Nuclear Factor-κB (NF-κB) Mediates a Protective Response in Cancer Cells Treated with Inhibitors of Fatty Acid Synthase*

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The efficacy of drugs used to treat cancer can be significantly attenuated by adaptive responses of neoplastic cells to drug-induced stress. To determine how cancer cells respond to inhibition of the enzyme fatty acid synthase (FAS), we focused on NF-κB-mediated pathways, which can be activated by various cellular stresses. Treating lung cancer cells with C93, a pharmacological inhibitor of FAS, results in changes indicative of a rapid initiation of NF-κB signaling, including translocation of RelA/p65 NF-κB to the nucleus, activation of a transfected NF-κB-luciferase reporter, and increased expression of NF-κB-dependent transcripts, IL-6, IL-8, and COX-2. Verifying that these responses to C93 are specifically related to inhibition of fatty acid synthase (FAS), we focused on attenuation by adaptive responses of neoplastic cells to drug-induced stress. Treating lung cancer cells with C93, a pharmacological inhibitor of FAS, results in changes indicative of a rapid initiation of NF-κB signaling, including translocation of RelA/p65 NF-κB to the nucleus, activation of a transfected NF-κB-luciferase reporter, and increased expression of NF-κB-dependent transcripts, IL-6, IL-8, and COX-2. Verifying that these responses to C93 are specifically related to inhibition of FAS, we confirmed that levels of these same transcripts increase in response to siRNA targeting FAS. Inhibiting this NF-κB response (either by transfecting a mutant IκBα or treating with bortezomib) resulted in increased cell killing by C93, indicating that the NF-κB response is protective in this setting. Because inhibiting FAS leads to accumulation of intermediate metabolites of fatty acid biosynthesis, we then questioned whether protein kinase C (PKC) is involved in this response to metabolic stress.

Overexpression of fatty acid synthase (FAS)4 is common in aggressive human cancers, and blocking FAS inhibits growth and leads to apoptosis in these cancer cells (1, 2). Although it is now well accepted that increased activity of this enzyme plays a key role in maintaining the metabolic stability of cancer cells, the molecular consequences of inhibiting FAS are still not well understood.

One central molecular network commonly altered in cancer pathogenesis and also affected by cancer treatment is that regulated by the transcription factor NF-κB. NF-κB signaling is constitutively activated in many epithelial solid tumors and hematologic malignancies (3–5), and this activation appears to generally confer resistance to apoptosis as well as enhance growth properties of cancer cells. Consequently, numerous inhibitors of NF-κB are being investigated for potential use in treating cancer (6, 7), and bortezomib, a proteosome inhibitor widely used for treating myeloma, is thought to act largely through inhibition of NF-κB by stabilization of the IκBα subunit (8, 9). Interestingly, NF-κB activity is also inhibited by a number of naturally occurring lipid compounds that have recognized anti-neoplastic properties, including curcumin, resveratrol, and coix seed extract (10–12), providing additional evidence to suggest that NF-κB activity supports or promotes the malignant phenotype.

NF-κB activity does not uniformly contribute to malignancy, however, and in some situations, increased NF-κB activity may actually suppress malignant characteristics of cells (13). For example, it has been shown that induction of p53 leads to activation of NF-κB, correlating with the ability of p53 to induce apoptosis (14). Thus, at least in some cellular settings, inhibition or loss of NF-κB activity abrogates p53-induced apoptosis, indicating that NF-κB can be functional in p53-mediated cell death.

The role of NF-κB signaling in the response of cancer cells to chemotherapy also appears to depend on variables of the particular situation. In many circumstances, activation of NF-κB by therapeutic agents appears to inhibit apoptosis and thus attenuates the response to these agents (15–17). However, activation of NF-κB by cancer therapeutic agents appears to mediate cell death in other circumstances, including treatment with UV light (18), doxorubicin (19), and paclitaxel (20). In light of the general importance of NF-κB to cellular physiology and response to stress and the expectation that manipulations of lipid metabolic pathways could affect NF-κB signaling, we investigated the effects of inhibiting FAS on NF-κB and the role of NF-κB signaling in the response of lung cancer cells to this inhibition.

EXPERIMENTAL PROCEDURES

Cell Culture—Human lung cancer cell lines A549 and H1975 (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C/5%
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CO₂. Cultures were screened periodically for mycoplasma contamination. For experiments using a constitutively active mutant IkBα to inhibit NF-κB, we stably transfected A549 cells with either the mutant IkBα (mIkBα; a gift of Drs. Yi Huang and Weimin Fan (21)) or pcDNA3.1A(−) control vector (Invitrogen). In brief, 1 × 10⁵ cells were transfected with 2 μg of mIkBα plasmid encoding a G418 resistance gene with 6 μl of Lipofectamine (Invitrogen) for 4 h. The transfection mixture was replaced with RPMI supplemented with 10% serum, and incubation was continued for 2 days before initiating selection with G418 (300 μg/ml). Resistant clones were selected at 4 weeks and screened for mIkBα protein expression by Western blot using 1κBα antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cell lines transfected with empty vectors, pcDNA3.1A(−), were also screened by G418 in parallel for controls.

Reagents—The specific FAS inhibitor C93, supplied by FASgen (Baltimore, MD), was dissolved in DMSO at a stock concentration of 50 mg/ml. Bortezomib (Millennium, Cambridge, MA) was dissolved in distilled H₂O at a stock concentration of 1 mg/ml. RO-31-8220, SC-791, and NS-398 (Calbiochem) were prepared at stock dilutions of 2 mm, 10 mm, and 10 μM, respectively, in DMSO. Prostaglandin E₂ (PGE₂) (Sigma-Aldrich) was prepared as a 2 μM stock in distilled H₂O. Fluorescein-tagged small interfering RNA (siRNA) against FAS was generated using combinations of sequences corresponding to nucleotides 1212–1231 (AACCCTGAGATCCCAGCGCTG) and 329–348 (AAGCAGGCACACACGATGGAC) of human FAS. For PKCα, siRNA was generated using a sequence corresponding to nucleotides 513–533 (AAGCTCCATGTCA-CCAAGCTGGCCGT) and non-targeting control siRNA was made using the sequence AATTCTCCGAACGTGTCACGT (all siRNA provided by Invitrogen). Dharmacon SMART Pool (Lafayette, CO) was used for PKCβ siRNA. All siRNA transfections were performed over 48 h using oligofectamine (Invitrogen) according to the manufacturer’s recommendations.

Immunoblot Analysis—For measurements of specific protein levels in cultured cells, samples were collected in lysis buffer (50 mMol/liter Tris-Cl (pH 7.0), 1 mmol/liter EDTA, 1% Triton X-100) and sonicated until clear. Protein concentration was determined by the Pierce BCA assay (Thermo Fisher Scientific, Rockford, IL). Protein samples (25 μg) were separated on 10% SDS-PAGE gels. Proteins were then transferred to Trans-Blot membranes. Membranes were blocked with 5% non-fat milk, incubated with isotype-specific, affinity cross-adsorbed anti-IgG antibodies (Chemicon). 4,6-Diamidino-2-phenylindole (DAPI) was used to highlight cell nuclei, and images were acquired using epifluorescence microscopy (Eclipse 200, Nikon) with a 60× objective (openlab software (Improvision, Lexington, MA), and an internally cooled 12-bit CCD camera (CoolSnapHQ, Photometrics, Tucson, AZ).

RT-PCR/Quantitative PCR—Transcript levels were measured by quantitative real-time PCR using previously described methods (22) using the following primer sequences: IL-6, 5'-CCTTCTCCACAAAGCGCTCTC-3' (forward) and 5'-GGCAAGTCTCCCTCATTGAATC-3' (reverse); IL-8, 5'-ATGACTTCACCGTTGGCCG-3' (forward) and 5'-CCTTCTCACA- ACTTCTCCACAC-3' (reverse); COX-2, 5'-TCTCAATGAGATTGTGGGAAAAT-3' (forward) and 5'-AGATCATCCTC- TGCTCTGATATCTCTT-3' (reverse); β-actin, 5'-TCCGGAGACGCGGTGTC-3' (forward) and 5'-CCTGCTGCTGATTCTC- CCA-3' (reverse). Quantitative PCRs were performed in triplicate using an iCycler (Bio-Rad). The amplified products were quantified by fluorescence intensity of SYBR Green I (Molecular Probes, Inc., Eugene, OR). Average -fold changes were cal-
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Measurements of PGE2 Release—A549 cells were cultured at ~80% density in 12-well plates with or without C93 (3 μg/ml) in RPMI medium containing 0.5% FBS for 24 h. Medium was then changed and collected at the indicated time intervals for measuring PGE2 using an enzyme immunoassay (Arbor Assays, Ann Arbor, MI) according to the manufacturer’s instructions. Briefly, control and samples were added to each well and incubated for 15 min at room temperature and overnight at 4 °C with primary antibody and conjugate. After washing, substrate solution was added to each well for 30 min at room temperature. Finally, “stop solution” was added, and the optical density of each well was determined within 30 min using a microplate reader (wavelength 450 nm). The standards used were in the range of 12.5–400 pg/ml (detection limit of 16.8 pg/ml) for PGE2 (sensitivity of 10.9 pg/ml).

Protein Kinase Activity Assays—Bacterial expression vector for GST-1kBα (amino acids 1–54) was a gift of M. Karin (University of California, San Diego). Plasmids were transformed into the bacterial strain BL21(DE3) and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at room temperature. The bacterial pellet from 250 ml of culture was suspended in 50 ml of PBS containing lysozyme, sonicated for 20 s, and frozen and thawed twice. The lysates were then cleared by centrifugation at 12,000 rpm for 30 min at 4 °C and incubated with precleared glutathione-Sepharose 4B (GE Healthcare) for 4 h at 4 °C. After rinsing, bound proteins were eluted with 20 mM reduced glutathione and desalted with a desalt spin column (Pierce).

For the in vitro kinase assays, recombinant PKCα (Sigma) was incubated for 45 min at 30 °C in a 50-μl reaction mixture containing 20 mM Hepes (pH 7.4), 1.3 mM CaCl₂, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP, and 0.1 mg of GST-1kBα substrate. The reactions were terminated by the addition of SDS-sample buffer and heating for 5 min at 95 °C. The level of 1kBα phosphorylation was evaluated by immunoblotting with anti-phospho-1kBα (Ser-32/36) (Cell Signaling) as described above.

To test the effects of C93 treatment, cells were lysed and precleared for 3 h with Protein G-agarose (Roche Applied Science), followed by incubation for 1 h in the presence of an anti-PKCα antibody (Santa Cruz Biotechnology, Inc.). Protein G-agarose was added and incubated overnight at 4 °C. The samples were then washed three times with buffer (50 mM Tris-Cl, 150 mM NaCl, and 0.1% Nonidet P-40, pH 7.5). Immunoprecipitate of PKCα was used as kinase, and purified GST-1kBα was used as a substrate in vitro. The level of phosphorylated 1kBα was evaluated by immunoblotting with anti-phospho-1kBα (Ser-32/36) (Cell Signaling) as described above.

Statistical Analysis of Quantitative Data—Quantitative data were graphed and analyzed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). By convention, heights of columns represent means, and error bars represent S.E. values. Student’s t tests were used for two-way comparisons of categories, and significance was noted for p < 0.05.

RESULTS

Activation of NF-κB in Response to C93—To explore how inhibiting fatty acid synthase affects the NF-κB signaling pathway, we first evaluated the effects of C93, a second generation pharmacological inhibitor of FAS, on subcellular distribution of the p65/RelA subunit of NF-κB. Cultured A549 and H1975 non-small cell lung cancer cells were exposed to 5 μg/ml C93, stained using p65/RelA-specific antibodies, and evaluated by immunofluorescence imaging. At 1 h following C93 treatment, we observed translocation of p65/RelA from cytoplasm to the nucleus in both A549 cells and H1975 lung cancer cells (Fig. 1). This change occurred within a period of time that precedes measurable effects on cell survival, suggesting that NF-κB is activated early in the response of these cells to FAS inhibition.

Functional activation of NF-κB in response to C93 treatment was measured using an NF-κB-responsive reporter assay and...
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To verify that the activation of NF-κB signaling is specifically a result of inhibition of FAS and not an off target effect of C93, we used siRNA to specifically reduce levels of FAS in A549 cells. Because this approach cannot produce the rapid reduction of FAS activity seen with the pharmacological agent, we focused on measuring NF-κB-dependent transcripts to evaluate the effects of this siRNA treatment on NF-κB. As seen in Fig. 2F, transcript levels of IL-6, IL-8, and COX-2 were all increased after levels of FAS protein were reduced using siRNA, indicating that the same response typical for NF-κB activation could be induced by either pharmacological or molecular inhibition of FAS.

**Activation of NF-κB and COX-2 Are Protective in Cells Treated with FAS Inhibitors**—As noted above, NF-κB has been found to have either prosurvival or proapoptotic effects, depending on the stimulus and possibly the cell environment. To determine the cellular effects of NF-κB activation in this setting, we transfected A549 cells with mLkβα, which diminishes NF-κB signaling (21). This mLkβα construct has a deletion of the 36-amino acid sequence that contains the serine phosphorylation sites (Ser-32 and Ser-36) responsible for targeting Ikβ for degradation. Thus, cells transfected with this degradation-resistant form of mLkβα trap NF-κB in an inactive state. For our experiments, transfection was confirmed by RT-PCR with primers specific to the mutant or wild-type Ikβα, and an empty pcDNA3.1A(−) vector was used as control. To confirm functional efficacy of the transfection, transcripts of IL-6, IL-8, and COX-2 were measured using reverse transcription/quantitative PCR at 4, 8, and 24 h following C93 treatment and compared with levels in untreated control cells (Fig. 3, A–C). Although A549 cells transfected with empty pcDNA3.1A(−) vector showed increased transcript levels of all three genes after C93 treatment, these increases were significantly attenuated in cells transfected with the mutant Ikβα. These data are consistent with our findings that these three prosurvival target genes are up-regulated through activation of the NF-κB pathway after treatment with C93.

Experiments also showed that A549 cells are significantly more sensitive to cell killing by inhibition of FAS activity when cellular NF-κB activity is reduced by mLkβα. First, MTS assays (Fig. 3D) showed increased dose-dependent cell cytotoxicity by C93 in A549 mLkβα compared with A549 empty pcDNA3.1A(−) transfected cells. Corroborating these results, mLkβα-transfected A549 cells treated with 3 μg/ml C93 for 4 h showed 85.1% reduction in cloning efficiency compared with cells transfected with empty pcDNA3.1A(−) vector (Table 1). Finally, MTS assays showed increased time-dependent cell cytotoxicity by FAS siRNA in A549 mLkβα compared with A549 empty pcDNA3.1A(−) transfected cells (Fig. 3E). These data are consistent with activation of NF-κB having a protective function in the setting of inhibited FAS.

Similar results were observed in cultures of cells treated with bortezomib, a 26 S proteasome inhibitor that is believed to block the degradation of Ikβ, thus trapping NF-κB in an inactive state in the cytoplasm (24). Although bortezomib does not specifically target the NF-κB pathway, the use of this agent does result in a rapid change in NF-κB signaling in cultured cells. Using low doses of bortezomib alone, we observed min-

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**FIGURE 2. Inhibiting FAS by C93 causes functional activation of NF-κB.**

A, A549 cells were treated with C93, and luminescence from a NF-κB reporter construct was monitored in parallel with Renilla lucinescence (internal control) over a period of 16 h. The ratio of NF-κB luminescence to Renilla luminescence significantly increased in a time-dependent manner in response to C93 treatment, indicating an increase in NF-κB activity. Error bars, S.E.; n = 6; *, p < 0.05. A549 (B) and H1975 cells (C) were treated with C93 (μg/ml) for 4, 8, or 24 h, and real-time PCR was performed to monitor transcription of downstream NF-κB-regulated transcripts, IL-6, IL-8, and COX-2. Results show significant increases in all three downstream targets of NF-κB signaling (A549: p < 0.05, n = 3; H1975: p < 0.01, n = 3), with variability between cell lines and among target genes with respect to timing of maximal changes. D, Western blot analysis confirms increased expression of COX-2 in A549 cells after treatment with C93 (5 μg/ml). E, levels of PGE2 released into culture medium after treatment of A549 cells with C93 (5 μg/ml), beginning 24 h prior to designated collection periods. Measurements were performed in triplicate; *, p < 0.05; **, p < 0.01 relative to appropriate control for time of collection. F, siRNA-mediated reduction in levels of FAS transcript levels also leads to increased transcript levels of COX-2, IL-6, and IL-8. FAS and COX-2 were measured by immunoblot in cell lysates, with actin levels measured as a control. IL-6 and IL-8 transcript levels were measured by reverse transcription PCR, with GAPDH measured in these samples as a reaction control.
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FIGURE 3. Inhibiting NF-κB reduces C93-dependent induction of cell survival gene expression and enhances the cell killing of C93. A–C, A549 cells were transfected with either control pcDNA 3.1A(−) or a mutant IκBα construct that blocks NF-κB activity. Cells were then treated with C93 (3 μg/mL), and real-time PCR was performed to measure mRNAs levels of IL-6 (A), IL-8 (B), and COX-2 (C). Results, expressed as levels relative to untreated cells, indicate that cells with transfected mutant IκBα construct have reduced induction of these transcripts by C93 treatment. Measurements were performed in triplicate and compared within specific time categories. *, p < 0.05; **, p < 0.01. D, MTS assays show the effects of inhibiting FAS and NF-κB pathways, individually or in combination, on survival of lung cancer cells. A549 cells stably transfected with either control pcDNA 3.1A(−) (black bars) or mutant IκBα (light gray bars) were compared with 3, 5, or 7 μg/mL C93 for 20 h, and MTS assays were performed. Data show reduction in cell survival in cells expressing the IκBα mutant. *, p < 0.001, n = 10. E, similarly, toxicity induced by reducing FAS levels with siRNA is greater in cells with inhibited NF-κB as a result of transfection with a mutant IκBα construct. Immunoblot demonstrating knockdown of FAS levels by siRNA is shown above the graph for the MTS assay results. MTS results are plotted for days 2, 3, and 5 after transfection with FAS siRNA. *, p < 0.05; **, p < 0.01. F, co-treatments (SC-791 and C93) compared with C93 alone. *, p < 0.005, n = 10. C, in addition, exogenous PGE2 (10−5 or 10−8 M) added to culture medium of A549 cells during exposure to C93 (5 μg/mL) attenuates cytotoxicity of C93 as measured by an MTT assay. *, p < 0.05, n = 10. Error bars, S.E.

TABLE 1
Survival of lung cancer cells treated with combinations of bortezomib and C93

| Cloning efficiency | Percentage reduction versus control |
|-------------------|-----------------------------------|
| H1975 + 3 μg/mL C93 | 0.0098 ± 0.0012 | 94.9% |
| H1975 + 3 μg/mL C93 + 10 nM bortezomib | 0.0004 ± 0.0007 | 99.7% |
| A549 pcDNA + 3 μg/mL C93 | 0.0017 ± 0.0003 | 98.3% |
| A549 millBα + 3 μg/mL C93 | 0.0002 ± 0.0002 | 99.9% |

Cox2

FIGURE 4. Increased COX2 mediates some of the protective effects in response to C93 treatment. A, to determine whether PGE2 affects survival after C93 treatment, A549 cells were treated with the COX2 inhibitor NS-398 (10 μM) and C93 (3 or 5 μg/mL), individually or in combination, for 20 h. Note that NS-398 alone at this level does not reduce survival. B, similarly, A549 cells were treated with the COX2 inhibitor SC-791 (10 μM) and C93 (7 μg/mL), individually or in combination, for 20 h. SC-791 alone at this level does not reduce survival. However, results show a significant decrease in cell survival with co-treatments (SC-791 and C93) compared with C93 alone. *, p < 0.005, n = 10. C, in addition, exogenous PGE2 (10−5 or 10−8 M) added to culture medium of A549 cells during exposure to C93 (5 μg/mL) attenuates cytotoxicity of C93 as measured by an MTT assay. *, p < 0.05, n = 10. Error bars, S.E.

NF-κB-mediated effects on lung cancer cell survival; for example, at 20 h post-treatment, more than 92% of cells were still viable according to MTS assay results. However, as shown in Fig. 3F, this level of bortezomib results in significant sensitization of A549 and H1975 lung cancer cells to C93. Thus, these experiments are consistent with the results of experiments using mutant IκBα transfection and suggest that activation of NF-κB has a protective effect on lung cancer cells treated with an FAS inhibitor.

We then investigated the potential role of COX-2 as a specific NF-κB-regulated transcript in protecting lung cancer cells from the metabolic stress initiated by inhibiting FAS, recognizing that a number of other NF-κB-dependent transcripts could also have cytoprotective effects. First, A549 cells were treated with combinations of C93 and either SC-791 or NS-398, both of which block COX-2 activity. Neither of these COX-2 inhibitors showed a significant effect on cell viability when used alone at low doses (Fig. 4, A and B), but as expected, C93 alone at doses of 3 or 5 μg/mL resulted in decreased cell viability. This C93-induced cell killing was significantly enhanced when cells were co-incubated with either of the COX-2 inhibitors (Fig. 4, A and B). By contrast, adding additional PGE2 to the culture medium offered A549 cells some protection from the C93 exposure (Fig. 4C). Thus, COX-2 probably represents one of the NF-κB-regulated transcripts that mediate the protective response initiated by inhibition of FAS.
**Activation of PKC in Response to C93 Mediates the Activation of NF-κB**—We then explored the possibility that the activation of NF-κB induced by inhibiting fatty acid synthase is mediated by protein kinase C (PKC), which can be activated by a variety of lipid mediators (25) and in turn is reportedly capable of phosphorylating and inactivating IκBα (26, 27). We first used isoform-specific antibodies to study localization of two classical PKC isoforms (α and β) and one novel PKC isoform (δ) after treatment with C93 (5 μg/ml) in lung cancer cell lines (Fig. 5). Immunofluorescence images show that PKCα rapidly translocates from the cytoplasm to the nucleus within 1 h of C93 treatment in A549 cells (Fig. 5A). By contrast, PKCβ translocates in the opposite direction, from the nucleus to the cytoplasm (Fig. 5B). Treatment with C93 had no discernible effects on the subcellular distribution of the novel PKCδ isoform (Fig. 5C). These changes in cellular localization are typically indicative of phosphorylation and activation of PKC, and we confirmed that the PKC is phosphorylated after C93 treatment by Western blot analysis using a non-isof orm-specific anti-phosphorylated PKC antibody (Fig. 5D).

Recognizing that C93 treatment results in changes in the phosphorylation and cellular distribution of PKCα and PKCβ, we next investigated whether these effects on PKC are related to the effects on NF-κB. First, we co-treated cells with combinations of C93 and RO-31-8220, a pharmacological inhibitor of classic PKC isoforms, at a concentration of RO-31-8220 (100 nM) that alone does not affect cell growth. In these experiments, we observed that RO-31-8220 prevented C93-induced translocation of NF-κB to the nucleus (Fig. 5E), and RO-31-8220 also significantly attenuated the rapid C93-dependent increases in mRNA expression of IL-6, IL-8, and COX-2 (Fig. 5F).

We further examined the roles of PKCα and PKCβ as activators of NF-κB activity using a luciferase reporter assay that links a firefly luciferase reporter to an NF-κB response element. Using the ratio of firefly luciferase to control Renilla luciferase luminescence as a measure of NF-κB activity, we observed that activation of NF-κB by C93 and PMA was significantly attenuated in cells that were co-transfected with siRNA specific to PKCα (Fig. 5G). By contrast, siRNA to PKCβ appeared to have no significant effects on activation of NF-κB by C93 (Fig. 5G). Together, these results indicate that the activation of NF-κB following inhibition of FAS by C93 is largely a result of PKC (particularly PKCα) activation. siRNA specific to PKCα also prevented C93-induced translocation of NF-κB to the nucleus (Fig. 5E).
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To explore mechanisms of this PKC-dependent activation of NF-κB, we focused on the IκBα subunit of NF-κB, which reportedly can be phosphorylated by PKC (26, 27). We first sought to determine whether PKC could phosphorylate IκBα in vitro, using recombinant PKCa protein as kinase and recombinant GST-IκBα as substrate. Probing an immunoblot of this reaction mixture with an anti-phospho-IκBα antibody (Fig. 6A) confirmed previous reports that PKCa enzyme is capable of directly phosphorylating IκBα in a dose-dependent manner. Next, to test the potential effect of C93 on activating phosphorylation of IκBα by PKC, an in vitro kinase assay was performed using PKCa immunoprecipitated from A549 cells (C93-treated or control) and recombinant GST-IκBα as substrate. Phosphorylation of IκBα was measured using phosphospecific antibody. C, siRNA-mediated decreases in PKCa levels lead to decreases in phosphorylation of IκBα (pIκBα) but no measurable decreases in phosphorylation of IKK (pIKKα/β) in intact cells treated with C93 or PMA. Cultures of A549 cells were transfected with PKCa siRNA as described under “Experimental Procedures” and probed for pan-phosphorylated PKC, total PKCa, phosphorylated IκBα (pIκBα), and phosphorylated IKKα/β (pIKK α/β). Total IκBα, IKKα/β, and actin were also probed as loading controls.

FIGURE 6. C93-activated PKCa phosphorylates IκBα. A, PKCa phosphorylates IκBα in vitro. Various concentrations of recombinant PKCa were added to a kinase reaction with recombinant IκBα substrate, and phosphorylation of IκBα was measured by immunoblot. B, in vitro kinase assay was performed using PKCa immunoprecipitated from A529 cells (C93-treated or control) and recombinant GST-IκBα as substrate. Phosphorylation of IκBα was measured using phosphospecific antibody. C, siRNA-mediated decreases in PKCa levels lead to decreases in phosphorylation of IκBα (pIκBα) but no measurable decreases in phosphorylation of IKK (pIKKα/β) in intact cells treated with C93 or PMA. Cultures of A549 cells were transfected with PKCa siRNA as described under “Experimental Procedures” and probed for pan-phosphorylated PKC, total PKCa, phosphorylated IκBα (pIκBα), and phosphorylated IKKα/β (pIKK α/β). Total IκBα, IKKα/β, and actin were also probed as loading controls.

DISCUSSION

We have investigated how inhibiting FAS affects the PKC and NF-κB signaling pathways and how these signaling pathways, in turn, affect the cellular response to decreased FAS activity. Using a pharmacological approach, we note that inhibiting FAS with C93 leads to a rapid translocation of the p65/RelA subunit of NF-κB to the nucleus, an increase in NF-κB reporter activity, and increased cellular transcription of prosurvival target genes that are downstream of NF-κB signaling. Although siRNA is not capable of inducing as rapid a response as possible with a pharmacological agent, the changes in NF-κB-dependent transcripts seen after treating cells with siRNA specific to FAS are again consistent with activation of NF-κB. The significance of this NF-κB was demonstrated by the finding that pharmacologically inhibiting NF-κB and FAS simultaneously in vitro leads to more effective cell killing than inhibiting either of these pathways individually. Together, these results indicate that NF-κB activity is induced in response to inhibiting FAS as a protective response by these cancer cells.

C93 alone (Fig. 7, A and B). At doses used in these experiments, RO alone had no significant effect on cell death. A clonogenic assay yielded results consistent with MTS assay results, with 99 and 77.6% reductions in clonogenic efficiency for A549 and H1975 cells, respectively, when treated with a combination of the two drugs, compared with C93 treatment alone (Table 2). Thus, these results indicate that inhibiting PKC results in enhanced killing of lung cancer cells by C93, most likely as a consequence of PKC leading to activation of NF-κB.

TABLE 2

Clonogenicity of lung cancer cells after treatments with combinations of C93 and PKC inhibitor RO-31-8220

| Condition | Cloning efficiency | Percentage reduction versus C93 alone |
|-----------|--------------------|--------------------------------------|
| H1975 + 3 μg/ml C93 | 0.010 ± 0.001 | 99.2% |
| H1975 + 3 μg/ml C93 + 100 nM RO-31-8220 | 0.002 ± 0.0001 | 77.6% |
| A549 + 3 μg/ml C93 | 0.706 ± 0.116 | 99.9% |
| A549 + 3 μg/ml C93 + 100 nM RO-31-8220 | 0.006 ± 0.001 | 99.2% |

Clonogenic growth assays measure effects of C93 (3 μg/ml) alone or in combination with 100 nM RO-31-8220, a PKC inhibitor. This concentration of RO-31-8220 alone results in no measurable change in cloning efficiency, but the combination of agents is significantly more effective in reducing clonogenicity than C93 alone (p < 0.01, n = 3).

FIGURE 7. Activation of PKC is protective in cells treated with FAS inhibitors. Shown is an MTT assay measuring short term survival of A549 (A) and H1975 (B) cells exposed to 100 nM RO-31-8220, C93 (7 μg/ml for A549, 3 μg/ml for H1975), or combinations of agents. Error bars, S.E.; *, significance by Student’s t test at p < 0.001, n = 10.
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We extended these studies to the evaluate the PKC pathway in this response, because 1) a variety of lipids affect PKC activity, and 2) PKCα is known to activate NF-κB by phosphorylation of IkBα or through activation of the MEK-ERK pathway via Raf1 (28, 29), which in turn lead to phosphorylation of IkBα and nuclear translocation of NF-κB (30). Using immunofluorescence as a sensitive means to detect PKC activity, we noted translocation of both PKCα and PKCβ isoforms but not the PKCδ isoform. Immunoblot analysis confirmed autophosphorylation of PKC after C93 exposure.

A mechanistic link between this PKC activation with subsequent activation of NF-κB was shown by preventing the C93-dependent increases in IL-6, IL-8, and COX-2 mRNA levels with RO-31-8220, a PKC inhibitor, and by attenuating C93-dependent activation of an NF-κB-dependent luciferase reporter construct with siRNA specific to PKCα. More specifically, C93-dependent activation of PKCα leads to increased IkBα phosphorylation, and this IkBα phosphorylation can be attenuated by pretreating cells with PKCα-specific siRNA. Although our data suggest that PKCα directly phosphorylates IkBα, we cannot exclude the possibility that other serine/threonine kinases, known to phosphorylate IkBα under physiological conditions, are involved in this process. Finally, C93-induced cell killing is increased by inhibiting PKC with RO-31-8220, suggesting that the NF-κB-mediated protective response is initiated by PKC activation and IkBα phosphorylation.

Our finding that NF-κB is activated by inhibitors of FAS is not unexpected, based on previously reported observations (31) that treating SK-BR3 cells with cerulinin results in the translocation of RelA/p65 to the nucleus. However, the role of this activation could not be accurately predicted because extensive work has shown that NF-κB activation, which commonly occurs in response to cellular stress, can have diverse consequences. Although NF-κB activation is generally considered to inhibit apoptosis and thus attenuate cellular responses to anti-cancer therapy, several examples of NF-κB activation actually functioning as a mediator of apoptosis have been documented. For example, in SH-SY5Y neuroblastoma cells, inhibiting NF-κB by either pyrrolidine dithiocarbamate treatment or transfecting mutant IkBα rendered cells resistant to killing by doxorubicin (19), and BCap37 breast cancer cells stably transfected with antisense IkBα expression vectors have remarkably increased sensitivity to paclitaxel-induced apoptosis (20). The role of NF-κB as a mediator of apoptosis induced by chemotherapeutic drugs is probably dependent on the cellular background, as suggested by experiments that found NF-κB to be essential for apoptosis induced by DNA damage in Saos-2 cells with wild-type p53 but not in p53-deficient Saos-2 cells (14).

In light of the ability of NF-κB to function in either proapoptotic or antiapoptotic roles, it was not initially apparent to us how the activation of this pathway would function in the setting of metabolic stress initiated by the inhibition of FAS. Using multiple experimental approaches, we were able to prevent activation of NF-κB during treatment with FAS inhibitors, and we consistently observed increased cell killing by these inhibitors, when NF-κB was also inhibited. Thus, our experiments provide evidence that NF-κB signaling provides a protective function in the setting of treatment with metabolic inhibitors such as inhibitors of fatty acid synthase.

The present work could have implications for classifying cellular responses to inhibitors of FAS or even eventually predicting which cancers are more likely to respond to this class of agents. Moreover, these findings could be particularly significant for enhancing the efficacy of fatty acid synthase inhibitors in cancer treatment, and the possibility that low levels of NF-κB inhibitors (such as bortezomib) could significantly enhance the antineoplastic efficacy of FAS inhibitors deserves further investigation and consideration for clinical testing. Interestingly, bortezomib appears to have limited activity as a single agent in non-small cell lung cancer, but it has shown encouraging activity in combination with chemotherapy without significantly adding to toxicity (32, 33). Thus, inhibiting NF-κB might prove to be generally more important for sensitizing lung cancer cells than for providing the primary lethal injury in cancer chemotherapy.

Similarly, inhibiting downstream pathways of NF-κB, such as COX-2, could also potentially increase the efficacy of FAS inhibitors. Clinical trials are ongoing to evaluate COX-2 inhibitors as adjuvants to chemotherapy in patients with lung cancer (34), although initial studies did not find improved survival in patients with docetaxel combined with celecoxib, as opposed to docetaxel alone (35). Based on our findings, however, the combination of celecoxib with FAS inhibitors for lung cancer treatment deserves further evaluation.

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