions resulted in a 2-fold higher IC₅₀ value of SFN with respect to inhibition of NO release. Identical results were reported when diethylmaleate was employed to deplete GSH *via* a glutathione *S*-transferase-mediated step (55). We quantitatively compared dose-dependent effects of SFN on GSH and nitrite levels at each time point by area under (induction or inhibition) curve values and obtained a significant correlation between both parameters ($r^2 = 0.96$) (Fig. 12B). Based on these observations, we speculated that elevated GSH levels might compete with essential Cys residues either of NF- κ B subunits (56) or other redox regulators relevant for NF- κ B function for interaction with SFN. At conditions of low GSH, SFN would predominantly interact with these factors and exert a stronger inhibitory effect, whereas higher GSH concentrations would render it less accessible for other reaction partners. The fact that elevated GSH concentrations weaken but do not totally abolish the inhibitory effect of SFN favors this possibility.

NF- κ B is a redox-sensitive transcription factor tightly regulated by the intracellular redox status, which is maintained mainly by the ratio of reduced and oxidized GSH. NF- κ B requires a high GSSG/GSH ratio for activation and nuclear translocation but depends on a reducing milieu for binding to its consensus site (57, 58). Because SFN did not inhibit nuclear translocation, we hypothesized that disturbance of intracellular redox conditions might contribute to the inhibition of NF- κ B DNA binding. Reducing conditions in the nucleus are modulated by redox regulators including thioredoxin (TRX) and redox factor-1 (Ref-1) (59). TRX has been found to be particularly important for gene expression because it facilitates protein-nucleic acid interactions by reducing Cys residues in the DNA binding loop of several transcription factors essential for recognition of binding sites through electrostatic interactions with specific DNA bases (60, 61). A direct association between TRX and the NF- κ B p50 subunit was suggested by *in vitro* cross-linking assays (62). Our own preliminary results underline the importance of reduced TRX in LPS-mediated iNOS induction, because 1-chloro-2,4-dinitrobenzene, an inhibitor of thioredoxin reductase (63), inhibited NO release after LPS stimulation with an IC₅₀ value of 5.5 μ M and synergistically lowered the IC₅₀ value obtained with SFN.² Ref-1 is a multifunctional protein that not only functions as a redox factor maintaining transcription factors in an active reduced state but is also responsible for repair of apurinic sites as a part of base excision repair (64). Active Ref-1 is recycled via its interaction with TRX, which acts as a hydrogen donor polypeptide. TRX and Ref-1 were found to act synergistically in the regulation of p50 DNA binding activity (65). A recent study employing high performance affinity bead chromatography suggested a direct interaction of Ref-1 with the quinone derivative E3330 (66). E3330 was originally developed as an anti-inflammatory drug and, in good agreement with our results with SFN, suppressed NF- κ B transactivation without affecting degradation of I κ B or nuclear translocation of NF- κ B (67). Considering the similarities between SFN and E3330 with regard to inhibition of NF- κ B activity, Ref-1 could be regarded as a potential target of SFN.

Taken together, our data indicate that SFN possesses anti-inflammatory activity, resulting in down-regulation of LPS-stimulated iNOS, Cox-2, and TNF- α expression in Raw macrophages. We conclude that the major mechanism of SFN action in this model is inhibition of NF- κ B DNA binding and of trans-

activation of κ B-dependent genes, presumably through modulation of intracellular redox conditions *via* dithiocarbamoylation of essential thiol groups involved in the activation of NF- κ B. Further studies are warranted to elucidate the relevance of these anti-inflammatory properties for SFN-mediated cancer chemopreventive efficacy.

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