Role of 5-Lipoxygenase in Interleukin-4-Induced Oxidative Stress and Inflammation in Vascular Endothelium

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ABSTRACT

Cardiovascular disease (CVD) including atherosclerosis is the leading cause of illness and death in the United States. The American Heart Association indicated that an estimated 81,100,000 American adults have one or more types of CVD and the estimated direct and indirect cost of CVD for 2010 is $503.2 billion, which is $27.9 billion more than last year. Although the exact cause of this disease remains unsolved, previous studies have demonstrated that pro-oxidative and pro-inflammatory pathways in vascular endothelium play a critical role in early pathological events of atherosclerosis. However, the detailed molecular signaling mechanisms underlying this process are not yet completely understood. Recently, the role of interleukin-4 (IL-4) in atherogenesis became controversial and gained attention. IL-4 is a pleiotropic immunomodulatory cytokine secreted by T-helper 2 (Th2) lymphocytes, eosinophils, and mast cells. It was traditionally believed to be an anti-inflammatory cytokine. Increasing evidence, however, has suggested that IL-4 contributes to the initiation and progression of atherosclerosis by oxidative stress-mediated up-regulation of pro-inflammatory mediators such as vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6) in vascular endothelium.

5-Lipoxygenase (5-LOX) is one of the key sources that generate reactive oxygen species (ROS) via metabolic pathways of arachidonic acid. Although 5-LOX has been implicated in the development of atherosclerosis, it remains unclear whether 5-LOX-mediated ROS generation is associated with IL-4-induced MCP-1 expression in vascular endothelium. The present study was focused on the oxidative mechanisms by which IL-4 induces vascular inflammation as well as how 5-LOX is involved in this process.

The results showed that IL-4 significantly up-regulates mRNA and protein expression of MCP-1 in vascular endothelium. In addition, DHE and DCF fluorescence staining demonstrated that IL-4 increases ROS production in human vascular endothelial cells. We have also provided the first novel evidence that 5-LOX, one of the enzymes associated with arachidonic acid metabolism, is responsible for the IL-4-induced ROS generation and MCP-1 expression in human vascular endothelial cells.
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List of Abbreviations

AA: Arachidonic acid
AP-1: Activator protein-1
APO: Apolipoprotein
cPLA2: Cytosolic phospholipase A2
CVD: Cardiovascular disease
DCF: Dichlorofluorescein
DHE: Dihydroethidium
ELISA: Enzyme-linked immunosorbent assay
eNOS: Endothelial nitric oxide synthase
FLAP: 5-lipoxygenase activating protein
HAEC: Human aortic endothelial cells
IL-4: Interleukin-4
LOX: Lipoxygenase
LTB4: Leukotriene B4
MAPK: Mitogen-activated protein kinase
MCP-1: Monocyte chemoattractant protein-1
NADPH: Nicotinamide adenine dinucleotide phosphate
NF-κB: Nuclear factor-κB
NO: Nitric oxide
NOX: NADPH oxidase
PDGF: Platelet-derived growth factor
ROS: Reactive oxygen species
RT-PCR: Reverse transcriptase-polymerase chain reaction
TNF-α: Tumor necrosis factor-α
VCAM-1: Vascular cell adhesion molecule-1
1. Introduction

1.1. Cardiovascular disease: atherosclerosis

Cardiovascular disease (CVD) including atherosclerosis is the leading cause of death in the United States and other developed countries. In fact, there are about 81.1 million American adults who are diagnosed with one or more types of CVD and the estimated cost of CVD for 2010 is $503.2 billion (American Heart Association Statistics Committee and Stroke Statistics Subcommittee, 2010). CVD continues to bring tremendous suffering to our society despite the developments in diagnosis and management of CVD.

CVD results from complications of atherosclerosis, which is a progressive disease caused by the accumulation of lipids, calcium, and cellular waste products in the subendothelial layer of arteries (Ross and Harker, 1976). When this accumulation, called plaque, grows and hardens, it narrows the arteries and therefore decreases the flow of oxygen-rich blood to the organs and other part of the body. Depending on severity and location of blockage, atherosclerosis leads to heart attack and stroke.

1.2. Causes and risk factors for atherosclerosis

While the exact cause of atherosclerosis still remains unknown, there are major conditions that are known to be associated with increased cardiovascular risk. Such conditions include hypercholesterolemia, hypertension, diabetes mellitus, obesity, and cigarette smoking (Fernando et al., 2009). Compelling evidence has indicated that all of these conditions are involved in increasing vascular reactive oxygen species (ROS) production, which plays a pivotal
role in the development of CVD (Bouloumie et al., 1999; Hennig et al., 1996). For example, increased production of superoxide anion was observed in human atherosclerotic coronary arteries. In addition, it is well known that elevated production of ROS is linked to over expression of pro-inflammatory mediators, which play a critical role in early steps in atherogenesis (Guzik et al., 2006; Lee and Hirani, 2006; Sorescu et al., 2002).

1.3. Overproduction of ROS: oxidative stress

Oxidative stress is created in our body when there is a disturbance in the balance between ROS and antioxidants in favor of the former. Excess amount of ROS compared to antioxidants exerts detrimental effects on cells. Most ROS are free radicals that have one or two unpaired electrons; most of the molecules in our body are non-radical or neutral species such as lipids, proteins, nucleic acids, and carbohydrates. Some ROS react with non-radical species by means of abstracting a hydrogen from a C-H or O-H bond of non-radical species. One such reaction is lipid peroxidation. In addition, two ROS such as superoxide anion and nitric oxide may react together and create peroxynitrite, which is detrimental to cells (Papaharalambus and Griendling, 2007).

Vascular endothelial cells produce ROS through several oxidases, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase, cyclooxygenase, lipoxygenase and cytochrome P450. ROS also can be produced through the uncoupling of the mitochondrial respiratory chain and endothelial nitric oxide synthase (eNOS) (Papaharalambus and Griendling, 2007) (Figure 1).
Figure 1. The ROS generating pathways in vascular endothelial cells.
1.4. Oxidative stress and atherosclerosis

Oxidative stress has been implicated in the initiation and progression of atherosclerosis. One of the main targets of oxidative stress during this process is the vasculature. Vascular endothelial cells are very sensitive to disturbances in the redox steady state (Thomas et al., 2008).

Vascular endothelium does not simply function as a selective permeable barrier between the bloodstream and vascular wall. It also plays an important physiological role in maintaining vascular homeostasis by regulating vascular tone, maintaining vascular shape and flow of blood through production of anti-thrombosis molecules, and regulating inflammation (Simionescu, 2007). The maintenance of vascular homeostasis is accomplished by the production and release of nitric oxide (NO) by vascular endothelial cells (Anderson, 2003). Presence of oxidative stress could result in impaired NO bioavailability due to the breakdown of NO by ROS (Taddei et al., 1998). If the oxidative stress due to excess amounts of ROS is not removed efficiently, then it could cause endothelial dysfunction, which is a crucial step in the development of atherosclerosis.

One of the most important functions of the vascular endothelium is to regulate inflammatory reactions. Endothelial dysfunction, characterized by loss of maintaining vascular homeostasis, brings changes to vascular endothelial cells such as overexpression of adhesion molecules and cytokines as well as neutrophil activation (De Caterina et al., 1995). The consequences of these changes are leukocyte adhesion to the vascular endothelium and initiation of inflammatory responses. It has been suggested that increases in intracellular oxidative stress may regulate the expression of redox-sensitive genes, including those encoding for pro-inflammatory mediators (Ylä-Herttuala, 1992). For example, it has been described that oxidative stress activates redox-sensitive transcription factors such as nuclear factor κB (NF-κB) and
activator protein-1 (AP-1), both of which are largely responsible for the overexpression of inflammatory mediators including cytokines, chemokines, and adhesion molecules (Taniyama and Griendling, 2003).

1.5. Inflammation and atherosclerosis

The deposition of lipids as well as the accumulation of foam cells and lipid stored macrophages in the subendothelial layer of arteries is a major morphological hallmark of atherosclerosis. The positive relationship between high levels of cholesterol and atherosclerosis has dominated our thoughts for several decades (Ross and Harker, 1976). But failure in maintaining vascular homeostasis in the vascular endothelium, therefore, producing pro-inflammatory mediators such as adhesion molecules, cytokines, and chemokines are now recognized as the important factors for atherosclerosis (Bijl, 2003). In fact, studies using apoE-knockout mice have found out that lymphocytes were present in atherosclerotic lesions as well as overexpression of cytokines and adhesion molecules (Pepine, 2009). It also has been widely known that the interaction between chemokines and adhesion molecules facilitates recruiting and adhering blood leukocytes to vascular endothelial cells, and subsequently stimulates transendothelial migration, a process that can be found during atherogenesis (Lee and Hirani, 2006) (Figure 2). These findings suggest that inflammatory processes are essential for atherogenesis and it is now generally believed that atherosclerosis is chronic inflammatory disease of the arterial wall (Clark, 2002; Libby et al., 2002).
Figure 2. An illustration of leukocyte adhesion to vascular endothelium and transendothelial migration.
1.6. Role of IL-4 in vascular inflammation and atherosclerosis

Interleukin-4 is a pleiotropic immunomodulatory cytokine secreted by T-helper 2 (Th2) lymphocytes, eosinophils, and mast cells (Lee and Hirani, 2006). It was traditionally considered as an anti-inflammatory cytokine (Paul, 1991; Rocken et al., 1996). A growing body of evidence, however, has indicated that IL-4 is a pro-inflammatory cytokine and plays a crucial role in the progression of atherosclerosis. For example, high levels of IL-4 were present in tissues of patients with chronic inflammatory disease and elevated levels of IL-4 were detected in atherosclerotic lesions (Walch et al., 2006). In addition, IL-4 induced pro-inflammatory environments via overexpression of a number of pro-inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1), and vascular cell adhesion molecule-1 (VCAM-1) in human vascular endothelial cells (Tedgui and Mallat, 2006; Lee et al., 2010b). Furthermore, animal model studies have shown pro-atherogenic effects of IL-4. For instance, a significant reduction in atherosclerotic lesion area was observed in IL-4-knockout/ApoE-knockout mice compared to ApoE-knockout mice (Davenport and Tipping, 2003). This evidence showed that IL-4 may have potential to exert its pro-atherogenic effects by enhanced expression of pro-inflammatory mediators such as chemokines and adhesion molecules in vascular endothelium.

1.6.1. Effects of IL-4 on pro-inflammatory mediators in vascular endothelium

MCP-1, a member of the CC chemokine family, is known to be expressed in response to extracellular stimuli such as cytokines and platelet-derived growth factor (PDGF) and released by both endothelial cells and smooth muscle cells located in vessel wall (Braunersreuther et al.,
MCP-1 plays a crucial role in recruiting monocytes onto the vascular endothelium as well as transmigrating into the subendothelial layer of the vasculature. Previous studies have indicated the pivotal role of MCP-1 in the pathogenesis of atherosclerosis. For example, both MCP-1 mRNA and protein expression were detected in early atherosclerotic lesions (Nelken et al., 1991; Takeya et al., 1993). In addition, MCP-1 deficiency significantly reduced atherosclerosis in LDLR-deficient mice fed a high cholesterol diet. Deletion of MCP-1 receptor (CCR2) also markedly decreased atherosclerotic lesion formation in ApoE-deficient mice (Gosling et al., 1999; Gu et al., 1998). Furthermore, patients with coronary artery disease had significantly elevated MCP-1 serum levels (Martinovic et al., 2005). This evidence clearly indicates that MCP-1 is one of the contributing factors to atherosclerosis.

It has been reported that IL-4 induces the synthesis of MCP-1 mRNA and secretion of MCP-1 protein by human umbilical vein endothelial cells (HUVEC) (Lee et al., 2004; Lee and Hirani, 2006; Rollins and Pober, 1991). It also was found that production of MCP-1 was amplified in TNF-α- or IL-1-activated HUVEC when treated with IL-4 (Colotta et al., 1992; Paleolog et al., 1992). Additionally, recent studies by our group have demonstrated that IL-4 significantly up-regulates both mRNA and protein expression of MCP-1 in vascular endothelium in vitro and in vivo (Lee et al., 2010b).

Enhanced expression of adhesion molecules was found in early phase of atherosclerosis. Adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and VCAM-1 facilitate adherence of circulating blood leukocytes to vascular endothelium and their transendothelial migration. VCAM-1 is a member of the immunoglobulin gene superfamily first described as cytokine-inducible endothelial adhesion protein and is expressed primarily on endothelial cells (Osborn et al., 1989; Blankenberg et al., 2003). Evidence has indicated that
VCAM-1 is one of the most important adhesion molecules involved in leukocyte infiltration to atherosclerotic lesions. Increased levels of VCAM-1 expression have been detected on human arteries with atherosclerotic lesions as well as on established lesions in animal models of atherosclerosis (Davis et al., 1993; Nakashima et al., 1998). In addition, blockage of VCAM-1 by monoclonal antibody abrogated monocyte adhesion on early atherosclerotic endothelium (Huo et al., 2000). These data strongly support an important role of VCAM-1 in the development of atherosclerosis.

There are several reports demonstrating that IL-4 stimulates the adhesiveness of blood leukocytes to vascular endothelium via overexpression of VCAM-1. IL-4 increased endothelial cell adhesiveness for peripheral blood lymphocytes by up-regulating mRNA and protein expression of VCAM-1 in HUVEC (Galea et al., 1992; Galea et al., 1993). In addition, treatment of HUVEC with IL-4 and TNF-α synergistically induced VCAM-1 expression and resulted in enhanced adhesiveness of HUVEC to T lymphocytes (Paleolog et al., 1992). Moreover, IL-4 significantly and dose-dependently induced VCAM-1 expression in HUVEC and increased the adherence of human monocytes to HUVEC monolayer (Lee et al., 2004; Lee et al., 2001).

1.6.2. Effects of IL-4 on ROS-generating pathways in vascular inflammation

IL-4 may be considered as a pro-oxidative cytokine that can increase the oxidative potential of target cells (Park et al., 2008). For example, HUVEC treated with IL-4 induced the generation of ROS such as superoxide anion and hydrogen peroxide (Lee and Hirani, 2006). In addition, pre-treatment with antioxidant such as pyrrolidine dithiocarbamate (PDTC), N-acetylcysteine (NAC) or epigallocatechin gallate (EGCG) resulted in a marked attenuation of IL-
4-mediated overexpression of IL-6, MCP-1, and VCAM-1 in human vascular endothelial cells (Lee et al., 2001; Lee et al., 2003; Lee et al., 2010a; Lee et al., 2010b). These findings suggest that intracellular ROS generation is associated with IL-4-induced vascular inflammation.

ROS generating pathways including NADPH oxidase, mitochondrial electron transport chain, xanthine oxidase, and arachidonic acid metabolism are known to be involved in the signal transduction cascade of vascular inflammation and progression of atherosclerosis (Basta et al., 2005; Thomas et al., 2008). Recent studies have described the intracellular sources of IL-4-induced ROS generation in vascular endothelium. NADPH oxidase (NOX) is a key source of enzymatic generation of ROS in a variety of cell types including endothelial cells. In fact, gp91ds-tat, a selective NOX inhibitor, significantly attenuated ROS generation and MCP-1 overexpression in IL-4-stimulated HUVEC (Walch et al., 2006). In addition, pre-treatment of primary human aortic endothelial cells (HAEC) with other NOX inhibitors such as apocynin and diphenylene iodonium (DPI) significantly suppressed IL-4-induced ROS generation and overexpression of pro-inflammatory mediators such as IL-6 and MCP-1 (Lee et al., 2010a; Lee et al., 2010b). These studies suggest that NOX is involved in ROS generation and overexpression of pro-inflammatory mediators in IL-4-stimulated vascular endothelium.

It is widely accepted that mitochondria generate ROS during normal respiration. Indeed, mitochondrial electron transport chain is one of the major sources of intracellular ROS production in vascular endothelium (Basta et al., 2005; Thomas et al., 2008). Recent studies have shown that inhibitions of mitochondrial electron transport chain did not have any significant effects on IL-4-induced IL-6 and MCP-1 expression (Lee et al., 2010a; Lee et al., 2010b). These results suggest that mitochondrial electron transport chain-mediated ROS
generation is not involved in IL-4-induced expression of pro-inflammatory mediators in vascular endothelium.

Many studies have indicated that metabolic pathways of arachidonic acid (AA) by several enzymes, including cyclooxygenase (COX) and lipoxygenase (LOX), are associated with the generation of ROS in vascular endothelium (Thomas et al., 2008). However, the potential role of AA metabolism in IL-4-induced ROS generation and inflammation in vascular endothelium has not yet been investigated.

1.6.3. Signaling mechanisms of IL-4-induced pro-inflammatory pathways in vascular endothelium.

Previous studies have demonstrated that activation of mitogen-activated protein kinase (MAPK) induced by pro-inflammatory cytokines results in up-regulation of a wide variety of inflammatory genes, including cytokines, chemokines, and adhesion molecules (Davis, 1993; Seger and Krebs, 1995). There are three MAPK pathways; extracellular signal-regulated kinases (ERK-1/2), c-Jun N-terminal kinases/stress-activated protein kinases (JNK/ SAPKs), and p38 MAPK (Pearson et al., 2001). Much attention was given to p38 MAPK since it has been identified as an important signaling molecule in various inflammatory diseases (Kumar et al., 2003). Evidence indicates that p38 MAPK plays an important role in the regulation of the pro-inflammatory mediators such as chemokines and adhesion molecules. For example, MCP-1 and interleukin-8 (IL-8) production are mediated via p38 MAPK signaling pathway upon inflammatory stimuli in various cell types such as vascular smooth muscle cells, endothelial cells, and monocytes/macrophages (Hall et al., 2005; Westra et al., 2005). In addition, pre-treatment of
activated endothelial cells with p38 MAPK inhibitors significantly reduced the mRNA and protein expression of adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 (Ju et al., 2003; Westra et al., 2005). These findings suggest crucial role of p38 MAPK in vascular inflammation.

A growing body of evidence has indicated that p38 MAPK might be involved in the IL-4 signaling cascade. For example, IL-4 activates p38 MAPK in human primary monocytes, CT6 T-cell line and BA/F3 pro-B cells (Hunt et al., 2002). This indicates an important role of p38 MAPK in the IL-4-mediated signaling cascade which leads to up-regulation of pro-inflammatory mediators in vascular endothelium.

1.6.4. Involvement of 5-lipoxygenase in ROS generation, vascular inflammation and atherosclerosis

5-LOX is an enzyme that plays a key role in the biosynthesis of leukotrienes, which are pro-inflammatory mediators as well as ROS from arachidonic acid (Kim et al., 2008) (Figure 3). Several studies have demonstrated that there is a relationship between 5-LOX and atherosclerosis. 5-LOX was abundantly expressed in atherosclerotic lesions of ApoE- and LDLR-deficient mice as well as human atherosclerotic arteries (Zhao et al., 2004). In addition, alteration in 5-LOX gene in LDLR-deficient mice markedly reduced atherosclerotic lesions (Mehrabian et al., 2002). Moreover, an enhanced level of ROS by TNF-α in Jurkat cells was markedly decreased when the cells were pre-treated with 5-LOX inhibitor (Kim et al., 2008). Further, products of 5-LOX pathways, leukotrienes, were detected in atherosclerotic lesions in mice. Among them, LTB₄ is known as a potent chemoattractant and proinflammatory mediator (Werz and Steinhilber, 2006). Indeed, increased levels of LTB₄ were detected in atherosclerotic lesions in mice (Huang et al.,
These findings suggest the possible involvement of 5-LOX in the ROS generation and progression of atherosclerosis.

In resting cells, 5-LOX is localized in cytoplasm. Increased in Ca$^{2+}$ level and activation of p38 MAPK upon leukotriene biosynthesis stimuli activate 5-LOX (Werz, 2002). Upon activation, the non-heme iron in the 5-LOX is oxidized by lipid hydroperoxide from Fe$^{2+}$ to Fe$^{3+}$ and 5-LOX translocates to the nuclear membrane where cytosolic phospholipase A$_2$ (cPLA$_2$) and 5-LOX activating protein (FLAP) help 5-LOX in leukotriene biosynthesis. cPLA$_2$ liberates arachidonic acid from phospholipids and membrane-bound FLAP facilitates the transfer of arachidonic acid to 5-LOX (Werz and Steinhilber, 2006; Radmark and Samuelsson, 2005) (Figure 4).

Although a growing body of evidence has indicated the presence of IL-4 and 5-LOX in atherosclerotic lesions, their possible contribution to the ROS generation, and vascular inflammation in pathophysiological process of atherosclerosis remains largely unknown. In the present study, we hypothesize that 5-LOX is involved in IL-4-induced ROS generation and MCP-1 expression in vascular endothelium. In order to fully address this hypothesis, there were two main objectives: (1) To determine whether IL-4, as a pro-inflammatory cytokine, induces ROS generation and MCP-1 expression in vascular endothelium; (2) To elucidate the involvement of p38 MAPK and 5-LOX in IL-4-induced ROS generation in vascular endothelium.
Figure 3. Generation of ROS via 5-LOX pathway.

Figure 4. 5-LOX activation in the cell. Abbreviations: arachidonic acid (AA), five-lipoxygenase activating protein (FLAP), cytosolic phospholipase A2 (cPLA2), 5-lipoxygenase (5-LOX), leukotriene (LT), mitogen-activated protein kinase (MAPK).
2. Materials and Methods

2.1. Cell cultures

Primary human aortic endothelial cells (HAEC) were purchased from Cascade Biologics™ (Portland, OR) and cultured in Medium 200 supplemented with Low Serum Growth Supplement (LSGS) in a 37 °C, 5% CO₂/95% air, humidified cell culture incubator.

Figure 5. Micrographs of human aortic endothelial cells employed in the present study. (A) Phase-contrast microscopic image. (B) Fluorescence microscopic image showing calcein-labeled HAEC.
2.2. Animals

C57BL/6 male mice were purchased from Harlan (Indianapolis, IN). Animals were housed in facilities at Virginia Polytechnic Institute and State University. They were maintained in a controlled environment and subjected to a 12 hour light/dark cycle with food and water *ad libitum*. The mice were administered a single intraperitoneal injection of either PBS (control) or 100 µg/kg of IL-4, and exposed for 4, 8, and 24 hours. After the exposure, the aortas and blood plasma were collected and frozen at -80 °C until analyses. All experiments were performed with the approval of Virginia Tech Institutional Animal Care and Use Committee (IACUC).

2.3. Interleukin-4

Recombinant human and mouse IL-4 were purchased from R&D Systems Inc. (Minneapolis, MN) and dissolved in sterile PBS. Based on previous literature data from our group and others (Lee *et al.*, 2010a; Lee *et al.*, 2010b; Lee *et al.*, 2003; Barks *et al.*, 1997; Blease *et al.*, 1998; Lee *et al.*, 2001; Walch *et al.*, 2006; Wright *et al.*, 1999; Rollins and Prober, 1991), concentrations of 0.1-10 ng/ml and 100 µg/kg of IL-4 were employed for *in vitro* and *in vivo* studies, respectively.

2.4. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs from HAEC and mouse aortas were isolated using the RNeasy Mini Kit from Qiagen (Germantown, MD) and Trizol reagent from Invitrogen (Grand Island, NY) according to
the protocol of the manufacturer, respectively. One μg of total RNA was reverse transcribed at
25 °C for 15 min, 42 °C for 45 min, and 99 °C for 5 min in 20 μl of 5 mM MgCl₂, 10 mM Tris-
HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/μl of recombinant RNasin
ribonuclease inhibitor, 15 units/μg of AMV reverse transcriptase and 0.5 μg of random hexamers.
Amplification of individual genes was performed on the Applied Biosystems 7300 Real-Time
PCR System using TaqMan® Universal PCR Master Mix and a standard thermal cycler protocol
(50 °C for 2 min before the first cycle, 95 °C for 15 sec, and 60 °C for 1 min, repeated 45 times).
For specific probes and primers, TaqMan® Gene Expression Assay Reagents for human
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene), human MCP-1,
human 5-LOX, mouse GAPDH, and mouse MCP-1 were obtained from Applied Biosystems.
The threshold cycle (Cₜ) was determined from each well using the Applied Biosystems Sequence
Detection Software v1.2.3. Cₜ indicates the fractional cycle number at which the amount of
amplified target gene reaches a fixed threshold. Relative quantification, or the change in gene
expression from real-time quantitative PCR experiments between control and treatment groups,
was calculated by the comparative Cₜ method (Livak and Schmittgen, 2001). Data was analyzed
using equation $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = [C_T$ of target gene - $C_T$ of housekeeping gene] treated group -
[Cₜ of target gene - Cₜ of housekeeping gene] control group. Evaluation of $2^{-\Delta\Delta C_T}$ indicates the fold
change in gene expression, which is normalized to a housekeeping gene (GAPDH) and relative
to the control group.

2.5. **Enzyme-linked immunosorbent assay (ELISA)**
The protein expression levels of human MCP-1 in cell culture supernatants and mouse MCP-1 in mouse aortas and blood plasma were determined using MCP-1 Immunoassay Kit (R&D Systems) according to the protocol of the manufacturer. This assay employs the quantitative sandwich enzyme immunoassay technique using a murine monoclonal antibody against MCP-1 and a polyclonal secondary antibody conjugated with horseradish peroxidase.

2.6. siRNA transfection

Transient siRNA transfection was performed using Negative Control siRNA (Silencer® Negative Control siRNA) as well as 5-LOX siRNA-#1 and #2 (Silencer® Select siRNA) obtained from Applied Biosystems. Briefly, HAEC were seeded on 100-mm dishes (10ml/dish) and incubated for 72 h in 37 °C, 5% CO2/95% air, humidified cell culture incubator. 1,000 pmol siRNA was mixed with OPTI-MEM® I Reduced Serum Medium. The mixture was then combined with a solution of Lipofectamine RNAiMAX in OPTI-MEM® I Reduced Serum Medium. The mixture was then applied to each dish to make the final concentration of 100 nM siRNA. After incubation for 6 h at 37 °C in 5% CO2/95% air, humidified cell culture incubator, transfection solution was replaced with HAEC growth media. Cells were then incubated for an additional 24 h at 37 °C in 5% CO2/95% air, humidified cell culture incubator before analysis. Figure 6 depicts real-time RT-PCR analyses demonstrating that transfection of HAEC with 5-LOX siRNAs reduced mRNA expression levels of 5-LOX by 14% (5-LOX siRNA #1) and 70% (5-LOX siRNA #2) compared to those from scrambled siRNA-transfected HAEC (Negative control).
Based on these preliminary data, we employed siRNAs #2 to knockdown 5-LOX expression in the present study.

![Bar graph showing the reduction of 5-LOX mRNA levels by siRNAs](image)

**Figure 6.** 5-LOX siRNA #2 markedly reduced mRNA expression levels of 5-LOX in HAEC.

2.7. **DHE and DCF fluorescence staining**

The intracellular levels of ROS such as superoxide anion and hydrogen peroxide were measured by DHE and DCF fluorescence staining using a Zeiss AXIO Imager A1m fluorescence microscope equipped with AxioCam MRc5 Digital Imaging System (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Briefly, HAEC were grown on the glass slide in the Lab-Tek® II Chamber Slide™ System (Nalge Nunc International Corp., Naperville, IL). After treatment with
IL-4, the cells were loaded with either dihydroethidium (DHE) or dichlorofluorescin diacetate (carboxy-H2DCF-DA) (Invitrogen Corp., Carlsbad, CA) at concentration of 5 µM in PBS for 30 min at 37 ºC in 5% CO2/95% air, humidified cell culture incubator. HAEC monolayers were washed with PBS and examined on a Zeiss AXIO Imager A1m fluorescence microscope. Cell images were acquired with 20x objective by AxioCam MRc5 Digital Imaging System. The DHE or DCF fluorescence intensity of acquired digital images was quantified by MATLAB® Imaging Processing Software (The MathworksTM, Natick, MA). Data were expressed in fluorescence arbitrary units (x10^9). The imaging software, Image J, was also utilized to quantify relative fluorescence intensity of acquired digital images.

2.8. Statistical analysis

Quantitative data was obtained and analyzed using SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA). One-way ANOVA was used to compare mean responses among the treatments. For each endpoint, the treatment mean was compared using Bonferroni least significant difference procedure. Data was presented as means ± SEM of at least three independent determinations, and statistical probability of p < 0.05 is considered significant.
3. Results and discussion

3.1. IL-4 induces ROS generation in vascular endothelium

It is widely known that ROS play a critical role in the development of atherosclerosis by up-regulating the expression of pro-inflammatory mediators such as cytokines, chemokines, and adhesion molecules in vascular endothelium (Bouloumìe et al., 1999; Hennig et al., 1996). In order to examine whether IL-4 induces ROS generation in vascular endothelial cells, DHE and DCF fluorescence staining were employed to measure levels of intracellular ROS in IL-4-stimulated HAEC. When DHE is oxidized to ethidium by superoxide anion, it intercalates within the cell’s DNA and stains its nucleus a bright fluorescent red (Lee et al., 2010a). Dichlorofluorescin diacetate (H₂DCF-DA) is a stable, non-polar compound that readily diffuses into the cells and is converted to a non-fluorescent polar derivative dichlorofluorescin (DCF-H) by intracellular esterases. DCF-H can be oxidized to the highly fluorescent compound DCF by hydrogen peroxide or other peroxides produced by the cells (Lee et al., 2010a). As illustrated in Figure 7 and Figure 8, the levels of both DHE and DCF fluorescence were markedly and time-dependently increased after exposure of HAEC to 10 ng/ml of IL-4. The quantitative analysis of DHE (Figure 7E) and DCF (Figure 8E) fluorescence staining further elucidates the involvement of IL-4 in the production of ROS in vascular endothelium. There was a significant increase of ROS generation in vascular endothelial cells in both DHE and DCF fluorescence staining after
incubation with 10 ng/ml of IL-4. These results demonstrated the possible involvement of IL-4 in the generation of ROS in vascular endothelium.

3.2. IL-4 induces MCP-1 expression in vascular endothelium in vitro and in vivo.

Since MCP-1, has been shown to play an important role in inflammatory responses in vascular endothelium by recruiting monocytes onto the vascular endothelium as well as transmigrating into the subendothelial layer, the effects of IL-4 on the mRNA and protein expression of MCP-1 were explored in vitro and in vivo. Quantitative real-time RT-PCR showed a significant and dose-dependent up-regulation of mRNA expression of MCP-1 when HAEC were treated with 0.1, 1.0, and 10 ng/ml of IL-4 (Figure 9A). Consistent with the data on gene expression, exposure of HAEC to IL-4 resulted in a significant and dose-dependent up-regulation of MCP-1 protein expression (Figure 9B). In addition, animal experiments were performed to further verify the fact that IL-4 induces MCP-1 expression in vascular endothelium. Mice were administered a single intraperitoneal injection of either PBS (control) or 100 µg/kg of IL-4, and exposed for 4, 8, and 24 h. As shown in Figure 10A and Figure 10B, real-time RT-PCR and ELISA analyses demonstrated a significant up-regulation of MCP-1 mRNA and protein expression in mouse aortas. It also was found that markedly increased expression of MCP-1 protein levels were observed in blood plasmas collected from IL-4-injected mice (Figure 10C). These results provide solid evidence that IL-4 induces MCP-1 mRNA and protein expression in vascular endothelium in vitro and in vivo.
3.3. 5-LOX mediated IL-4-induced ROS generation and MCP-1 expression in vascular endothelium

It has been proposed that metabolic pathways of arachidonic acid by several enzymes, including cyclooxygenase (COX) and lipoxygenase (LOX), are associated with the generation of ROS (Thomas et al., 2008). To determine whether arachidonic acid metabolism is involved in IL-4-induced MCP-1 expression in human vascular endothelial cells, HAEC were pre-treated with several pharmacological inhibitors, such as eicosatetraynoic acid (ETYA; 10 and 50 µM), nordihydroguaiaretic acid (NDGA; 1.0 and 10 µM), and N-2-(cyclohexyloxy)-4-nitrophenylmethanesulfonamide (NS-398; 0.1 and 1.0 µM), for 30 min and then incubated with either PBS (control) or 10 ng/ml of IL-4 for 16 h. As indicated in Figure 11A, ETYA, a combined inhibitor of COX and LOX, significantly attenuated IL-4-induced MCP-1 protein expression in HAEC. To verify whether this effect was caused by inhibition of either COX or LOX, more selective inhibitors for COX and LOX were employed. As shown in Figure 11A, NDGA, a selective 5-LOX inhibitor, significantly decreased IL-4-induced MCP-1 protein expression levels in HAEC. The involvement of 5-LOX in IL-4-induced MCP-1 expression in HAEC was further investigated by using real-time RT-PCR. MCP-1 mRNA levels in IL-4-stimulated HAEC were significantly and dose-dependently attenuated by pre-treatment with NDGA (Figure 11B). Additionally, pre-treatment of HAEC with NDGA at 10 µM significantly suppressed IL-4-induced ROS generation as assessed by DHE and DCF fluorescence staining (Figure 12 and Figure 13). On the other hand, NS-398, a selective inhibitor of COX-2, did not exert any significant effect (Figure 11A), indicating COX-2 is not associated with IL-4-induced MCP-1 expression in vascular endothelial cells. Moreover, RNA interference (RNAi) gene silencing
technique using 5-LOX siRNA were employed to further examine the critical role of 5-LOX in IL-4-induced MCP-1 expression in human vascular endothelial cells. As depicted in Figure 14, IL-4 resulted in a significant attenuation in MCP-1 protein expression in the 5-LOX siRNA-transfected HAEC compared to the cells transfected with negative control siRNA with nucleotide sequences that do not target any gene product. These results from both pharmacological and genetic approaches provide robust evidence that 5-LOX plays a pivotal role in IL-4-induced ROS generation and MCP-1 expression in human vascular endothelial cells.

3.4. p38 MAPK mediates IL-4-induced ROS generation in vascular endothelium

Emerging evidence has suggested that one of the mitogen-activated protein kinase (MAPK) signaling pathways, p38 MAPK, is involved in the IL-4 signaling cascade (Hunt et al., 2002). To assess whether IL-4-induced ROS generation in vascular endothelium is mediated by p38 MAPK signaling pathway, HAEC were pre-treated with three complementary pharmacological inhibitors of p38 MAPK such as SB202190, SB203580, and PD169316, and then incubated with IL-4. Intracellular levels of ROS were determined by DHE and DCF fluorescence staining, respectively. As illustrated in Figure 15 and Figure 16, inhibitions of p38 MAPK by SB202190 (C), SB203580 (D), and PD169316 (E) completely abolished IL-4-induced ROS production in HAEC. These results indicate the potential role of p38 MAPK signaling pathway in IL-4-induced ROS generation in vascular endothelium.
Figure 7. IL-4 induces ROS (superoxide anion) in HAEC in a time-dependent manner. (A-D) The representative fluorescence microscopic images showing intracellular levels of superoxide anion induced by IL-4 in HAEC (n=4). The cells were treated with either PBS (A) or 10 ng/ml of IL-4 for 15 (B), 30 (C), and 60 (D) min. Intracellular levels of ROS in HAEC were determined by DHE fluorescence staining as described in Materials and Methods. (E) The fluorescence intensity quantified by MATLAB. ROS values are in arbitrary units of fluorescence intensity (x10^9). Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05).
Figure 8. IL-4 induces ROS (hydrogen peroxide and other peroxides) in HAEC in time-dependant manner. (A-D) The representative fluorescence microscopic images showing intracellular levels of hydrogen peroxide and other peroxides induced by IL-4 in HAEC (n=4). The cells were treated with either PBS (A) or 10 ng/ml of IL-4 for 15 (B), 30 (C), and 60 (D) min. Intracellular levels of ROS in HAEC were determined by DCF fluorescence staining as described in Materials and Methods. (E) The fluorescence intensity quantified by MATLAB. ROS values are in arbitrary units of fluorescence intensity (x10^9). Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05).
Figure 9. IL-4 up-regulates MCP-1 expression in human vascular endothelial cells. HAEC were incubated with either PBS (control) or indicated concentrations of IL-4 for 4 h (A) or 16 h (B). The mRNA and protein expression levels of MCP-1 were determined by real-time RT-PCR (A) and ELISA (B) as described in Materials and Methods. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05).
Figure 10. IL-4 up-regulates MCP-1 expression in mice. Animals were administered a single intraperitoneal injection of either PBS (control) or 100 µg/kg of IL-4, and exposed for 4, 8, and 24 h. The mRNA expression levels of MCP-1 in mouse aortas were determined by real-time RT-PCR (A). Protein expression levels of MCP-1 in mouse aortas (B) and mouse blood plasma(C) were determined by ELISA as described in Methods and Materials. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05).
Figure 11. A selective 5-LOX inhibitor, NDGA, attenuates IL-4-induced mRNA and protein expressions of MCP-1. HAEC were pre-treated with arachidonic acid metabolism inhibitors, such as ETYA (10 and 50 µM), NDGA (1.0 and 10 µM), and NS-398 (0.1 and 1.0 µM), for 30 min and then incubated with either PBS (control) or 10 ng/ml of IL-4 for 16 h (A). HAEC were pre-treated with (NDGA; 1.0 and 10 µM) for 30 minutes and incubated with either PBS (control) or 10 ng/ml of IL-4 for 4 hours (B). The protein and mRNA expression levels of MCP-1 were determined by ELISA and real-time RT-PCR, respectively, described in Materials and Methods. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05). #Statistically significant compared with IL-4 treated group (p < 0.05).
Figure 12. NDGA attenuates IL-4-induced ROS (superoxide anion) production in HAEC. (A-D) The representative fluorescence microscopic images showing on intracellular levels of superoxide anion induced by IL-4 in HAEC (n=4). The cells were pre-treated with 10 µM NDGA for 30 min and incubated with 10 ng/ml of IL-4 for 1 h. Intracellular levels of ROS were determined by DCF fluorescence staining as described in Materials and Methods. (E) The fluorescence intensity quantified by Image J. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05). #Statistically significant compared with IL-4 treated group (p < 0.05). A. Control; B. NDGA, 10 µM; C. IL-4, 10 ng/ml; D. NDGA, 10 µM + IL-4, 10 ng/ml.
Figure 13. NDGA attenuates IL-4-induced ROS (hydrogen peroxide and other peroxides) production in HAEC. (A-D) The representative fluorescence microscopic images showing intracellular levels of hydrogen peroxide and other peroxides induced by IL-4 in HAEC (n=4). The cells were pre-treated with 10 µM NDGA for 30 min and incubated with 10 ng/ml of IL-4 for 1 h. Intracellular levels of ROS were determined by DCF fluorescence staining as described in Materials and Methods. (E) The fluorescence intensity quantified by Image J. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05). #Statistically significant compared with IL-4 treated group (p < 0.05).
A. Control; B. NDGA, 10 µM; C. IL-4, 10 ng/ml; D. NDGA, 10 µM + IL-4, 10 ng/ml.
Figure 14: 5-LOX siRNA attenuates IL-4-induced MCP-1 expression in HAEC. MCP-1 protein expression levels were determined by ELISA as described in Materials and Methods. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05).
Figure 15. p38 MAPK inhibitors attenuate IL-4-induced ROS (superoxide anion) production in HAEC. (A-E) The pictures representative fluorescence microscopic images showing intracellular levels of superoxide anion in HAEC (n=4). The cells were pre-treated with 10 µM p38 MAPK inhibitors, such as SB202190, SB203580, and PD169316 for 30 min and incubated with 10 ng/ml of IL-4 for 1 h. Intracellular levels of ROS were determined by DHE fluorescence staining as described in Materials and Methods. (F) The fluorescence intensity quantified by Image J. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05). #Statistically significant compared with IL-4 treated group (p < 0.05). A. Control; B. IL-4 10 ng/ml; C. SB202190, 10 µM + IL-4, 10 ng/ml; D. SB203580, 10 µM + IL-4, 10 ng/ml; E. SB169316, 10 µM + IL-4, 10 ng/ml.
Figure 16. p38 MAPK inhibitors attenuate IL-4-induced ROS (hydrogen peroxide and other peroxides) production in HAEC. (A-E) The pictures representative fluorescence microscopic images showing intracellular levels of hydrogen peroxide and other peroxides in HAEC (n=4). The cells were pre-treated with 10 µM p38 MAPK inhibitors, such as SB202190, SB203580, and PD169316 for 30 min and incubated with 10 ng/ml of IL-4 for 1 h. Intracellular levels of ROS were determined by DHE fluorescence staining as described in Materials and Methods. (F) The fluorescence intensity quantified by Image J. Values represent means ± SEM (n=4). *Statistically significant compared with control group (p < 0.05). #Statistically significant compared with IL-4 treated group (p < 0.05). A. Control; B. IL-4 10 ng/ml; C. SB202190, 10 µM + IL-4, 10 ng/ml; D. SB203580, 10 µM + IL-4, 10 ng/ml; E. SB169316, 10 µM + IL-4, 10 ng/ml.
4. Conclusion

Atherosclerosis is the underlying cause of cardiovascular diseases including heart attack and stroke. Since atherosclerosis is becoming a major public health problem and the exact cause of this disease still remains unclear, elucidating the complex cellular and molecular mechanisms involved in atherosclerosis is highly significant.

Evidence indicates that the common denominator for cardiovascular risk factors such as hypercholesterolemia, hypertension, diabetes mellitus, obesity, and cigarette smoking is ROS generation. Overproduction of ROS and induction of pro-inflammatory mediators are associated with progression of atherosclerosis. Among those pro-inflammatory mediators, IL-4 has gained attention recently. Although IL-4 was traditionally believed to be an anti-inflammatory cytokine, growing body of evidence has raised the possibility that IL-4 may play an important role in the initiation and progression of atherosclerosis via induction of oxidative stress-mediated pro-inflammation environments in vascular endothelium.

The present study focused on the pro-oxidative and pro-inflammatory effects of IL-4 as well as the involvement of 5-LOX in IL-4-induced ROS generation and MCP-1 expression in vascular endothelium. Both real-time RT-PCR and ELISA analysis clearly showed that IL-4 significantly up-regulates mRNA and protein expression of MCP-1 in vascular endothelium. In addition, DHE and DCF fluorescence staining demonstrated that IL-4 increases ROS production in human vascular endothelial cells. We have also provided the first novel evidence that 5-LOX, one of the enzymes associated with arachidonic acid metabolism, is responsible for the IL-4-induced ROS generation and MCP-1 expression in human vascular endothelial cells. Furthermore, the potential involvement of p38 MAPK signaling pathway in IL-4-induced ROS
generation in human vascular endothelial cells was demonstrated. These data strongly suggest that 5-LOX mediates IL-4-induced ROS generation and MCP-1 expression in vascular endothelium (figure 17).

Even though it has been implicated the involvement of both Th1 and Th2 cells in the progression of atherosclerosis, most previous studies have focused mainly on Th1 cell-dependent mechanisms in the process. However, little is known concerning the possible contribution of Th2 cytokines to the vascular inflammation and atherosclerosis. Therefore, the present study provided an excellent balance to the more extensive studies on Th1 cell responses in the pathophysiological processes of atherosclerosis. Elucidation of novel cellular and molecular signaling mechanisms by which IL-4, a Th2 cytokine, induces oxidative stress and inflammation in vascular endothelium will contribute to new early, preventative therapies for atherosclerosis.

Future work will include (1) elucidation of the critical role of 5-LOX in IL-4-induced ROS generation and MCP-1 expression in vivo using 5-LOX knockout mice, (2) verification of 5-LOX-mediated LTB4 production in IL-4-stimulated human vascular endothelial cells, and (3) investigation of the mechanistic links among p38 MAPK signaling pathway, 5-LOX, ROS generation, MCP-1 expression in vascular endothelium in vitro and in vivo.
Figure 17: Schematic diagram illustrating the molecular signaling mechanisms of IL-4-induced vascular inflammation and development of atherosclerosis.


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Superoxide production and expression of Nox family proteins in human atherosclerosis. 
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