Vitamin C Inhibits NF-κB Activation by TNF Via the Activation of p38 Mitogen-Activated Protein Kinase[1]

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The transcription factor NF-κB is a central mediator of altered gene expression during inflammation, and is implicated in a number of pathologies, including cancer, atherosclerosis, and viral infection. We report in this study that vitamin C inhibits the activation of NF-κB by multiple stimuli, including IL-1 and TNF in the endothelial cell line ECV304 and in primary HUVECs. The induction of a NF-κB-dependent gene, IL-8, by TNF was also inhibited. The effect requires millimolar concentrations of vitamin C, which occur intracellularly in vivo, particularly during inflammation. Vitamin C was not toxic to cells, did not inhibit another inducible transcription factor, STAT1, and had no effect on the DNA binding of NF-κB. Inhibition by vitamin C was not simply an antioxidant effect, because redox-insensitive pathways to NF-κB were also blocked. Vitamin C was shown to block IL-1- and TNF-mediated degradation and phosphorylation of I-κBα (inhibitory protein that dissociates from NF-κB), due to inhibition of I-κB kinase (IKK) activation. Inhibition of TNF-driven IKK activation was mediated by p38 mitogen-activated protein kinase, because treatment of cells with vitamin C led to a rapid and sustained activation of p38, and the specific p38 inhibitor SB203580 reversed the inhibitory effect of vitamin C on IKK activity, I-κBα phosphorylation, and NF-κB activation. The results identify p38 as an intracellular target for high dose vitamin C. *The Journal of Immunology,* 2000, 165: 7180–7188.

V itamin C (ascorbic acid) is required in the diets of humans and several other species that lack the terminal enzyme in its synthetic pathway, L-gulonolactone oxidase (1). It is involved in a number of vital functions within cells, including the hydroxylation reactions of collagen biosynthesis (2) and the facilitation of iron transport (3), as well as being one of the most important physiological antioxidants (4). The current recommended dietary allowance (1) for vitamin C is 60 mg/day for nonsmoking adult males, which is sufficient to prevent scurvy (5). However, vitamin C has been suggested as having both a preventative and therapeutic role in a number of other pathologies when administered at much higher-than-recommended dietary allowance levels, including cancer (6), atherosclerosis (7), and viral infections (8, 9).

The inducible, higher eukaryotic transcription factor NF-κB regulates the expression of a number of cellular genes involved in immune and inflammatory responses (10), has antiapoptotic effects (11–13), is involved in the replication of several viruses (10), most notably HIV-1 (14), and has been implicated in the initiation and development of atherosclerosis (15, 16). It is activated by diverse pathogenic signals, including the proinflammatory cytokines IL-1 and TNF (10). NF-κB exists in a latent form in the cytoplasm of unstimulated cells, comprising a transcriptionally active dimer bound to an inhibitor protein, I-κB[3] (inhibitory protein that dissociates from NF-κB). The currently known subunit members of the NF-κB family in mammals are p50, p65 (RelA), c-Rel, p52, and RelB, while multiple mammalian forms of I-κB also exist, namely I-κBα, β, γ (p105), δ (p100), and ε, and Bcl-3 (17). Most work has focused on the p50/p65 dimer, the predominant form of NF-κB activated in many cells, and its association with I-κBα. Upon stimulation with many NF-κB inducers, I-κBα is rapidly phosphorylated on Ser^{32} and Ser^{36}, which targets the inhibitor protein for ubiquitination and subsequent degradation by the 26S proteasome (18). A specific E3 ligase that recognizes doubly phosphorylated I-κBα has now been identified (19). The released NF-κB dimer can then translocate to the nucleus and activate target genes by binding with high affinity to κB elements in their promoters. The phosphorylation and degradation of I-κBα are tightly coupled events, and recently two I-κB kinases (IKKs), termed IKKα and IKKβ, have been identified (reviewed in Ref. 20). IKKα and β have been shown to be activated by IL-1 and TNF, to specifically phosphorylate Ser^{32} and Ser^{36} of I-κBα, and to be crucial for NF-κB activation by these cytokines (20–22). The IKKs are part of a larger multiprotein complex called the IKK signalsome, which contains a core of IKKα and β together with IKKγ (or NF-κB essential modulator, NEMO) which is essential for NF-κB activation (23, 24).

Upstream of IKK activation, binding of TNF to TNFR1 aggregates three TNFR1s together, inducing the association of TNFR-associated death domain protein with the death domains in the cytosolic region of TNFR1s (reviewed in Ref. 25). TNFR1-associated death domain protein then recruits TNFR-associated factor 2 (TRAF2) and receptor-interacting protein, both of which are essential for NF-κB induction. TRAF2 can then interact with and activate NF-κB-inducing kinase (NIK), which can activate the IKKs (25). In contrast, binding of IL-1 to its type 1 receptor (IL-1R1) recruits the IL-1R accessory protein, which leads to recruitment of IL-1R-associated kinase (25), via the adaptor molecule MyD88 (26). IL-1R-associated kinase then activates TRAF6, which can associate with the mitogen-activated protein kinase (MAPK) kinase kinase TGF-β-activating kinase-1, leading to activation of NIK (27), and subsequently the IKKs. MAPK/extracellular signal-regulated kinase kinase kinase-1 has also been implicated

in activation of IKKs by IL-1 and TNF (28), although the physiological relevance of both NIK and MAPK/extracellular signal-regulated kinase kinase kinase-1 in IKK activation remains to be determined (20).

We decided to examine the effect of vitamin C on NF-κB, because although there has been much interest in the effect of redox-modulating compounds on pathways to NF-κB, there are little data on the direct effect of this antioxidant vitamin on NF-κB activation. Furthermore, NF-κB has been implicated in pathologies for which high dose vitamin C has been suggested as a therapy. In this study, we show for the first time that millimolar doses of vitamin C inhibit multiple pathways to NF-κB, including IL-1 and TNF, in endothelial cells. Cytokine stimulation of NF-κB was inhibited before phosphorylation of I-κBα and activation of IKK. This inhibition was not simply due to an antioxidant effect. Rather, for TNF, inhibition was mediated by vitamin C-induced activation of the stress-activated protein kinase p38 MAPK.

Materials and Methods

Reagents

Human rIL-1α was a gift from the National Cancer Institute (Frederick, WA), while human rTNF-α was a gift from Dr. Steve Foster (Zeneca Pharmaceuticals, Macclesfield, U.K.). The NF-κB consensus oligonucleotide was from Promega (Madison, WI). The STAT1 consensus and mutant oligonucleotides, and the rabbit polyclonal Ab to human I-κBα were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-I-κBα (Ser^{32}), anti-p38 MAPK, and anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) polyclonal Abs were from New England Biolabs (Beverly, MA). Anti-NEMO antiserum and plasmids encoding GST/I-κBα fusion proteins GST/I-κBα (1–72) and mutant GST/I-κBα (1–72)S32A/S36A were a gift from Dr. Alain Israel and Dr. Shoji Yamaoka (Institut Pasteur, Paris, France). GST/I-κBα fusion proteins were overexpressed and purified by GST-affinity chromatography using standard techniques. The p38 MAPK inhibitor SB203580 was purchased from Calbiochem (San Diego, CA) and dissolved in DMSO. Vitamin C (sodium salt), vitamin E analogues, PMA, and hydrogen peroxide (H$_2$O$_2$) were from Sigma (Poole, U.K.). Vitamin C was dissolved in PBS, pH 7.4, PMA in DMSO, Trolox in ethanol, α-tocopherol acetate in 50% (v/v) ethanol, and α-tocopherol phosphate in 25 mM Tris-HCl, pH 7.4.

Cell culture

The human cell line ECV304 and HUVECs were grown and passaged as described previously (29). All experiments were conducted in complete medium at 37°C. Cell integrity was assessed by measuring lactate dehydrogenase (LDH) release. After treatment of cells, a 100-μl aliquot of medium was removed from each experimental well and assayed for LDH by monitoring spectrophotometrically the decrease in absorbance at 340 nm in the presence of 75 mM Tris-HCl, pH 7.2, containing 150 mM KCl, 0.2 mM NADH, and 4.8 mM sodium pyruvate.

Cell fractionation and preparation of nuclear and cytosolic extracts

Nuclear extracts were prepared as described previously (29). Cytosolic extracts were prepared by removing the supernatant from pelleted nuclei, further centrifugation of this supernatant (13,000 × g, 10 min, 4°C), and removal of the ensuing supernatant into 100 μl storage buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% v/v glycerol, 0.5 mM PMSF, 0.5 mM DTT). Protein concentrations were determined using the method of Bradford (30).

Electrophoretic mobility shift assay

Nuclear extracts (2 μg protein) were assayed for NF-κB DNA-binding activity in the EMSA, as described previously (29). For STAT1 EMSA, 4 μg protein and 50,000 cpm of radiolabeled STAT1 consensus oligonucleotide were used. For competition analysis, unlabeled consensus or mutant STAT1 oligonucleotide was incubated with nuclear extract protein in binding buffer for 20 min at room temperature, before addition of radiolabeled oligonucleotide.

IL-8 ELISA

HUVECs were seeded in 96-well plates (5 × 10^4 cells in 200 μl) and 36 h later treated with 20 ng/ml TNF for 6 h with or without a 1-h pretreatment

with vitamin C. Cell supernatants were harvested and assayed for IL-8 by ELISA using the DuoSet ELISA Development System for human IL-8 (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Detection of IL-8 mRNA by RT-PCR

HUVECs were seeded in 10-cm dishes (1 ×10^6 cells in 6 ml) and 24 h later treated with 20 ng/ml TNF for 1 h with or without a 1-h pretreatment with vitamin C. Total RNA was isolated using Tri reagent (Sigma), according to the manufacturer's recommendations. RT-PCR was then performed using the enhanced avian RT-PCR kit (Sigma) with 2.5 μg total RNA as template. The PCR was performed with an annealing temperature of 55°C for 35 cycles, using primers to IL-8 (human IL-8 PCR Primer Pair; R&D Systems) or to the housekeeping gene aldolase as control.

Anti-I-κBα immunoblot analysis

Confluent ECV304 cells in six-well plates (3-ml vol) were treated as described in figure legends. Treatment was terminated by washing monolayers twice with ice-cold PBS. Total I-κBα was then detected, as described previously (29). For detection of phospho-I-κBα, 100 μl SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue) was added directly to cells, which were then sonicated. Lysates were then resolved by SDS-PAGE, transferred to polyvinylidine difluoride membranes, and probed with anti-phospho-I-κBα Ab according to manufacturer's instructions.

p38 MAPK assay

Cells were harvested as above, and p38 activation was determined by the appearance of a band on immunoblots specifically recognized by an Ab to phosphorylated p38, because phosphorylation of p38 correlates with kinase activity (31, 32). In each experiment, extracts were also probed using anti-p38 Ab to demonstrate equal protein loading (not shown).

Determination of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation was assessed by the TBARS assay, as described previously (29).

IKK assay

Confluent ECV304 cells in 100-mm dishes were treated as described in figure legends, and monolayers then washed twice with ice-cold PBS. Cells were lysed in 400 μl lysis buffer (20 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 2 mM DTT, 10 mM MgCl$_2$, 50 mM NaCl, 100 μM sodium orthovanadate, 20 mM β-glycerol phosphate, 1% Nonidet P-40). Clarified lysates were precipitated with 1.5 μl anti-NEMO for 1 h at 4°C, and then 35 μl of 50% protein G-Sepharose was added for 1 h at 4°C. Immune complexes were washed twice with lysis buffer, and then three times with kinase buffer (20 mM HEPES, pH 7.5, 2 mM DTT, 10 mM MgCl$_2$, 50 mM NaCl, 100 μM sodium orthovanadate, 20 mM β-glycerol phosphate). Kinase assays were then performed for 30 min at 30°C in kinase buffer containing 20 μM ATP, 5 μCi [γ^{32}-P]ATP, and 1 μg of bacterially expressed GST/I-κBα (1–72) or mutant GST/I-κBα (1–72)S32A/S36A. The reaction mixtures were resolved by SDS-PAGE, followed by autoradiography.

Results

Inhibition of multiple pathways to NF-κB activation by vitamin C in endothelial cells

We investigated the effect of vitamin C on a number of stimulants of NF-κB in ECV304 cells. The response of NF-κB to all those stimulants used below have been previously extensively characterized in both these cells and HUVECs (29). First, cells were treated with vitamin C for 1 h before stimulation with TNF. Fig. 1A shows that vitamin C inhibited TNF-mediated NF-κB activation in a dose-dependent manner, with the effect being apparent at 5 mM (compare lanes 2 and 3), and potent at 10–20 mM (compare lanes 4 and 5 with lane 2). Activation of NF-κB by TNF was completely abolished upon exposing cells to 5 mM vitamin C for 16 h (Fig. 1A, lanes 6–8). Fig. 1B shows that IL-1, PMA, or H$_2$O$_2$, all of which activate NF-κB in these cells (29), were also sensitive to a 1-h pretreatment with vitamin C: 10–20 mM blocked the IL-1
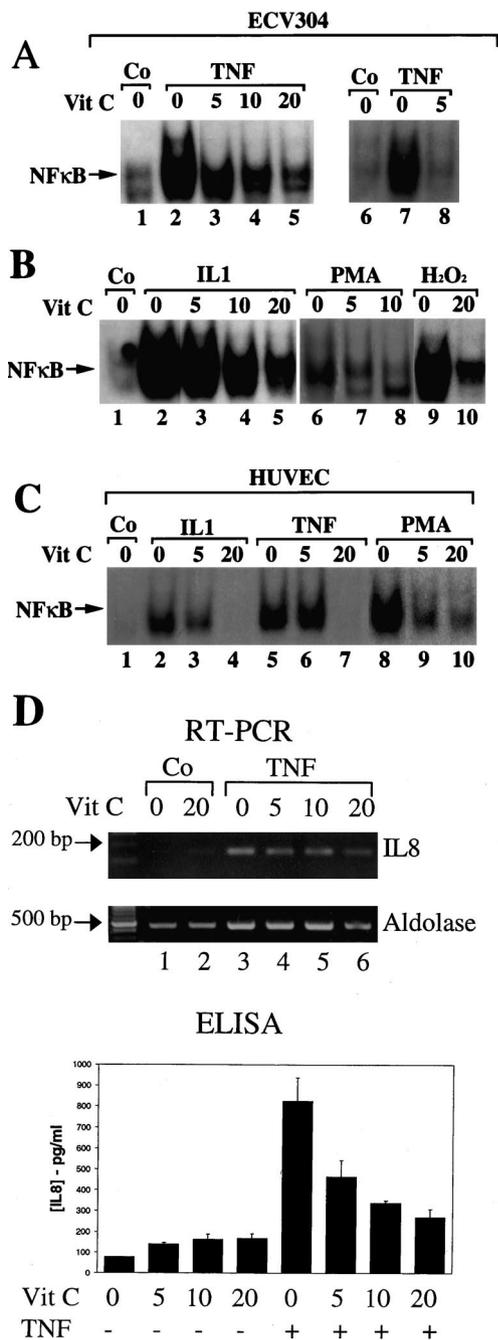
FIGURE 1. Vitamin C inhibits NF-κB activation by multiple stimuli. *A*, Confluent monolayers of ECV304 cells in six-well plates were pretreated with 0–20 mM vitamin C for 1 h before stimulation with 10 ng/ml TNF for 1 h. In *lanes 6–8*, cells were pretreated or not with 5 mM vitamin C for 16 h before stimulation with 10 ng/ml TNF for 1 h. Control cells (Co) were left unstimulated. *B*, ECV304 cells were pretreated with vitamin C for 1 h before stimulation with 10 ng/ml IL-1 or 100 ng/ml PMA for 1 h, or 0.2 mM H₂O₂ for 4 h. *C*, Monolayers of HUVECs were pretreated with vitamin C 1 h before stimulation with IL-1, TNF, or PMA, as above. In all cases (*A–C*), nuclear extracts were prepared and assessed for activated NF-κB by EMSA. Retarded protein-DNA complexes are shown and are representative of two to four experiments. *D*, For RT-PCR, confluent monolayers of HUVECs in 10-cm dishes were pretreated with 0–20 mM vitamin C for 1 h before stimulation with 20 ng/ml TNF for 1 h. Total RNA was then isolated and analyzed by RT-PCR, for IL-8 and Aldolase. Representative of two experiments. For ELISA, confluent monolayers of HUVECs in 96-well plates were pretreated with 0–20 mM vitamin C for 1 h before stimulation with 20 ng/ml TNF for 6 h. Cell supernatants were then collected and analyzed for the presence of IL-8 by ELISA. Representative of two experiments, each performed in triplicate.

response (compare *lanes 4* and *5* with *lane 2*), 5–10 mM was active against PMA (compare *lanes 7* and *8* with *lane 6*), while 20 mM inhibited H₂O₂ (compare *lanes 9* and *10*). Fig. 1*C* shows that inhibition of NF-κB by vitamin C was not a cell line-specific phenomenon, because primary HUVECs were also sensitive. Activation by IL-1, TNF, and PMA was all inhibited by 5–20 mM vitamin C. Other cell types tested that were also sensitive include EL4.NOB1 T lymphoma cells, and human 132INI astrocytoma cells (not shown).

Vitamin C was also capable of inhibiting TNF induction of IL-8, an important NF-κB-dependent gene, in HUVECs. Fig. 1*D* shows that TNF treatment of HUVECs led to an increase in both IL-8 mRNA (measured 1 h post-TNF stimulation by RT-PCR) and secreted IL-8 protein (measured 6 h post-TNF stimulation by ELISA of cell supernatants). In both cases, pretreatment with 5–20 mM vitamin C for 1 h before TNF stimulation led to a dose-dependent inhibition, while there was no inhibitory effect on constitutive levels of IL-8 release, demonstrating the relevance of vitamin C-mediated NF-κB inhibition to downstream gene induction in endothelial cells.

Specificity of the effect of vitamin C on NF-κB activation

We next tested the specificity of the effect of vitamin C on NF-κB. Because vitamin C inhibited all agents tested, the effect may simply have been due to a general toxic effect on cells. However, this was found not to be the case, based on a number of indices of toxicity. Morphologically, cell monolayers of ECV304s and HUVECs appeared normal, with no detachment of the cells observable after exposure to the doses of vitamin C used (not shown). Furthermore, as mentioned above, 5–20 mM vitamin C did not inhibit basal levels of IL-8 release during a 7-h exposure period in which inhibition of an increase in TNF-mediated IL-8 release was observed (Fig. 1*D*). Fig. 2*A* shows that membrane integrity was not affected because there was no increase in LDH release in response to up to 100 mM vitamin C in ECV304 cells over the 2-h time course of the experiments shown in Fig. 1, *A* and *B*. Additionally, vitamin C did not induce necrosis or apoptosis of cells over the course of the experiment shown in Fig. 1*A*, *lanes 6–8*, because no DNA smearing or laddering was detected under these conditions (not shown). A general effect on inducible transcription factors was also unlikely. Fig. 2*B* shows that the induction of STAT1 DNA binding by IFN-γ was not inhibited by 5–40 mM vitamin C, under identical conditions to Fig. 1 in which strong inhibition of NF-κB was observed.

Some inhibitors of NF-κB have been shown to act by directly modifying NF-κB and so interfere with its binding to DNA (33, 34). To explore this possibility for vitamin C, we incubated nuclear extracts from TNF-stimulated cells with vitamin C for 2 h at 37°C. Fig. 2*C* shows that this treatment had no inhibitory effect on NF-κB DNA binding in the EMSA at the highest dose of 40 mM (*lane 5*), while concentrations of 5–20 mM slightly potentiated DNA binding (*lanes 2–4*). This strongly suggested that vitamin C does not inhibit NF-κB by modifying the complex directly or by interfering with DNA binding. Another possibility investigated was whether vitamin C chemically interfered with the release of NF-κB from I-κB. Fig. 2*D* shows that crude cytosolic extracts from control cells had a basal level of NF-κB activity (*lane 1*). Treatment of these extracts with a sodium deoxycholate/3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate detergent mix led to increased detection of NF-κB activity in the EMSA due to an in vitro release of NF-κB from I-κB (*lane 2*). The profile of NF-κB detected in crude cytosolic extracts, and in the presence of detergent differed somewhat from that detected in nuclear extracts, in that an extra lower band seemed to be revealed
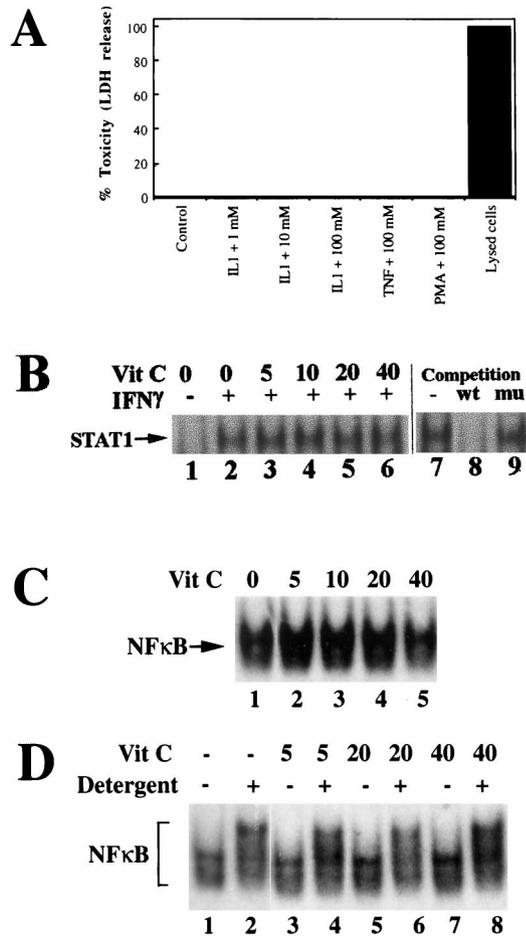
FIGURE 2. Specificity of the effect of vitamin C on NF-κB activation. *A*, Vitamin C does not affect cell integrity. ECV304 cells were left untreated (Control) or pretreated with 1–100 mM vitamin C for 1 h before stimulation with IL-1 (10 ng/ml), TNF (10 ng/ml), or PMA (100 ng/ml) for 1 h. Toxicity was assessed by spectrophotometrically measuring LDH release, and expressed as a percentage of control levels, taking the level of LDH released from fully lysed cells as 100% toxicity. *B*, Vitamin C does not inhibit IFN-γ-induced STAT1 DNA-binding activity. ECV304 cells were pretreated with 0–40 mM vitamin C for 1 h before stimulation with 200 U/ml IFN-γ for 15 min. STAT1 was measured by EMSA using a specific probe containing a STAT1 binding site. Detected complexes were specific for STAT1, as shown by competition analysis with mutant and wild-type unlabeled STAT1 probe (*lanes 7–9*). *C*, Vitamin C does not inhibit NF-κB DNA binding in vitro. Nuclear extracts from TNF-stimulated cells were incubated in vitro with 0–40 mM vitamin C for 2 h at 37°C before measurement of NF-κB DNA-binding activity in the EMSA. *D*, Treatment of intact cells with vitamin C does not prevent chemical dissociation of I-κB from NF-κB. Cytosolic extracts from cells treated with 5, 20, or 40 mM vitamin C for 1 h were prepared. Extracts were incubated for 20 min at room temperature with or without a detergent mix of sodium deoxycholate/3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (final concentration of 0.34% and 2%, respectively) to reveal NF-κB masked by the cytoplasmic inhibitor I-κB, before assay in EMSA. All panels are representative of at least two experiments.

(*lane 1*). Importantly, detergent treatment of extracts from cells treated with 5–40 mM vitamin C led to a release of similar levels of NF-κB to control cells (compare *lanes 4*, *6*, and *8* with *lane 2*). This demonstrated that vitamin C was not altering the NF-κB/I-κB complex in a manner that would render it insensitive to stimulant-induced dissociation, as has been shown for some NF-κB inhibitors (33).

Inhibition of NF-κB by vitamin C is not primarily due to its antioxidant properties

Because vitamin C was inhibiting some pathways to NF-κB in ECV304s and HUVECs which we have previously shown to be insensitive to antioxidants (particularly IL-1) (29), we suspected that the effect of vitamin C was not dependent on its antioxidant properties. To address this, we first determined whether millimolar vitamin C could act as an antioxidant in our system, by assessing its ability to block lipid peroxidation. Lipid peroxidation was assessed by the TBARS assay, a sensitive index of lipid peroxidation that detects mainly malondialdehyde, an end product of the peroxidation of polyunsaturated fatty acids and related esters (35). Table I shows that, as previously reported (29), stimulation of ECV304 cells with TNF for 30 min led to a small but significant increase in levels of TBARS, here of $123 \pm 8\%$ ($p < 0.05$) above control levels. Pretreatment of cells with 10 mM vitamin C decreased basal levels of TBARS to $68 \pm 4\%$ of control values, and also completely blocked the TNF-mediated increase. Hence, vitamin C was capable of acting as an antioxidant in our experimental system. However, Table II shows that other antioxidants failed to mimic the general inhibitory effect of vitamin C on NF-κB, in that they either only inhibited the TNF, and not the IL-1 pathway, or else were ineffective against both cytokines. As reported previously (29), butylated hydroxyanisole, a chain-breaking antioxidant that inhibits lipid peroxidation at the concentrations tested in this study ((29), and not shown), inhibited TNF, but not IL-1. *N*-acetyl-L-cysteine, a radical scavenger and glutathione precursor, inhibited neither IL-1 nor TNF. Furthermore, the water-soluble vitamin E analogues Trolox, α-tocopherol phosphate, and α-tocopherol acetate were also ineffective against both cytokines (Table II). At the concentrations and contact times used in this study, α-tocopherol acetate has been shown previously to be active against NF-κB in other systems (36). Additionally, the vitamin E analogues failed to further potentiate vitamin C-mediated inhibition of IL-1 or TNF (not shown), even though vitamin C and E have a cooperative antioxidant effect (4). Together, these results suggested that the inhibition of NF-κB by vitamin C was not dependent on its antioxidant properties.

Vitamin C blocks phosphorylation of I-κBα by inhibiting IKK activity

Because degradation of I-κBα is a common key event in the activation of NF-κB by diverse stimuli, the effect of vitamin C on this stimulant-induced degradation was next determined. Fig. 3A shows that treatment of ECV304 cells with IL-1 or TNF for 1 h led to degradation of I-κBα, as measured by the disappearance of a band specifically detected by a polyclonal Ab to I-κBα (compare

Table I. *Effect of vitamin C on basal and TNF-stimulated TBARS[a]*

Treatment	TBARS (% Control)
Vitamin C	$68 \pm 4 (8)^b$
TNF	$123 \pm 8 (6)^b$
TNF + Vitamin C	$66 \pm 17 (6)^{b,c}$

[a] Confluent monolayers of ECV304 cells were pretreated with 10 mM vitamin C for 1 h before stimulation with or without 40 ng/ml TNF for 30 min. Control cells were left untreated. TBARS levels were measured in triplicate and results were calculated as nmol malondialdehyde equivalents/mg protein. Control levels were 0.23 ± 0.02 nmol malondialdehyde equivalents/mg protein ($n = 8$). Results shown are mean ± SE TBARS expressed as a percentage of control. The number of experiments is indicated in parentheses.
[b] $p < 0.05$ vs control.
[c] $p < 0.05$ vs TNF.

Table II. *Effect of antioxidants on IL1- and TNF-stimulated NFκB activation*[a]

Antioxidant	IL1	TNF
Vitamin C (20)	+	+
Butylated hydroxyanisole (0.5)	−	+
N-acetyl-L-cysteine (40)	−	−
Trolox (2)	−	−
α-Tocopherol phosphate (0.5)	−	−
α-Tocopherol acetate (0.1)	−	−

[a] Confluent monolayers of ECV304 cells were pretreated for 1 h (vitamin C, *N*-acetyl-L-cysteine), 2 h (butylated hyroxyanisole) or 16 h (Trolox, α-tocopherol phosphate, α-tocopherol acetate) with a range of doses of each antioxidant, before stimulation with 10 ng/ml IL-1 or TNF for 1 h. Nuclear extracts were prepared and assessed for the presence of NF-κB. The effect of the antioxidants on cytokine-stimulated NF-κB was then recorded: (−), no inhibition; (+), inhibition. The highest concentration of antioxidant tested is indicated in parentheses (mM). Results are representative of at least three experiments.
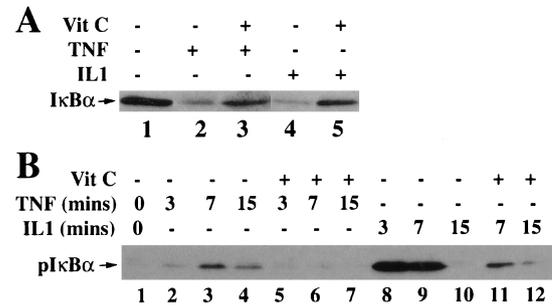

FIGURE 3. Vitamin C inhibits cytokine-mediated phosphorylation and degradation of I-κBα. *A*, To measure I-κBα degradation, ECV304 cells were pretreated with 20 mM vitamin C for 1 h before stimulation with 10 ng/ml IL-1 or TNF for 1 h. Cell lysates were probed by Western blotting with a polyclonal Ab to I-κBα. *B*, To measure phosphorylation of I-κBα, ECV304 cells were pretreated with 20 mM vitamin C for 1 h before stimulation with IL-1 or TNF for the times indicated. Cell lysates were probed by Western blotting with an Ab that specifically recognizes Ser[32]-phosphorylated I-κBα. Representative of three experiments.

lanes 2 and 4 with lane 1). Pretreatment of both IL-1- and TNF-stimulated cells with 20 mM vitamin C for 1 h inhibited this degradation (*lanes 3 and 5*). Because a critical step in many pathways to I-κBα degradation (including IL-1 and TNF) is the phosphorylation of Ser[32] and Ser[36] on I-κBα, we tested whether vitamin C could block this phosphorylation. Fig. 3*B* shows that treatment of cells with IL-1 or TNF led to a rapid and transient increase in phosphorylated I-κBα, as measured by the appearance of a band detected by an Ab that specifically cross-reacts with I-κBα phosphorylated on Ser[32], but does not react with unphosphorylated I-κBα. The increase in phospho-I-κBα in response to IL-1 was both more rapid and potent than that seen for TNF (compare *lanes 8–10* with *lanes 2–4*). In both cases, the presence of 20 mM vitamin C for 1 h before cytokine treatment of cells inhibited the appearance of phospho-I-κBα. For TNF, this was seen as a complete absence of phospho-I-κBα in the presence of vitamin C (*lanes 5–7*), while for IL-1 the appearance of phospho-I-κBα was both reduced (compare *lane 11* with *9*) and delayed (compare *lane 12* with *10*).

Hence, vitamin C inhibited cytokine-induced NF-κB activation and I-κBα degradation by interfering with I-κBα phosphorylation. This strongly suggested that vitamin C would have an effect on the activity of the IKK complex, given that it contains the specific I-κB kinases IKKα and IKKβ that can phosphorylate I-κBα on Ser[32] and Ser[36] (20). To investigate this possibility, endogenous IKK kinase activity was measured by an in vitro anti-NEMO immunocomplex kinase assay using bacterially expressed GST/I-κBα (1–72) as a substrate. NEMO (or IKKγ) forms a core IKK complex together with IKKα and IKKβ (20). ECV304 cells were treated with IL-1 or TNF for different times, and then lysates were immunoprecipitated using anti-NEMO and assayed for IKK activity. Fig. 4*A* shows that both IL-1 and TNF treatment induced a rapid increase in phosphorylation of the I-κBα substrate. The response to IL-1 was more sustained, and was still increasing after 30 min (*lane 7*), while TNF was more transient, peaking at 15 min (*lanes 3*) and decreasing again by 30 min (*lane 4*). When a mutant substrate, GST/I-κBα (1–72)S32A/S36A, in which the critical Ser residues phosphorylated by IKKs were replaced with Ala, was used in the assay, no phosphorylated band was detected in response to IL-1 or TNF (*lanes 9 and 10*), confirming the specificity of the cytokine-induced IKK activity. When cells were pretreated with 20 mM vitamin C for 1 h, the effect of a subsequent 15-min stimulation with IL-1 or TNF on IKK activity was potently inhibited (Fig. 4*B*, compare *lane 2* with *3* and *4* with *5*). Conversely, when 20 mM vitamin C was incubated with immunocomplexes

from IL-1- and TNF-stimulated cells directly for 1 h at 37°C just before kinase assay, there was only a marginal inhibitory effect on IKK activity (Fig. 4*B*, *lanes 7–10*). Hence, vitamin C was inhibiting IKK activity by a mechanism unlikely to involve a direct effect on components of the IKK complex.

Vitamin C potently activates p38 MAPK

Although vitamin C was not directly affecting general kinase activity, because it didn't inhibit the IKK kinase assay in vitro, there was still a possibility that exposing cells to vitamin C led to an in vivo nondirect effect that could inhibit kinases, such as depletion of intracellular ATP. Therefore, to confirm the specificity of the inhibitory effect of vitamin C on IKK, we examined the effect of vitamin C on the activity of another TNF-responsive kinase, p38 MAPK. Because phosphorylation of p38 tightly correlates with kinase activity (31, 32), p38 activation was determined by the appearance of a band specifically recognized by Ab to phosphorylated p38. Fig. 5*A* shows an experiment in which both NF-κB and p38 activity were measured in response to TNF, both in the presence and absence of vitamin C. Under the same experimental conditions, vitamin C markedly inhibited TNF-stimulated NF-κB activity (*top panel*), while having no inhibitory effect on TNF-mediated p38 activation (*bottom panel*). This confirmed that vitamin C was not inhibiting kinases nonspecifically either in vivo or in vitro. Surprisingly, when cells were pretreated with vitamin C before addition of TNF, p38 activity was increased substantially over cells stimulated with TNF alone (Fig. 5*A*, *bottom panel*, compare *lanes 3 and 4* with *lane 2*). Furthermore, Fig. 5*B* shows that vitamin C alone could activate p38. Treatment of cells with as low as 2 mM vitamin C led to a modest activation of p38 (compare *lane 2* with *lane 1*), which increased dose dependently with increasing concentrations of up to 20 mM vitamin C (*lanes 3–5*). Pretreatment of cells with the specific p38 inhibitor SB203580 (37, 38) at a concentration of 3 μM for 1 h before addition of 20 mM vitamin C completely blocked p38 phosphorylation (compare *lane 6* with *lane 5*). Although SB203580 has been characterized as an inhibitor of p38 kinase activity, it also inhibits phosphorylation (i.e., activation) of p38, as has been previously reported and discussed (39). The basis for this effect is still unexplained. A comparison of the time course of p38 activation by TNF and vitamin C shows the potency of the effect of vitamin C on p38. Compared with TNF, induction of p38 activity by vitamin C was more rapid (Fig. 5*C*, compare *lanes 6* and

A

TNF (mins) 0 5 15 30 - - - 0 - 15
IL1 (mins)   0 - - - 5 15 30 0 15 -

GST-IκBα →

1 2 3 4 5 6 7 8 9 10

B

	In vivo					*In vitro*			
Vit C	-	-	+	-	+	-	-	+	-	+
IL1	-	+	+	-	-	-	+	+	-	-
TNF	-	-	-	+	+	-	-	-	+	+

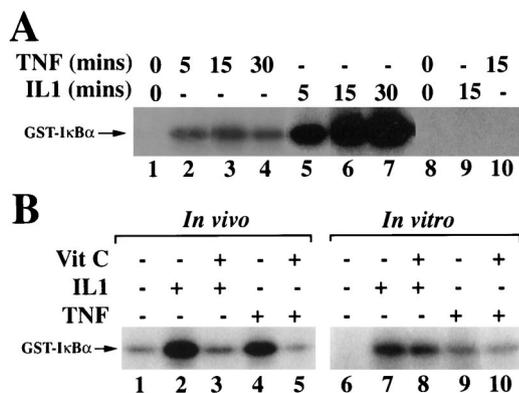GST-IκBα →

1 2 3 4 5 6 7 8 9 10

FIGURE 4. Vitamin C inhibits cytokine-mediated IKK activity. *A,* Time course of IKK activity in response to TNF and IL-1 in ECV304 cells. Cells were stimulated with 10 ng/ml TNF or IL-1 for the times indicated before ex vivo IKK assay, as described in *Materials and Methods*. In *lanes 1–9,* wild-type GST/I-κBα (1–72) was used as substrate, while in *lanes 10–12,* a mutant GST/I-κBα (1–72) containing the amino acid substitutions S32A and S36A was used. *B,* Addition of vitamin C to whole cells, but not to anti-NEMO immunocomplexes, inhibits IL-1- and TNF-stimulated IKK activity. In *lanes 1–5,* ECV304 cells were pretreated with 20 mM vitamin C for 1 h before a 15-min stimulation with IL-1 or TNF, before measurement of IKK activity. In *lanes 6–10,* anti-NEMO immunocomplexes from IL-1- or TNF-stimulated cells were incubated with 20 mM vitamin C for 1 h at 37°C after washing and just before IKK assay. Representative of two experiments.

2), more intense (compare *lanes 14* and *11*), and more sustained (compare *lanes 16* and *13*).

Activation of p38 MAPK by vitamin C has a role in inhibition of TNF-stimulated IKK activity

It has been previously shown that rapid activation of p38 MAPK can be inhibitory toward TNF signaling even though TNF itself activates p38 (40, 41). Because vitamin C potently and rapidly activated p38 MAPK at concentrations that inhibited IKK activity, we examined whether IKK inhibition was mediated by p38 MAPK activation. Given that pretreatment of cells with 3 μM SB203580 effectively blocked activation of p38 by 20 mM vitamin C (Fig. 5*B, lane 6*), we tested the effect of this pretreatment on the ability of vitamin C to inhibit the NF-κB system. When cells were incubated with SB203580 before addition of 20 mM vitamin C, the inhibitory effect of vitamin C on TNF-mediated NF-κB activation was relieved (Fig. 6, *top panel*). Densitometric analysis of the bands detected in the EMSA shows that vitamin C reduced the relative intensity of TNF-induced bands from 4.2 (*lane 2*) to 3 (*lane 3*). The presence of SB203580 prevented this decrease, and slightly enhanced the relative intensity to 5.6 (*lane 4*). In the absence of vitamin C, SB203580 had no effect on either constitutive or TNF-stimulated NF-κB EMSA activity (not shown). Furthermore, this relief of vitamin C inhibition by SB203580 was also apparent when TNF-stimulated I-κBα phosphorylation was measured (*middle panel*). SB203580 prevented the ability of vitamin C to block the appearance of phospho-I-κBα induced by TNF (compare *lane 4* with *lane 3*). Importantly, the effect of SB203580 was shown to be at the level of IKK activation, because it also relieved vitamin C inhibition of TNF-stimulated IKK activity (*bottom panel*). As in the case of the EMSA, SB203580 had no effect alone on IKK activity (not shown). Thus, p38 MAPK, activated by vitamin C, can negatively regulate TNF-mediated NF-κB activation by preventing IKK activation.
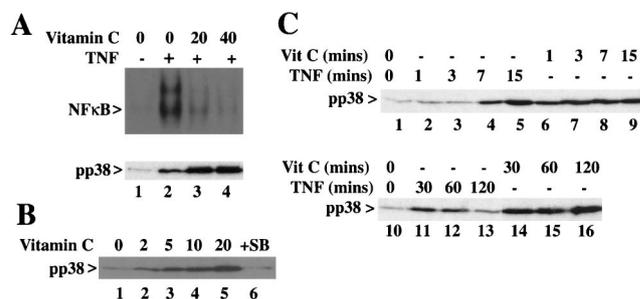
A

Vitamin C 0 0 20 40
TNF   - + + +

NFκB >

pp38 >

1 2 3 4

B

Vitamin C 0 2 5 10 20 +SB
pp38 >

1 2 3 4 5 6

C

Vit C (mins)   0 - - - 1 3 7 15
TNF (mins)   0 1 3 7 15 - - - -

pp38 >

1 2 3 4 5 6 7 8 9

Vit C (mins)   0 - - - 30 60 120
TNF (mins)   0 30 60 120 - - -

pp38 >

10 11 12 13 14 15 16

FIGURE 5. Vitamin C potently and rapidly activates p38 MAPK. *A,* Vitamin C blocks NF-κB activation without inhibiting p38 MAPK. ECV304 cells were pretreated with 0, 20, or 40 mM vitamin C before stimulation with 10 ng/ml TNF for 1 h. *Top panel,* Nuclear extracts were prepared and assessed for NF-κB activity. *Bottom panel,* Cell lysates were prepared and immunoblotted with an anti-phospho-p38 MAPK Ab. The band shown was the only one detected and corresponds to phospho-p38 (pp38). Equal protein loading was demonstrated by using an anti-p38 Ab (not shown). *B,* Activation of p38 by vitamin C. ECV304 cells were treated with 0–20 mM vitamin C for 1 h before immunoblotting for phospho-p38 as above. In *lane 6,* cells were pretreated with 3 μM SB203580 before addition of 20 mM vitamin C. *C,* ECV304 cells were stimulated with 10 ng/ml TNF or 20 mM vitamin C for the indicated times, and cell lysates were immunoblotted for phospho-p38 as above. Representative of two experiments.

Discussion

We have demonstrated for the first time that a physiologically relevant vitamin, vitamin C, inhibits NF-κB activation by diverse stimuli in endothelial cells. Inhibition was found to be independent of cell type and activating stimulus, because pathways encompassing IL-1, TNF, PMA, and H_2O_2, in transformed and primary endothelial cells (Fig. 1), and other cell types (a T cell line and an astrocytoma cell, not shown) proved sensitive to vitamin C. Another group has reported that in Jurkat T cells, vitamin C actually enhanced TNF-mediated NF-κB activation (42). However, only one dose of vitamin C was used, 0.2 mM, which is 10–100-fold lower than the concentrations tested in this study. Lack of inhibition by vitamin C on NF-κB in another T cell line has also been reported (43), but again the dose was lower; in this study, 1.5 mM. Therefore, it is possible that inhibition by millimolar vitamin C is a more general phenomenon, although it is also conceivable that T cells are less sensitive than endothelial cells to inhibition by vitamin C.

The stimulus-independent inhibition by vitamin C is in contrast to the more stimulus-specific effects of the commonly used antioxidant pyrrolidine dithiocarbamate (29), and the general lack of effect of *N*-acetyl-L-cysteine on most of the pathways tested in this study (29, 44, 45). Moreover, we found that although vitamin C was redox active in ECV304s, being capable of suppressing lipid peroxidation, the antioxidant properties of vitamin C were unlikely to be critical to its inhibitory effect, because it inhibited redox-insensitive pathways to NF-κB, and because other antioxidants failed to mimic or potentiate vitamin C inhibition. This has important implications for the purported role of oxidative stress in NF-κB activation (46, 47), and in fact we and others find no compelling evidence for a central role for oxidative stress in diverse pathways to NF-κB (48, 49), and we have consistently found many pathways to NF-κB to be insensitive to antioxidants (29, 44, 45). Additionally, where we do see an inhibitory effect with an antioxidant on a pathway to NF-κB, often multiple or unexpected targets are involved, including direct chemical modification of the NF-κB
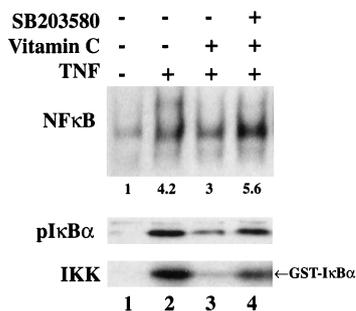
FIGURE 6. SB203580 relieves vitamin C inhibition of TNF-stimulated NF-κB activation, I-κBα phosphorylation, and IKK activity. ECV304 cells were pretreated with vehicle or 3 μM SB203580 for 1 h before addition of 20 mM vitamin C for 1 h. Cells were subsequently stimulated with 10 ng/ml TNF for 30 min (*top panel*), 7 min (*middle panel*), or 15 min (*bottom panel*) before assay of NF-κB (*top panel*), phospho-I-κBα (*middle panel*), or IKK activity (*bottom panel*). The figures below the *top panel* are the relative intensity of the EMSA bands, as assessed by densitometric analysis. Representative of two experiments.

complex itself (29, 34, 50). Although the kinases that phosphorylate I-κB are now well characterized, there is a notable silence in relation to the predicted effect of redox modulation on them.

Vitamin C inhibited the increased phosphorylation and degradation of I-κBα, and also inhibited the activation of the I-κB kinase complex. To assay IKK activity, antiserum to NEMO (IKKγ) was used to immunoprecipitate the IKK complex, because IKKα, β, and γ most likely form a core complex that is responsive to IL-1 and TNF, and critical for I-κB phosphorylation (20, 23). IKKα and IKKβ are responsible for specifically phosphorylating Ser[32] and Ser[36] in response to IL-1 and TNF (21, 22). Importantly, vitamin C almost completely blocked this IL-1- and TNF-mediated IKK phosphorylation of I-κBα. Because the IKKs also specifically phosphorylate I-κBβ, leading to its degradation (21, 22), vitamin C would probably inhibit these events as well. Inhibition by vitamin C was not a direct effect on the IKK complex, because the NEMO-precipitated complex was not sensitive to direct treatment with vitamin C in vitro. However, the possibility still exists that a cellular metabolite of vitamin C was directly targeting the complex.

Surprisingly, vitamin C was shown to be a potent activator of p38 MAPK. When compared with TNF, a known physiological activator of p38, vitamin C was a more potent, rapid, and sustained activator. Thus, p38 MAPK is a novel intracellular target of vitamin C action. This revealed an important clue as to how vitamin C might inhibit TNF-mediated IKK activity and NF-κB activation, because other groups have recently shown that rapid activation of p38 by sodium salicylate, sorbitol, or H_2O_2 could inhibit TNF-stimulated I-κBα and β degradation, and I-κBα phosphorylation (40, 41, 51). Interestingly, p38 has a well-defined positive regulatory role in the induction of NF-κB-dependent genes regulated by proinflammatory cytokines such as TNF, but this is downstream of NF-κB activation via IKK, at the levels of transactivation and transcription. For example, it has been demonstrated that p38 can regulate activation of the RNA polymerase II general transcription factor TFIID during NF-κB-dependent gene transcription (52) and also can stabilize proinflammatory cytokine-induced mRNA (53). However, it seems that the kinetics of p38 activation are critical, in that a rapid and sustained activation of p38 can lead to inhibition of NF-κB, upstream of transactivation and gene induction. Thus, p38 has a dual role, depending on the kinetics of activation. The fact that vitamin C inhibited TNF-induced IL-8 gene expression

demonstrates that initial activation of p38 can override the potential positive effect of p38 downstream of NF-κB activation.

In this study, blocking vitamin C-stimulated p38 activation by using the specific pharmacological p38 inhibitor SB203580 relieved the inhibition of TNF-mediated NF-κB activation, I-κBα phosphorylation, and IKK activation. Furthermore, the kinetics of activation of p38 by vitamin C were consistent with an inhibitory effect because both p38 activation and the appearance of phospho-I-κB were only strongly apparent after 7-min treatment with TNF, while p38 activation in response to vitamin C was apparent after just 1 min. Hence, our results confirm the ability of p38 to exert a negative regulatory effect on TNF-mediated NF-κB activation, and also implicate this effect in the mechanism of vitamin C inhibition. The inability of TNF to activate IKK in vitamin C-treated cells provides a rationale for the reported inhibition of TNF-stimulated I-κBα and β degradation, and I-κBα phosphorylation by p38 (40, 41, 51). Although p38 seemed to be acting at a point between TNF engaging its receptor and IKK activation, the specific target for p38 in this system is not known.

How vitamin C activates p38 remains to be determined, but it is unlikely to be due to a general stress effect on cells, such as hyperosmolarity or low pH, because activation was easily detectable at 2 mM vitamin C (Fig. 5*B*), while activation by sorbitol is typically seen at about 300 mM (41), and the vitamin C solution was buffered. It also remains to be determined as to which isoforms of p38 are activated in response to vitamin C and involved in NF-κB inhibition. There are four known isoforms of p38, namely α, β, γ, and δ (54). Generally, p38α is the most abundant form (55). However, endothelial cells were found to have higher levels of p38β, compared with other inflammatory cell lineages (55), and both p38α and p38β are inhibited by SB203580, while the other two isoforms are not (54), so either or both of p38α and p38β could be involved here.

Although the concentration of vitamin C used in this study was in the millimolar range, our observations are likely to have relevance during inflammation in vivo. Cells in culture are deficient in vitamin C, unlike in vivo, in which intracellular concentrations are typically 2–5 mM, due to active accumulation into cells against circulating levels of the order of 0.1 mM (56). Some immunologically relevant cells such as neutrophils can accumulate vitamin C up to concentrations of 14 mM when activated during inflammation (57). Vitamin C (5 mM) was inhibitory in our study when added to cells overnight, while shorter incubations required 10–20 mM, which would allow acute accumulation.

Inhibition of NF-κB by millimolar vitamin C may also be relevant to the purported beneficial effects of megadose therapy, whereby exogenous administration of high dose vitamin C might have an immunomodulatory effect by subtly suppressing NF-κB at sites of inflammation, due to a greater availability of vitamin C to the plasma during times of acute intracellular accumulation. High intakes of vitamin C have been associated with the treatment of viral infections, most notably the common cold and HIV-1. Rhinovirus is reliant on NF-κB-dependent processes both for cell invasion and for the pathogenesis of infection (58, 59). As regards HIV-1, clinical improvement was claimed in AIDS patients who ingested high doses of vitamin C (9), and it has been shown to suppress HIV-1 replication in chronically and acutely infected cells (60, 61). NF-κB has a central role in the activation and replication of HIV-1 (10, 14). Our data would also explain a report demonstrating inhibition of HIV long terminal repeat-directed transcription by 3–5 mM vitamin C in 293 cells (62). However, it must be noted that others have also shown that vitamin C can also suppress HIV expression in an NF-κB-independent manner (43). Other NF-κB-dependent pathologies in which beneficial effects for

vitamin C have been claimed include cardiovascular disease (7, 16) and cancer (6, 10–13).

In conclusion, we have demonstrated for the first time that millimolar doses of vitamin C inhibit multiple pathways to NF-κB. This inhibition is mediated by the prevention of IKK activation, which in the case of TNF is dependent on activation of p38 MAPK. p38 MAPK is thus a novel intracellular target of vitamin C action. The results may contribute toward providing a rationale for the purported benefits of megadose therapy, and suggest that the relationship between vitamin C, p38 MAPK, and NF-κB in vivo warrants further investigation.

Acknowledgments

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