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Nitric Oxide Inhibits NF-κB-mediated Survival Signaling: Possible Role in Overcoming TRAIL Resistance

JOSEPH A. BAUER[1], JOSEPH A. LUPICA[2], JOSEPH A. DIDONATO[3] and DANIEL J. LINDNER[4]

[1]*Nitric Oxide Services, LLC, St. John Paul II Center for Science Innovation, North Canton, OH, U.S.A.;*
[2]*Department of Math & Sciences, Walsh University, North Canton, OH, U.S.A.;*
[3]*Department of Cardiovascular & Metabolic Sciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, U.S.A.;*
[4]*Translational Hematology Oncology Research (THOR), Taussig Cancer Center, Cleveland Clinic Foundation, Cleveland, OH, U.S.A.*

Abstract. *Background/Aim: Chemoresistance is a major consequence of multicycle chemotherapy and can be attributed to constitutive activation of pro-survival signaling pathways. Nitric oxide is a ubiquitous signaling molecule which has been shown to inhibit several pathways involved with survival signaling in cancer cells. We have previously demonstrated the anti-tumor activity of a nitric oxide-donor, nitrosylcobalamin (NO-Cbl), mediated by increased expression of tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) and its receptors in human tumors. We also demonstrated that a functional Apo2L/TRAIL receptor is necessary for the induction of cell death by NO-Cbl and the Apo2L/TRAIL death receptor DR4 (TRAIL R1) is S-nitrosylated. The aim of the study was to examine the effects of nitric oxide (NO) on nuclear factor kappa B (NF-κB) and determine whether nitric oxide could sensitize drug-resistant melanomas to Apo2L/TRAIL via inhibition of NF-κB or inhibitor kappa B kinase (IKK). Materials and Methods: 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 the TUNEL assay. The activation status of NF-κB was established by assaying luciferase reporter activity, the phosphorylation status of IκBα, and in vitro IKK activity. Results: 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. NO-Cbl suppressed Apo2L/TRAIL- and TNF-α-mediated activation of a transfected NF-κB-driven luciferase reporter. NO-Cbl inhibited IKK activation, characterized by decreased phosphorylation of IκBα. Conclusion: 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 nitric oxide to inhibit NF-κB and potentiate the effects of chemotherapeutic agents, such as Apo2L/TRAIL, represents a promising anti-cancer combination based on recent clinical investigations of anti-TRAIL antibodies for cancer treatment strategies.*

Correspondence to: Daniel J. Lindner, MD, Ph.D., Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH 44195, U.S.A. Tel: +1 2164450548, e-mail: lindned@ccf.org, and Joseph A. DiDonato, Assistant Professor, Department of Cardiovascular & Metabolic Sciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44106, U.S.A. Tel: +1 2164448178, e-mail: didonaj@ccf.org

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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 *in vitro* tumor-specific properties of tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) have been investigated since the 1990s (2-5). Apo2L/TRAIL has been evaluated *in vitro* as an anti-cancer agent alone and in combination with other agents (6-10) including ionizing radiation (11-13) which has helped elucidate its mechanism of action. Recently, wild-type p53 expression has been associated with increased TRAIL-receptor mediated apoptosis but also has been shown to activate survival signaling (14). Additionally, Apo2L/TRAIL can initiate apoptosis in cells that overexpress the survival factors Bcl-2 and Bcl-X_L, and may represent a treatment strategy for tumors that have acquired resistance to chemotherapeutic drugs (15).

Apo2L/TRAIL binds to two TRAIL receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) that mediate apoptotic signaling (16) which have been shown to be present on cancer cells and

not normal cells. Studies have shown that Map kinase Activating death domain containing protein (MADD) is a pro-survival mediator of TRAIL signaling and a MADD knockout model demonstrated sensitivity to TRAIL in aplastic thyroid cancer (17). In addition, the inhibitor of TRAIL-mediated apoptosis c-FLIP has been identified as a major factor in the resistance of ER-positive breast cancer patients (18). Oncogenes such as RAS, MYC and HER2 have been shown to sensitize cancer cells to TRAIL-mediated cell death (19). Inhibition of XIAP has also shown increased antitumor activity of TRAIL in leukemia cells (20). TRAIL receptor agonists are being evaluated clinically, however, resistance is a major factor involved in their efficacy (20, 21) which can be attributed to the dual nature of apoptosis and protection inherent with TRAIL signaling (16).

Nitric oxide is a key signaling molecule involved in almost every aspect of human physiology as well as pathology. The dichotomous nature of nitric oxide is determined based on 3 principle factors, 1) site of NO production/delivery 2) NO concentration, and 3) duration of NO production. NO produced *in vivo* has been shown to prevent tumorigenesis (22, 23) and numerous studies have shown the anti-tumor effects of nitric oxide donor compounds in cancer therapy (24-27).

Nitrosylcobalamin (NO-Cbl), is an analogue of vitamin B12 (cobalamin, Cbl) coordinated with nitric oxide (NO) as a ligand (28). Similar to Apo2L/TRAIL, the CD320 receptor specific for NO-Cbl, is overexpressed on cancer cells compared to normal cells (29, 30). Accordingly, we pursued a target for possible nitrosylation on TRAIL R1 and identified the Cys-336 residue of the Apo2L/TRAIL death receptor TRAIL R1 as a target for S-nitrosylation by NO-Cbl. We assessed wild-type DR4 C336 compared to mutant DR4 C336A which did not result in S-nitrosylation by NO-Cbl and was more resistant to growth inhibition by NO-Cbl (31).

Several studies have shown that TRAIL induces apoptosis in cancer cells but simultaneously activates NF-κB (32-36). Additionally, we showed that several chemotherapeutic agents activate NF-κB survival signaling (37) and agents, including nitric oxide-donors, that inhibit NF-κB can potentiate anti-tumor activity (37-39). NO has been to shown to sensitize TRAIL-resistant tumors to TRAIL *via* inhibition of NF-kB (40, 41).

In this study we pre-treated cells with the nitric oxide donor, NO-Cbl, 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-Cbl and Apo2L/TRAIL in Apo2L/TRAIL-resistant cell lines, and to 2) determine the mechanism by which NO-Cbl inhibits NF-κB activation.

Materials and Methods

Synthesis of nitrosylcobalamin. Nitrosylcobalamin was synthesized as previously described (28, 42). Hydroxocobalamin (vitamin B12a) acetate (George Uhe Company, Paramus, NJ, USA) was dissolved in dichloromethane (Burdick and Jackson, Muskegon, MI, USA) and exposed to CP grade NO gas (Praxair, Danbury, CT, USA). The reaction proceeds in a closed system within a high-pressure gas cylinder (Midwest Process Controls, Avon Lake, OH, USA). The system was nitrogen-purged daily and evacuated prior to NO exposure. The NO gas was scrubbed prior to entering the system using a stainless-steel cylinder (Midwest Process Controls) containing NaOH pellets. The solid NO-Cbl product was collected following rotary evaporation of the solvent and stored at –80˚C prior to use.

Cell culture and cytokine treatment. Human melanoma tumor cell lines, WM9 and WM3211 (Wistar Institute, Philadelphia, PA, USA), and A375 (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle medium (DMEM; Life Technologies Inc., Rockville, MD, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% Antibiotic-Antimycotic (GIBCO BRL, Invitrogen, Carlsbad, CA, USA). Cells were maintained in 5% CO_2 at 37˚C in a humidified tissue culture incubator. Primary non-tumorigenic melanoma cell lines (DMN-1 and CMN-1, A. Gudkov, CCF, Cleveland, OH, USA), and human foreskin fibroblasts (HFF; CCF, Cleveland, OH, USA) were cultured in DMEM-F12 medium supplemented with 10% FBS. Cells were confirmed as mycoplasma free.

All experiments were performed using trimeric recombinant human Apo2L/TRAIL (43) (Genentech Inc, San Francisco, CA, USA) and were independently confirmed using recombinant Apo2L/TRAIL from another source (Peprotech Inc, Rocky Hill, NJ, USA). Apo2L/TRAIL (Genentech), consisted of >99% trimeric protein with Zn^{+2}, which is necessary for optimal biologic activity of Apo2L/TRAIL (43).

Sulforhodamine B cell growth assay. Cells were harvested with 0.5% trypsin/0.53 mM EDTA, washed with PBS and resuspended in media containing 10% FBS. Cells were plated in 96-well plates in 0.2-ml aliquots containing 10^4 cells. Cells adhered to the plate for 4 h and then NO-Cbl was added in different dilutions (25, 50 and 100 μM) to the assay plate. Replicates of four were performed for each treatment. After 16 h, recombinant human Apo2L/TRAIL was added at different concentrations (25-100 ng/ml). Growth was monitored by the sulforhodamine B (SRB; Sigma Chemical, St. Louis, MO, USA) colorimetric assay (44). 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 Multiscan RC, Thermo Lab Systems, Franklin, MA, USA). To quantify the growth of the cells, the experimental absorbance values (Aexp) were compared with initial absorbance readings representing the starting cell numbers (A^{ini}). To determine the starting cell number, an additional 96-well plate was seeded with cells and fixed at the beginning of the experiment. The absorbances derived from the initial plate and from the untreated cells at the end of the growth period (A^{fin}) were defined as 0% and 100% growth, respectively. The percentage control growth ($100\% \times [A^{exp} - A^{ini}]/[A^{fin} - A^{ini}]$) is expressed as a percentage of untreated controls.

In vivo experiments. The Institutional Animal Care and Use Committee at the Cleveland Clinic Foundation approved all procedures for animal experimentation (IACUC approval 2017-

1863). Mice were housed in the Biological Resources Unit in a HEPA air-filtered rack in cages with microisolator tops at a maximum density of five mice per cage. Mice had ad libitum access to standard chow and water (*via* automatic cage watering system) and kept in a temperature and humidity-controlled environment with a 12 h:12 h light dark cycle. Mice were observed once daily by BRU personnel and once daily by research personnel. Five-week-old NCR male athymic nude homozygous (*nu/nu*) mice (Taconic, Germantown, NY, USA) were inoculated with A375 tumors. There were four experimental groups (untreated, single agents, and the combination) n=8. Cultured tumor cells (4×10^6) were inoculated into flanks in the mid-axillary line. NO-Cbl was given twice daily (50 mg/kg *s.c.*) and recombinant trimeric Apo2L/TRAIL (50 mg/kg *s.c.*) (43) was administered every other day, starting on day 2. Tumor volume was measured three times a week using the formula for a prolate spheroid: $(4/3)\ \pi ab^2$ where 2a=major axis, 2b=minor axis. Mice were euthanized when tumors reached experimental endpoint (maximum 17 mm diameter), or if there was statistical difference in tumor size between experimental groups, or if mice exhibited >15% weight loss, or if mice exhibited lethargy, loss of limb function, vocalization, or other generalized signs of distress. Mice were euthanized using controlled gradient delivery of CO_2, followed by cervical dislocation. 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-Cbl, Apo2L/TRAIL and NO-Cbl + 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, USA). Cells were processed according to the manufacturer's recommended protocol. The percentage of FITC-positive cells was analyzed by fluorescent activated cell scanning (FACS, Becton Dickinson, FACSVantage, San Diego, CA, USA).

Gel electrophoresis and immunoblot analyses. Whole-cell lysates were prepared in 1x lysis buffer (50 mM Tris-Cl, pH 8.0, 1% Triton X 100, 10% glycerol, 1 mM EDTA, 250 mM NaCl, 1 mM DTT, 1 mM PMSF, 10 μg/ml 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 PVDF membranes by the semidry method (Trans Blot SD, BioRad, Hercules, CA, USA). Membranes were immunoblotted with, pIκBα and IκBα (Cell Signaling, Beverly, MA, USA), anti-IKKα/β (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with HRP-conjugated secondary antibodies (Pierce, Rockford, IL, USA). Immunoreactive bands were visualized by using enhanced chemiluminescence (Perkin Elmer, Waltham MA, USA). Equal protein loading was confirmed by re-probing with monoclonal anti-actin antibody (Sigma Chemicals Co, St. Louis, MO, USA).

Dual luciferase NF-κB reporter assay. The NF-κB-luciferase (NF-κB-luc) reporter plasmid, containing a 2× NF-κB response element fused to luciferase, has been previously characterized (45). Renilla luciferase (pRL-TK, Promega, Madison, WI, USA) was co-transfected to normalize for transfection efficiency. A375 cells were co-transfected with 20 μg NF-κB-luc and 10 μg pRL-TK using Lipofectamine plus (Gibco BRL/Life Technologies, Invitrogen). After transfection cells recovered overnight and were plated in 6 well plates. Cells were pre-treated 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 in 1× passive lysis buffer and luciferase activity was measured according to the manufacturer's protocol (Promega,) using a Wallac 1420 multilabel counter (Perkin Elmer). Fold induction of NF-κB-luciferase for each treatment was based on untreated values normalized to the fold induction of pRL-TK reporter values.

IκB kinase (IKK) assay. Whole-cell extracts (300 μg) were supplemented with 150 ul of Buffer A (20 mM Hepes, pH 7.9, 20 mM beta-glycerophosphate, 10 mM NaF, 0.1 mM orthovanadate, 5 mM para-nitrophenyl phosphate (pNPP), 10 mM 2-mercaptoethanol (BME), 0.5 mM PMSF, and protease cocktail), 2 μl normal rabbit serum (NRS), and mixed by rotation at 4˚C for 1h as previously described (46). A 50% slurry of Protein G Sepharose (80 μl) (Amersham-Pharmacia, Piscataway, NJ, USA) prepared in Buffer A (without BME or PMSF) 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, San Diego, CA, USA), 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 $MgCl_2$, without BME and PMSF) was added and mixed by rotation in the cold for 30 min. Protein G immunopellets were collected by centrifugation at 800 x g for 30 sec, washed 3 times with Buffer B (Buffer A plus 250 mM NaCl), and once with Buffer C (Buffer A plus 50 mM NaCl and 10 mM $MgCl_2$). Immunopellets were re-suspended in 30 μl kinase buffer with 0.1 mM orthovanadate, 50 μM unlabeled ATP, 5 μCi γ32P-ATP, 2 mM DTT, and 2 μg of recombinant GST-IκBα1-54 (47) and incubated at 30˚C for 30 min. Reactions were stopped by the addition of 15 μl 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, NY, USA) 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 (48). A combination index (CI) >1 indicates antagonism, CI=1 indicates additivity, and CI <1 indicates synergy. Differences in mean tumor volume between groups were compared using the unpaired two-tailed Student's *t*-test, using a pooled estimator of variance to determine statistical significance.

Results

Anti-tumor effects of NO-Cbl, Apo2L/TRAIL, and their 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

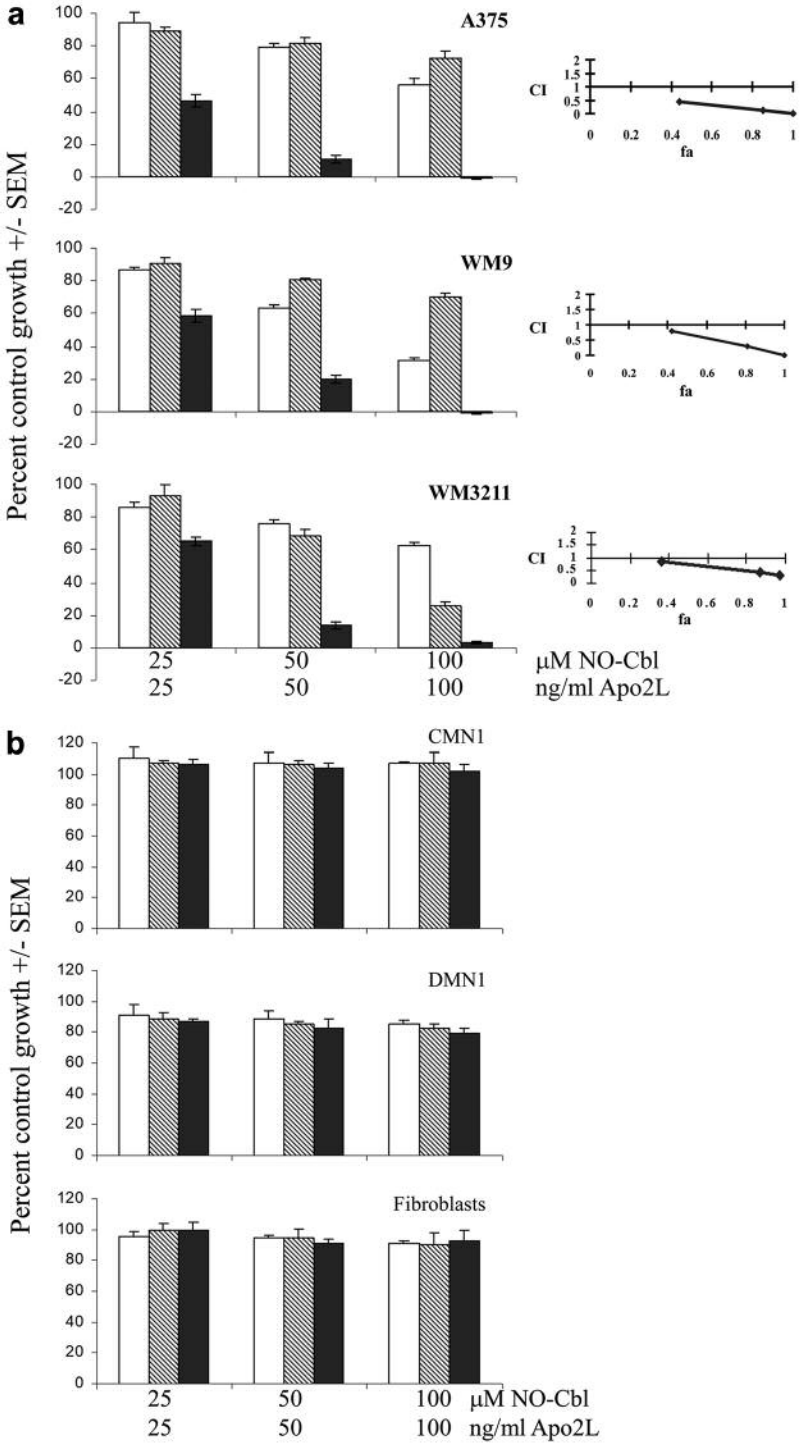

Figure 1. *Effects of nitrosylcobalamin (NO-Cbl), Apo2L/TRAIL, and their combination on the proliferation of melanoma cell lines A375, WM9, and WM3211 and normal cell lines CMN1, DMN1, and primary HFF fibroblasts. Left panels: Cells were treated with NO-Cbl (open bars), Apo2L/TRAIL (hatched bars), or pre-treated with NO-Cbl followed by Apo2L/TRAIL (solid bars) for three days, and growth was measured by the colorimetric sulforhodamine B assay (44). Data points represent the mean of four replicates±standard error of the mean (SEM). Right panels: Synergy between NO-Cbl and Apo2L/TRAIL was determined by median effect analysis (48), (combination index >1 indicates antagonism, =1 indicates additivity, and <1 indicates synergy). (a) The sequential treatment of NO-Cbl and Apo2L/TRAIL induced synergistic antiproliferative activity in A375, WM9 and WM3211 cells at each combined dose. (b) Normal melanocyte CMN1 and DMN1 cell lines, and normal HFF fibroblasts were completely resistant to simultaneous exposure to NO-Cbl, Apo2L/TRAIL, or the pre-treatment with NO-Cbl followed by Apo2L/TRAIL.*

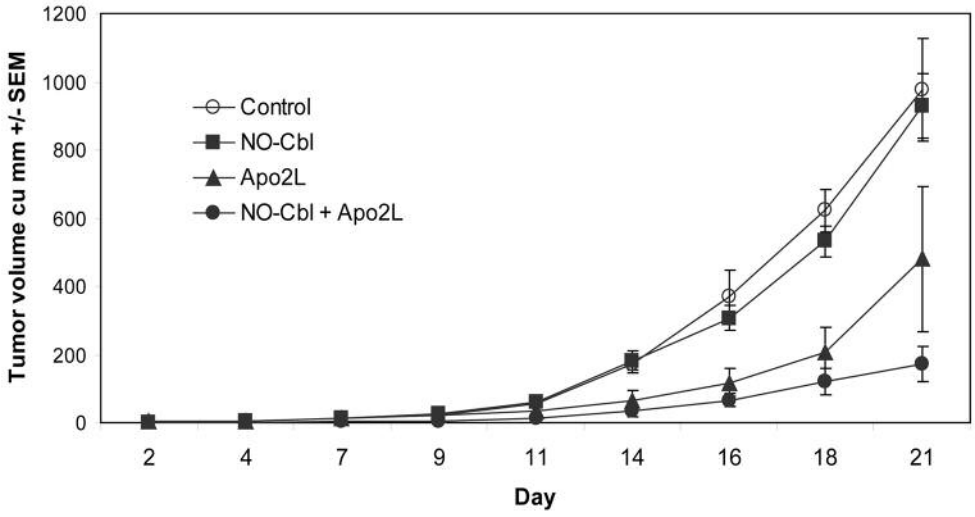

Figure 2. *Effect of NO-Cbl, Apo2L/TRAIL and the combination on the growth of A375 melanoma xenografts. NCR male athymic nude (nu/nu) mice were injected subcutaneously with 4×10^6 A375 cells (n=8 per group). Drug treatments began on day 2 after injection of tumor cells. NO-Cbl was administered twice daily for the duration of the study. Apo2L/TRAIL was administered every other day. Control mice received phosphate buffered saline. Tumor volume was measured three times per week. Data points represent the mean tumor volume (mm^3)±SEM.*

melanoma lines A375, WM9, and WM3211. 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 (44). Median effect analysis was used to characterize the interaction between NO-Cbl and Apo2L/TRAIL (48). Cells were pre-treated 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 (Figure 1a). Non-malignant cells were resistant to the antiproliferative effects of NO-Cbl, Apo2L/TRAIL and their combination (Figure 1b). This is consistent with the tumor-specific properties of both NO-Cbl and Apo2L/TRAIL (28, 43).

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 (Figure 2). After 21 days, the tumors from mice treated with single-agent NO-Cbl or Apo2L/TRAIL were not significantly smaller than controls. However, the tumors from mice treated with the combination of NO-Cbl and Apo2L/TRAIL were 82.42% smaller than control tumors ($p\leq0.00016$). The mice maintained their weight and activity and exhibited no adverse side-effects due to single agents or their combination. Compared to the *in vitro* activity of Apo2L/TRAIL, the enhanced anti-tumor activity observed *in vivo* likely results from multiple biological effects. In addition, our group confirmed (unpublished data) that Apo2L/TRAIL upregulates NK activity *in vivo* resulting in synergistic anti-tumor effects (49). Though athymic nude mice lack T-cells, they possess robust NK cell activity.

Mechanism of NO-Cbl/Apo2L/TRAIL-initiated apoptosis. We performed TUNEL assays of A375 cells treated *in vitro* with NO-Cbl, Apo2L/TRAIL, or their 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 (Figure 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.

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 pre-treated 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 pre-treatment caused a 34% and 51% inhibition of NF-κB activity in response to Apo2L/TRAIL and TNF-α, respectively (Figure 4).

We next determined whether NO-Cbl treatment could affect the degradation of IκBα, the prototypic inhibitor of NF-κB (50).

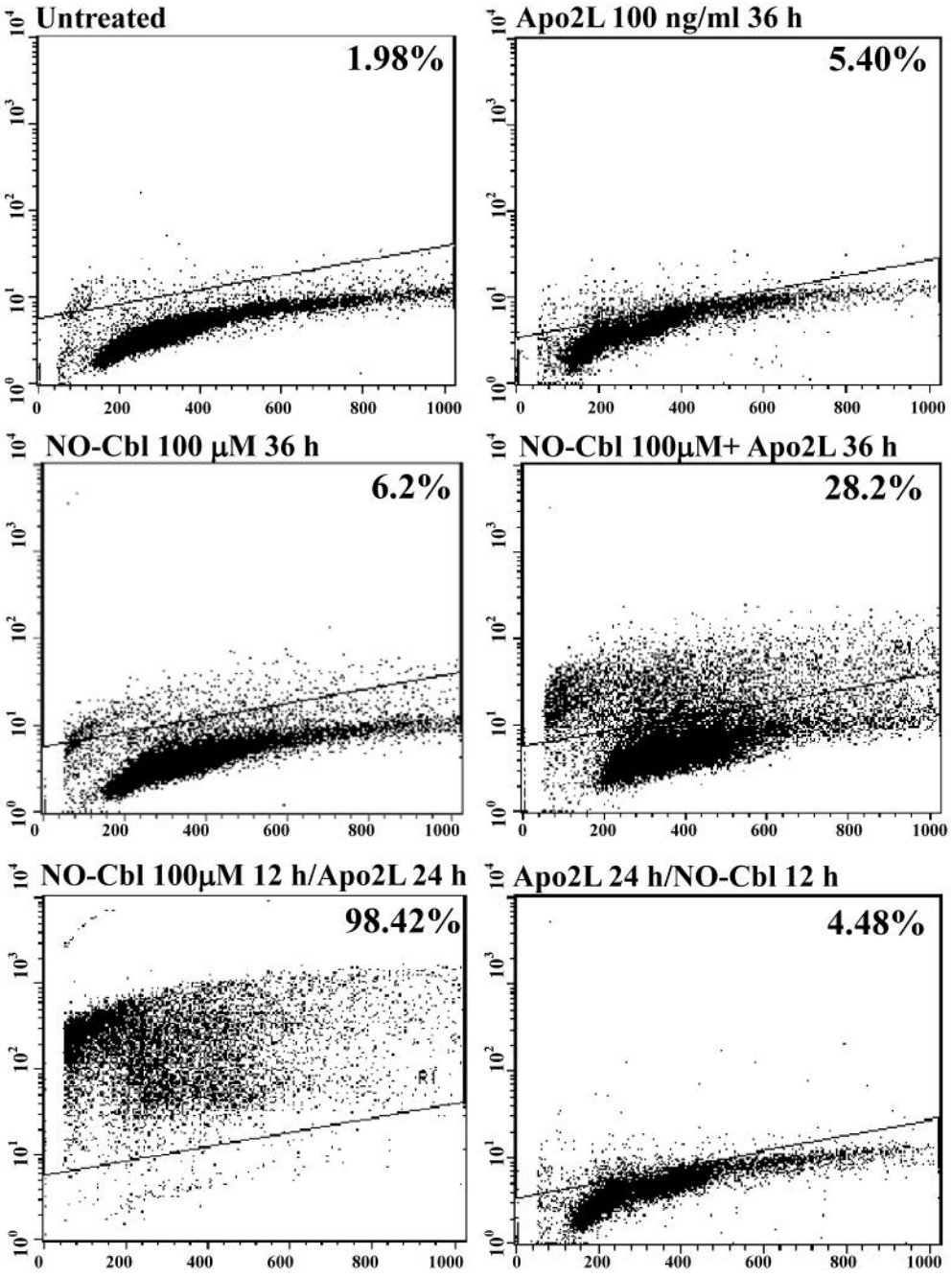

Figure 3. *TUNEL apoptosis assay. A375 cells were treated with NO-Cbl, Apo2L/TRAIL, and their combination. NO-Cbl and Apo2L/TRAIL were minimally effective as single agents but demonstrated greater apoptosis when administered concomitantly. The highest levels of apoptosis were observed when cells were pre-treated 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.*

After 15 min stimulation with TNF-α (20 ng/ml) or Apo2L/TRAIL (100 ng/ml, 30 min), IκBα was almost completely degraded (Figure 5a). However, NO-Cbl pre-treatment for 16 h (100 μM) completely blocked IκBα degradation following stimulation with Apo2L/TRAIL. NO-Cbl was much less efficient at blocking IκBα degradation following TNF-α stimulation. Pre-treatment with NO-Cbl for 16 h (100 μM) completely blocked IκBα phosphorylation induced by 1 h

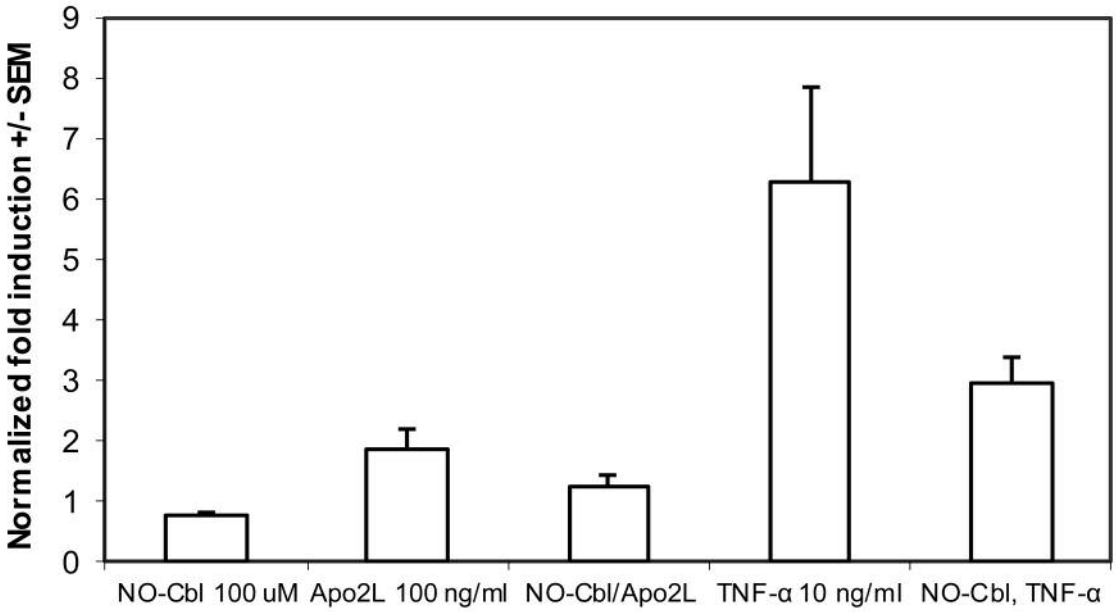

Figure 4. *NF-κB-luc reporter assay. NF-κB-luc transfected A375 cells were pre-treated with NO-Cbl for 1 h followed by Apo2L/TRAIL or TNF-α for 4 h. Renilla luciferase was co-transfected to normalize samples for transfection efficiency. Cell lysates were analyzed for NF-κB-luc reporter activity. NO-Cbl pre-treatment inhibited Apo2L/TRAIL- and TNF-α-induced activation of the NF-κB-luc reporter.*

stimulation using Apo2L/TRAIL (100 ng/ml) and decreased that induced by TNF-α (20 ng/ml) (Figure 5b). At 1 h following TNF-α stimulation, IKK remains activated, albeit at reduced levels compared to15 min (47). Phosphorylation of IκBα (all of which has been newly synthesized by 1 h) is evident. After 1 h, total IκBα levels were comparable between treatment groups. Accordingly, phospho-IκBα migrates slower than IκBα (Figure 5b, compare lanes 3 and 4 to other lanes).

Inactivation of IκB kinase activity by NO-Cbl. IκB kinase (IKK) is responsible for phosphorylation and activation of IκBα, we therefore examined the effect of NO-Cbl upon IKK activity. A375 cells were pre-treated with NO-Cbl (100 μM) for 16 h followed by stimulation with Apo2L/TRAIL (100 ng/ml) or TNF-α (20 ng/ml) and whole-cell extracts were prepared at 30 min and 15 min after treatment, respectively. IKKα was immunoprecipitated from A375 whole-cell extracts and IKK activity was assessed using recombinant GST-IκBα as a substrate (47). NO-Cbl effectively inhibited IKK activity induced by TNF-α and Apo2L/TRAIL by 22% and 92%, respectively (Figure 6a). Anti-β-actin antibody was used as an irrelevant antibody control for immunoprecipitation and yielded no signal. The kinase assay gel was stained with Coomassie blue to visualize total protein and demonstrated equal loading of the substrate, GST-IκBα (Figure 6b). The same cell extracts were probed for total IKK by immunoblot analysis and demonstrated equal loading of IKK (Figure 6c).

Discussion

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 TRAIL-R1 and TRAIL-R2 receptors are ubiquitously expressed in malignant cells. In the current study, we demonstrated the anti-tumor activity of NO-Cbl as mediated in part by nitric oxide-induced inhibition of NF-κB activation. Specifically, we showed that NO-Cbl inhibited IKK enzymatic activity, preventing phosphorylation of IκB in response to Apo2L/TRAIL.

Studies conducted in the early 1990s demonstrated that nitric-oxide donor compounds effectively inhibited NF-κB-mediated survival signaling (51) which has been confirmed by recent studies that have established the role of nitric oxide as an inhibitor of NF-κB (52). In addition, a current strategy to enhance TRAIL-mediated cell death is to sensitize TRAIL-resistant tumors by inhibiting TRAIL-mediated survival pathways (53) this is especially important with the clinical use of anti-TRAIL receptor antibodies which has been plagued by TRAIL resistance (54, 55).

Researchers have shown that activation of NF-κB is mediated through activation of IKK (56). Remarkably, in our study, NO-Cbl was more effective at inhibiting Apo2L/TRAIL-induced IKK activity compared to activation by TNF-α which may suggest strong vs. weak activators of IKK. We hypothesize that NO-Cbl may nitrosylate and deactivate a component of the

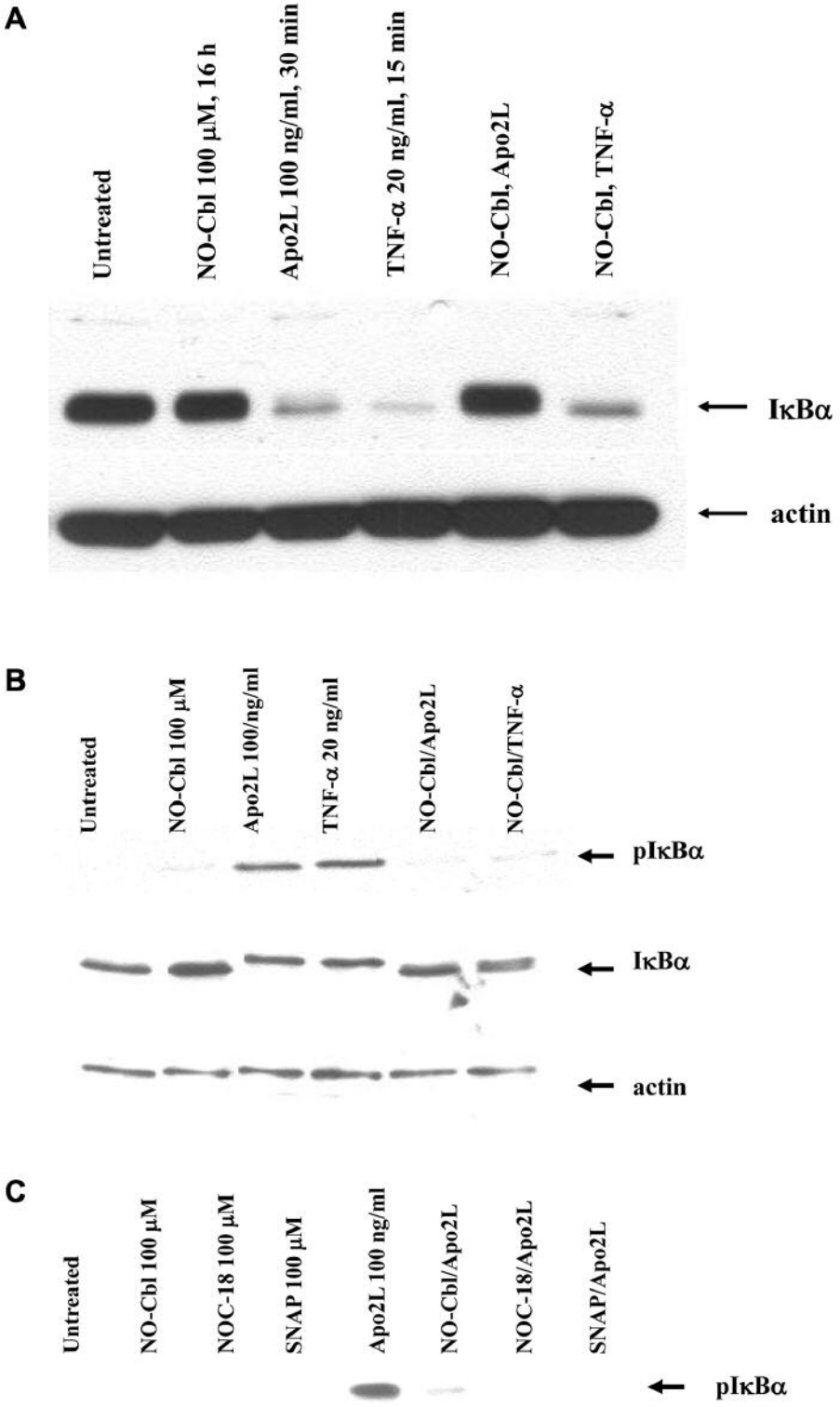

Figure 5. *Western blot analysis of IκBα and phospho-IκBα. A375 cells were pre-treated for 16 h with NO-Cbl followed by Apo2L/TRAIL or TNF-α stimulation. IκBα and phospho-IκBα protein levels were determined in A375 whole-cell lysates. (a) After stimulation with Apo2L/TRAIL (30 min) or TNF-α (15 min), IκBα was almost totally degraded. NO-Cbl efficiently blocked IκBα degradation following Apo2L/TRAIL, but only partially blocked IκBα degradation following TNF-α. (b) After 1 h, cellular levels of IκBα are restored as a result of re-synthesis. NO-Cbl blocks the phosphorylation of newly translated IκBα. Band retardation of IκBα is evident following Apo2L/TRAIL or TNF-α stimulation. Phospho-IκBα migrates slower than IκBα (compare middle two lanes to other four lanes). c, NO-Cbl, NOC-18, and SNAP pre-treatment all inhibited Apo2L/TRAIL-induced IκBα phosphorylation.*

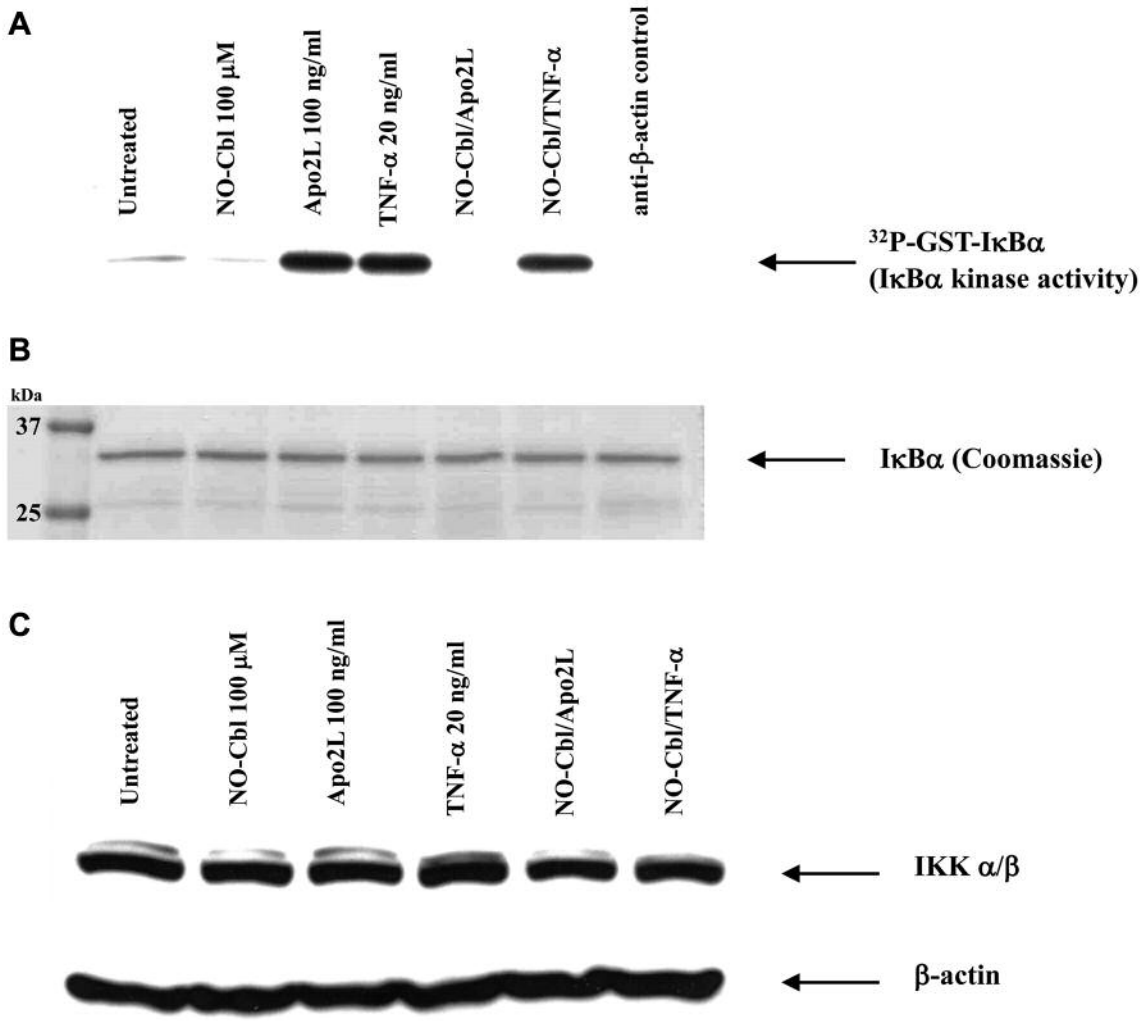

Figure 6. *IκB kinase (IKK) activity. IKK activity was assessed using recombinant GST-IκBα(1-54) and γ32P-ATP as substrates. The phosphorylated GST fusion protein was detected by autoradiography. (a) IKK activity was determined in A375 cells pre-treated with NO-Cbl followed by Apo2L/TRAIL or TNF-α stimulation for 30 min and 15 min, respectively. NO-Cbl treatment inhibited IKK activity more effectively when Apo2L/TRAIL was the stimulus, compared to 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.*

Apo2L/TRAIL pathway that is absent from the TNF-α pathway. This functional divergence is under active investigation.

Similarly, others have shown that NO can inhibit NF-κB by nitrosylating critical cysteine residues (57-59). Interestingly, prostaglandins (PGA1 and 15dPGJ2) can inhibit IKK by covalently modifying a critical cysteine residue (C179) within the activation loop (60). 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.

A major advantage of the pro-drug NO-Cbl is its tumor-specific accumulation (28). Cobalamin (Cbl) is avidly taken up by tumor cells relative to most normal tissues (61-63). Unlike other nitric oxide donors, NO-Cbl releases NO inside the cell (28), and therefore minimizes systemic toxicity. NO-Cbl is relatively tumor-specific due to transcobalamin receptor (TCII-R; CD320)-mediated uptake and intercellular transport by TCII, which are overexpressed in cancer compared to normal tissues (29, 30).

Chemoresistance is a major problem for multi-cycle chemotherapy and involves multiple pathways and molecular factors (64, 65) which has resulted in the use of immunotherapies and combination treatments (65). NF-κB plays a major role in survival signaling and is a promising

target to potentiate the anti-tumor effects of chemotherapy and radiotherapy as demonstrated in several review articles (66-73). Currently, several clinical trials evaluating or involving NF-κB are recruiting patients in the cancer setting (74). The use of NO to inhibit NF-κB activation is a favorable strategy as the use of NO is becoming more readily available clinically (75, 76).

Conclusion

The use of NO-Cbl to deliver intra-tumor NO and inhibit survival signaling represents a promising approach to anti-tumor therapy to help mitigate TRAIL resistance. Our study provides the rationale for the use of NO, generated by nitric oxide donors, nitric oxide synthase activators, and NO-precursors with the goal of potentiating anticancer therapies. However, further studies are required to determine the exact mechanism of action by which NO inhibits NF-κB. Our study demonstrates the potential of nitric oxide in the multimodal treatment of cancer and highlights the need to develop NF-κB-pathway inhibitors which merits future exploration especially in the context of TRAIL cancer treatments.

Conflicts of Interest

JAB is employed by Nitric Oxide Services, LLC. No competing interests for DJL, JAD, or JAL.

Authors' Contributions

JAB, DJL, JAD: Conceived and designed the analysis; JAB, JAL: Performed the analysis; JAB, DJL, JAD: Wrote and edited the paper.

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