Suppression of NF-κB Survival Signaling by Nitrosylcobalamin Sensitizes Neoplasms to the Anti-tumor Effects of Apo2L/TRA企业提供  

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We have previously demonstrated the anti-tumor activity of nitrosylcobalamin (NO-Cbl), an analog of vitamin B12 that delivers nitric oxide (NO) and increases the expression of tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAiL) and its receptors in human tumors. The specific aim of this study was to examine whether NO-Cbl could sensitize drug-resistant melanomas to Apo2L/TRAiL. Antiproliferative effects of NO-Cbl and Apo2L/TRAiL were assessed in malignant melanomas and non-tumorigenic melanocyte and fibroblast cell lines. Athymic nude mice bearing human melanoma A375 xenografts were treated with NO-Cbl and Apo2L/TRAiL. Apoptosis was measured by TUNEL and confirmed by examining levels and activity of key mediators of apoptosis. The activation status of NF-κB was established by assaying DNA binding, luciferase reporter, and the phosphorylation status of IκBα, and in vitro IKK activity. NO-Cbl sensitized Apo2L/TRAiL-resistant melanoma cell lines to growth inhibition by Apo2L/TRAiL but had minimal effect on normal cell lines. NO-Cbl and Apo2L/TRAiL exerted synergistic anti-tumor activity against A375 xenografts. Treatment with NO-Cbl followed by Apo2L/TRAiL induced apoptosis in Apo2L/TRAiL-resistant tumor cells, characterized by cleavage of caspase-3, caspase-8, and PARP. NO-Cbl inhibited IKK activation, characterized by decreased phosphorylation of IκBα and inhibition of NF-κB DNA binding activity. NO-Cbl suppressed Apo2L/TRAiL- and TNF-α-mediated activation of a transfected NF-κB-driven luciferase reporter. XIAP, an inhibitor of apoptosis, was inactivated by NO-Cbl. NO-Cbl treatment rendered Apo2L/TRAiL-resistant malignancies sensitive to the anti-tumor effects of Apo2L/TRAiL in vitro and in vivo. The use of NO-Cbl and Apo2L/TRAiL catalyzes the tumor-specific properties of both agents and represents a promising anti-cancer combination.  

Apoptosis is the rigorously controlled process of programmed cell death. Current trends in cancer drug design focus on selective targeting to activate the apoptotic signaling pathways within tumors while sparing normal cells (1). The tumor-specific properties of tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAiL)1 have been widely reported (2–5). Apo2L/TRAiL has been used as an anti-cancer agent alone and in combination with other agents (6–10) including ionizing radiation (11–13). Apo2L/TRAiL can initiate apoptosis in cells that overexpress the survival factors Bcl-2 and Bel-XL, and may represent a treatment strategy for tumors that have acquired resistance to chemotherapeutic drugs (14).  

Apo2L/TRAiL binds its cognate receptors and activates the caspase cascade utilizing adapter molecules such as FADD (5, 15). TRAIL receptors, type II membrane-bound proteins, are members of the tumor necrosis factor (TNF) superfamily of receptors (2). To date, five Apo2L/TRAiL receptors have been identified (5). Two receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) mediate apoptotic signaling, and three non-functional receptors, DcR1, DcR2, and osteoprotegerin (OPG) may act as decoy receptors (5). Agents that increase expression of DR4 and DR5 may exhibit synergistic anti-tumor activity when combined with Apo2L/TRAiL (16).  

Recently we demonstrated the anti-tumor effects of nitrosylcobalamin (NO-Cbl), an analogue of vitamin B12 (cobalamin, Cbl) coordinated with nitric oxide (NO) as a ligand (17). Anti-tumor activity correlated with the expression of the transcobalamin II receptor (TCII-R) on the plasma membrane of tumor cells. NO-Cbl is an ideal candidate to be used in combination with Apo2L/TRAiL because NO-Cbl induced the mRNAs of DR4, DR5, and Apo2L/TRAiL in ovarian carcinoma cells (17). Treatment of leukemia cells with Apo2L/TRAiL resulted in increased Apo2L/TRAiL mRNA and protein, suggesting autocrine regulation that can function in a positive feedback loop (18). Transfecting ovarian carcinoma cells with a non-functional, dominant negative DR5 receptor (DR5a) (19) abrogated increases in DR4, DR5, and Apo2L/TRAiL cuando treated with NO-Cbl.2 This suggested that a functional Apo2L/TRAiL receptor was necessary for the autoinduction of Apo2L/TRAiL, and that DR5a interfered with positive feedback signaling.  

Cytokines of the TNF superfamily, upon receptor ligation, simultaneously induce an apoptotic signal (mediated via caspase-8) in addition to a survival signal (mediated via caspase-8)
tion of NF-κB) (20). NF-κB is a transcription factor that generally functions to suppress apoptosis (20, 21). Binding of TNF-α (22) or Apo2L/TRAIL (23) to their cognate receptors results in the phosphorylation and activation of the inhibitor of κB kinase (IKK). In its quiescent state, NF-κB is complexed to the inhibitor of κB (IκB) in the cytoplasm (24). Activated IKK phosphorylates serine 32 and serine 36 of IκB (25). Once phosphorylated, IκB is ubiquitinated and targeted for proteolysis as it remains complexed to NF-κB (24). Within the proteosome IκB is degraded, while NF-κB is released from the inhibitor to translocate to the nucleus where it binds to NF-κB response elements, which activate transcription of target genes (22, 24). NF-κB stimulates transcription of genes such as Bcl-XL and cIAP that function as survival factors (26, 27). Therefore, agents that inhibit NF-κB may have anti-tumor activity.

NO is a ubiquitous, multifaceted signaling molecule (28, 29) that inhibits NF-κB DNA binding activity (30, 31) and suppresses the cell survival function of NF-κB (32, 33). Sulfasalazine, an anti-inflammatory agent, inhibits NF-κB activity, enhancing Apo2L/TRAIL-induced apoptosis in human leukemia cells (34). Furthermore, Apo2L/TRAIL-induced apoptosis was increased in prostate carcinoma cells that were infected with a mutant IκB, supporting the role of NF-κB as a TRAIL-induced survival factor (35). The use of NO-Chl to deliver nitric oxide and suppress the survival arm of NF-κB is a promising strategy to enhance the anti-tumor effects of Apo2L/TRAIL in resistant tumors.

In this study we pretreated cells with NO-Chl to inhibit NF-κB activity and enhance the apoptotic signal of Apo2L/TRAIL. Our specific aims were to: 1) measure the anti-tumor effects of NO-Chl and Apo2L/TRAIL in Apo2L/TRAIL-resistant cell lines, and to 2) determine the mechanism by which NO-Chl inhibits NF-κB activation.

EXPERIMENTAL PROCEDURES

Synthesis of Nitrosylcobalamin—Nitrosylcobalamin was synthesized as previously described (17, 36). Hydroxocobalamin (vitamin B12a) was purchased from Sigma Chemical Co. Hydroxocobalamin (vitamin B12a) and sodium nitrite were stored in 80% aqueous methanol (Burdick and Jackson, Muskegon, MI) and exposed to CPromiscuous gases (Burdick and Jackson, Muskegon, MI) and exposed to CPromiscuous gases (Burdick and Jackson, Muskegon, MI) and exposed to C-sulfuric acid and stored at −30 °C prior to use.

Cell Culture and Cytokine Treatments—Human melanoma tumor cell lines, WM9 and WM211 (Wistar Institute, Philadelphia, PA), and A375 (ATCC, Manassas, VA) were grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% Antibiotic-Antimycotic (Invitrogen). Cells were maintained in 5% CO2 at 37 °C in a humidified tissue culture incubator. Primary non-tumorigenic melanoma cell lines (DM1-1 and DM1-1-A, A. Gudkov, CCF, Cleveland, OH), and human foreskin fibroblasts (HFF; CCF, Cleveland, OH) were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum. Cells were confirmed as mycoplasma free.

All experiments were performed using trimeric recombinant human Apo2L/TRAIL (37) (Genentech Inc., San Francisco, CA) and were independent of the source (Peprotech Inc, NJ). Apo2L/TRAIL (Genentech Inc.), consisted of more than 99% trimeric protein with Zn2++, which is necessary for optimal biologic activity of Apo2L/TRAIL (37).

Sulforhodamine B Cell Growth Assay—Cells were harvested with 0.5% trypsin/0.53 mM EDTA, washed with phosphate-buffered saline and resuspended in media containing 10% fetal bovine serum. Cells were plated in 96-well plates for 20 min and then Apo2L/TRAIL was added at different dilutions (25, 50 and 100 μM) to the assay plate. Repliques of four were performed for each treatment. After 16 h, recombinant human Apo2L/TRAIL was added at different concentra-

tions (25–100 nM). Growth was monitored by the sulforhodamine B (SRB; Sigma Chemical) colorimetric assay (38). After 40 h growth, the medium was removed, and the cells were fixed with 10% trichloroacetic acid and stained with SRB. Bound dye was eluted from the cells with 10 mM Tris-HCl (pH 10.5) and absorbance was measured at 570 nm using a Lab systems Multiskan RC 96-well plate reader (Lab Systems Multispec, RC, Thermo Lab Systems, Franklin, MA). To quantify the growth of the cells, the experimental absorbance values (Aexp) were compared with initial absorbance readings representing the starting cell numbers (A0).

In Vivo Experiments—The Institutional Animal Care and Use Committee at the Cleveland Clinic Foundation approved all procedures for animal experimentation. 5-week-old NCR male athymic nude homozygous (nu/nu) mice (Taconic, Germantown, NY) were inoculated with A375 tumors. There were four experimental groups (untreated, single agents, and the combination) n = 8. Cultured tumor cells (4 × 106) were inoculated in flanks in the mid-axillary line. NO-Chl was given twice daily (50 mg/kg s.c.) and recombinant trimeric Apo2L/TRAIL (50 mg/kg s.c.) (37) was administered every other day, starting on day 2. Tumor volume was measured three times a week using the formula: π/6 × major axis × minor axis. Formalin-fixed sections were processed by the Cleveland Clinic Histology Core. Sections were stained with hematoxylin and eosin and evaluated for pathologic changes in a blinded fashion.

TUNEL Assay—A375 cells were cultured for 36 h and exposed to various treatments (control, NO-Chl, Apo2L/TRAIL, and NO-Chl + Apo2L/TRAIL). Apoptotic cells were detected by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end-labeling) staining using a commercially available kit (APO-BRDU kit, BD PharMingen, San Diego, CA). Cells were processed according to the manufacturer’s recommended protocol. The percentage of fluorescein isothiocyanate-negative-positive cells was analyzed by fluorescent activated cell-scanning (FACS, Becton Dickinson, San Jose, CA).

Electrophoresis and Immunoblot Analyses—Whole cell lysates were prepared in 1× lysis buffer (50 mM Tris-Cl, pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 250 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) for subsequent immunoblotting studies. SDS-PAGE was conducted by using the Laemmli buffer system and 12% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes by the semidyry method (Trans Blot S.D., BioRad, Hercules, CA). Binding of the primary and secondary antibodies was performed according to standard protocols (39). Membranes were im-

munoblotted with mouse antibodies against PARP (BioMOL, FLIP (Calbiochem), p53, IκBα (Cell Signaling), cIAP-1, anti-Iκκα/β (Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Pierce). Immunoreactive bands were visualized by using enhanced chemilumi-

nescence (PerkinElmer). Equal protein loading was confirmed by reprobing with monoclonal anti-actin antibody (Sigma Chemical Co.). All immunoblots in this study were repeated 3 times with reproducible results.

Electrophoretic Mobility Shift Assay (EMSA)—A375 cells were treated with NO donors (NO-Chl, NOC-18, SNAP, 100 μM, 16), or with NO donors (16 h) followed by Apo2L/TRAIL or TNF-α for 16 h. Plates were washed twice with ice-cold phosphate-buffered saline. Cells were resuspended in cold 1× lysis buffer (20 mM HEPES, 20 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 10% glycerol, and protease inhibitors) as previously described (40) and incubated on ice for 30 min followed by centrifugation at 4 °C at 10,000 rpm for 10 min. Supernatants were transferred to 100 μl microcentrifuge tubes and protein concentrations were assessed using the Bradford method (Bio-

rad protein assay, BioRad). The NF-κB consensus binding sequence (5′-AGTGGAGGGGACCTTCCAGGCGC-3′) from the IFN-β gene promoter was end-labeled with [γ-32P]ATP (3000 Ci/mmol) using T4 polynu-

cleotide kinase. DNA binding reactions were performed in 20 μl reac-

tions containing 10 mM HEPES, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, and 10% glycerol. Complexes were separated from the free probe on 6% non-

denaturing polyacrylamide gels in 0.5× TBE buffer at 200 V for 2 h. Gels were dried and exposed to film. To verify the identity of the band
observed lysates from A375 cells stimulated for 15 min with TNF-α (20 ng/ml) were incubated with anti-NF-κB p50 or p65 antibodies (Santa Cruz Biotechnology).

**Dual Luciferase NF-κB Reporter Assay—**The NF-κB-luciferase (NF-κB-luc) reporter plasmid, containing a 2xNF-κB response element fused to luciferase, has been previously characterized (41). Renilla luciferase (pRL-TK, Promega, Madison, WI) was co-transfected to normalize for transfection efficiency. A375 cells were co-transfected with 20 μg of NF-κB-luc and 10 μg of pRL-TK using Lipofectamine plus (Invitrogen). After transfection cells were allowed to recover overnight and were plated in 6-well plates. Cells were pretreated with NO-Cbl (100 μM) for 16 h followed by TNF-α (10 ng/ml) or Apo2L/TRAIL (100 ng/ml) for 4 h. Cells were then harvested and luciferase activity was measured according to the manufacturer’s protocol (Promega, Madison, WI) using a Wallac 1420 multilabel counter (PerkinElmer). Fold induction of NF-κB-luciferase for each treatment was based on untreated values normalized to the fold induction of pRL-TK reporter values. The assays were performed in triplicate.

**IkB kinase (IKK) assay—**Whole cell extracts (300 μg) were supplemented with 150 μl of Buffer A (20 mM HEPES, pH 7.9, 20 mM β-gerophosphate, 10 mM NaF, 0.1 mM orthovanadate, 5 mM p-nitrophenyl phosphate (pNPP), 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and protease mixture), 2 μl of normal rabbit serum, 0.5 μg of anti-IKKα monoclonal antibody (0.5 μg, BD PharMingen), or anti-β-actin epitope antibody was added and mixed by rotation at 4 °C for 1 h as previously described (42). A 50% slurry of protein G-Sepharose (80 μl) (Amersham Biosciences) prepared in Buffer A (without mercaptoethanol or phenylmethylsulfonyl fluoride) was added and mixed by rotation at 4 °C for 1 h. Protein G-Sepharose was removed by centrifugation at 800 × g for 1 min and discarded. Anti-IKKα monoclonal antibody (0.5 μg, BD PharMingen), or anti-β-actin epitope antibody was added to the supernatant and mixed by rotation at 4 °C for 2 h. A 50% slurry of protein G-Sepharose (60 μl) prepared in Buffer C (Buffer A plus 50 mM NaCl and 10 mM MgCl₂) without mercaptoethanol and phenylmethylsulfonyl fluoride) was added and mixed by rotation in the cold for 30 min. Protein G immunopellets were collected by centrifugation at 800 × g for 30 s, washed three times with Buffer B (Buffer A plus 250 mM NaCl), and once with Buffer C (Buffer A plus 50 mM NaCl and 10 mM MgCl₂). Immunopellets were resuspended in 30 μl of kinase buffer with 0.1 mM orthovanadate, 50 μM unlabeled ATP, 5 μCi of γ[32P]ATP, 2 mM dithiothreitol, and 2 μg of recombinant GST-IκBα-1–54 (22) and incubated at 30 °C for 30 min. Reactions were stopped by the addition of 15 μl of 4X SDS-PAGE loading buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.2% 2-mercaptoethanol), heated at 95 °C for 10 min, and resolved by SDS-PAGE on a 12% acrylamide gel by standard procedures. Gels were rinsed, stained with Bio-Safe Coomassie (BioRad) to visualize protein bands, rinsed, photographed, then dried and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, New York) to detect substrate phosphorylation. IKK activation was quantified by PhosphorImage analysis on a Storm-840 Imager using Image Quant v 4.2 software (Molecular Dynamics, Amersham Biosciences).

**Statistical Analysis—**Median effect analysis was used to characterize the interaction between NO-Cbl and Apo2L/TRAIL (43). A combination index (CI) > 1 indicates antagonism, CI = 1 indicates additivity, and CI
A375 cells were treated with NO-Cbl, Apo2L/TRAIL, and the combination. NO-Cbl and Apo2L/TRAIL were minimally effective as single agents but demonstrated greater apoptosis when administered concomitantly. The highest levels of apoptosis were observed when cells were pretreated with NO-Cbl for 12 h followed by Apo2L/TRAIL treatment for 24 h. Conversely, the effect of Apo2L/TRAIL followed by NO-Cbl was no different than Apo2L/TRAIL alone.
Non-malignant cells were resistant to the antiproliferative effects of NO-Cbl, Apo2L/TRAIL and the combination (Fig. 1b). This is consistent with the tumor-specific properties of both NO-Cbl and Apo2L/TRAIL (17, 37).

To test drug activity in vivo, subcutaneous A375 xenografts were established in nude mice. Daily drug treatments began on day 2 following implantation, at which time tumors were both visible and palpable (Fig. 2). After 21 days, the tumors in mice treated with single agent NO-Cbl or Apo2L/TRAIL were not significantly smaller than controls. However, the tumors in mice treated with the combination of NO-Cbl and Apo2L/TRAIL were 82.42% smaller than control tumors (p = 0.00016). The mice maintained their weight and activity and exhibited no adverse side effects due to single agents or the combination. Compared with the in vitro activity of Apo2L/TRAIL, the enhanced anti-tumor activity observed in vivo likely results from multiple biological effects. Recently, our laboratory confirmed3 that Apo2L/TRAIL up-regulates NK activity in vivo resulting in synergistic anti-tumor effects (44). Though athymic nude mice lack T-cells, they possess robust NK cell activity.

Mechanism of NO-Cbl/Apo2L/TRAIL-initiated Apoptosis—We focused our studies on A375 because this cell line has a defect in Apo2L/TRAIL gene induction (45). Therefore, additive cellular responses from endogenous Apo2L/TRAIL were avoided. We performed TUNEL assays of A375 cells treated in vitro with NO-Cbl, Apo2L/TRAIL, or the combination. Treatment with NO-Cbl (100 μM) or Apo2L/TRAIL (100 ng/ml) for 36 h induced 6.2% and 5.4% TUNEL-positive cells, respectively (Fig. 3). The simultaneous co-treatment of A375 cells with NO-Cbl (100 μM) and Apo2L/TRAIL (100 ng/ml) for 36 h resulted in 28.2% TUNEL-positive cells. However, sequential pre-treatment of A375 cells with NO-Cbl (100 μM) for 12 h, followed by Apo2L/TRAIL (100 ng/ml) for an additional 24 h induced 98.4% TUNEL-positive cells, suggesting that NO-Cbl primes cells to Apo2L/TRAIL-induced apoptosis. In contrast, pre-treatment with Apo2L/TRAIL followed by NO-Cbl did not enhance TUNEL staining.

To further examine apoptosis pathways, we performed immunoblot analysis using antibodies to various components of the apoptosis signaling cascade. A375 cells were treated with TNF-α (20 ng/ml), Apo2L/TRAIL (100 ng/ml) for 6–12 h. Whole cell lysates were probed for caspase-8, caspase-3, and PARP cleavage. Cells pre-treated with NO-Cbl followed by Apo2L/TRAIL demonstrated enhanced cleavage of caspase-8, caspase-3, and PARP, indicating activation of initiators and effectors of apoptosis (Fig. 4a). In addition, cleavage of the X-linked inhibitor of apoptosis (XIAP) was enhanced by NO-Cbl pre-treatment followed by Apo2L/TRAIL (Fig. 4b), indicating that NO-Cbl promoted degradation of an apoptosis inhibitor. Some melanomas have increased XIAP activity which may contribute to their resistance (46). This effect was specific to XIAP, as there was no change in the levels of CIAP-1 or FLIP.

RESULTS

Anti-tumor Effects of NO-Cbl, Apo2L/TRAIL, and the Combination In Vitro and in Vivo—To test our hypothesis that NO-Cbl would enhance the anti-cellular effects of Apo2L/TRAIL against malignant Apo2L/TRAIL-resistant cell lines, we measured the antiproliferative effects of three melanoma lines A375, WM9, and WM3211 (previously shown to be resistant to Apo2L/TRAIL) (39). Three non-malignant human cell lines CMN1 and DMN1 (normal melanocytes) and primary human foreskin fibroblasts (HFF) were examined to demonstrate the tumor-specific effects of NO-Cbl and Apo2L/TRAIL. We used the SRB antiproliferative assay, used by the National Cancer Institute (NCI) to evaluate new chemotherapeutic agents (38). Median effect analysis was used to characterize the interaction between NO-Cbl and Apo2L/TRAIL (43). Cells were pretreated with NO-Cbl for 16 h followed by Apo2L/TRAIL for 24 h. Sequential drug treatment resulted in synergistic antiproliferative activity in all three malignant cell lines (Fig. 1a).

Inhibition of NF-κB Survival Signaling by NO-Cbl—We next used EMSA to examine the effects of NO-Cbl on NF-κB activation. The NF-κB binding element from the IFN-β gene promoter was used as a probe to assess DNA binding activity. A375 cells were treated with TNF-α (20 ng/ml), Apo2L/TRAIL (100 ng/ml) or NO-Cbl (100 μM). Pretreatment with NO-Cbl (16 h) completely inhibited NF-κB DNA binding activity induced by 1 h stimulation with Apo2L/TRAIL (Fig. 5a, lanes 4 and 6). NO-Cbl only partially inhibited NF-κB DNA binding activity induced by TNF-α at 15 min and 1 h (Fig. 5a, compare lanes

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\[ p \leq 0.00016 \]
Antibodies to NF-κB p50 or p65 induced a supershift and confirmed the presence of the p50 and p65 components of the NF-κB complex. Compared with TNF-α, which strongly activates NF-κB after 15 min, Apo2L/TRAIL is a weaker activator of NF-κB; band shifts are not detectable 15-min post-stimulation with Apo2L/TRAIL, but do appear by 1 h. This effect has previously been observed in renal cell carcinomas (47). Pretreatment with other NO donors including NOC-18 (100 μM) and SNAP (100 μM) also inhibited NF-κB DNA binding activity induced by Apo2L/TRAIL (Fig. 5b).

Transient transfection assays were performed to assess NF-κB transcriptional activity. A375 cells were co-transfected with a NF-κB-luciferase reporter (NF-κB-luc) and Renilla luciferase (to assess transfection efficiency). Cells were pretreated with NO-Cbl (100 μM) for 16 h followed by treatment with Apo2L/TRAIL (100 ng/ml) or TNF-α (10 ng/ml) for 4 h. NO-Cbl pretreatment caused a 34 and 51% inhibition of NF-κB activity in response to Apo2L/TRAIL and TNF-α, respectively (Fig. 5c).

We next determined whether NO-Cbl treatment could affect the degradation of IkBα, the prototypic inhibitor of NF-κB (24). After a 15-min stimulation with TNF-α (20 ng/ml) or Apo2L/TRAIL (100 ng/ml, 30 min), IkBα was almost completely degraded (Fig. 6a). However, NO-Cbl pretreatment for 16 h (100 μM) completely blocked IkBα degradation following stimulation with Apo2L/TRAIL. NO-Cbl was much less efficient at blocking IkBα degradation following TNF-α stimulation. Pretreatment with NO-Cbl for 16 h (100 μM) completely blocked IkBα phosphorylation induced by 1 h stimulation using Apo2L/TRAIL (100 ng/ml) and decreased that induced by TNF-α (20 ng/ml).
Suppression of NF-κB Survival Signaling by Nitrosylcobalamin

Induction of apoptosis by exogenous Apo2L/TRAIL requires effective activation of the Apo2L/TRAIL receptors and downstream signaling components. Apo2L/TRAIL, as well as the DR4 and DR5 receptors are ubiquitously expressed in malignant cells. Moreover, sensitivity of melanoma cell lines to Apo2L/TRAIL correlated with levels of death receptor expression. Cellular resistance to Apo2L/TRAIL may be due to defects in caspase signaling or caspase inhibition rather than over-expression of decoy receptors. We have shown that defects in Apo2L/TRAIL gene induction as well as overexpression of inhibitors of apoptosis (such as XIAP) in melanoma cell lines which express Apo2L/TRAIL receptors, may account for resistance to Apo2L/TRAIL. Additionally, Apo2L/TRAIL resistance has been reported in nasopharyngeal carcinomas due to a homozygous deletion of DR4. Conversely, enhanced survival signaling may also confer a growth advantage. Certain renal cell carcinomas may be resistant to Apo2L/TRAIL as a result of constitutively activated NF-κB.

We demonstrated that IFN-β treatment increased expression of endogenous Apo2L/TRAIL and thus sensitized melanoma lines to the anti-tumor effects of exogenously administered recombinant Apo2L/TRAIL. IFN-β did not alter the DNA binding activity of NF-κB in melanoma cells. We previously demonstrated that the anti-tumor effects of IFN-β and NO-Cbl were synergistic in vitro and in vivo. Treatment with NO-Cbl increased the expression of Apo2L/TRAIL, DR4, and DR5 mRNAs, and caspase-8 enzymatic activity, indicating activation of the extrinsic apoptotic pathway. Thus, IFN-β mediates anti-growth effects in melanoma by enhancing Apo2L/TRAIL expression, rather than by inhibiting NF-κB activation.

In the current study we have shown that the anti-tumor activity of NO-Cbl was mediated in part by inhibition of NF-κB activation. NO-Cbl inhibited IKK enzymatic activity, preventing phosphorylation of IκB in response to Apo2L/TRAIL. Remarkably, NO-Cbl was more effective at inhibition of Apo2L/TRAIL-induced IKK activity compared with activation by TNF-α. We hypothesize that NO-Cbl may nitrosylate and deactivate a component of the Apo2L/TRAIL pathway that is absent from the TNF-α pathway. This functional divergence is under active investigation.

NO can inhibit NF-κB by nitrosylating critical cysteine residues (30, 31, 51). Interestingly, prostaglandins (PGA3 and 15dPGJ2) can inhibit IKK by covalently modifying a critical cysteine residue (C179) within the activation loop. In a similar manner, NO-Cbl may inhibit IKK, or an IKK-related kinase which is critical for Apo2L/TRAIL signaling, but is less important for TNF-α signaling.

Although SNAP and NOC-18 can inhibit NF-κB downstream signaling, these NO donors release NO indiscriminately and completely lack any tumor specificity. High concentrations of the NO donor sodium nitroprusside (SNP, 1 mM) in combination with Apo2L/TRAIL was effective at killing human colorectal carci-
Fig. 7. IkB kinase (IKK) activity. IKK activity was assessed using recombinant GST-IκBα(1–54) and γ-32P-ATP as substrates. 

The phosphorylated GST fusion protein was detected by autoradiography. a, IKK activity was determined in A375 cells pretreated with NO-Cbl followed by Apo2L/TRAIL or TNF-α stimulation for 30 min and 15 min, respectively. NO-Cbl treatment inhibited IKK activity more effectively when Apo2L/TRAIL was the stimulus, compared with stimulation by TNF-α. Anti-β-actin antibody served as the irrelevant antibody with no phosphorylation of GST-IκBα(1–54) observed. b, Coomassie Blue-stained gel shows equal loading of GST-IκBα(1–54) substrate. c, immunoblot analysis shows the presence of equal amounts of total IKK in the lysates. β-actin was used as a loading control.

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