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Lovastatin inhibits Oxidized- L-A-phosphatidylcholine B-arachidonoyl-gamma-palmitoyl (ox-PAPC)-Stimulated Interleukin-8 mRNA and Protein Synthesis in Human Aortic Endothelial Cells by Depleting Stores of Geranylgeranyl Pyrophosphate

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Abstract

Human aortic endothelial cells (HAEC) exposed to 50 µg/ml oxidized L-A-phosphatidylcholine B-arachidonoyl-gamma-palmitoyl (ox-PAPC) for 6 hours increased in interleukin-8 mRNA and protein levels. Preincubation of HAEC with the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) inhibitor, (20 µM), significantly inhibited ox-PAPC-stimulated interleukin-8 mRNA and protein levels. Mevalonate (200 µM) reversed the inhibition of ox-PAPC-stimulated mRNA and protein levels by lovastatin, indicating the inhibitory effect of lovastatin was due to inhibition of mevalonate synthesis. Addition of the geranylgeraniol (GGOL, 10 µM) but not farnesol (FOL, 10 µM), reversed the inhibitory effect of lovastatin on interleukin-8 mRNA and protein levels stimulated by ox-PAPC, indicating that lovastatin exerted its effect by inhibiting stores of geranylgeranyl pyrophosphate (GGPP) which are necessary for geranylgeranylation of proteins. These results suggest a new mechanism for lovastatin in preventing atherosclerosis by inhibiting the inflammatory response that takes place in the vascular wall.

Keywords

atherosclerosis; ox-PAPC; interleukin-8; lovastatin; geranylgeranyl; pyrophosphate

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Reduction in coronary blood flow resulting from atherosclerotic coronary artery obstruction is the primary cause for more than 90% of ischemic heart disease cases. It used to be thought that atherosclerosis was simply a lipid storage disease, with build-up of fat in the vessel wall. It is now known that its pathogenesis is much more complicated. Inflammation plays a key role in the initiation and development of atherosclerosis 1. Normally the endothelium that lines the vessel wall resists adhesion of monocytes; however, triggers of atherosclerosis, such as the activation of endothelial cells by oxidized LDL (ox-LDL) and C-reactive protein (CRP) results in monocyte migration into the subendothelial space 2, 3. Ox-LDL contains biologically active molecules that induce endothelial cells to produce chemokines that attract monocytes such as interleukin-8, and growth factors 3. Further, it has been shown that phospholipid oxidation products of L- α -phosphatidylcholine B-arachidonoyl-gamma-palmitoyl (ox-PAPC) found in minimally modified/oxidized LDL activate monocyte/endothelial cell interactions 4 and has been identified in atherosclerotic lesions 2. The chemokine interleukin-8 mRNA is stimulated in endothelial cells treated with ox-PAPC and has been shown to be a critical determinant of monocyte/endothelial cell interactions 5. In the intima, monocytes mature into tissue macrophages which bind and phagocytose ox-PAPC (transforming into lipid-laden macrophages). This is referred to as the development of the fatty streak. The monocytes continue to increase in the intima and the fatty streak becomes a complex plaque. The mechanisms by which ox-PAPC exerts its effect on stimulating interleukin-8 production are unknown; however it has been shown to be independent of NF- κ B promoter activation 6. Here we show for the first time that statins, by depleting intracellular stores of geranylgeranyl pyrophosphate, inhibit interleukin-8 mRNA and protein in human aortic endothelial cells (HAEC) treated with ox-PAPC. A recent report released from the Jupiter Trial supports our results that statins play a role beyond inhibiting cholesterol synthesis 7. Study participants in the Jupiter Trial had normal levels of cholesterol but increased levels of CRP, a protein associated with inflammation. Participants on statin had significantly reduced risk of heart attack and stroke 7. The results were so striking that the trial was stopped after 2 years so the group on placebo could be treated with the statin.

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitor, lovastatin, has been is a well known inhibitor of cholesterol synthesis and it, along with second generation statins, are the first line treatment for hypercholesterolemia. Statins have also generated interest as possible chemotherapeutic agents in the treatment of cancer 8 as well as inhibitors of inflammation 9. At high concentrations, lovastatin prevents the conversion of HMG CoA to mevalonate and thereby depletes the stores of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) 8· 10⁻¹³ which is necessary for protein prenylation. Protein prenylation is an important mechanism of post translational modification of proteins. Many of prenylated proteins are small GTPases, which include member of the Ras and Rho families. Prenylation of small G-proteins with FPP and GGPP is essential for their localization to cellular membranes and hence for their activation. The Rho family of proteins is generally geranylgeranylated while H-Ras is selectively farnesylated 8· 10⁻¹³. The small GTPases act as molecular switches, trafficking from a membrane-bound (active) GTP-bound state, to a cytosolic (inactive) GDP-bound state 8· 10⁻¹³. Thus, statins affect multiple cellular functions by interfering with basic cell signaling pathways. Our results suggest an important role for geranylgeranylated proteins of the Rho subfamily, including RhoA, Rac1 and Cdc42Hs, which have been shown to be involved in cell shape regulation and actin filament assembly 11· 12, and, as we show here, in mediating the effect of ox-PAPC on interleukin-8 transcription and protein synthesis.

Material and Methods

Cell Culture

Human aortic endothelial cells (HAECs; Cascade Biologics) were cultured in Cascade Biologics™ Medium 200 (Portland, Oregon) supplemented with low serum growth supplement. Cultures were maintained at 37°C and 5% CO₂. Medium was changed every other day or more frequently for highly confluent cultures.

Oxidation of LDL

L- α -phosphatidylcholine, B-arachidonoyl-gamma-palmitoyl (PAPC; Sigma, St. Louis, MO) was oxidized by exposure to air for 72h; ox-PAPC was suspended in 100% ethanol ¹⁴.

HAEC treatments

HAECs were treated with vehicle (ethanol), or 50 μ g/ml PAPC or ox-PAPC for 6h. Prior to ox-PAPC exposure, HAEC were incubated in Cascade Biologics™ Medium 200 supplemented with low serum growth supplement plus or minus 20 μ M lovastatin (A.G. Scientific, Inc., San Diego, CA), mevalonate (200 μ M), 10 μ M GGOL (Sigma), or 10 μ M FFOL (Sigma). Culture medium for all experiments was harvested prior to lysis of HAECs. Culture medium and lysates were stored at -70°C.

RNA Isolation

RNA isolation was performed using Sigma®'s GenElute™ Mammalian Total RNA Miniprep Kit (St. Louis, MO). Briefly, thawed cell lysates were processed through filtration and binding columns, genomic DNA was removed by incubation with a DNase 1/Digest Buffer mixture, and RNA was isolated. All steps were completed at room temperature; isolated RNA was stored at -70°C. RNA concentration for each sample was determined using a Nanodrop® ND-1000 spectrophotometer.

RT-qPCR

RNA samples (100ng) were reverse transcribed using Promega's Plexor™ Two-Step qRT-PCR System (Madison, WI). RNA samples were combined with 8.1 μ L RT Reaction Mix (4 μ L ImPromII 5x Rxn Buffer, 1.6 μ L MgCl₂, 1 μ L dNTP mix, 0.5 μ L RNasin Plus, and 1 μ L ImPromIIRT and 1 μ L of 0.5 μ g/ μ L Oligo(dT)). RNase-free water was added for a final reaction volume of 20 μ L. Samples were reverse transcribed in a Perkin Elmer GeneAmp PCR System 2400 for 5 minutes at 25°C, 60 minutes at 45°C, and 15 minutes at 70°C. The reaction was held at 4°C prior to qPCR. For qPCR, 20 μ L of PCR reaction mix (12.5 μ L Plexor™ master mix, 4.5 μ L nuclease-free water, and 1 μ L of each of the following 5 μ M primers: 5' IL-8, 3' IL-8, GAPDH (5' and 3')) was added to each test well of a 96-well plate. Sample cDNA (5 μ L) was added to each well prior to sealing the plate and amplifying in an Applied Biosystems 7500 Real Time PCR System. Samples were amplified in three stages: Stage 1, 2 minutes at 95°C; Stage 2, 5 seconds at 95°C and 35 seconds at 60°C, 40 cycles; and Stage 3, 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 75°C.

Interleukin-8 ELISA

RayBio® Human IL-8 ELISA Kit (Norcross, GA) was used according to manufacturer's instructions to measure interleukin-8 concentrations in culture supernatants. Positive and negative controls were added, diluted 1:30, to a 96-well plate along with samples in triplicate. The plate was incubated at room temperature for 2.5 h. The plate was washed 4 times with 1X wash solution. Biotinylated antibody (1X) was added to each well and incubated at room temperature for 1 h. The plate was washed 4 times with 1X wash solution. Streptavidin solution diluted 30,000 fold was added to each well and incubated at room temperature for 45 minutes.

The plate was washed 5 times with 1X wash solution. One hundred microliters TMB One-Step Substrate Reagent was added to each well and incubated at room temperature for 30 minutes in the dark. Fifty microliters Stop solution was added to each well and the plate was read at 450 nm immediately on ELX808 Ultra Microplate Reader from Bio-Tek Instruments Inc. Interleukin-8 concentrations were determined by comparing absorbance values against an interleukin-8 standard curve.

Statistics

Delta delta C_t ($\Delta\Delta C_t$) for each sample was determined by subtracting the average of the no treatment group GAPDH C_t from each sample GAPDH C_t . These values were subtracted from the difference between each sample IL-8 C_t and the average of the No Treatment C_t . A summary of the derivation of $\Delta\Delta C_t$ is illustrated in the equation below¹⁵

$$\Delta\Delta C_t = (X - X_{NT}) - (Y - Y_{NT})$$

$X = \text{IL-8 } C_t \text{ for each sample}$
 $X_{NT} = \text{Average of IL-8 } C_t \text{ from No Treatment group}$
 $Y = \text{GAPDH } C_t \text{ for each sample}$
 $Y_{NT} = \text{Average of GAPDH } C_t \text{ from No Treatment group}$

An average $\Delta\Delta C_t$ was based on six replicate assays. IL-8 expression ratios are equal to $2^{-\text{average}\Delta\Delta C_t}$. P values were obtained by performing two-sample Student T-Tests on the $\Delta\Delta C_t$ values for each treatment

Results

HAEC treated with ox-PAPC, but not PAPC or vehicle control resulted in a time and dose-dependent increase in interleukin-8 mRNA and protein (not shown). Based on these experiments, HAEC were treated for 6 hours with 50ug/ml ox-PAPC prior to harvesting for mRNA and protein. Ox-PAPC caused greater than a 5 – 15 fold increase in mRNA levels in HAEC (Figures, 1 and 2) which was sustained through 24 hours (not shown). Treatment with lovastatin (20 μ M) inhibited ox-PAPC-stimulated interleukin mRNA levels by 60% to 300% (Figures 1 and 2). Addition of mevalonate (200 μ M) reversed the inhibiting effect of lovastatin, indicating lovastatin exerted its effect by inhibiting the mevalonate pathway and depleting stores of farnesyl pyrophosphate (FPP) and/or geranylgeranyl pyrophosphate (GGPP) for protein prenylation (Figure 1). To determine whether FPP or GGPP played a role in ox-PAPC-stimulated interleukin-8 mRNA stimulation, we pre-treated the cells with FOL (10 μ M) or GGOL (10 μ M) prior to incubation with lovastatin. GGOL, but not FOL inhibited lovastatin's effect on ox-PAPC-stimulated interleukin-8 transcription, indicating a role for GGPP in ox-PAPC's stimulatory effect (Figures 2 and 3). Treatment of HAEC with 50ug/ml ox-PAPC increased interleukin-8 protein production 50 to 80% (Figures 4 and 5). Lovastatin inhibited ox-PAPC stimulated-interleukin-8 production, as shown by a 79% decrease in interleukin-8 production (Figures 3 and 4). Similar to what was found with mRNA levels, mevalonate and GGOL, but not FOL, significantly reversed the observed inhibition of interleukin - 8 production by lovastatin (Figures 4 and 5).

Discussion

Atheromatous plaques would not necessarily be a major health issue as long as they remained intact. It is now known that the physical rupture of the atherosclerotic plaque, which results in blood contact with the lipid core, is the cause of acute coronary syndrome, resulting from thrombus formation and sudden expansion of the lesion to the extent that blood flow through the affected artery becomes compromised and even blocked¹. Lipid-laden macrophages

residing in the intima are believed to be the culprits behind plaque rupture. Release of matrix metalloproteinase 9 (MMP-9) into the intima is believed to be the mechanism leading to plaque instability and rupture¹. Therefore, treatments which reduce inflammation in the vessel wall should not only decrease the development of the atheromatous plaque, but should also stabilize the plaque and reduce the risk of heart attack.

Recent data, including our own, have shown statins have direct effects on vascular endothelial cells and macrophages independent from its cholesterol lowering effect². Statins inhibit cholesterol synthesis by inhibiting HMG-CoA which undeniably reduces the risk of plaque accumulation by lowering serum cholesterol. Statins used to treat hypercholesterolemia may also attenuate inflammation of the vascular wall by inhibiting prenylation of proteins involved in chronic inflammation. One such statin, lovastatin, which prevents the conversion of HMG CoA to mevalonate, which inhibits the synthesis of the other products of the mevalonate pathway besides cholesterol. These include GGPP and FPP¹¹, which modify and target small GTPases, including Ras and Rho, to their site of action¹². The small GTPases act as molecular switches, trafficking from a membrane-bound (active) GTP-bound state, to a cytosolic (inactive) GDP-bound state¹³. Lovastatin, by inhibiting the synthesis of GGPP and FPP, prevents the activation of these small GTPases. This affects multiple cellular functions by interfering with basic cell signaling pathways. The results presented here point to a role for geranylgeranylated proteins in endothelial cell signaling in response to ox-PAPC. The geranylgeranylated proteins of the Rho subfamily, including RhoA, Rac1 and Cdc42Hs, are involved in cell shape regulation and actin filament assembly^{8, 16, 17}. Protein prenylation has been shown to be involved in cell adhesion⁸, cell proliferation^{12, 16}, malignant transformation^{8, 16}, cell survival^{10, 18}, endothelial cell barrier dysfunction¹⁸, vascular smooth muscle cell DNA synthesis and migration, angiogenesis¹⁹⁻²¹, and, as reported here, interleukin-8 production by HAEC treated with ox-PAPC. A variety of putative RhoA effectors have been identified^{8, 17}. Among these effectors, the serine/threonine kinase, RhoA kinase, which mediates actin filament assembly, focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K).

Rho proteins act in concert and function in a cascade where activation of Rac by Ras causes membrane ruffling which in turn activates RhoA-mediated actin stress fiber assembly and cell shape and adhesion^{10, 18, 22}. When cells are stimulated, GDP-RhoA is believed to be converted to GTP-RhoA, which binds to specific membrane targets to exert its biological functions. RhoA activates FAK and PI3K, both of which have been shown to be important proteins involved in endothelial cell and macrophage activation^{5, 10, 23-25}. One of the products generated by PI3K activation, PI^{3, 4}P₂, has been shown to increase the kinase activity of the PI3K effector ribosomal p70 S6 kinase. PI3K is also an effector of Ras. GTP-bound Ras interacts with the catalytic subunit of PI3K and cooperates with tyrosine phosphorylation of the regulatory subunit of the enzyme to give optimal activation. Recently, ox-PAPC has been reported to activate the Ras/PI3K signaling pathways following the clustering of receptors²⁵. Lovastatin may reduce monocyte adhesion and spreading on activated endothelial cells which is dependent on RhoA-regulated receptor clustering into focal adhesions with myosin light chain phosphorylation and stress fiber assembly. Statins may play a key role in reducing the inflammation in the vessel wall that leads to myocardial infarction by inhibiting receptor clustering into focal adhesions. Elucidation of the mechanisms of endothelial cell activation could lead to new therapies to reduce plaque formation or increase plaque stability; one of these treatments could be statins as shown recently in the results of the Jupiter Trial⁷.

Summary Paragraph

The fact that lovastatin inhibits the synthesis of inflammatory cytokines independently of its effects on cholesterol lowering supports recently reported results from the Jupiter Trial⁷. The

Jupiter Trial looked into the effects of statins on people without high cholesterol or histories of heart disease. It involved 17,802 people — men 50 and older and women 60 and older — in 26 countries who took either a statin or a placebo. The main Jupiter were that statins lowered the risk of heart attack by more than half and significantly lowered the risk of stroke in people without high cholesterol, or histories of heart problems, but with high levels of the inflammatory protein, CRP, indicating another role for statins besides its effects on cholesterol lowering. In a more recent report from the Trial, after an average of less than two years, it was found that the people taking the statin who had the lowest risk of heart attack, stroke and other problems were those who wound up not only with very low cholesterol but also very low CRP levels²⁶. Indeed, the lead author of the study, said the findings indicated that people with high CRP levels should be taking statins, a recommendation that the national medical panels are considering. These results along with those presented in this manuscript show that inflammation, not just high cholesterol, are involved in the pathology of the vascular wall.

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Figure 1. Ox-PAPC (50 μ g/ml) increased interleukin-8 mRNA levels 5 fold compared to no treatment (NT) or unoxidized PAF. The error bars indicate a transformed 95% confidence interval around the original $\Delta\Delta C_t$ means.

Figure 2.

ox-PAPC (50 ug/ml) increased interleukin-8 mRNA levels by 15 fold ($p < 0.0001$). Lovastatin inhibited ox-PAPC's effect on interleukin-8 mRNA ($p < 0.0005$) which was reversed by mevalonate ($p < 0.0005$). GGOL likewise inhibited lovastatin's effect ($p < 0.05$). These results indicate that lovastatin is exerting its inhibitory effect on interleukin-8 mRNA by inhibiting the mevalonate pathway and depleting stores of GGPP. The error bars indicate a transformed 95% confidence interval around the original $\Delta\Delta\text{Ct}$ means. N=6

Figure 3.

ox-PAPC increased interleukin-8 mRNA levels by 9-fold ($p < 0.0001$). Lovastatin inhibited ox-PAPC's effect on interleukin-8 mRNA ($p < 0.0005$) which was not reversed by FOL, suggesting that the mechanism of lovastatin's effect was not by depleting stores of FPP. The error bars indicate a transformed 95% confidence interval around the original $\Delta\Delta C_t$ means. $N=6$

Figure 4.

Treatment of HAEC with 50ug/ml ox-PAPC increased interleukin-8 production by a 80% ($p<0.0001$). Lovastatin inhibited ox-PAPC-stimulated interleukin 8 production ($p<0.0001$). Mevalonate and GGOL reversed lovastatin's effect, suggesting lovastatin exerted its effect by depleting stores of FPP. The error bars indicate a 95% confidence interval around the concentration means. N=6

Figure 5.

Ox-PAPC stimulated interleukin-8 production by 50% ($p < 0.0005$). Unlike GGOL, FOL failed to reverse lovastatin's effect on inhibiting ox-PAPC-stimulated interleukin-8 production, suggesting that lovastatin did not exert its effect by depleting stores of FPP. The error bars indicate a 95% confidence interval around the concentration means. $N=6$