Forum Original Research Communication

Time-Dependent Modulation of Thioredoxin Reductase Activity Might Contribute to Sulforaphane-Mediated Inhibition of NF-κB Binding to DNA

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ABSTRACT

The chemopreventive agent sulforaphane (SFN) exerts anti-inflammatory activity by thiol-dependent inhibition of nuclear factor κB (NF-κB) DNA binding. To further analyze the underlying mechanisms, we focused on the thioredoxin/thioredoxin reductase (TrxR) system as a key redox mechanism regulating NF-κB DNA binding. Using cultured Raw 264.7 mouse macrophages as a model, 1-chloro-2,4-dinitrobenzene (CDNB), a known inhibitor of TrxR, was identified as an inhibitor of lipopolysaccharide (LPS)-mediated nitric oxide (NO) production and of NF-κB DNA binding. CDNB and SFN acted synergistically with respect to inhibition of LPS-induced NO release, and we consequently identified SFN as a novel inhibitor of TrxR enzymatic activity *in vitro*. Short-term treatment of Raw macrophages with SFN or CDNB resulted in the inhibition of TrxR activity *in vivo* with half-maximal inhibitory concentration of 25.0 ± 3.5 μM and 9.4 ± 3.7 μM, respectively, whereas after a 24-h treatment with 25 μM SFN, TrxR activity was >1.5-fold elevated. In additional experiments, we could exclude that inhibition of *trans*-activating activity of NF-κB contributed to the reduced expression of pro-inflammatory proteins by SFN, based on transient transfection experiments with a $(\kappa B)_2$-chloramphenicol acetyltransferase construct and a lack of inhibition of protein kinase A activity. These findings further emphasize the importance of redox modulation or thiol reactivity for the regulation of NF-κB-dependent transcription by SFN. *Antioxid. Redox Signal.* 7, 1601–1611.

INTRODUCTION

THE TRANSCRIPTION FACTOR nuclear factor-κB (NF-κB) plays a central role in immunological and inflammatory processes and is involved in carcinogenesis and several chronic degenerative diseases like atherosclerosis (1). It affects a wide range of responsive genes involved in a variety of cellular mechanisms, including proliferation, differentiation, angiogenesis, and metastasis. NF-κB shows an intricate complexity of subunits and regulation, and its activity is strictly controlled and finely tuned. In quiescent cells, the transcription factor is sequestered in the cytosol by interaction with the inhibitor protein IκB (inhibitor of NF-κB). Upon exposure of cells to lipopolysaccharide (LPS) or inflammatory cytokines

including tumor necrosis factor-α (TNF-α) and interleukins, activation and nuclear translocation of NF-κB are controlled by targeted phosphorylation and subsequent degradation of IκB. Once in the nucleus, NF-κB dimers bind to consensus DNA elements and transactivate transcription of genes encoding proteins involved in immune and inflammatory responses (1) such as inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase 2. Transactivating activity of NF-κB is enhanced by phosphorylation of the p65 subunit and by acetylation of p50, p65, and histones (summarized in 10). One key phosphorylation event involves the action of protein kinase A (PKA), which is activated by LPS and mediates phosphorylation at Ser[276], thus promoting recruitment of the transcriptional co-activators CREB-binding protein and p300 (54).

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Using LPS-induced murine Raw 264.7 macrophages as a model to study inflammatory response in cell culture, we have recently demonstrated that sulforaphane (SFN) [1-isothiocyanato-(4R)-(methylsulfinyl)butane:$CH_3S(O)(CH_2)_4$–N=C=S], an isothiocyanate found as the precursor glucosinolate in broccoli, inhibits κB-dependent iNOS and COX-2 protein expression and TNF-α secretion (15). SFN prevented active NF-κB from binding to its nuclear DNA site *via* a thiol-mediated mechanism, presumably by dithiocarbamoylation of NF-κB subunits or of factors involved in the redox regulation of NF-κB. NF-κB requires oxidative conditions for activation and nuclear translocation, but a reductive milieu for DNA binding and transactivation, ensuring that cysteine residues pivotal for DNA binding, like Cys[62] of the p50 subunit, are in a reduced state (2, 26, 39). The redox regulator thioredoxin (Trx), a ubiquitous protein with two redox-active half cystine residues, was shown to play dual and opposing roles in regulating NF-κB action. Trx undergoes NADPH-dependent reduction by Trx reductase (TrxR, EC 1.8.1.9), a selenocysteine-containing oxidoreductase, and in turn reduces oxidized cysteine groups on proteins. In the cytosol, it interferes with signals activating IκB kinases and consequently blocks degradation of IκB, whereas in the nucleus it locally promotes a reducing environment to avoid oxidation of cysteine residues in the DNA-binding domain of numerous transcription factors including those involved in cancer promotion and progression, like the activator protein-1 (AP-1) and NF-κB. Recent *in vitro* cross-linking assays have indicated that Trx and the NF-κB p50 subunit might be directly associated (19). To characterize the influence of TrxR inhibition in our experimental setup, we included the TrxR inhibitor 1-chloro-2,4-dinitrobenzene (CDNB), which was shown to inhibit TrxR activity by irreversible alkylation of essential selenocysteine and cysteine residues (30). CDNB is often used as a substrate for the determination of glutathione *S*-transferase activity by conjugation to GSH; however, CDNB was reported to possess a 10,000-fold higher affinity to TrxR than to GSH (3). Furthermore, CDNB was shown to conjugate with the p50 subunit of NF-κB (9) and to suppress NF-κB-dependent luciferase activity after transfection of HeLa cells with a 5× κB-luciferase reporter gene plasmid and stimulation with TNF-α (19).

In this report, we provide evidence that SFN also modulates the Trx/TrxR system. These effects might transiently influence the intranuclear redox potential to disfavor NF-κB DNA binding.

MATERIALS AND METHODS

Chemicals

All cell culture media and supplements were obtained from Invitrogen GmbH (Karlsruhe, Germany). Fetal bovine serum was from Greiner Labortechnik GmbH (Frickenhausen, Germany). Oligonucleotide probes for NF-κB (sc-2505) and AP-1 (sc-2501) were obtained from Santa Cruz (Heidelberg, Germany). [γ-^{32}P]ATP was purchased from ICN (Costa Mesa, CA). All other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). The PepTag® non-radioactive cyclic AMP (cAMP)-dependent kinase assay was from Promega (Mannheim, Germany). The chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay was acquired from Roche Diagnostics (Mannheim, Germany). All electrophoresis equipment was obtained from Bio-Rad (München, Germany). SFN was synthesized as described previously (15). Auranofin was kindly provided by Dr. R.H. Schirmer, Biochemie-Zentrum Heidelberg.

Cell culture

Raw 264.7 murine macrophages were obtained from the Tumorbank (German Cancer Research Center, Heidelberg) and maintained in Dulbecco´s minimal essential medium (DMEM) 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% CO_2 atmosphere. Cells were preincubated in DMEM containing fetal bovine serum for 24 h. Then, the medium was replaced by serum-free DMEM, and LPS, (from *Escherichia coli*, serotype O111:B4) was added at a final concentration of 500 ng/ml.

Inhibition of LPS-mediated iNOS induction

Treatment of Raw 264.7 macrophages, quantification of NO production *via* nitrite levels, and determination of cell growth by sulforhodamine B staining have been described in detail by Heiss *et al.* (15).

Electrophoretic mobility shift assay

Cultivation and treatment of Raw 264.7 macrophages, extraction of cytosolic and nuclear protein fractions, binding reactions, and native polyacrylamide gel electrophoresis were performed as described previously (15).

Synergism studies between SFN and CDNB

Raw 264.7 macrophages were plated at a density of 2×10^5 cells per well in 96-well plates and incubated overnight. Cells were treated with combinations of SFN and CDNB (molar ratio: 4:1, 2:1, 1:1, 1:2, and 1:4) for 45 min, before LPS (500 ng/ml) was added for 24 h. NO production was determined *via* nitrite levels in cell culture supernatants by the Griess reaction (15). Median effect plots and isobolograms were generated according to the method described by Chou and Talalay (11). Briefly, for median effect plots, log D was plotted against log f_a/f_u, where D represents the concentration of each single compound or the mixture of both, and f_a and f_u stand for the affected (inhibited NO production) and unaffected fraction, respectively, at each concentration D. An isobologram was obtained by plotting the fractional half-maximal inhibitory concentration (IC_{50}) values of SFN against the fractional IC_{50} values of CDNB. Fractional IC_{50} values are defined as the ratio between the IC_{50} value of one compound in combination with a second mutually exclusive compound and the IC_{50} value of the compound alone.

Determination of total GSH levels

Raw 264.7 macrophages were cultured as described above. For the determination of GSH levels, cells were

seeded into 96-well plates (1 × 10⁵ cells per well). Six serial twofold dilutions of SFN or CDNB were added in duplicates. After incubation periods of 45 min, 2 h, 4 h, or 24 h, plates were washed three times with phosphate-buffered saline and stored at −80°C until analyzed. GSH was measured essentially as described previously using 5,5′-dithio*bis*-(2-nitrobenzoic acid) (DTNB) (the Ellman reagent) (15) and normalized to protein concentrations determined according to the method of Smith *et al.* (41). To compare the GSH-depleting potential of SFN and CDNB after various incubation periods, half-maximal depleting concentration values were computed.

TrxR activity

The determination of TrxR activity was based on the NADPH-dependent reduction of DTNB according to a protocol of Hill *et al.* (16). The activity was corrected for non-TrxR-dependent DTNB reduction by addition of Auranofin, a gold compound that selectively inhibits TrxR (5). To assess the influence of SFN or CDNB on TrxR activity *in vitro*, Raw macrophages were grown to 80% confluence, scraped into phosphate-buffered saline, adjusted to 2.5×10^7 cells/ml, and stored in aliquots at −80°C. After thawing, cells were homogenized by passing through a 16-gauge injection needle and centrifuged at 13,000 rpm for 5 min. The supernatant served as the source for TrxR. Cell lysate (25 μl, 45–75 μg of protein) was incubated with different concentrations of test compounds (as indicated) in the presence or absence of 1 μ*M* Auranofin (dissolved in dimethylsulfoxide [DMSO]) for 20 min at room temperature (RT). Then, 225 μl of reaction mixture, containing 70 m*M* of potassium phosphate, 1.5 m*M* EDTA, 3 m*M* DTNB, 10 μg of bovine serum albumin (BSA), and 0.2 m*M* NADPH, was added, and 2-nitro-5-thiobenzoic acid formation was monitored at 412 nm every 15 s for 10 min. To calculate TrxR-specific activities, the rate of unspecific DTNB reduction of Auranofin-containing (TrxR-inhibited) samples was subtracted from that of Auranofin-free samples and normalized for protein content. A reduction in TrxR activity by test compounds was expressed as a percentage of a DMSO-treated solvent control. For *in vivo* experiments, cells were grown on three 96-well plates (1 × 10⁵ cells per well) and incubated with five to six serial twofold dilutions (concentration range: 45 min; 1.56–50 μ*M*; 24 h, 3.1–50 μ*M*) of test compounds in duplicates for 45 min or 24 h, respectively, before plates were washed three times with phosphate-buffered saline and stored at −80°C. After thawing, 25 μl of 250 m*M* phosphate buffer, pH 7.4, was added to each well, and the plates were shaken vigorously. Cells on one plate were incubated with 1 μl per well of 250 μ*M* Auranofin for 20 min at RT, before 225 μl of reaction mixture was added to two plates, and 2-nitro-5-thiobenzoic acid formation was monitored at 412 nm as described above. Protein was determined according to the method of Bradford (7) on the third plate. Auranofin-inhibitable TrxR activity was expressed as a percentage in comparison with a DMSO-treated solvent control.

Transient transfection experiments

Raw 264.7 macrophages were seeded at a density of 2 × 10⁶ cells in 6-cm-diameter cell culture dishes and incubated overnight. Transient transfection experiments were performed by the calcium phosphate procedure according to Sambrook *et al.* (36). At 3 h prior to transfection, medium was replaced with fresh growth medium. Raw cells were transfected with 5 μg of a (κB)₂-CAT construct (kindly provided by L. Schmitz, University of Berne, Berne, Switzerland) and 2.5 μg of β-gal plasmid for normalization. At 4 h after addition of the DNA precipitate, cells were treated with 10% glycerol in growth medium for 2 min. The medium was changed, 5 μ*M* SFN was added, and the cells were stimulated with 500 ng/ml LPS for an additional 24 h. The amount of CAT protein was determined in cell lysates using a CAT enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer's protocol. The obtained values were normalized for β-galactosidase activity (37) and the protein content (41).

PKA activity

Inhibitory effects on PKA activity were determined using the PepTag non-radioactive cAMP-dependent protein kinase assay by Promega according to the manufacturer's instructions. The assay is based on the PKA-dependent phosphorylation of the specific fluorescent PepTag-A1 peptide substrate (LRRASLG, Kemptide). Phosphorylation alters the net charge of the peptide and allows the separation of the phosphorylated and nonphosphorylated versions by agarose gel electrophoresis under neutral conditions. Briefly, PKA catalytic subunit (0.01 μg in 350 m*M* K₃PO₄, pH 7.5, and 0.1 m*M* dithiothreitol) was preincubated with test compounds for 5–10 min at RT before the substrate and cofactors were added. Final reaction mixtures contained 20 m*M* Tris HCl, pH 7.4, 10 m*M* MgCl₂, 1 m*M* ATP, 1 μ*M* cAMP, and 2 μg of PepTag-A1 peptide, PKA (as indicated), and test compounds or DMSO, respectively, in a total volume of 25 μl. Reaction mixtures were then incubated at 30°C for 30 min. The reaction was stopped by heating the samples to 95°C for 10 min. Samples were separated on a 0.8% agarose gel (in 50 m*M* Tris HCl, pH 8.0) at 100 V for 15 min. According to its phosphorylation status, the substrate migrates towards the cathode (nonphosphorylated form) or the anode (phosphorylated form). For quantification of PKA activity, gel bands containing the nonphosphorylated and phosphorylated fractions of the peptide were excised, melted at 95°C, and measured in a microplate fluorimeter (Cytofluor 4000, PE Applied Biosystems, Foster City, CA; excitation at 530 ± 25 nm, emission at 620 ± 40 nm). Results were compared with a DMSO control, and the percentage of PKA inhibition was calculated. IC₅₀ values were generated from testing six to seven serial twofold dilutions of test compounds in a concentration range up to 1 m*M* as indicated. To correct for unspecific protein binding, a 500-fold excess of BSA (5 μg) was added to the incubation mixtures in one experiment.

RESULTS

We used cultured LPS-stimulated Raw 264.7 murine macrophage cells as a model to study NF-κB-mediated reactions and mechanisms regulating its activity. Early reactions after

LPS stimulation, *i.e.,* NF-κB activation and nuclear translocation, were not inhibited by SFN, although later effects, *i.e.*, DNA binding and the induction of transcription of pro-inflammatory proteins, were potently reduced (15). Since SFN transiently depleted GSH levels, we concluded that a thiol-dependent influence on NF-κB or on redox regulators facilitating its DNA binding could contribute to the inhibitory mechanism of SFN.

Inhibition of NO production and NF-κB DNA binding by CDNB

Based on these observations, we were interested whether a disturbance of the Trx/TrxR system would result in a reduction of nitrite levels as a measure for the inhibition of iNOS induction. We treated Raw macrophages with CDNB as a selective TrxR inhibitor. After induction with 500 ng/ml LPS for 24 h, NO was measured *via* nitrite levels in cell culture supernatants by the Griess reaction. Interestingly, CDNB caused dose-dependent inhibition of NO production in a range of 1.56–50 μ*M* (Fig. 1A), and the IC_{50} value was determined as 12.1 ± 0.9 μ*M*. Reduction of NO levels was not due to toxicity as demonstrated by concomitant sulforhodamine B staining of intact cells, and the IC_{50} value for toxicity was significantly higher than that for NO inhibition (Table 1). To examine whether the observed potential of CDNB to inhibit LPS-mediated NO production was regulated at the level of NF-κB DNA binding, we performed electrophoretic mobility shift assay analyses with extracts of CDNB-treated cells. Cultured Raw 264.7 macrophages were pretreated for 60 min with the indicated concentrations of CDNB before NF-κB was activated by LPS stimulation for another 45 min. Nuclear protein was isolated and subjected to NF-κB-specific DNA binding analysis. We could demonstrate that similar to SFN, CDNB at concentrations of 20 μ*M* and 40 μ*M* potently suppressed DNA binding of NF-κB (Fig. 1B).

Synergistic inhibition of iNOS induction by SFN and CDNB

Our own preliminary results underline the importance of redox effects on NF-κB DNA binding and subsequent effects. A redox milieu in favor of transcription factor DNA binding is maintained by redox regulators like reduced Trx (3). Consequently, we tested combinations of SFN and CDNB for synergistic inhibitory effects on LPS-stimulated NO production. For this purpose, we incubated Raw 264.7 macrophages with mixtures of SFN and CDNB, in various molar ratios (4:1, 2:1, 1:1, 1:2, 1:4). Parallel median effect plots for SFN, CDNB, and the mixture of SFN and CDNB (at a molar ratio of 1:2), obtained as described in detail by Chou and Talalay (11), were an indication for mutual exclusion of the two compounds and pointed to a similar mechanism in inhibiting LPS-stimulated NO production (Fig. 2A). Therefore, we utilized the isobologram method for the evaluation of synergistic effects between these two compounds. By plotting the fractional IC_{50} values of SFN versus those of CDNB, we could demonstrate synergistic inhibition of LPS-stimulated iNOS induction for the combination of SFN and CDNB, especially at proportionally higher molar concentrations of
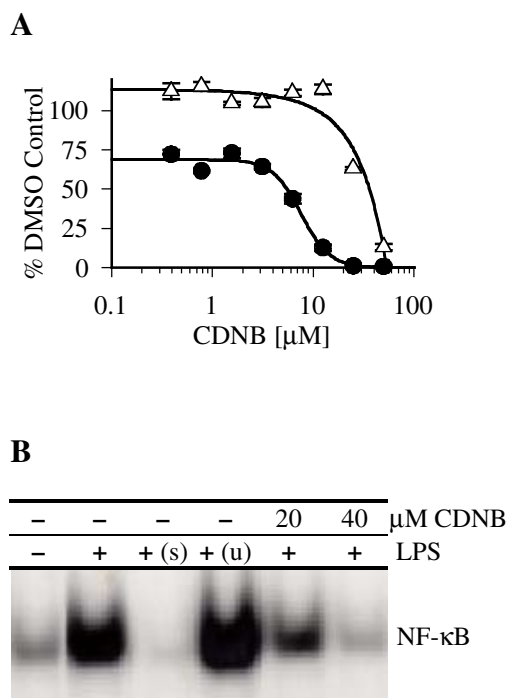
A

B

FIG. 1. Inhibition of NO production and NF-κB DNA binding by CDNB. (**A**) Raw 264.7 macrophages were incubated with 500 ng/ml LPS. Eight serial twofold dilutions of CDNB in the range of 0.4–50 μ*M* were added in duplicate for 24 h before NO was determined *via* nitrite levels in cell culture supernatants and expressed as a percentage of LPS-stimulated DMSO-treated control samples (●; nitrite levels in supernatants of unstimulated cells, 0.5 ± 0.1 nmol of nitrite/ml; after LPS stimulation, 32 ± 1.2 nmol of nitrite/ml; $n = 2$). An effect on cell growth was estimated by sulforhodamine staining (Δ). (**B**) Electrophoretic mobility shift assay of NF-κB DNA-binding capacity. Raw 264.7 macrophages were incubated with DMSO (−) or CDNB as indicated and stimulated with LPS (+) for 45 min, before nuclear protein was isolated. DNA binding was analyzed using a specific ^{32}P-labeled oligonucleotide probe for NF-κB. Specificity was demonstrated by co-incubation with a 25-fold excess of unlabeled specific (s) or unspecific probe (u, oligonucleotide probe for AP-1).

SFN. At a fourfold molar excess of CDNB, the plotted fractional IC_{50} values resulted in a point located over the isoeffect line of additivity, indicating additive or weak antagonistic effects (Fig. 2B).

Effects on cellular GSH levels

CDNB can react with GSH in a glutathione *S*-transferase-catalyzed reaction and thus lead to a reduction in cellular GSH levels. Therefore, we next compared the effects of CDNB and SFN with respect to GSH depletion. We incubated Raw macrophages with six serial twofold dilutions of CDNB or SFN, respectively, in a concentration range of 1.25–50 μ*M* for 45 min, 2 h, and 4 h (short-term) and 24 h (long-term), respectively, before GSH levels were analyzed. Both compounds dose-dependently depleted intracellular GSH already within the first 45 min of treatment (Table 1). The GSH-

TABLE 1. COMPARISON OF INHIBITORY EFFECTS OBTAINED WITH SFN AND CDNB

	SFN (μM)	CDNB (μM)
Inhibition of NO production[a]		
NO levels	0.9 ± 0.1	12.1 ± 0.9
Viability	41.2 ± 5.0	41.4 ± 7.0
GSH depletion[b]		
45 min	29.8 ± 6.6	13.3 ± 0.8
2 h	18.0 ± 0.5	4.1 ± 0.6
4 h	11.7 ± 0.4	7.6 ± 0.6
TrxR inhibition[a]		
In vitro	37.2 ± 1.5	22.5 ± 2.3
In vivo	25.0 ± 3.5	9.4 ± 3.7

[a]$IC_{50} \pm$ SD.
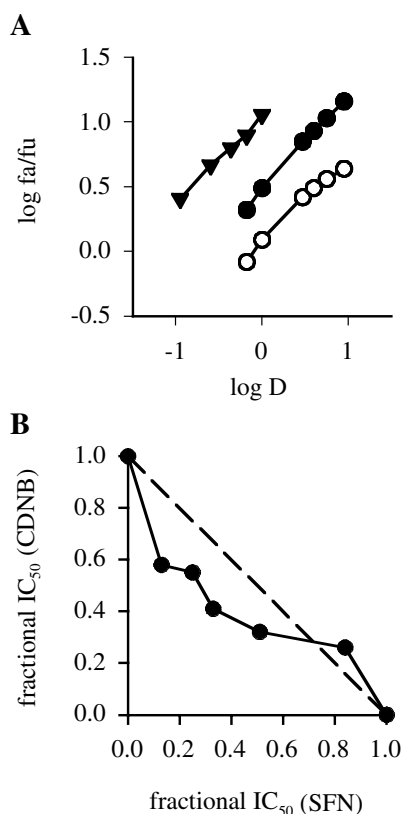[b]Half-maximal GSH-depleting concentration $\pm$ SD.

FIG. 2. Synergistic inhibition of LPS-induced NO levels after co-treatment of Raw macrophages with SFN and CDNB. (A) median effect plots of SFN (○), CDNB (▼), and a combination of SFN and CDNB in a molar ratio of 1:2 (●) to demonstrate mutual exclusion. *D* represents the concentration of each single compound or the mixture of both; f_a and f_u stand for the affected (inhibited NO production) and unaffected, respectively, fraction at each concentration *D*. (B) Isobolographic plot of fractional IC_{50} values of SFN against fractional IC_{50} values of CDNB (solid line). Fractional IC_{50} values describe the ratio of IC_{50} values of defined molar combinations of the two compounds and the IC_{50} values of each single compound alone. The dashed line indicates the isoeffect line of additivity. The graph represents one of three identical experiments.

lowering capacity of SFN was most pronounced after an incubation time of 4 h, whereas long-term treatment for 24 h significantly increased GSH levels to a maximum 1.5-fold elevation in comparison with DMSO-treated controls (Fig. 3A). With CDNB, the strongest depleting effects were measured after 2 h of incubation (Table 1). GSH depletion was only transient, and GSH levels began to increase after a treatment period of 4 h and reached DMSO-control levels after 24 h (Fig. 3B).

Influence of SFN on TrxR activity

The results of SFN and CDNB synergism studies provided evidence that TrxR might be a target of SFN. Thus, we performed *in vitro* TrxR inhibition experiments using homogenates of Raw macrophages as an enzyme source, and DTNB as a substrate. Non–TrxR-dependent reduction of DTNB was corrected by addition of Auranofin as a selective TrxR inhibitor. CDNB served as a positive control and demonstrated an IC_{50} value of 22.5 ± 2.3 μ*M*. Interestingly, SFN was identified as an inhibitor of TrxR enzymatic activity with an IC_{50} value of 37.2 ± 1.5 μ*M* (Table 1 and Fig. 4). Subsequently, we tested the influence of our test compounds on TrxR activity in Raw macrophages. Similar to the results observed in the *in vitro* experiment, short-term treatment with
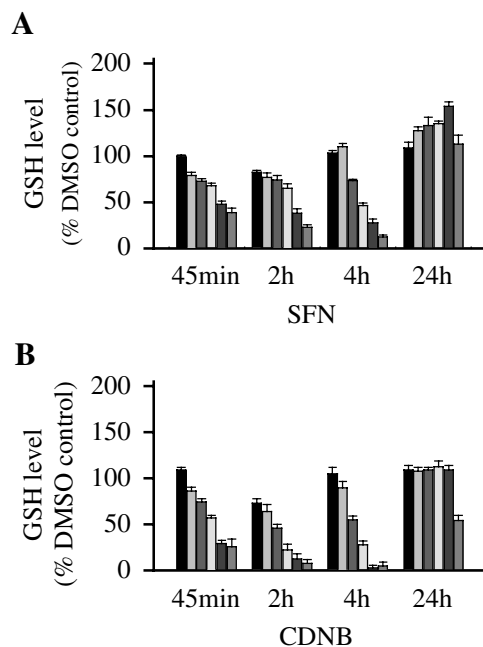

FIG. 3. Effects on cellular GSH levels. Raw 264.7 macrophages were treated with 1.56 (■), 3.1 (□), 6.25 (▨), 12.5 (□), 25 (▨), or 50 μ*M* (□) concentrations of SFN (A) and CDNB (B) in duplicates for 45 min, 2 h, 4 h, or 24 h as indicated, before GSH levels were determined. Values for each tested concentration were expressed as a percentage of DMSO-treated control cells: (GSH levels after a 45-min treatment, 65.3 ± 6.9 nmol of GSH/mg of protein ($n = 2$); 2-h treatment, 72.8 ± 5.6 nmol of GSH/mg of protein ($n = 2$); 4-h treatment, 69.4 ± 4.9 nmol of GSH/mg of protein ($n = 2$); 24-h treatment, 77.9 ± 2.8 nmol of GSH/mg of protein ($n = 2$).
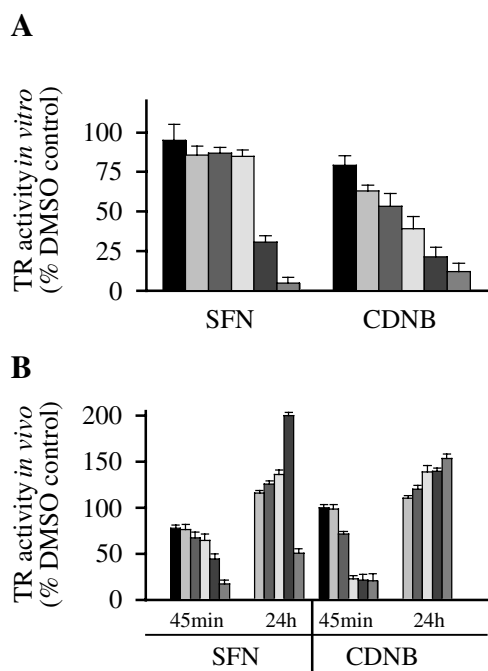
FIG. 4. Effects on TrxR (TR) activity *in vitro* and in treated Raw macrophages. (**A**) Cytosolic preparations of Raw macrophages served as an *in vitro* source of TR. TR activity was calculated from the Auranofin-inhibitable NADPH-dependent reduction of DTNB. The effects of SFN and CDNB were assessed at 1.56 (■), 3.1 (□), 6.25 (▨), 12.5 (□), 25 (▨), or 50 μ*M* (□) concentrations in duplicates. The values for each tested concentration were expressed as a percentage of a DMSO-treated control sample (TR activity of control, 1.14 ± 0.28 nmol of DTNB reduced/min/mg of protein; $n = 3$). (**B**) Dose-dependent effects of SFN and CDNB on TR activity after treatment of Raw macrophages for 45 min (short-term, concentration range 1.56–50 μ*M*) or 24 h (long-term, concentration range 3.1–50 μ*M*, labels as described above). TR activity in DMSO-treated control cells after a 45-min treatment, 0.96 ± 0.26 nmol of DTNB reduced/min/mg of protein ($n = 3$); after a 24-h treatment, 1.35 ± 0.18 nmol of DTNB reduced/min/mg of protein ($n = 3$). Graphs show one of three identical experiments.

SFN and CDNB for 45 min resulted in dose-dependent inhibition of TrxR activity, with IC_{50} values of 25 ± 3.5 μ*M* and 9.4 ± 3.7 μ*M*, respectively (Table 1 and Fig. 4B). However, after an incubation period of 24 h, CDNB and SFN (at concentrations ≥ 25 μ*M*) dose-dependently increased TrxR activity by maximally 1.5- and twofold (Fig. 4B). In close agreement with the results observed with the modulation of GSH levels, an influence of LPS on TrxR activity or on the inhibitory capacity of the test compounds could be excluded (data not shown).

Inhibition of PKA activity

In addition to redox regulation, NF-κB activity is controlled at the transactivational level by phosphorylation of its p65 subunit. To examine whether inhibition of κB-dependent gene expression by SFN might be due to an inhibitory effect on PKA activity, we employed an *in vitro* PepTag non-radioactive cAMP-dependent PKA assay (example in Fig. 5A). *N*-Ethylmaleimide (NEM), known to inhibit PKA activity by modification of a Cys residue at the catalytic site, served as the positive control, and we obtained an IC_{50} value of 130 μ*M* (Fig. 5B). SFN dose-dependently inhibited PKA activity with an IC_{50} value of 226 μ*M* (Fig. 5B). To exclude that the observed inhibitory effects were due to an unspecific interaction with the highly purified PKA catalytic subunit provided with the test system, we added a 500-fold excess of BSA (5 μg) to the reaction mixtures. BSA addition prevented PKA inhibition by SFN, whereas the NEM-mediated reduction of PKA phosphorylation was not affected (Fig. 5C). This pointed to a specific inhibition of PKA by NEM and a rather unspecific reversible protein binding by the isothiocyanate. With respect to the molecular inhibition of κB-dependent gene transcription, we therefore excluded that SFN impairs
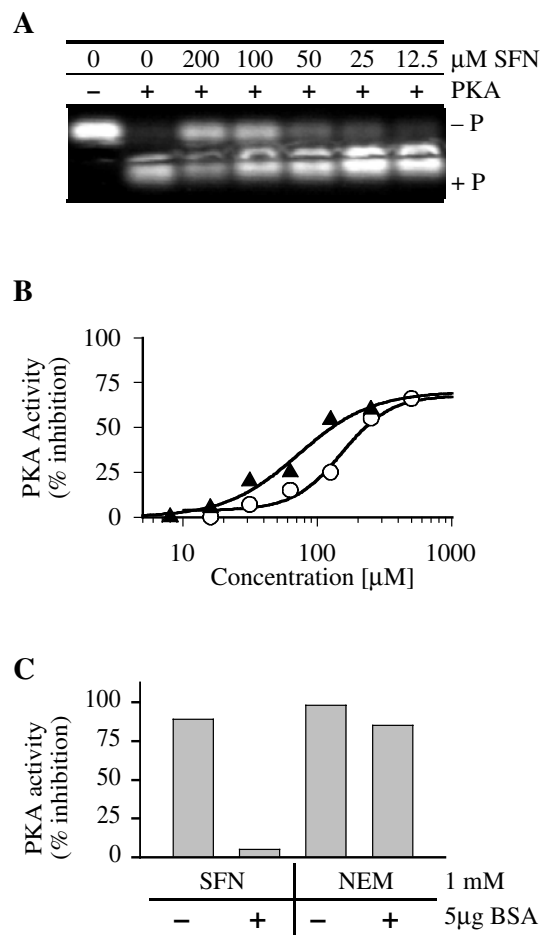

FIG. 5. Inhibition of PKA activity. (**A**) Example of a gel analysis of the PKA activity assay. Reaction mixtures contained PKA (+) and DMSO (−) or various concentrations of SFN as indicated. (−P), nonphosphorylated peptide; (+P), phosphorylated peptide. (**B**) Dose-dependent inhibition of PKA activity by SFN (○) and NEM (▲). (**C**) Reversion of SFN-mediated inhibition of PKA activity by addition of a 500-fold excess of BSA (+).

transactivating activity of NF-κB *via* inhibition of PKA-mediated p65 phosphorylation.

Transient transfection experiments

To verify that SFN does not interfere with NF-κB transactivating activity independent of phosphorylation by PKA by other pathways, *e.g.,* by inhibition of phosphorylation by other kinases or acetylation of p65 and histones, we performed transient transfection experiments with Raw 264.7 macrophages and (κB)₂-CAT plasmid constructs containing two κB elements ligated to the reporter gene. LPS treatment of transfected cells led to a 4.1-fold increase in CAT expression (normalized to co-transfected β-galatosidase activity and protein levels), from 3.5 ± 0.8 ng of CAT/mg of protein in unstimulated cells to 14.4 ± 1 ng of CAT/mg of protein in stimulated cells. Concomitant treatment with LPS and 5 μ*M* SFN resulted in a 2.9-fold induction of CAT expression, corresponding to a 32.3% inhibition in comparison with the LPS-stimulated DMSO-treated control (Fig. 6). Thus, 5 μ*M* SFN does not severely interfere with the transactivating activity of NF-κB. Concentrations higher than 5 μ*M* were not suitable for studying the effect of SFN with this respect because they would lead to an impaired DNA binding of NF-κB as reported previously (15).

DISCUSSION

NF-κB-dependent overexpression of pro-inflammatory proteins such as TNF-α, cyclooxygenase 2, and iNOS leads to elevated tissue levels of TNF-α, prostaglandins, and NO, which in turn facilitate tumor initiation and promotion. Therefore NF-κB represents an attractive target in chemoprevention research (1, 4, 45). The activity of NF-κB is controlled at four levels: activation and cellular localization, redox status, phosphorylation, and acetylation (10). Many studies have concentrated on the inhibitory influence of compounds on the activation process of NF-κB. In this respect, resveratrol and aspirin were shown to inhibit the degradation of IκB and thus activation and nuclear translocation of NF-κB (20, 48). Redox modulation as a mechanism of NF-κB inhibition by chemopreventive agents, however, has been un-
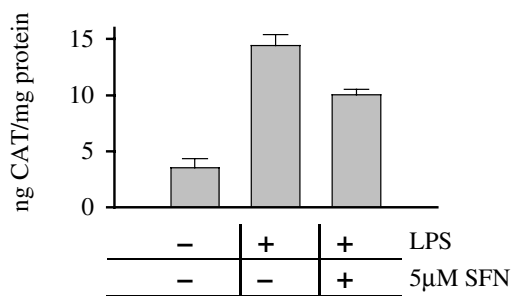

FIG. 6. Transient transfection experiments. Raw 264.7 macrophages were transfected with a (κB)₂-CAT construct, stimulated with LPS (+), and treated with DMSO (−) or 5 μ*M* SFN (+) before CAT was assayed (CAT level of DMSO control, 3.5 ± 0.8 ng/mg of protein; *n* = 2).

derestimated so far. NF-κB is a redox-sensitive transcription factor whose activation process can be separated in two stages with respect to the required redox state (2). Initiation of the signal transduction machinery leading to IκB degradation and subsequent nuclear translocation of NF-κB requires an oxidative potential such as after stimulation with H_2O_2 or an oxidative burst induced by phorbol esters (47). For binding to its DNA consensus sequence and transactivation of transcription, NF-κB requires conditions that ensure specific Cys residues to be in the reduced state. Thus, transcription of κB-dependent genes can be prevented by either antioxidants such as pyrrolidine dithiocarbamate (24), vitamin C (6), or pro-oxidants such as diamide (8). Redox regulators of NF-κB function include GSH, constituents of the Trx system composed of Trx, TrxR, and NADPH, and Ref-1 (27).

We have recently reported that SFN inhibits NF-κB binding to DNA by a thiol-dependent effect (15). In the present study, we showed that CDNB effectively inhibited NO production and NF-κB DNA binding in LPS-stimulated Raw macrophages, and we could demonstrate that CDNB and SFN acted synergistically with respect to inhibition of LPS-induced NO production. CDNB, apart from covalently conjugating with GSH and with the p50 subunit of NF-κB, specifically inhibits TrxR, which is required to keep Trx and the essential Cys[62] of p50 in a reduced state (3). Recently, aurothiomalate, another inhibitor of TrxR, was shown to inhibit TNFα-induced NF-κB activation (35). In a previous report, inhibition of NF-κB activation by the TrxR inhibitor auranofin was attributed to a thiol-mediated inhibition of IκB kinase by metal compounds (22). Since neither SFN (15) nor CDNB (9) prevented IκB degradation and subsequent translocation of NF-κB to the nucleus, we focused on their effects on GSH levels and the Trx/TrxR system as a key redox mechanism regulating NF-κB DNA binding.

Both compounds led to a dose-dependent GSH depletion after short-term treatment for 45 min and 2 h, favoring an oxidative cellular potential and activation and nuclear translocation of NF-κB. SFN was shown previously to form dithiocarbamates with GSH in murine hepatoma and MCF-7 human breast carcinoma cells (50–53). This might also account for the observed GSH depletion in isothiocyanate-treated Raw 264.7 macrophages. CDNB, in contrast to our observations, has been reported to affect the redox system around NF-κB in the absence of significant changes in total GSH levels in TNF-α-activated Jurkat lymphoma cells (9). This apparent discrepancy might be due to cell-specific differences in cellular uptake and metabolism. Modification of GSH levels as the most abundant intracellular thiol by highly SH-reactive compounds such as CDNB (*via* thioether formation) is likely, although CDNB was shown to possess a 10,000-fold higher affinity to TrxR than to GSH (3).

Interestingly, we identified SFN as a novel inhibitor of TrxR *in vitro*. Similar to the results observed with GSH levels, short-term treatment (45 min) of Raw macrophages with SFN resulted in inhibition of TrxR activity *in vivo*, whereas after a 24-h treatment with 25 μ*M* SFN, TrxR activity was elevated.

An increase in TrxR activity can be due to enhanced enzyme activity or to elevated levels of TrxR protein. Relatively little is known concerning the mechanism of up-regulation of

the TrxR protein. Rundlöf and Arner (32) have recently summarized the current knowledge on regulation of TrxR by exogenous stimuli. The core promoter of human TrxR1 was described and further characterized by Rundlöf *et al.* (33, 34). According to its structure and function, TrxR was categorized as a housekeeping gene, and basal expression was found to involve transcription factors Sp-1 and Oct-1, both known to be sensitive to redox modulation. Induction of TrxR might be mediated at the post-transcriptional level, due to the presence of 3′-untranslated AU-rich elements, which contribute to a fast stabilization of mRNA.

Rundlöf *et al.* (33, 34) proposed a model of TrxR regulation that supports our results obtained with SFN: Exogenous stimuli, leading to the production of reactive oxygen species (ROS), might result in a transient inhibition of TrxR activity by ROS, but then activate a mitogen-activated protein kinase (MAPK) cascade, finally resulting in the up-regulation of MAPK-activated protein kinase 2, which could stabilize AU-rich element-containing mRNAs including TrxR mRNA. This would result in a fast response of TrxR expression to ROS, counteracting the inhibitory effect of ROS on TrxR activity. Importantly, in this respect, SFN has been described as influencing the MAPK pathway in human HepG2 hepatoma cells by an increase in extracellular signal-regulated protein kinase 2 activity (49).

Regarding the inhibition of TrxR activity, SFN could reversibly interact with two redox-active Cys residues in the N-terminus (13) by dithiocarbamoylation, or with an essential selenocysteine residue in the C-terminal tail (38). Additionally, SFN was reported recently to stimulate cellular production of ROS in primary rat and human hepatocytes, determined by intracellular oxidation of dihydrorhodamine-123 (DHR) to the fluorescent rhodamine-123 (31). DHR oxidation induced by SFN was specific for ROS formation, as it was preventable by co-treatment with 2% DMSO used as a radical scavenger, indicating the generation of hydroxyl radicals. Tobi *et al.* (42) have established that DHR oxidation induced by ultraviolet A radiation was insensitive to reduced GSH levels and a consequent shift in the intracellular redox milieu by treating a human bladder carcinoma cell line with DL-buthionine *S,R*-sulfoximine. Therefore, it is unlikely that the observed increase in DHR oxidation induced by SFN was due to its GSH-depleting activity. A recent report by Singh *et al.* (40) confirms the production of ROS by SFN, detected in PC-3 human prostate cancer cells using dichlorodihydrofluorescein diacetate as a sensor. Kim and Gates (25) have provided a possible explanation for *in vitro* ROS formation by the chemopreventive sulfur-containing agents oltipraz and 1,2-dithiole-3-thione: These compounds in conjunction with thiols including GSH were shown to convert molecular oxygen to a peroxide species that was then converted to oxygen radicals in a Fenton-type reaction. Another possibility of ROS generation might be related to the inhibition of TrxR activity: Arner *et al.* (3) and Nordberg *et al.* (30) found that TrxR modification by CDNB induced a superoxide producing NADPH oxidase activity in the enzyme (reviewed in 29). It remains to be elucidated whether a similar mechanism might apply for SFN. Taken together, these reports demonstrate that an influence of SFN on TrxR activity and expression mediated by the putative ROS-dependent mechanism described above is feasible.

SFN-induced ROS could also account for the observed induction of GSH levels after long-term treatment. Oxidative stress generated by treatment of H4IIE rat hepatoma cells with the pro-oxidant *tert*-butylhydroquinone, again measured by the DHR method, was identified as an important regulating mechanism in the expression of the γ-glutamylcysteine synthetase heavy subunit, the rate-limiting enzyme in GSH synthesis (46). This might be mediated *via* AP-1- or NF-κB-binding to the γ-glutamylcysteine synthetase heavy subunit promoter region (14).

Recent evidence suggests that, in addition, the glutamylcysteine synthetase subunit gene expression is activated by transcription factor Nrf2 *via* the antioxidant responsive element (ARE) (reviewed in 44). Nrf2 has also been shown to play an important role in the induction of phase 2 enzymes by Michael acceptor type-chemopreventive compounds, and sulfhydryl reactivity was identified as the key determinant of inducing capacity (12). Nrf2, similar to NF-κB, is kept in the cytosol of quiescent cells by a redox-sensitive molecule called Keap1. Oxidative stress or treatment with compounds able to influence the redox state of or to directly bind to Cys residues is followed by conformational changes in Keap1 and a subsequent release of Nrf2 into the nucleus (21, 23), where transcription of phase 2 enzymes is initiated. Direct or indirect modification of thiol groups can thus be regarded as a common principle of chemopreventive agents contributing to anti-inflammatory or antioxidant and detoxifying activity. Interestingly, Hintze *et al.* (17, 18) recently demonstrated by reporter gene studies that TrxR expression is also regulated *via* a putative ARE in the 5′ promoter region of the gene. Increased luciferase activity in HepG2 cells transfected with the reporter construct was measured after treatment with electrophilic compounds including SFN, and mutation of the core sequence of the putative ARE in the promoter drastically decreased inducibility by SFN. Consequently, TrxR might also belong to the group of Nrf2-regulated genes.

As a final observation of our study, we could exclude that inhibition of NF-κB transactivation contributed to the reduced expression of pro-inflammatory proteins by SFN. Nuclear translocation and DNA binding are not sufficient for full transactivating activity of NF-κB. Rather, phosphorylation of the p65 subunit at Ser^{276} mediated by PKA is required and leads to recruitment of the transcriptional co-activators CREB binding protein and p300, acting as histone acetyltransferase (2) and facilitating access of the transcription machinery. Whereas NEM irreversibly inhibited PKA activity by alkylation of a Cys residue in the catalytic site of the enzyme (43), SFN inhibited PKA activity in a BSA-reversible manner, indicating unspecific protein binding.

To further confirm that SFN did not interfere with NF-κB transactivation, we performed transient transfection experiments with a (κB)$_2$-CAT plasmid construct. We were able to demonstrate that SFN does not markedly suppress κB-dependent expression of the CAT reporter gene in a concentration where DNA binding of NF-κB is not impaired by SFN. The observed 32% inhibition of CAT expression in transiently transfected macrophages may be explained by weak inhibition of NF-κB DNA binding, not recognizable in electrophoretic mobility shift assay analyses. SFN might also interfere with the phosphorylation of Ser^{529} of p65 mediated by

TNF-α-induced casein kinase II activity, that also enhances NF-κB trans-activation (54). We have previously reported that SFN mediated down-regulation of TNF-α secretion in LPS-stimulated Raw 264.7 macrophages with an IC$_{50}$ value of 7.8 μM. This could result in impaired phosphorylation of Ser[529] and NF-κB transactivation and thus account for the moderately reduced CAT expression seen with 5 μM SFN. In addition, SFN metabolites have recently been identified as inhibitors of histone deacetylase (28). It remains to be shown whether this novel mechanism is also involved in the inhibition of NF-κB activity by SFN.

In summary, it can be stated that modulation of the redox control of NF-κB by modification of Cys residues either of NF-κB subunits themselves or of redox regulators could constitute a mechanism that underlies the inhibition of NF-κB activity by SFN and CDNB. The reactivity profile resulting in the most potent inhibition of the κB-dependent gene observed with SFN included (a) transient GSH depletion and (b) inhibition of TrxR activity after short-term treatment, resulting in (c) reversible thiol modification and impaired NF-κB DNA binding, and (d) GSH and TrxR induction after long-term treatment to counteract potential harmful pro-oxidative redox conditions. Synergistic enhancement of inhibitory activities of SFN and CDNB with respect to LPS-mediated NO production might indicate that at least two sulfhydryl targets need to be affected coordinately to result in a pronounced redox-dependent inhibition. Combination of agents with similar mechanisms but differences in their affinity to thiol groups as well as in dissociation kinetics could then result in a potentiation of inhibitory effects.

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ABREVIATIONS

AP-1, activator protein-1; ARE, antioxidant response element; BSA, bovine serum albumin; cAMP, cyclic AMP; CAT, chloramphenicol acetyltransferase; CDNB, 1-chloro-2,4-dinitrobenzene; DHR, dihydrorhodamine-123; DMEM, Dulbecco's minimal essential medium; DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GSH, glutathione; IC$_{50}$, half-maximal inhibitory concentration; IκB, inhibitor of nuclear factor κB; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NEM, N-ethylmaleimide; NF-κB, nuclear factor κB; NO, nitric oxide; PKA, protein kinase A; ROS, reactive oxygen species; RT, room temperature; SFN, sulforaphane; TNF-α, tumor necrosis factor-α; Trx, thioredoxin; TrxR, thioredoxin reductase.

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