Vitamin C Protects Against Lysophosphatidylcholine-Induced Expression of Monocyte Chemoattractant Protein-1 in Cultured Human Umbilical Vein Endothelial Cells

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Background and Purpose: It is well documented that oxidized low-density lipoproteins (LDLs) can stimulate human vascular endothelial cells to produce monocyte chemoattractant protein-1 (MCP-1). Vitamin C is known to be an important antioxidant for vasodilation. The purpose of this study was to determine whether pretreatment with vitamin C could protect against oxidized–LDL-induced expression of MCP-1 in cultured human umbilical vein endothelial cells (HUVECs).

Methods: Cultured HUVECs were used for desired experiments before passage 4. Lysophosphatidylcholine (lysoPC), an oxidized component of LDL, was designated as the stimulator for MCP-1 synthesis from cultured HUVECs. MCP-1 concentrations in the cultured media were determined by enzyme-linked immunosorbent assay. MCP-1 RNA was evaluated by a semi-quantitative reverse transcriptase-polymerase chain reaction.

Results: HUVECs secreted MCP-1 within 30 minutes after exposure to 50 µM lysoPC. Compared with samples treated with lysoPC alone, pretreatment with vitamin C in concentrations of 50, 100, 150, and 200 µM, reduced levels of MCP-1 in the culture medium by 44%, 51%, 60%, and 67%, respectively, while levels of MCP-1 mRNA decreased by 15%, 18%, 80%, and 82%, respectively.

Conclusions: Our findings imply that pretreatment with vitamin C can suppress lysoPC-induced expression and secretion of MCP-1 in cultured HUVECs. Therefore, vitamin C is protective against lysoPC-mediated inflammatory insults to the vascular endothelium in vitro.

Key words: Cell culture; Lysophosphatidylcholines; Monocyte chemoattractant protein-1; Protein kinase C; Ascorbic acid

Monocyte chemotactrant protein-1 (MCP-1) is a proinflammatory chemokine, the mRNA of which has been shown to be present in the atherosclerotic plaques of humans and cholesterol-fed animals. Oxidation of low-density lipoproteins (LDLs) appears to play an important role in the stimulation of endothelium-monocyte interaction, and the development and progression of the atherosclerotic lesions.

A marked increase of MCP-1 mRNA by minimally modified (oxidized) LDL was found in human endothelial and smooth muscle cells. Through the activation of phospholipase A2, lysophosphatidylcholine (lysoPC) is produced during oxidation of LDL, and a several-fold increment of lysoPC content is found in atherosclerotic arterial walls. The increase of lysoPC could impair endothelium-dependent relaxation and modulate the expression and production of MCP-1 from vascular endothelial cells. Therefore, the biological effects of lysoPC on vascular cells may play an important role in the pathogenesis of atherosclerosis.

Vitamin C (ascorbic acid) is a water-soluble antioxidant and cannot be synthesized in human cells. In vitro studies have shown that vitamin C and alpha-tocopherol could act synergistically in the prevention of LDL peroxidation. Jialal et al. found that vitamin C can suppress 93% of the uptake of LDL
by macrophages and that the suppression efficiency of α-tocopherol was 49%. The endothelial cells lining the blood vasculature play a vital role in the absorption of vitamin C from the circulatory system to the underlying tissues.

An in vitro study showed that the uptake of vitamin C into human umbilical vein endothelial cells may protect them from oxidant insult. Clinically, vitamin C could reverse endothelial vasmotor dysfunction in patients with coronary artery disease. However, the protective effect of vitamin C on the oxidative response of MCP-1 gene expression from human vascular endothelial cells is not clear. The object of this study was to assess the effect of vitamin C pretreatment on the lysoPC-mediated expression of MCP-1 in cultured human umbilical vein endothelial cells (HUVECs).

**Methods**

Dipalmitoyl lysoPC, vitamin C (L-ascorbic acid), phorbol 12-myristate 13-acetate (PMA) and calphostin-C were obtained from Sigma Chemical Co. Cell culture media and supplements and recombinant Taq DNA polymerase and M-MLV reverse transcriptase were purchased from GIBCO BRL. RNase and Oligod (T) were purchased from Pharmacia. Human MCP-1 immunoassay kits were from R & D Systems. LysoPC was delivered to cell cultures as aqueous solutions in phosphate-buffered saline. Vitamin C was dissolved immediately before use in metal-free distilled water (> 18 Mohm).

**Cell culture**

HUVECs were isolated and cultured in M199 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), gentamicin (25 µg/mL), cytosine arabinoside (Ara-C, 5 µg/mL), and 25 µg/mL endothelial cell growth factor. HUVECs were allowed to grow to confluence in 10-cm petri dishes at 37°C for 5 to 6 days in a humidified atmosphere of air containing 5% CO₂. During the culture, fresh endothelial growth medium was changed every 2 to 3 days. Then, HUVECs were subcultured by exposure to 0.025% trypsin-0.01% EDTA and seeded on to 6-well petri dishes until the monolayer became confluent. The medium for the cultured HUVECs was then changed to the same medium containing only 2% lipoprotein-free FBS, and the cells were incubated for 8 hours before the experiments. Cells were stimulated by the addition of test substances and incubated for indicated periods of time at 37°C. The cells were used before passage 4 in all experiments.

**MCP-1 enzyme-linked immunosorbent assay**

MCP-1 levels in the culture supernatants of HUVECs were determined by enzyme-linked immunosorbent assay (ELISA). The reaction products were measured at 450 nm with a microplate reader (Molecular Device). The standard concentration curve for MCP-1 measured by this ELISA was linear from 0.15 to 15 ng/mL.

**RNA isolation and analysis**

Total RNAs were extracted from cultured cells with a TRIZOL reagent (Gibco, Grand Island, NY). The RNA...
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Biotech). Two steps were taken to ensure that the reverse transcriptase-PCR amplification was quantitative (Fig. 1). The first step was to look for an exponential phase in which the amount of the products increased linearly with the concentration of total RNA with fixed amplification cycle. The goal of this step was to determine the optimal concentration of total RNA in the amplification. The second step was to look for an exponential phase in which the amount of the products increase linearly with the number of PCR cycles. Knowing the exponential phase for both target MCP-1 and control \( \beta \)-actin can avoid spurious results due to analysis during the plateau phase of amplification. The mRNAs of MCP-1 of conditioned experiments were expressed as a ratio of the simultaneously amplified products of MCP-1 and \( \beta \)-actin.

Statistical analysis

All quantitative assays were done in triplicate and data were expressed as the mean \( \pm \) SEM. The significance of differences between 2 groups was estimated by Student’s \( t \) test. A \( p \) value < 0.05 was considered significant.

Results

The time course of MCP-1 secretion from HUVECs after exposure to a 50 \( \mu \)M lysoPC is shown in Fig. 2. At different time points, an aliquot of the medium was collected. A significant increase of MCP-1 was noted
beginning 30 minutes after lysoPC stimulation and this increase was maintained for 36 hours.

HUVECs were pretreated with vitamin C in various concentrations (50, 100, 150, and 200 µM) for 8 hours, with 50 µM lysoPC added for an additional 8 hours. Treatment of HUVECs with lysoPC alone (control) significantly increased MCP-1 secretion in the culture medium.

In various concentrations of vitamin C pretreatment, the levels of MCP-1 decreased by 44%, 51%, 60%, and 67%, respectively, versus the control level (Fig. 3A). The level of MCP-1 mRNA was expressed as the ratio between the simultaneously PCR-amplified MCP-1 and β-actin genes, and decreased by 15%, 18%, 80%, and 82%, respectively, versus the control level (Fig. 3B). The above results indicate that vitamin C significantly suppressed lysoPC-induced secretion and expression of MCP-1 in HUVECs.

Pretreatment of HUVECs with 200 µM vitamin C effectively suppressed the secretion (Fig. 4A) and

![Fig. 3. Vitamin C inhibits lysophosphatidylcholine (lysoPC)-induced production of monocyte chemoattractant protein-1 (MCP-1) in cultured human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated with vitamin C (vit. C) in various concentrations (50, 100, 150, and 200 µM) for 8 hours and then exposed to 50 µM lysoPC for an additional 8 hours. A) Enzyme-linked immunosorbent assay in the culture medium; control panel was freshly prepared HUVECs without adding either lysoPC or vitamin C. *p < 0.05 and **p < 0.01, compared with lysoPC treatment alone. B) Expression of MCP-1 mRNA was measured by reverse transcriptase polymerase chain reaction (PCR). The bars represent the ratio between the simultaneously amplified PCR products of MCP-1 and β-actin genes obtained from individual samples. Results are mean ± SD from 3 separate experiments.](image1)

![Fig. 4. The role of protein kinase C and effect of vitamin C treatment on lysophosphatidylcholine (lysoPC)-induced secretion of monocyte chemoattractant protein-1 (MCP-1) from human umbilical vein endothelial cells. Cells were preincubated with calphostin C (Cal-C, 2.5 µM) for 30 minutes, or with phorbol 12-myristate 13-acetate (PMA, 50 nM) and vitamin C (vit. C, 200 µM) for 8 hours before 50 µM lysoPC treatment. A) Enzyme-linked immunosorbent assay in the culture medium. *p < 0.05 and **p < 0.01, for comparisons between the conditioned experiments and the lysoPC treatment alone. B) Expression of MCP-1 mRNA was measured by reverse transcriptase polymerase chain reaction (PCR). The bars represent the ratio between the simultaneously amplified PCR products of MCP-1 and β-actin genes obtained from individual samples. Results are mean ± SD from 3 separate experiments.](image2)
expression (Fig. 4B) of MCP-1, whether stimulated from the protein kinase C (PKC) activator PMA, or from lysoPC, compared with lysoPC treatment alone (control). We also found that the inhibitory effect of vitamin C on lysoPC-mediated secretion of MCP-1 was equivalent to that of the PKC antagonist calphostin C.

**Discussion**

This study found that lysoPC, a component of oxidized LDL, can induce MCP-1 production in cultured HUVECs. However, pretreating cells with vitamin C may attenuate the lysoPC-induced production of MCP-1. Chisolm et al suggested that lysoPC and other toxic reactive substances are formed upon the oxidation of LDL, and have been shown to be cytotoxic to endothelium leading to cellular dysfunction and injury. Studies have shown that lysoPC causes impairment of endothelium-dependent vasorelaxation, and the induction of various proatherogenic and proinflammatory molecules in endothelial cells. Frie found that vitamin C acts as a primary defensive agent against free radical attack and protects LDL against oxidation modification.

Vitamin C can regenerate α-tocopherol and, hence, prevent tocopherol-mediated peroxidation of LDL. Coexistence of large amounts of oxidized cholesterol esters with significant concentrations of vitamin C and vitamin E in their reduced, antioxidant-active form has been found in human atherosclerotic plaque. A recent study in human umbilical vein endothelial cells suggests a cytoprotective role for vitamin C against hypochlorous acid-mediated apoptosis.

Jenner et al also confirmed that pretreatment with physiological levels of vitamin C significantly reduced the extent of hypochlorous acid-induced DNA and protein damage in human vascular smooth muscle cells. Langlois et al reported that vitamin C concentration is lower in patients with peripheral arterial disease in association with higher C-reactive protein levels and severity of atherosclerosis. Vita et al reported that vitamin C concentration and total plasma thiols predict the presence of an unstable coronary syndrome. A human study found that monocytes isolated from smokers exhibit increased adhesion to cultured endothelial cells versus monocytes isolated from non-smokers.

Supplementing smokers with vitamin C 2 g/day for 10 days elevated plasma vitamin C levels almost 2-fold and significantly reduced monocyte adhesion to cultured endothelial cells. Huang et al showed that physiological concentrations of vitamin C increase the synthesis and biological activity of nitric oxide (NO) in cultured endothelial cells by increasing intracellular tetrahydrobiopterin. Supplementing coronary artery disease patients with vitamin C 500 mg/day for 30 days improved endothelial function through ultrasound-detected flow-mediated dilation of the brachial artery.

Steinbrecher reported that vitamin C, at concentrations of 50 µM, could effectively inhibit endothelium-mediated LDL modification. Alexander et al observed that the uptake of vitamin C by incubated HUVECs is time- and concentration-dependent. It may be saturated between 100 µM and 200 µM, and is optimal at extracellular concentrations between 50 µM and 100 µM, matching the normal levels of vitamin C in human plasma. They also found that intracellular levels of vitamin C fell rapidly within the first hour of re-incubation in a vitamin C-free medium, indicating that vitamin C undergoes either rapid breakdown or oxidation.

Expression of the MCP-1 gene is regulated by transcription factors of the nuclear factor-κB (NF-κB) family. Activation of NF-κB has been associated with gene expression in response to increased PKC activity. LysoPC has been shown to modulate multiple signal transduction pathways, with effects including stimulation of protein kinase C activity and regulation of transcription factor NF-κB activity.

Takahara et al showed that treating HUVECs with lysoPC for 6 hours results in a 6-fold increase in MCP-1 mRNA levels, and this increased lysoPC-induced expression of MCP-1 mRNA is attenuated by 53% with a PKC inhibitor. Bowie and O’Neill reported that vitamin C can inhibit NF-κB activation in endothelial cells. In the present study, the lysoPC-induced MCP-1 levels in the culture medium were attenuated by 60% with a PKC inhibitor, calphostin, compared with the 67% decrement when cells were treated with 200 µM vitamin C. However, the present study has limited implications concerning the role of PKC on the effect of vitamin C treatment on lysoPC-induced expression and secretion of MCP-1 from HUVECs for several reasons. First, we did not investigate the combined effect of vitamin C and PKC inhibitor (calphostin C) on lysoPC-induced MCP-1 mRNA and protein secretion. Second, at their maximum effective concentrations, both vitamin C and calphostin C might have no additive effects with combined treatment if PKC indeed mediates the effect of vitamin C. Third, we did not analyze the effect of vitamin C on NF-κB activation in endothelial cells.

Formation of atherosclerotic lesions is a multifaceted process that is initiated with the oxidative modification of LDL. Oxidized LDLs cause foam cell formation, leukocyte adhesion to the endothelium, and vascular endothelial dysfunction. The present study has shown that in addition to its antioxidant...
role, vitamin C can suppress lysoPC-induced expression and secretion of MCP-1, which leads to cytotoxic insult to the vascular endothelium. These data suggest that an optimal intake of vitamins C may attenuate the progression of atherogenic processes.

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References


