

Vitamin C suppresses lipopolysaccharide-induced procoagulant response of human monocyte-derived macrophages

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Abstract. – OBJECTIVE: Although vitamin C is a strong antioxidant, the epidemiologic evidence to support its role in lowering risk of cardiovascular disease is inconsistent. In order to define the role of vitamin C in vascular pathophysiology, we have investigated the effect of vitamin C on the tissue factor (TF) and Factor VII Activating Protease (FSAP) expression induced by lipopolysaccharide (LPS) in human monocyte-derived macrophages.

MATERIALS AND METHODS: Vitamin C at clinically relevant doses was tested to its ability to influence the LPS- and reactive oxygen species (ROS) – generating system of xanthine/xanthine oxidase (X/XO) NF-κB activity in human monocyte-derived macrophages.

RESULTS: Vitamin C-treatment prevents LPS- and ROS-induced DNA-binding activity of NF-κB in a concentration-dependent fashion. Vitamin C also inhibited the phosphorylation and proteolytic degradation of the inhibitor protein IκBα. In parallel to regulate NF-κB activity, vitamin C reduced the expression of TF and FSAP, genes known to be induced by bacterial LPS and triggered the extrinsic coagulation cascade and linked thrombosis with inflammation.

CONCLUSIONS: Vitamin C alters pro-inflammatory and pro-coagulatory processes via inhibition of NF-κB activation and exerts beneficial antiatherogenic effects on human monocyte-derived macrophages in addition to its anti-oxidant properties.

Key Words:

Vitamin C, Vascular inflammation, TF, FSAP, Monocyte-derived macrophages, NF-κB.

enhance immunity, and have anti-inflammatory properties^{1,2}. Epidemiologic studies have shown an association between increased intake of vitamin C and reduced morbidity and mortality from coronary heart disease³⁻⁵. It was demonstrated that use of oral contraceptives and smoking are associated with an increase in monocyte tissue factor (TF) expression in pre-menopausal women⁶. In the same study, the procoagulant activity of monocytes was significantly reduced by intake of 3 g of vitamin C⁶. Furthermore, the antioxidants also blocked NF-κB translocation induced by a wide range of stimuli, including reactive oxygen species (ROS), lipopolysaccharide (LPS), lectins, and the cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1)⁷. In addition, the ROS formed by the inflammatory response within atherosclerotic lesions may, in turn, reduce ascorbic acid antioxidant levels, further promoting atherosclerosis⁸. When considered together, these data suggest that oxidants may play a central role in the activation of various genes, particularly those endowed with NF-κB binding sites in their promoter/enhancer regions. The transcription factor NF-κB, shown to be aberrantly regulated in atherosclerosis, is a primary mediator of oxidative stress and plays a critical role in cell growth and inflammation⁹. NF-κB is also a ubiquitous transcription factor that is involved in the regulation of coagulation by increasing the expression of numerous genes, including cytokines and TF¹⁰. In unstimulated cells, NF-κB is bound to its inhibitor IκB and resides in an inactive state within the cytoplasm^{11,12}. Various inflammatory stimuli, including bacterial LPS, and cytokines like TNF-α,

Introduction

Vitamin C, the most important antioxidant, has been shown to exert marked modulation of inflammatory reactions and generally thought to

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induce rapid phosphorylation of I κ B by I κ B-kinase (IKK)¹². This process releases NF- κ B which then can translocate to the nucleus where it induces the transcription of its target genes^{11,12}.

The monocyte is a crucial cell involved in atherogenesis and is present during all stages of atherosclerosis¹⁰. TF, a monocytic cell-surface glycoprotein, regulated by NF- κ B, is the primary initiator of the extrinsic coagulation cascade and links thrombosis with inflammation¹²⁻¹⁴. TF, therefore, plays a central role in diverse pathologic processes including atherosclerosis, thrombosis, and sepsis¹⁵⁻¹⁷. Furthermore, next to hepatocytes, the major cellular source of intravascular Factor VII Activating Protease (FSAP) are monocytes, the only blood cells capable of synthesizing FSAP in response to various inflammatory mediators such as cytokines and bacterial endotoxins, nicotine and female hormones¹⁸⁻²⁰. FSAP is a serine protease involved in both coagulation and fibrinolysis, with the ability to activate the coagulation factor VII independent of TF and single-chain plasminogen activators (pro-urokinase)²¹⁻²³. Increased FSAP expression has been localized in human atherosclerotic plaques and associated with plaque instability^{18,24,25}. FSAP induced I κ B-dependent NF- κ B activation in a time- and concentration dependent fashion²⁶. Concomitantly, FSAP up-regulates the expression of proinflammatory cytokines, matrix metalloproteinases, cell adhesion molecules and TF²⁶.

Despite the important role of the NF- κ B pathway in atherothrombotic disease and the detection of activated NF- κ B in cells of the human monocyte-derived macrophages origin within atherosclerotic lesions, the relative potency of vitamin C in inhibiting NF- κ B pathway has not yet been investigated. We examined whether LPS- and reactive oxygen species (ROS)-generating system of xanthine/xanthine oxidase (X/XO) contribute to the NF- κ B activity in human monocyte-derived macrophages. Based on the demonstrated ability of oxidants to induce binding of these transcription factors to their respective DNA binding site and initiate gene transcription, we performed experiments to examine the effect of vitamin C on TF and FSAP functional activity and gene expression in stimulated human monocyte-derived macrophages.

Materials and Methods

Cell Isolation and Cell Culture

Human peripheral blood mononuclear cells were isolated from buffy coats obtained from

healthy blood donors by Ficoll-Hypaque density gradient centrifugation (Invitrogen, Darmstadt, Germany) and further fractionated by centrifugal elutriation as described previously¹⁸. The purity of isolated mononuclear cells was always > 95% as evidenced by fluorescence activated cell sorter (FACS) analysis. The final cultures, suspended at a density of 1×10^6 cells/mL in serum-free culture medium (Macrophage-SFM) supplemented with 10000 U/ml penicillin and 10000 μ g/ml streptomycin and plated in culture dishes. To exclude any side effects of serum growth or coagulation factors, the incubation and stimulation of cells was performed in serum-free culture medium. Monocyte-derived macrophages were differentiated from adherent monocytes as described previously¹⁸.

Cell Viability

Cell viability in the absence and presence of vitamin C was determined by ethidium bromide staining of cell aliquots and subsequent FACS analysis.

RNA Isolation and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total cellular RNA was isolated using the RNeasy[®] MiniElute spin column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using a kit from Perkin Elmer as described previously and the cDNA was stored at -20°C [18]. Relative mRNA quantification was performed by real-time RT-PCR using CFX 96 real-time system Bio-Rad (Bio-Rad, Munich, Germany) as described previously¹⁸. For FSAP, the following primer sequences were used: sense: CCCACTGAGCCATCAACCAA; antisense: TGGGGCATGGAGATGGTC. For TF, the following primer sequences were used: sense: TTTGGAGTGGGAACCCAAACCCGTCA; antisense: ACCCGTGCCAAGTACGTCTGCTTCACAT. The expression was normalized to the internal control (PBGD) sense: CCCACGC-GAATCACTCTCAT; antisense: TGTCTG-GTAACGGCAATGCG. The expression of target genes mRNA was normalized to PBGD mRNA as an internal standard to control for variability in amplification using the following equation: $T_0/R_0 = 2^{(CT - R)}$, where T_0 is the initial number of target gene copies; R_0 , the initial number of standard gene copies; CT, T, the threshold cycle of the target gene; CT, R, are the threshold cycle of the standard gene as described previously¹⁸.

Western Blot Analysis

The cytosolic and nuclear extracts were prepared as described previously^{18,26}. Ten micrograms of cytoplasmic protein extract were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting was done using rabbit polyclonal antibody specific for I κ B α (c-21, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and secondary goat anti-rabbit antibody (sc-2004, 1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies were diluted in phosphate buffered saline, 5% non-fat dry milk, and 0.05% Tween. For detecting the Ser32-phosphorylated I κ B α , rabbit anti-human I κ B α antibody (9241S, 1:1000, Cell Signaling Technology, Danvers, MA, USA) was used. To assess p-38 MAPK and NF- κ B activation we performed Western blot analysis, using monoclonal antibodies directed against anti-phospho p-38 MAPK rabbit antibody (20G11, 1:1000; Cell Signaling Technology, Danvers, MA, USA) and against total p-38 MAPK rabbit antibody (#9102, 1:1000; Cell Signaling Technology, Danvers, MA, USA). Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL) Plus detection kit (Invitrogen, Carlsbad, CA, USA).

Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded DNA probes for NF- κ B (5'-AGTTGAGGGGACTTTCCAGGC-3'), AP-1 (5'-CGCTTGATGACTCAGCCGGA-3') and CREB (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3') were labelled to a specific activity of greater than 5×10^7 cpm/ μ g DNA by end-labelling with [γ 33P] dATP using T4 kinase as described previously²⁶. Binding of probes was performed in 10 mM HEPES, pH 7.5, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 4% Ficoll, 100 mM phenylmethylsulfonyl fluoride, 0.02 U calf thymus DNA, and 0.01 U poly (dI-dC) in a total volume of 20 μ L. Nuclear extracts were incubated for 30 minutes at room temperature in the presence of 1 ng labelled probe (approximately 50 000 cpm). Protein-DNA complexes were separated from the free DNA probe by electrophoresis on a 4% native polyacrylamide gel in 0.25 $\times$ Tris-boric acid-EDTA buffer and subjected to autoradiography. Band intensities were quantified by densitometric analysis.

Measurement of Tissue Factor (TF) Activity

Procoagulant activity (PCA) was measured and characterized as TF in cell lysates using an one-stage clotting assay as described previously²⁷. Shortly, PCA in freeze-thawed macrophages was determined by measuring their capacity to shorten the spontaneous clotting time of normal citrated human plasma in an on-stage clotting assay. Clotting times were converted to milliunits of PCA by reference to a standard curve established by serial dilutions of a standard rabbit brain thromboplastin preparation (Sigma). This standard curve (double logarithmic plot) was linear between 1 mU (122 seconds) and 1.000 mU (40 seconds). Medium or medium with reagents alone did not show any PCA.

Statistical Analysis

For each intervention, multiple experiments were performed and data presented as mean $\pm$ SD. The number of samples (n) indicates the number of experiments done in separate days using different isolates of cells. Two-tailed unpaired Student *t*-test was used for comparison between two groups, whereas multi-group analyses were performed using 1-way ANOVA with Newman-Keuls post hoc test. Differences were assumed to be statistically significant at **p* < 0.05, ***p* < 0.01. NS indicates not significant.

Results

Vitamin C Inhibits ROS- and LPS-Induced Transcriptional Responses Mediated by NF- κ B in Human Monocyte-Derived Macrophages

In this study, we undertook to understand the role of vitamin C in ROS- and LPS-induced NF- κ B activation in human monocyte-derived macrophages. Vitamin C circulates in the plasma as ascorbic acid (AA)²⁸. However, it is generally transported into cells as dehydroascorbic acid through the facilitative glucose transporters²⁸. In this way cells can accumulate high intracellular concentrations of vitamin C. For example, the intracellular concentration of AA in mononuclear cells is reported to be about 3 mM, with circulating concentrations of AA in the range of 50 μ M²⁹. We therefore used vitamin C in the range from 5 until 100 μ M and investigated whether the inhibitory effect of vitamin C on the reactive oxygen species (ROS) – generating system of xanthine/xanthine oxidase (X/XO, 200 μ M/10

mU) – and LPS (10 µg/ml) – induced NF-κB-mediated transcription was due to inactivation of the DNA binding activity of NF-κB. This induction was shown by the appearance, in nuclear extracts, of the DNA-binding activity of NF-κB us-

ing electrophoretic mobility shift assays (EMSA, Figure 1A and B). Human monocyte-derived macrophages incubated with X/XO (200 µM/10 mU) and LPS (10 µg/ml) for different period of time showed a prominent increase of lu-

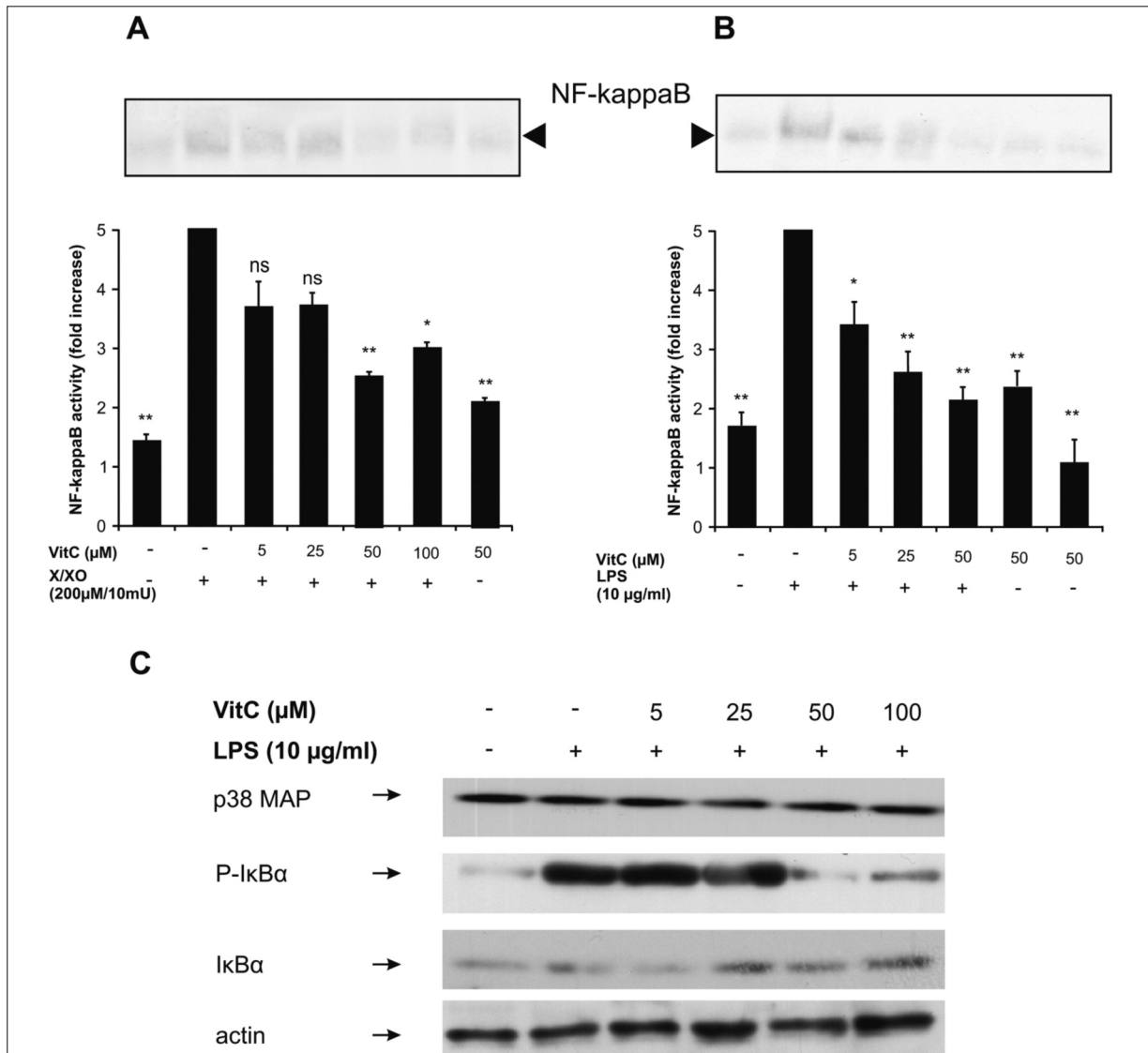


Figure 1. **A**, and **B**, Vitamin C inhibits ROS- and LPS-induced transcriptional responses mediated by NF-κB in human monocyte-derived macrophages. Human monocyte-derived macrophages were incubated for 1 h, in the absence or presence of vitamin C (5, 25, 50 or 100 µM), and with X/XO (200 µM/10mU for 24 h) or LPS (10 µg/ml for 2 h). The nuclear extracts were analyzed by EMSA for binding to a DNA probe containing the NF-κB consensus sequence. Representative photographs of EMSA gels are shown in the upper panels and a densitometric quantitation is shown in the lower panels. Unstimulated (lane 1); X/XO or LPS (lane 2); X/XO or LPS + vitamin C (lanes 3-6); vitamin C 50 µM (lane 7). Each panel is representative of at least 5 independent experiments performed in separate cell isolates. **p* < 0.05, ***p* < 0.01 versus stimulation with X/XO or LPS, respectively. **C**, Effect of vitamin C on the LPS-induced phosphorylation and degradation of IκBα in human monocyte-derived macrophages. Human monocyte-derived macrophages were stimulated at 37°C for 1 h with or without 10 µg/ml LPS, and then treated with various concentration of vitamin C (5, 25, 50 or 100 µM) for 1 h. Cytoplasmic proteins extracts were prepared and immunoblotting was performed for the phosphorylated form of IκBα (pIκBα, middle panel), for IκBα (middle panel), and phosphorylated p38 MAP kinase (upper panel) whereas actin was used as a loading control (lower panel). Relative changes in the pIκBα and IκBα protein bands were measured using densitometric analysis and optical densities. The results illustrated are from a single experiment, and are representative of three separate experiments.

ciferase activity. The maximum increase in luciferase activity was approximately 5-fold after 24 h incubation with X/XO and after 2 h incubation with LPS, respectively (Figure 1A and B). First, cells were treated with vitamin C for 1 h before and after stimulation with X/XO for 24 h and with LPS for 2 h, respectively. Figure 1A and B show that vitamin C inhibited X/XO- and LPS-mediated NF- κ B activation in a dose-dependent manner, with the effect being apparent at 5–25 μ M (compare lanes 2 and 4), and potent at 50–100 μ M (compare lanes 5 and 6 with lane 2). However, cells treated for 24 h with X/XO (200 μ M/10 mU) showed a 5-fold increase in NF- κ B activation, and vitamin C was more effective if preincubated with cells for 1 hour before to X/XO; little inhibition was seen if X/XO and vitamin C were added simultaneously (data not shown). We reasoned that the transient inhibitory activity of the vitamin was due to decrease in the intracellular content of the vitamin through efflux and perhaps hydrolysis intracellular by longer as four hours³⁰. Activation of NF- κ B by LPS (10 μ g/ml for 2 h) was completely abolished upon exposing cells to 50 μ M vitamin C for 1 h before or after to LPS. Moreover, in cells loaded with vitamin C (50 μ M), little induction was detected (Figure 1A and B, lanes 7). The inhibitory effect was not due to any toxic effect on cells, as evidenced by ethidium bromide staining (data not shown).

To test whether vitamin C is a specific inhibitor of NF- κ B, we also analyzed the same nuclear extracts from X/XO- and LPS-treated human monocyte-derived macrophages by EMSA with 33p-labeled DNA probes detecting active protein 1 (AP-1) and cAMP response element-binding protein (CREB) binding activities. In contrast, 50 μ M vitamin C could not prevent the induction of AP-1 and CREB binding activity in human monocyte-derived macrophages compared with cells stimulated with only LPS (data not shown).

Effect of vitamin C on the Agonist-Induced Phosphorylation and Degradation of I κ B α in Human Monocyte-Derived Macrophages

NF- κ B activation requires a sequential cascade with I κ B kinase (IKK)-dependent I κ B phosphorylation and its subsequent degradation⁷. This leads to the translocation of the cytosolic NF- κ B complex to the nucleus to affect target gene expression^{11,12}. Since vitamin C prevented the translocation of NF- κ B to the nucleus, we

reasoned that vitamin C might inhibit ROS- and LPS-dependent phosphorylation and degradation of I κ B α . To determine whether vitamin C affects the ROS- and LPS-induced phosphorylation and degradation of I κ B α , a Western blot assay was performed. Analysis of cell extracts using I κ B-specific antibodies showed that stimulation of the cells with LPS (10 μ g/ml for 2 h) caused rapid degradation of I κ B α compared to untreated cells. The stimulation of the cells with ROS (xanthine/xanthine oxidase (X/XO), 200 μ M/10 mU) after 4, 6 and 24 h showed increased degradation of I κ B α , which was not statistically significant compared to unstimulated cells (data not shown). When cells were treated with increasing concentrations of vitamin C (5–100 μ M for 1 h) after incubation with LPS (10 μ g/ml for 1 h) the I κ B α protein levels were significantly increased, indicating that the degradation of I κ B α was dose-related inhibited after pre-treatment of cells with LPS (Figure 1C). Preloading with the same dose of vitamin C (5–100 μ M for 1 h) had no effect on inhibition of degradation of I κ B α at this time point (data not shown), because of transient inhibitory activity of the vitamin, which decreases in the intracellular content through efflux or hydrolysis³⁰. To be certain that the inhibitory effect of vitamin C on I κ B α phosphorylation and degradation was an intracellular event, vitamin C and LPS were incubated simultaneously for 5 min prior to addition to human monocyte-derived macrophages. We found that vitamin C did not alter the activity of LPS (data not shown). Degradation of I κ B α is largely dependent on its phosphorylation by IKK activation and we found that five-fold increase of I κ B α phosphorylation in response to LPS was suppressed by vitamin C in a dose-dependent manner (Figure 1C, middle panel). The immunoblots were stripped and incubated with an anti-actin antibody, demonstrating that equal amounts of nuclear extracts were loaded on the gel (Figure 1C, lower panel). These results indicate that intracellular vitamin C after incubation with LPS can disrupt the signalling pathway involved in the phosphorylation and degradation of I κ B, impairing the activity of NF- κ B.

We also analyzed cytoplasmatic protein extracts from vitamin C-treated cells for phosphorylation of p38 MAP kinase and found that although LPS activated this pathway the phosphorylation of p38 is not involved in the vitamin C-mediated inhibition of the LPS-induced NF- κ B response (Figure 1C, upper panel).

Vitamin C Affects Lipopolysaccharide-Induced Procoagulant Response of Human Monocyte-Derived Macrophages

The impact of vitamin C observed on NF- κ B binding activity after treatment with bacterial LPS led us to investigate whether vitamin C affects the induction of LPS-controlled genes in human monocyte-derived macrophages. For this purpose, the transcription of the tissue factor (TF) as well as Factor VII Activating Protease (FSAP) gene and functional activity were analyzed and were assayed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and one-stage clotting assay, respectively, upon stimulation of cells in the presence or absence of vitamin C. As shown in Figure 2, stimulation of cells with LPS resulted in strong induction of TF gene expression and activity at 8 h (Figure 2A and B) in human monocyte-derived macrophages. TF activity, but not TF gene expression, was suppressed by vitamin C in a dose-independent manner to 50% (Figure 2A and B). Additionally, stimulation of cells with LPS re-

sulted in a rapid increase of FSAP mRNA expression already after 1 h (six-fold increase), reaching a plateau at 2 h (seven-fold increase) and gradually decreasing until returning to the base level at 24h¹⁸. Pretreatment of cells with vitamin C reduced LPS-induced FSAP gene expression in a dose-dependent manner to 60% (Figure 2C). This indicates that the pro-inflammatory and pro-coagulatory activation of cells is abolished if vitamin C is present in the culture medium in sufficient concentrations. As shown by real-time RT-PCR and clotting assay, respectively, FSAP gene as well as TF activity were affected by vitamin C in manner similar to that of NF- κ B (Figure 2).

Discussion

These studies showed that vitamin C inhibits the increase of tissue factor (TF) activity and Factor VII Activating Protease (FSAP) gene expression in human monocyte-derived macrophages induced by LPS via NF- κ B activa-

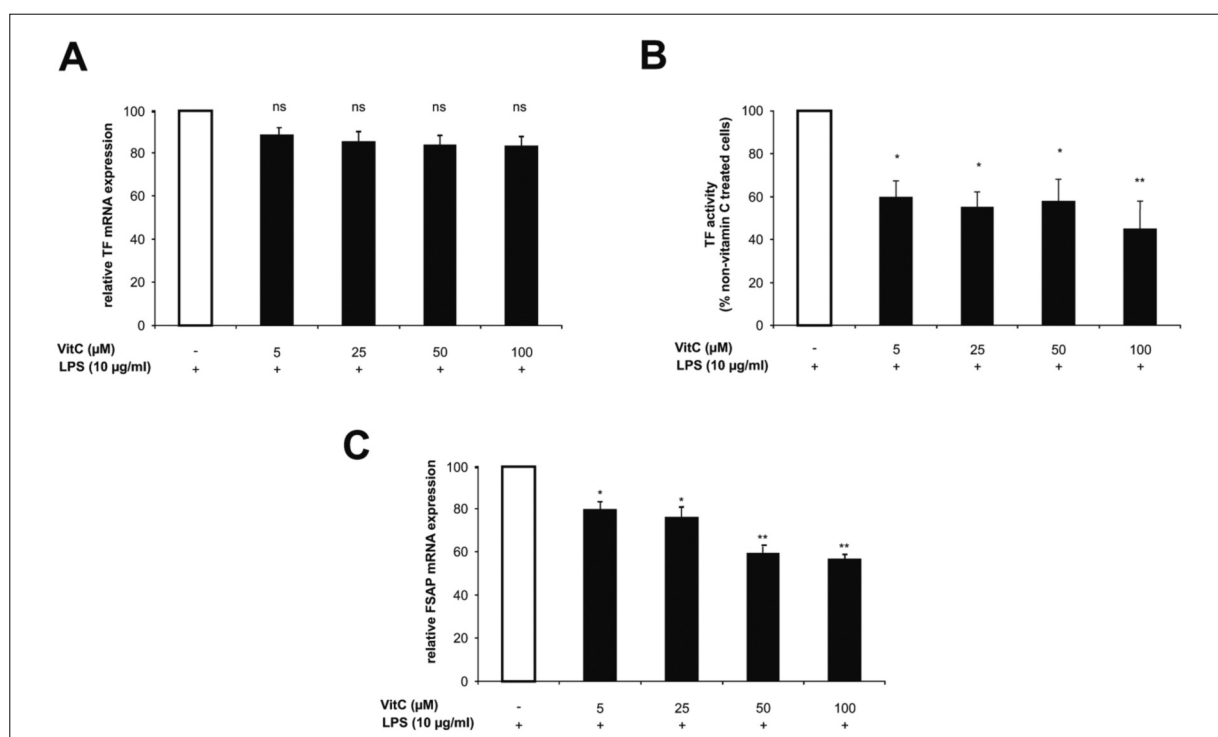


Figure 2. Effect of vitamin C on LPS-controlled genes expression in human monocyte-derived macrophages. Cells were stimulated with LPS (10 μ g/mL), followed by treatment with the indicated concentrations of vitamin C for 1 h. Cells were harvested and real-time RT-PCR was performed with primers specific for TF or FSAP gene. Relative values of TF and FSAP mRNA (normalized to PBGD) are given (A and C, respectively). TF activity was determined by one-stage clotting assay in cell lysates. Quantification of the inhibiting effects of the different concentration of vitamin C on LPS-induced TF-activity is given. The values represent means $\pm$ SEM of 4 independent experiments. * p < 0.05, ** p < 0.01, ns not significant versus stimulation in the absence of vitamin C.

tion. However, this effect was not mediated by a reduction in TF gene expression but rather by an alteration in the posttranscriptional modification of the protein that prevented its functional expression at the cell surface. In human monocyte-derived macrophages, vitamin C reduced LPS-induced expression of TF only 10%, whereas the mean reduction in functional activity by the addition of vitamin C was approximately 50% (Figure 2A&B). The observation that the accumulation of mRNA for TF in LPS-treated cells was equivalent in either the presence or absence of vitamin C suggests that an effect on mRNA stability is unlikely. Thus, it is unlikely that the vitamin C directly altered the activity of membrane-associated TF. The findings are more consistent with either reduced translation of the mRNA or some posttranslational alteration of the mRNA or some posttranslational alteration of the protein.

Furthermore, vitamin C treatment strongly modulates the classical pathway of I κ B-dependent NF- κ B activation and inhibits DNA-binding as well as transactivation activity of the transcription factor (Figure 1). The mechanism of inhibition of procoagulant activity by human monocyte-derived macrophages appears to be via decreasing DNA-binding activity of NF- κ B. As demonstrated by Western blotting, the inhibition of NF- κ B resulted from a reduced phosphorylation and degradation of its inhibitory protein I κ B α (Figure 1C). Our data indicate that vitamin C blocks the phosphorylation of I κ B α , a key step allowing the translocation of NF- κ B to the nucleus to activate TF gene expression. The phosphorylation of I κ B α is mediated by several kinases, and we demonstrate that vitamin C inhibits the activation of NF- κ B-inducing kinase and I κ B kinase complex independent of p38 Mitogen-Activated Protein Kinase (p38 MAP, Figure 1C). The experiments reported here also indicate that cellular loading of vitamin C for the short period resulted in inhibition of NF- κ B transcriptional activity induced by LPS. Treatment with vitamin C for a long period had no effect on LPS-dependent NF- κ B transcriptional reporter activity, because transient inhibitory activity of the vitamin, which due to decrease in the intracellular content of the vitamin through efflux and perhaps hydrolysis intracellularly by longer as four hours²³.

X/XO is well known to support lipid peroxidation and free radical generation and the TF induction was an oxidation-dependent process³. Accordingly, the ability of vitamin C to inhibit X/XO-induced NF- κ B was studied. As showed

by Figure 1A, vitamin C significantly blunted the effect of X/XO and inhibited the NF- κ B, but not the phosphorylation and degradation of its inhibitory protein I κ B α (data not shown). Vitamin C was more effective if preincubated with cells for 1 hour before to X/XO; little inhibition was seen if X/XO and vitamin C were added simultaneously. Further investigation with focus on redox-regulated I κ K complex is required to understand the molecular mechanisms how vitamin C exerts its inhibitory effects.

In conclusion, inhibition of NF- κ B activation by vitamin C via suppression the degradation of its inhibitory protein I κ B α , causing a suppression of LPS-stimulated TF functional activity in human monocyte-derived macrophages, suggests a potential cellular site that could prevent initial vascular events in atherosclerosis and other inflammatory diseases.

For comparison, the ability of vitamin C to inhibit FSAP expression in response to LPS was also studied (Figure 2C). Vitamin C significantly affected LPS-induced FSAP gene expression, producing approximately 45%. The inhibition was concentration-dependent, with the most pronounced effect at 100 μ M with a 1 hour incubation before and after to LPS. Further increase of the concentration to 250 μ M did not, however, result in enhanced inhibition. Under physiological condition, vitamin C circulates in the blood in its reduced form, ascorbic acid, at approximately 50 μ M^{28,29}. However, cells accumulate a wide range of intracellular concentrations of vitamin C, up to 6 mM in mononuclear leukocytes³¹. It is possible that inhibition of LPS induced FSAP expression was evident at an intracellular low concentration of vitamin C in human monocyte-derived macrophages.

The circulating FSAP/zymogen concentration is 12 μ g/ml and up to 30% can be activated *in vivo* after LPS application³². It has been identified as a protease that activates coagulation factor VII as well as pro-urokinase (pro-uPA)^{22,23}. FSAP also inactivates tissue factor pathway inhibitor (TFPI), which would also have a procoagulant effect on blood clotting²⁶. FSAP expression is also enhanced by differentiation of human circulating monocytes into macrophages¹⁸. The activation of circulating FSAP in patients with a bacterial infection as well as in mice after LPS application suggests that FSAP is activated in pro-inflammatory conditions^{32,33}. Furthermore, FSAP can enhance the activation of NF- κ B specifically by inducing phosphorylation and degradation of

I κ B α from the latent cytoplasmic form of NF- κ B²⁶. Since the effect of vitamin C on FSAP expression was closely paralleled by phosphorylation and degradation of I κ B α , our data suggest that vitamin C inhibits LPS-induced FSAP expression in human monocyte-derived macrophages by preventing DNA binding of NF- κ B. However, our data totally ruled out the possibility that the inhibition of TF and FSAP expression by vitamin C was due to the affect of the NF- κ B/ I κ B signalling pathway at the level (or upstream) of I κ B kinase and suggest a novel mechanism for the antiatherogenic effect of vitamin C on the vascular wall. We conclude that vitamin C inhibits the LPS-induced FSAP expression in human monocyte-derived macrophages and propose that vitamin C can play a role in modulating coagulation and inflammation mediated by NF- κ B.

In support of our interpretation that FSAP leads to an inflammatory state, we demonstrate that the cascade of events which lead to NF- κ B activation in human monocyte-derived macrophages, influences expression of pro-inflammatory factors and confirms a role for FSAP in regulating vascular inflammation²⁶. Furthermore, we present evidence that expression of IL-6, MCP-1 and MMP-9 in human monocyte-derived macrophages can be up-regulated by FSAP which then, directly or indirectly promotes monocyte chemotaxis, monocyte-endothelial adhesion and transmigration and thus play a role in atherogenesis²⁶. Since our study has shown that vitamin C inhibits NF- κ B/ I κ B signalling parallel to FSAP gene expression, our observations are likely to have relevance during inflammation *in vivo*.

Conclusions

As shown by clotting assay and real-time RT-PCR, respectively, TF activity and FSAP expression were affected by short-term treatment with vitamin C in a manner similar to that of NF- κ B. NF- κ B pathway is important for the release of extracellular DNA traps³⁴, and since vitamin C inhibits this pathway the possibility exists that vitamin C also regulates the release of extracellular traps and thus could influence both coagulation and inflammation. Thus, in addition to the decrease in oxidative stress resulting from vitamin C supplementation as evidenced by decreased NF- κ B activation by reactive oxygen species

(ROS)-generating system of xanthine/xanthine oxidase, its anti-coagulant activity on human monocyte-derived macrophages and exerts beneficial antiatherogenic effects on cells crucial in atherogenesis such as monocytes/macrophages could further explain its antiatherogenic effects. Furthermore, because FSAP and TF may also be expressed by macrophages in atherosclerotic plaques, the data also point to a mechanism whereby antioxidants might abrogate acute thrombosis and plaque instability caused by ulceration of a plaque. Study using *in vivo* models of these disease processes is required to explore these possibilities.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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