Vitamin C Suppresses TNFα-Induced NFκB Activation by Inhibiting IκBα Phosphorylation†

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ABSTRACT: Extracellular stimuli signal for activation of the transcription factor NFκB, leading to gene expression regulating processes involved in immune responses, inflammation, and cell survival. Tumor necrosis factor-α (TNFα) activates NFκB via a well-defined kinase pathways involving NFκB-inducing kinase (NIK), which activates downstream multisubunit IκB kinases (IKK). IKK in turn phosphorylates IκB, the central regulator of NFκB function. We found that intracellular vitamin C inhibits TNFα-induced activation of NFκB in human cell lines (HeLa, monocytic U937, myeloid leukemia HL-60, and breast MCF7) and primary endothelial cells (HUVEC) in a dose-dependent manner. Vitamin C is an important antioxidant, and most cells accumulate ascorbic acid (AA) intracellularly by transporting the oxidized form of the vitamin, dehydroascorbic acid (DHA). Because ascorbic acid is a strong pro-oxidant in the presence of transition metals in vitro, we loaded cells with vitamin C by incubating them with DHA. Vitamin C-loaded cells showed significantly decreased TNFα-induced nuclear translocation of NFκB, NFκB-dependent reporter transcription, and IκBα phosphorylation. Our data point to a mechanism of vitamin C suppression of NFκB activation by inhibiting TNFα-induced activation of NIK and IKKβ kinases independent of p38 MAP kinase. These results suggest that intracellular vitamin C can influence inflammatory, neoplastic, and apoptotic processes via inhibition of NFκB activation.

Ascorbic acid is an essential vitamin for humans, primates, guinea pigs, and few other animals and insects that lack the enzyme L-gulono-γ-lactone oxidase, the final enzyme in the biosynthetic pathway for vitamin C (*1*, *2*). Most animals synthesize ascorbic acid from glucose in the liver. Vitamin C is generally transported into cells in the oxidized form as dehydroascorbic acid (DHA)[1] via facilitative glucose transporters and as ascorbic acid in specialized cells by sodium-dependent ascorbic acid transporters (*3*−*6*). When DHA is transported via the glucose transporters, it is rapidly reduced and trapped inside the cell, where it accumulates as ascorbic acid (*3*, *7*). Although ascorbic acid circulates in human plasma at approximately 30−50 uM, it accumulates in mM concentrations in host defense cells (*8*, *9*). Mononuclear leukocytes, for example, may have intracellular ascorbic acid concentrations of 3.5−6 mM (*10*). Vitamin C is a strong antioxidant that sustains a balance of reactive oxygen species (ROS) generated in the course of aerobic ATP generations (*11*, *12*), inhibits cell death, and prevents mutations induced by reactive oxygen species (ROS) (*13*, *14*).

The dimeric eukaryotic transcription factor NFκB (Rel family of DNA-binding proteins) plays a central role in the regulation of host defense cells and is activated by a variety of inflammatory stimuli, including cytokines and viral proteins (*15*−*19*). In most cell types, the inactive form of NFκB is retained in the cytosol by association with inhibitory factors known as IκB proteins (*16*) which, when activated, are rapidly phosphorylated and degraded via proteasomal pathways (*20*). Several kinases are implicated in the activation of NFκB signaling pathways (*21*). The NFκB-inducing kinase (NIK), a member of the mitogen-activated protein kinase family, activates NFκB by inducing the phoshorylation of IκB (*21*−*23*); however, the downstream IκB kinase complex (IKKα, IKKβ) phosphorylates IκBα directly on serine 32 and 36 (*24*−*28*). The multiple subunit IKK is activated by phosphorylation in response to inflammatory signals (*20*−*22*, *26*, *29*).

There is now considerable evidence that reactive oxygen species (ROS) play an important role in cellular signaling systems linked to transcriptional machinery (*30*−*32*). For example, ROS are important in GM-CSF signal transduction, and antioxidants can inhibit GM-CSF signaling (*33*, *34*). Similarly, antioxidant thiols or iron chelators may inhibit NFκB activation induced by down regulating IκBα phosphorylation (*35*). An ample body of information exists concerning modulators of NFκB activation (*19*). For example, nonsteroidal antiinflammatory drugs inhibit the activity of NFκB (*36*, *37*), and aspirin and sodium salicylate inhibit ATP binding to IKKβ (*38*). The immunosuppressive drug methotrexate (MTX) inhibits cell replication by blocking dihydrofolate reductase (*39*) but also suppresses TNFα-induced NFκB activation by decreasing IκBα phosphorylation (*40*).

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[1] Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MBP, myelin basic protein.

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Antioxidants are obvious tools for targeting signal transduction pathways. Zhang and Frei reported that the thiol antioxidant α-lipoic acid inhibits TNFα-induced activation of NFκB in human aortic endothelial cells (*41*); however, these investigators found no effect of vitamin C. A recent study using endothelial cells reported that ascorbic acid inhibits TNFα-induced NFκB activation by inducing p38 mitogen-activated protein kinase (*42*). These contradictory results concerning the role of vitamin C on NFκB activation may be explained by the use of ascorbic acid in vitro, which is poorly transported, if at all, and has strong pro-oxidant properties in the presence of free-transition metals ubiquitous in in vitro culture (*43*, *44*). We directly investigated the effect of vitamin C on TNFα-induced NFκB activation by loading cells with vitamin C via incubation with DHA. Millimolar intracellular concentrations of AA were achieved, and the in vitro pro-oxidant effects of AA were avoided. We found that vitamin C loading suppressed TNFα-dependent activation of NFκB by inhibiting the activation of kinases involved in the phosphorylation of IκBα. Because of the established role of NFκB in inflammation and apoptosis, we propose that vitamin C may play a role in modulating inflammation and apoptosis, being inhibitory of both processes at high concentration.

EXPERIMENTAL PROCEDURES

Cell Lines. HeLa and MCF7 cells were obtained from the American Type Culture Collection (ATCC) and grown in Iscove's Modified Dulbecco's Medium (IMDM) and DME-HG F-12, respectively, containing 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 in air at 37 °C. Digitized images from the developed films were obtained, and the intensities of the individual bands were quantified using NIH Image software. HUVEC cells were obtained from Clonetics (Walskerville, MD) and grown in Endothelial Cell Growth Medium Kit with 2% fetal bovine serum (EGM Bullet Kit). Human HL-60 and U937 cells were obtained from ATCC and grown in IMDM and RPMI 1640, respectively, supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The p38 kinase inhibitor SB203580 was from Calbiochem (San Diego, CA).

AA, DHA, and TNFα Treatment. For AA and DHA treatment, cells were processed as previously described (*3*, *7*). In short, cells were incubated for 30 min with incubation buffer, pH 7.4 (IB) (15 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$; pH 7.4) and then treated with different concentrations of AA or DHA for 1 h at 37 °C. After vitamin C incubation, the cells were washed with PBS and incubated with 30 ng/mL recombinant human TNFα (R&D Systems, Minneapolis, MN). AA and DHA were obtained from Sigma. Cell extracts were prepared as previously described with modifications (*45*). In short, cell monolayers growing in 100 mm plates were washed twice with PBS, scraped, transferred to a 50 mL tube, centrifugated, and resuspended with buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.1% NP-40). Cells were incubated for 10 min on ice and pelleted at 12 000 rpm at 4 °C for 10 min. Supernatant was collected (cytoplasmic extract), and the nuclear pellet was extracted with 3 volumes of buffer C (20 mM HEPES (pH 7.9), 25% v/v glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT). Samples were incubated on ice for 30 min and then centrifugated at 12 000 rpm at 4 °C for 20 min. The supernatant (nuclear extract) was saved at −80 °C for further analysis.

Cell Volume Determination. Intracellular volume was estimated as described previously (*3*) with 30% correction for trapped extracellular radioactivity (*46*). In short, 1×10^6 cells were incubated for 60 min at room temperature with 200 μL of IB containing 1 mM 3-oxy-methyl-glucose (OMG) and 5 μCi of ^{3}H−OMG. During incubation, equilibrium (zero-trans) is established between intra- and extra-cellular concentrations of OMG. After incubation, 2 μL of 2 mM cytochalasin B was added to the cells to prevent efflux of trapped OMG during washing and the mixture incubated at room temperature for 5 min. The cells were then washed three times with cold Ca−Mg free PBS containing 20 μM of cytochalasin B to remove unincorporated radioactivity. After lysis in 10 mM Tris-HCl (pH 8.0) containing 0.2% SDS, the incorporated radioactivity was determined by liquid scintillation spectrometry. The amount of radioactivity accumulated inside the cells is in direct proportion to the intracellular volume.

Immunoblotting Analysis. Cell lysates were fractionated by SDS−PAGE and transferred to nitrocellulose membranes (0.45 μm, BIORAD). Immunoblot analysis was performed using the following primary polyclonal antibodies: rabbit anti-phospho-IκBα, anti-IκBα, anti-phospho-p38, anti-p38 (New England Biolabs Inc. Beverly, MA), anti-p50, anti-Ku86, and anti-tubulin antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA). The secondary antibody, horseradish peroxidase-coupled goat anti-rabbit (BioRad Laboratories, Hercules, CA), was used with the enhanced chemiluminescence assay (ECL) (Amersham Pharmacia Biotech., Piscataway, NJ).

Transfection and Luciferase Assays. HeLa and MCF7 cells were transiently transfected by the calcium phosphate method (*47*) in six well-plates at 80% of confluency with pRLTK (Promega, San Luis Obispo, CA) containing the herpes simplex virus thymidine kinase promoter region upstream of the renilla gene (to normalize for the frequency of transfections and protein content in each sample) and pNFκB-luc (Clontech, Palo Alto, CA) containing the NFκB responsive promoter upstream of luciferase gene. Cells treated with DHA or AA and/or TNFα in duplicate samples were washed with PBS and lysed with passive lysis buffer (Promega). Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) with a Berthold luminometer Lumet (B9501).

Vitamin C Uptake. Uptake assays were performed as described (*3*, *7*). In short, cells seeded at 3×10^5 cells per well were incubated with 1 mM ascorbic acid and 0.5 μCi of L-^{14}C ascorbic acid (specific activity, 8.0 mCi/mmol; DuPont NEN) for AA uptake and with 1mM ascorbic acid and 0.5 μCi of L-^{14}C ascorbic acid in the presence of 2 units of ascorbate oxidase (Sigma, Saint Louis, MO) for DHA uptake. Cells were incubated at room temperature for the periods of time indicated in the figures. Cells were washed twice with cold PBS before lysis with 10 mM Tris-HCl (pH 8.0) and 0.2% SDS, and cell-associated radioactivity was determined by scintillation spectrometry.

IKKβ and NIK Kinase Assays. IKKβ and NIK kinases were isolated from HeLa cell cytoplasmic extracts by

immunoprecipitation using 2 μg of anti-IKKβ antibody or 4 μg of anti-NIK antibody, respectively (both from Santa Cruz, CA). Beads containing IKKβ or NIK were washed twice with kinase buffer (50 mM Tris-HCl (pH 8.0), 100 μM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 mM β-glycerophosphate, 10 mM NaF, and 1 mM Na_3VO_4). The kinase activity of IKKβ was determined after 10 or 30 min at 30 °C in the same buffer and in the presence of 10 μM ATP, 1 μCi [γ-^{32}P]-ATP, and 1 μg of substrate (GST-IκBα). Beads were separated, and supernatants were incubated with glutathione sepharose beads at room temperature for 1 h and then washed twice with PBS. The phospho-labeled GST-IκBα was separated by SDS−PAGE, followed by autoradiography or counted with a Beckman LS 6000LL counter. NIK kinase activity was determined after 1 min incubation at 30 °C in the presence of 10 μM ATP, 1 μCi [γ-^{32}P]ATP, and 1 μg of myelin basic protein (MBP) as a substrate. The kinase reaction was stopped by placing the tubes on ice and the proteins precipitated with 10% TCA on ice for 30 min. Pellets of labeled substrate were washed twice with cold 20% TCA and the radioactivity determined with a Beckman LS 6000LL counter.

RESULTS

Vitamin C Inhibits TNFα-Induced Transcriptional Responses Mediated by NFkB. A time course analysis of vitamin C accumulation revealed that HeLa cells preferentially transported the oxidized form of vitamin C (dehydroascorbic acid, DHA) but there was little, if any, uptake of AA (Figure 1A). Transport studies over less than 60 s demonstrated rapid DHA transport and no AA transport, as previously seen in HL60 cells (insert, Figure 1A) (*7*). We estimated the intracellular volume of HeLa cells as 1.7 μL/10^6 cells base on tritiated methyl glucose equilibrium studies. HeLa cells incubated with 1 mM DHA for 1h and accumulated 4 mM intracellular vitamin C. Conversely, cells incubated with 1 mM AA for 1h under the same conditions accumulated only 0.2 mM intracellular vitamin C (Figure 1A), probably due to conversion of AA to DHA under aerobic conditions. After 30 min incubation with 1 mM DHA, uptake reached a plateau with no further increase in accumulation of vitamin C.

We investigated the effect of high intracellular concentrations of vitamin C on the activation of NFκB transcription induced by TNFα using the pNFκB-luc reporter plasmid. This reporter construct contains four tandem copies of the NFκB consensus-binding site upstream of a luciferase basic promoter. Cells incubated with 30 ng/mL TNFα for different periods of time showed a prominent increase of luciferase activity (Figure 1B). The maximum increase in luciferase activity was approximately 10-fold after 6 h incubation with TNFα. In cells loaded with vitamin C and incubated with TNFα, there was a significant inhibition of TNFα-dependent activation (Figure 1B). A 5-fold increase in luciferase activity was observed after 2 h incubation with TNFα; however, in cells loaded with vitamin C, little induction was detected (Figure 1B,C). Over a range of time periods, intracellular accumulation (4 mM) of vitamin C inhibited approximately 50% of the activation of luciferase activity induced by TNFα (Figure 1B,C). As shown in Figure 1B, the inhibitory effect of vitamin C on TNFα-dependent NFκB activation was

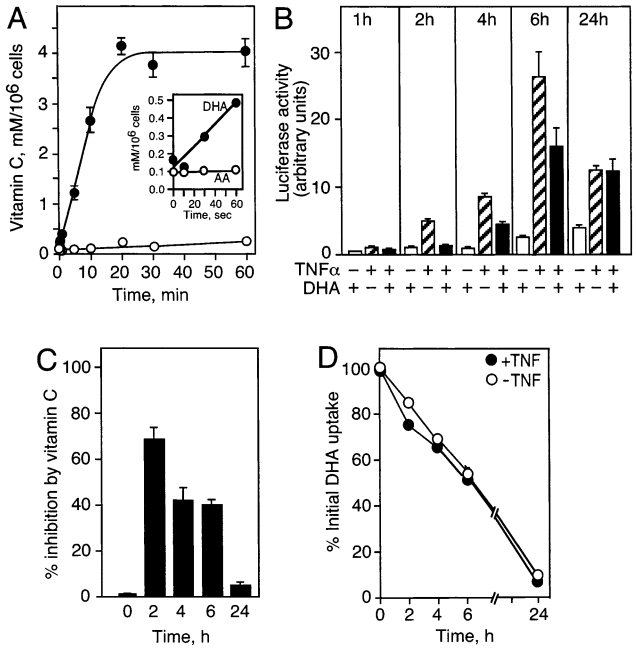

FIGURE 1: Vitamin C suppresses TNFα-induced NF-κB transcriptional activation. (A) HeLa cells transport vitamin C as dehydroascorbic acid (DHA). Cells were incubated for the time indicated with 1 mM AA (open circle) or 1 mM DHA (closed circle), and the accumulation of vitamin C was measured. The insert shows the transport of DHA and accumulation of vitamin C over short periods of time. (B) Vitamin C inhibits NFκB-mediated transcriptional activation. HeLa cells transfected with pNFκB-luc luciferase reporter construct treated with either buffer or 1 mM DHA for 60 min were incubated with TNFα (30 ng/mL) for the time indicated. Cell lysates were prepared and analyzed for luciferase activity. (C) Percentage of decrease on TNFα-dependent transcriptional activation in cells loaded with vitamin C. (D) Efflux of intracellular vitamin C. Cells incubated with 1 mM DHA for 60 min were washed with PBS and treated with (close circle) or without (open circle) TNFα for the time indicated. The amount of vitamin C inside the cells was measured and expressed as a percentage of the initial amount accumulated.

transient. Cells treated for 24 h with TNFα showed a 3-fold increase in luciferase activity, and preloading with the vitamin had no effect at this time point (Figure 1B). We reasoned that the transient inhibitory activity of the vitamin was due to a decrease in the intracellular content of the vitamin by 24 h. We therefore loaded the cells with ^{14}C-labeled vitamin C and measured the amount of vitamin C remaining inside the cells over a 24 h time period. After 4 h there was approximately 65% of the original amount of vitamin C taken up in the cells after 1 h incubation with 1 mM DHA (Figure 1D). After 24 h incubation at 37 °C, almost no intracellular vitamin C was detected, suggesting that the transient inhibitory activity observed was due to efflux of vitamin C and perhaps hydrolysis intracellularly (*13*). TNFα treatment did not change the rate of loss of vitamin C (Figure 1D).

These results indicated that vitamin C inhibits TNFα-dependent activation of NFκB transcription in a dose-dependent manner. Cells incubated with 0.5 and 1 mM DHA accumulated 3 and 4 mM, AA respectively (data not shown). The luciferase activity induced by TNFα in cells containing 3 and 4 mM DHA showed 55% and 72% decreases, respectively. No effect on NFκB-dependent luciferase was observed when HeLa cells were incubated with 0.5 or 1 mM DHA or AA (Figure 2). We found that cells exposed to 0.5

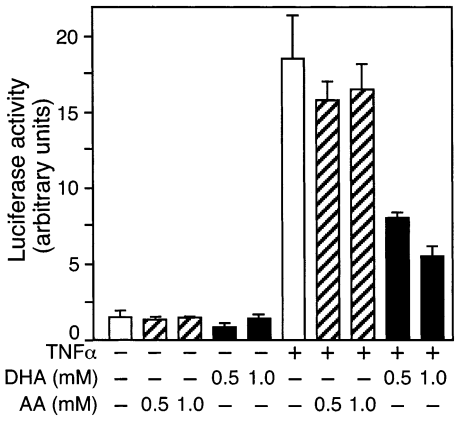

FIGURE 2: Ascorbic acid treatment has no effect on TNFα-induced NFκB transcriptional activation. HeLa cells cotransfected with pNFκB-luc (luciferase) and pRLTK (Renilla) were treated with either DHA (0.5 or 1mM) or AA (0.5 or 1mM) for 1 h and then incubated with TNFα (30 ng/mL) for 4 h. The activity of NFκB was estimated by the luciferase assay.

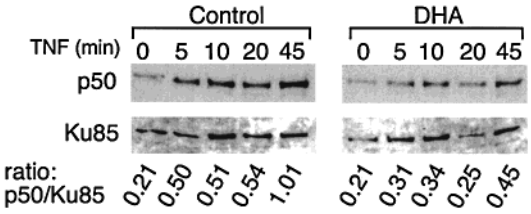

FIGURE 3: Vitamin C inhibits TNFα-dependent nuclear translocation of NFκB. HeLa cells were incubated with either buffer (control) or DHA for 60 min and treated with 30 ng/mL TNFα for the time indicated. Nuclear cell lysates were prepared, subjected to SDS−PAGE, and p50 was visualized by immunoblotting with an anti-p50 antibody (upper panel). Equal protein loading was demonstrated by immunoblotting the same membrane with an anti-ku85 antibody as shown in the lower panel.

or 1 mM AA for 1 h showed no significant decrease in TNFα-dependent luciferase activity compared to cells left untreated (Figure 2). DHA and AA were prepared under controlled pH conditions to avoid cellular responses induced by acidic solutions. As demonstrated for HL60 cells, incubation of HeLa cells with 1 mM or 2 mM DHA for 1 h had no deleterious effect on cell viability (data not shown) (*13*).

Vitamin C Inhibits TNF-Dependent Nuclear Translocation of NFκB. We investigated whether the inhibitory effect of vitamin C on TNFα-induced NFκB-mediated transcription was due to inhibition of NFκB nuclear translocation or inactivation of the DNA binding activity of NFκB. To distinguish between these two possibilities, we analyzed the nuclear translocation of NFκB p50 subunit by immunoblotting. TNFα stimulated nuclear translocation of NFκB and after 5 min treatment with 30 ng/mL TNFα the accumulation of p50 in the nucleus was evident (Figure 3). Cells loaded with vitamin C, however, had decreased nuclear translocation of p50. The inhibitory effect of vitamin C on nuclear migration of NFκB was evident even after 45 min treatment with TNFα . The immunoblots were striped and reprobed with anti-Ku86 antibody, demonstrating that equal amounts of nuclear extracts were loaded on the gel (Figure 3, lower panel). Analysis of the ratio p50/ku85 showed clearly that vitamin C inhibits NFκB nuclear translocation (Figure 3). These results indicate that vitamin C inhibited TNFα-induced NFκB-dependent transcription activity by blocking the signal transduction pathway involved in the activation nuclear translocation of NFκB.

Vitamin C Inhibits the TNFα-Induced Phosphorylation and Degradation of IκBα. Since vitamin C prevented the translocation of NFκB to the nucleus, we reasoned that vitamin C might inhibit TNFα-dependent phosphorylation of IκBα. Phosphorylation of IκBα is a key event in the activation of NFκB as it releases NFκB, allowing NFκB to translocate to the nucleus. To assess the ability of vitamin C to regulate the phosphorylation of IκBα, HeLa cells were incubated with buffer (control) or DHA, and TNFα-mediated phosphorylation of IκBα was analyzed (Figure 4). HeLa cells incubated with 30 ng/mL TNFα evidenced a prominent increase in phosphorylation of IκBα, as measured by immunoblotting with anti-phospho-IκBα antibody (Figure 4A,B). Loading cells with vitamin C caused dose-related inhibition of TNFα-dependent phosphorylation of IκBα (Figure 4A, panels I and III). HeLa cells exposed to 0.1 mM DHA showed a clear decrease in IκBα phosphorylation when treated for 5 min with 30 ng/mL TNFα; however, there was only a modest decrease in phosphorylation of IκBα when the cells were incubated with for 20 min with TNFα (Figure 4, panel I). To inhibit IκBα phosphorylation induced by 20 min incubation with 30 ng/mL TNFα required incubation for 1 h with 1 mM DHA prior to TNFα treatment (Figure 4A, panel III). Cells incubated with 0.1, 0.5, and 1 mM DHA accumulate 1, 3, and 4 mM, respectively. The immunoblots were striped and reprobed with anti-IκBα antibody, showing the levels of IκBα after TNFα treatment (Figure 4A, panel II). Because of proteasomal degradation, the amount of IκBα correlated inversely with the levels of phosphorylated IκBα (Figure 4, panel II). Reprobing the immunoblot (panel III) revealed that equal amounts of extracts were loaded on the gel, as detected by an anti-tubulin antibody (Figure 4, panel IV). These results indicate that HeLa cells required an intracellular concentration of vitamin C of approximately 4 mM to significantly inhibit IκBα phosphorylation induced by 30 ng/mL TNFα. Such concentrations of intracellular AA are achievable in host defense cells in vivo. For example, mononuclear leukocytes are reported to contain about 3.5−6 mM AA (*10*). Neutrophiles are generally thought to have about 1 mM (*8*, *9*).

To be certain that the inhibitory effect of vitamin C on IκBα phosphorylation was an intracellular event, DHA and TNFα were incubated simultaneously (DHA/TNFα) for 5 min prior to addition to HeLa cells. We found that mM concentrations of DHA did not alter the activity of TNFα. TNFα and TNFα incubated with DHA (DHA/TNFα) induced comparable levels of IkBα phosphorylation (Figure 4B). A summary diagram showing the experimental protocol and the outcome on IκBα phosphorylation are shown in Figure 4C. These results indicate that intracellular vitamin C can disrupt the signaling pathway involved in the phoshorylation of IκBα, impairing the activity of NFκB.

To further assess the inhibitory potential of vitamin C on phosphorylation of IκBα mediated by TNFα, we investigated suppression of IκBα phosphorylation in other cell types. Human MCF7 (breast cancer cells) and primary HUVEC (human umbilical vein endothelial cells) were incubated with DHA for 60 min prior to TNFα treatment (Figure 5). Similar to the results with HeLa cells, MCF7 cells showed a dose-dependent inhibitory effect of vitamin C on TNFα-induced

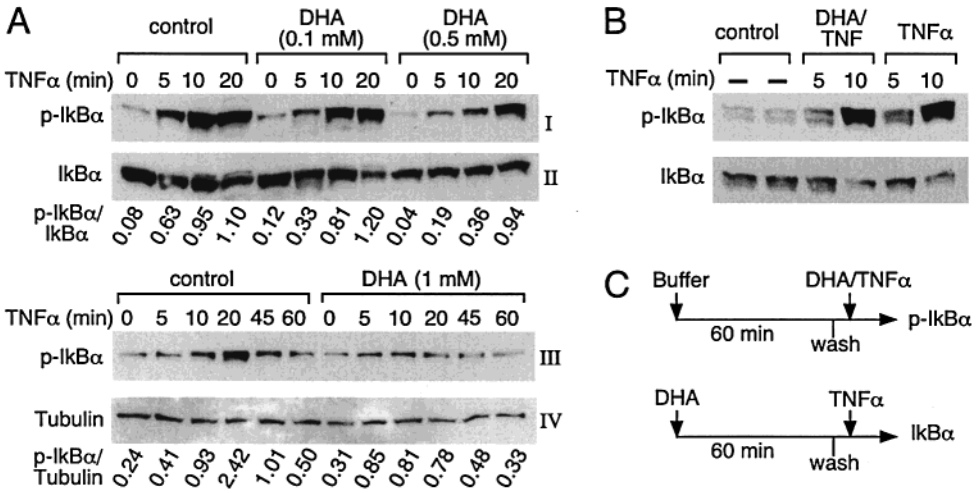

FIGURE 4: Vitamin C inhibits TNFα-induced phosphorylation of IκB in HeLa cells. (A) HeLa cells treated for 60 min with either buffer or different concentrations of DHA (0.1, 0.5, or 1 mM) were incubated with 30 ng/mL TNFα for the time period shown. Cell extracts were prepared, and phosphorylated IκBα (p-IκBα) was visualized by immunoblotting with an anti-phospho IκBα antibody (panel I and III). The nonphosphorylated IκBα is shown below the phosphorylated p-IκBα panel (panel II). Equal protein loading was demonstrated by immunoblotting with anti-tubulin (panel IV). (B) DHA does not affect TNFα ligand. DHA and TNFα were incubated together for 5 min prior addition to cells (DHA/TNFα). Cells were incubated with TNFα or DHA/TNFα for the time period indicated. The phosphorylated and nonphosphorylated IκBα are shown in the upper and lower panel, respectively. (C) A summary diagram of the experiments measuring inhibition of phosphorylation of IκBα is shown.

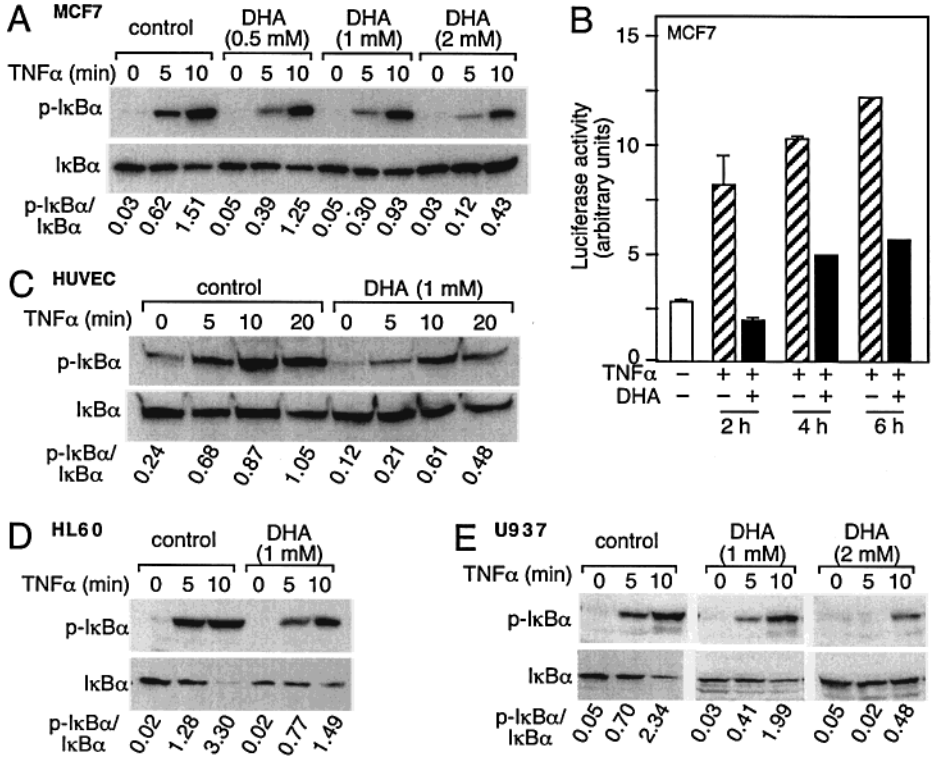

FIGURE 5: Vitamin C inhibits TNFα-mediated NFκB responses in MCF7, HUVEC, HL60 and U937 cells. (A) MCF7 cells treated for 60 min with buffer (control) or DHA were incubated with 30 ng/mL TNFα for the time indicated. Cell extracts were prepared, and the phosphorylated and nonphosphorylated IκBα is shown in the upper and lower panels, respectively. (B) MCF7 cells cotransfected with pNFκB-luc and pRLTK were incubated for 1 h with either 1 mM DHA (closed bars) or buffer (striped bars). Transfectants were incubated with 30 ng/mL TNFα for the time indicated in hours (h). (C) HUVEC, (D) HL60, and (E) U937 cells were treated with buffer or DHA (1 mM or 2 mM) for 60 min prior to the treatment with TNFα for the time indicated. Cell extracts were prepared, and the phosphorylated and nonphosphorylated IκBα is shown in the upper and lower panels, respectively.

phosphorylation of IκBα (Figure 5A). Vitamin C loading also inhibited NFκB transcriptional response induced by TNFα in MCF7 cells (Figure 5B). TNFα induced a 6-fold increase of NFκB-mediated luciferase activity in MCF7 cells transfected with pNFκB-luc (Figure 5B). In this cellular system, vitamin C inhibited approximately 50% of the transcriptional response mediated by TNFα. HUVEC cells incubated with DHA also had decreased levels of phospho-IkBα after treatment with TNFα as compared with cells treated with vehicle buffer (Figure 5C). Human myeloid HL60 and monocytic U937 cell lines incubated with TNFα also evidenced a prominent increase in phosphorylation of

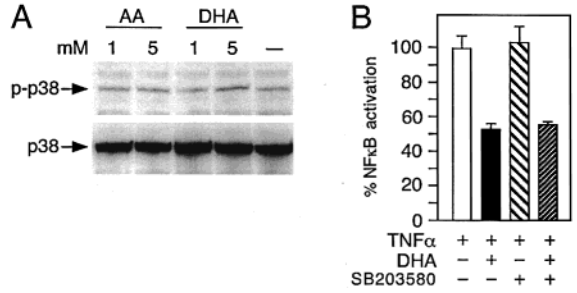

FIGURE 6: Intracellular vitamin C inhibits TNFα-mediated NFκB responses independent of p38 MAP kinase activation. (A) Phosphorylation of p38 MAP kinase was analyzed by immunoblotting in HeLa cells incubated for 1 h with AA (1 or 5 mM) or DHA (1 or 5 mM). The phophorylated (p-p38) and nonphosphorylated p38 MAP kinase (p38) are shown in the upper and lower panels, respectively. (B) The p38 MAP kinase inhibitor (SB203580) did not block TNFα inactivation by vitamin C loading. HeLa cells cotransfected with pNFκB-luc and pRLTK were incubated for 1 h with 1 mM DHA, 3 μM SB203580, or buffer. Transfectants were treated with 30 ng/mL TNFα for 5 h, and luciferase activity was measured. The TNFα-dependent NFκB transcriptional activity was measured and expressed as % NFκB activation.

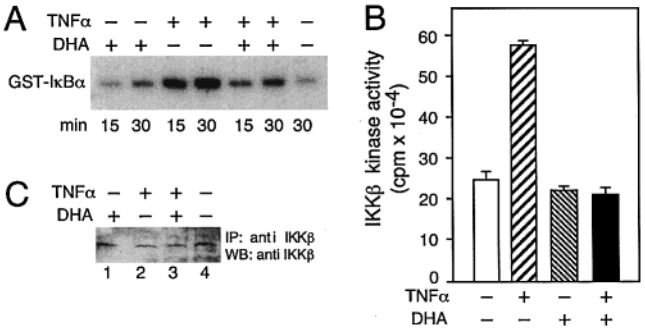

FIGURE 7: Vitamin C inhibits IKKβ kinase activation. HeLa cells incubated for 60 min with buffer or 1 mM DHA were treated for 10 min with 30 ng/mL TNFα. Cell extracts were prepared, and IKKβ was immunoprecipitated with an anti-IKKβ antibody. Immunoprecipitates were assayed for IKKβ kinase activity for the time indicated with GST-IκBα as substrate. (A) The kinase reaction was incubated with glutathione beads, subjected to SDS−PAGE, and the phosphorylated GST-IκBα was visualized by autoradiography. (B) The glutathione beads containing phosphorylated GST-IκB was quantified by scintillation counting. (C) The immunoprecipitated IKKβ was subjected to SDS−PAGE and visualized by immunoblotting with an anti-IKKβ antibody.

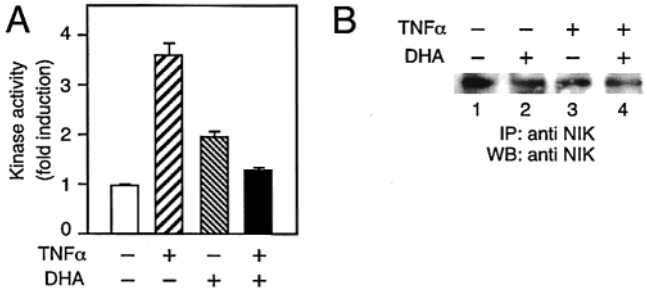

FIGURE 8: Vitamin C inhibits NIK kinase activation. HeLa cells incubated for 60 min with buffer or 1 mM DHA were treated for 10 min with 30 ng/mL TNFα. Cell extracts were prepared and NIK was immunoprecipitated with an anti-NIK antibody. NIK kinase activity was assayed using γP^{32}-ATP and myelin basic protein as substrate. The kinase reaction was incubated for 1 min at 30 °C, TCA precipitated, washed, and counted with a scintillation counter. (B) The immunoprecipitated NIK was subjected to SDS−PAGE, and it was visualized by immunoblotting with an anti-NIK antibody.

IκBα (Figure 5D,E). Similar to the previous results, HL60 and U937 cells loaded with vitamin C showed a clear decrease in IκBα phosphorylation when treated for 5 min with TNFα (Figure 5D,E).

Vitamin C Inhibits TNFα-Induced NFκB Responses Independent of p38 MAP Kinase Activation. It was reported that in the endothelial cell line ECV340, treatment with very high concentrations of ascorbic acid (20 mM) induced phosphorylation of p38 MAP kinase (p38) (*42*). To investigate if the phosphorylation of p38 is required for the inhibition of TNFα-dependent activation of NFκB by vitamin C, we analyzed the phosphorylation of p38 in HeLa cells incubated with 1 or 5 mM DHA or AA. Our results, in HeLa cells, showed that incubation with 1 or 5 mM DHA for 1 h did not induce phosphorylation of p38, as detected by immunoblotting with anti-phophorylated p38 antibody (Figure 6A, upper panel). Similarly, exposure to AA for 1 h did not induced phosphorylation of p38. The levels of p38 remained unaltered after 1 h treatments with AA or DHA (Figure 6A, lower panel). HeLa cells transfected with pNFκB-luc and incubated with 3.0 uM SB203580, a specific inhibitor of p38 showed no induction of luciferase activity. Also, the inhibitor did not alter the level of luciferase activity induced by TNFα. Similarly, the inhibitory effect of vitamin C loading with DHA was not altered by SB203580. These results indicate that p38 is not involved in the vitamin C-mediated inhibition of the TNFα-induced NFκB response (Figure 6B).

Vitamin C Inhibits TNF-Induced IKKβ and NIK Kinase Activation. IKKβ is the main catalytic subunit required for IκBα phosphorylation of NFκB, signaling activation (*20*). Since vitamin C inhibited TNFα-induced phosphorylation of IκBα, we hypothesized that the vitamin inhibited IKKβ activation. We therefore investigated vitamin C effects on the kinase activity of IKKβ after TNFα treatment of cells loaded with vitamin C. IKKβ was isolated as an immune complex from HeLa extracts and the kinase activity analyzed in vitro with GST-IκBα as substrate. We found that the activity of IKKβ from TNFα treated cell extracts was at least 3-fold more than that of the kinase isolated from cells not treated with TNFα (Figure 7A,B). Extracts from cells exposed to DHA showed IKKβ kinase activity equivalent to untreated cells (Figure 7A,B). Extracts from cells loaded with vitamin C and treated with TNFα showed a minimal activation of IKKβ (Figure 7A,B). The decrease in TNFα-induced IKKβ kinase activity in cells loaded with vitamin C and treated with TNFα is due to reduced kinase activity and not to degradation of the enzyme as evidenced by similar amounts of IKKβ in the immuno complexes (Figure 7C, lanes 2 and 3). Phosphorylation of IκBα is also induced indirectly by NIK in Hela cells. We observed a 3.5-fold increase in NIK kinase activity in cells treated with TNFα (Figure 8A); however, cells loaded with vitamin C showed a decreased NIK kinase activity (Figure 8A). DHA incubation itself caused a small increase in kinase activity, however, the reduced NIK kinase activity in cells loaded with vitamin C was due to inhibition of TNFα activation of NIK kinase, as similar quantities of the kinase were seen in the immune complexes (Figure 8B, lanes 2 and 4). NIK kinase is known to activate IKKβ, which then phosphorylates IκBα, suggesting that vitamin C inhibits TNFα-dependent activation of kinases that phosphorylates IκBα.

DISCUSSION

Nuclear factor-κB (NFκB) is a key transcription factor activated by several cellular signal transduction pathways critically important in host defense, inflammation, and apoptosis (*19*, *48*−*50*). NFκB exerts its effects by regulating genes encoding cytokines, chemokines, adhesion molecules, growth factors, and inducible pro-inflammatory enzymes such as cyclooxygenase 2 (Cox2) (*50*, *51*). The molecular mechanisms involved in NFκB activation have been well studied and involve a sequential activation of cytoplasmic protein kinases (*20*, *52*−*54*).

There is considerable evidence implicating reactive oxygen species (ROS) in cellular signaling and transcriptional regulation (*32*, *55*). NFκB has been considered a redox-sensitive transcription factor (*49*, *56*−*58*), however, reactive oxygen species may not be a universal mechanism of NFκB activation, as seen in human umbilical vein endothelial cells (HUVEC) (*59*), HeLa, and other cells where H_2O_2 did not activate NFκB (*49*, *60*, *61*). This limited literature is somewhat contradictory, and the mechanistic basis for effects of physiological antioxidants such as glutathione and vitamin C on cell signaling is poorly understood. We sought to define precisely the role of the antioxidant vitamin C in NFκB activation.

We previously described in detail a universal transport system of vitamin C into cells in the oxidized form (dehydroascorbic acid, DHA) through the facilitative glucose transporters (*3*, *7*, *62*). After transport through the glucose transporter, DHA is rapidly reduced to ascorbic acid (AA) inside the cells, where it can accumulate to mM concentrations (*7*). We refer to this system as "universal" because all cells have glucose transporters. AA is the species of vitamin C found in the plasma and intracellularly. While all cells can transport DHA, certain specialized cells can transport AA directly through sodium-dependent ascorbic acid co-transporters (*4*, *6*, *62*). The use of AA in vitro experiments, however, has led to artifacts and confounding results due to slow cellular accumulation and the ability of AA to act as a strong pro-oxidant in vitro in the presence of free transition metals (iron) ubiquitously found in tissue culture media (*12*, *63*). Zhang and Frei reported that α-lipoic acid inhibited TNFα-induced NFκB activation, whereas AA treatment had no effect (*41*). Bowie and O' Neill found inhibition of NFκB activation after very long exposure to mM concentration of AA and concluded that the inhibition was due to p38 MAP kinase activation (*42*). We loaded the cells with AA by brief exposure to DHA, thereby circumventing the issue of pro-oxidant effects of extra cellular AA and precisely controlling the intracellular concentration of AA. This methodology allowed us to show that vitamin C suppresses GM-CSF signaling through inhibition of the JAK-2 kinase activation step (*34*) and that vitamin C inhibits oxidative DNA mutations in a human cell system (*14*).

The experiments reported here indicate that cellular loading of vitamin C by exposure to DHA resulted in inhibition of NFκB transcriptional activity induced by TNFα. Treatment with AA for a short period had no effect on TNFα-dependent NFκB transcriptional reporter activity because the cells do not transport AA, or do so minimally. Our data indicate that vitamin C blocks the phosphorylation of IκBα, a key step allowing the translocation of NFκB to the nucleus to activate gene expression. The phosphorylation of IκBα is mediated by several kinases, and we demonstrate that vitamin C inhibits the activation of NIK and IKKβ kinases independent of p38 MAP kinase. The activation of p38 by mM concentration of AA observed by Bowie and O' Neill may be due to the long exposure to very high concentrations of AA. The inhibitory activity of vitamin C on TNFα-dependent phosphorylation of IκBα was observed in several cellular systems, including HeLa (human cervix adenocarcinoma), MCF7 (human breast adenocarcinoma) U937 (monocytic) and HL60 (myeloid leukemia) cell lines and in primary cultures of human umbilical vein endothelial cells (HUVEC), demonstrating the generality of the effect.

The molecular mechanisms by which vitamin C inhibits TNFα-induced NFκB activation are not completely resolved. We found that vitamin C suppressed the activation of kinases involved in the signaling pathway leading to NFκB activation. Our data support the hypothesis that vitamin C inhibits the activation of NIK kinase, as our in vitro kinase assays showed that AA has no direct inhibitory activity on IKKβ or NIK. We conclude that vitamin C inhibits the activation of NFκB and propose that ascorbic acid can play a role in modulating inflammation and apoptosis mediated by NFκB. Our in vitro experiments found effects of intracellular concentrations of AA of 1 mM and very prominent inhibition of NFκB activation at 4 mM. Host defense cells normally may have intracellular AA concentrations of 1−4 mM, and the brain is generally considered to have 1 mM AA. These findings have potential importance for human nutrition as well as the pharmacological use of DHA (*64*, *65*).

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