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Biosynthetic pathways of pro-resolving lipid mediators in vascular cells

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BIOSYNTHETIC PATHWAYS OF PRO-RESOLVING LIPID MEDIATORS IN VASCULAR CELLS

by

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ABSTRACT

Introduction: Specialized pro-resolving lipid mediators (SPM) such as resolvin-D1 (RvD1) act to resolve vascular inflammation and may guard against the progression of restenosis following cardiovascular interventions. Stimulating synthesis of these mediators directly in vascular cells may increase their local availability, and thus, protect against restenotic injury. However, the ability of endothelial (EC) and vascular smooth muscle cells (VSMC) to produce SPMs from their polyunsaturated fatty acid precursor decosahexaenoic acid (DHA) via lipoxygenase (LO) enzymatic transformation remains unknown. We sought to determine whether vascular cells produce SPMs from DHA and, if they do, how inflammation and mechanical injury of the vasculature alter biosynthesis.

Methods: Primary cultures of human saphenous vein endothelial and smooth muscle cells were treated with DHA in cell culture media (+10% serum) for 4h-24h. Freshly dissected rabbit aorta was incubated intact or following gentle endothelial denudation in cell culture media (+10% serum) with or without DHA for 48h. SPM levels in media were quantified by LC-MS/MS and ELISA and lipoxygenase expression and localization were assessed by western blotting and immunofluorescence staining, respectively.
Results: EC and SMC receiving media without DHA did not synthesize SPMs within the detection limits of the assay, whereas DHA treatment produced 17-HDHA, 14-HDHA, Mar1, RvD5, RvD2, and a dose and time-dependent increase in RvD1 production in EC (10.1 ±1.0 pg for 1000nM at 24h) and SMC (7.4 ± 0.2 pg for 1000nM at 24h). Intact rabbit aorta incubated in DHA\(^+\) media produced 0.24 ± 0.05 pg RvD1/mg tissue whereas aorta incubated in DHA\(^-\) media produced 0.13 ± 0.007 pg RvD1/mg tissue. Moreover, EC-denuded aortas produced less RvD1/mg tissue than intact aortas. 5-LO was expressed in both cell types, however DHA induced 5-LO expression in EC (1.3 fold -DHA) but not in SMC. DHA promoted a nuclear to cytoplasmic shift of 5-LO in both EC and SMC. Finally, TNF-\(\alpha\) stimulated an increase in RvD1 production in EC.

Conclusions: Human vascular cells and rabbit vascular tissue can biosynthesize SPMs de novo from their precursor DHA, signifying a new source of SPMs in the vasculature.
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# LIST OF ABBREVIATIONS

- **AA**: Arachidonic Acid
- **DES**: Drug Eluting Stent
- **DHA**: Docosahexaenoic Acid
- **EC**: Endothelial Cell
- **EPA**: Eicosapentaenoic Acid
- **IL**: Interleukin
- **LC-MS/MS**: Liquid Chromatography/Tandem Mass Spectrometry
- **LX**: Lipoxin
- **MaR**: Maresin
- **MMP**: Metalloproteinase
- **PD**: Protectin
- **PKA**: Protein Kinase A
- **PUFA**: Polyunsaturated Fatty Acid
- **RvD**: Resolvin
- **SPM**: Specialized Pro-resolving Lipid Mediator
- **TNF**: Tumor Necrosis Factor
- **VSMC**: Vascular Smooth Muscle Cell
- **17-HDHA**: 17-hydroxy-DHA
- **14-HDHA**: 14-hydroxy-DHA
- **15-LO**: 15-Lipoxygenase
- **12-LO**: 12-Lipoxygenase
5-LO .................................................................................................................. 5-Lipoxgenase
INTRODUCTION

Cardiovascular disease is the leading cause of mortality in the US, accounting for 600,000 deaths per year, and is on the verge of becoming the most common medical concern worldwide. Cardiovascular risk factors such as hypertension, diabetes, smoking and advancing age contribute to the development of atherosclerotic plaques, which cause narrowing of the cardiac and systemic vasculature. This manifests clinically as angina pectoralis and myocardial infarctions in the coronary system, transient ischemic attacks and ischemic strokes in the cerebral vascular system, and claudication and loss of limb in the peripheral vascular system. Atherosclerosis is a chronic and progressive disease which often requires surgical or endovascular intervention.

Although these interventions are initially effective in reintroducing circulation to obstructed arteries, they cause inflammation within the vessel wall. Inflammation is initially necessary for healing post-intervention. However, in excess it causes pathological changes within the vessel. The end product of these changes is neointimal hyperplasia, which causes restenosis. Thus, the interventions aimed at treating obstructed arteries often themselves lead to obstruction of the blood vessel.

Whereas we previously thought resolution of inflammation was a passive process, we now know that specialized lipid mediators play an active role in resolution. These appropriately named specialized pro-resolving lipid mediators (SPMs) are essential to avoiding neointimal hyperplasia and the associated clinical consequences of restenosis. There has recently been evidence that restenosis can be viewed as a “resolution deficit”
within the vessel wall.\textsuperscript{9} However, little is known about the biosynthetic pathways of SPMs in vascular cells. This knowledge would be of great clinical significance, as it would open the door for new therapeutic strategies.

We set out to establish SPM production in human endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), investigate the molecular pathways through which SPMs are produced in these cells, assess production of SPMs after administration of a precursor lipid, and define the effect of inflammation on SPM biosynthesis. By achieving these aims, we hope to ultimately introduce the foundation for new approaches to avoiding restenosis after vascular intervention.

**Atherosclerosis Pathophysiology**

Atherosclerosis is a chronic inflammatory disease of the vasculature characterized by lipid insudation and eventual fibrous accumulation in the intimal layer of large arteries.\textsuperscript{10,11} In the past atherosclerotic lesions, or atheromas, were thought to be composed solely of lipid stores, but it is now understood that chronic inflammation and the immune response play a critical role in atherogenesis.\textsuperscript{12} In fact, T cells, macrophages, and mast cells form major components of atheromas.\textsuperscript{13–15} Connective tissue elements, endothelial cells, smooth muscle cells, lipids, and debris constitute the remaining portion of the lesion.\textsuperscript{13}

The progression of atherosclerosis is a dynamic process dependent on the interplay of multiple inflammatory factors. When LDL cholesterol accumulates in the arterial intima and is subsequently oxidized through free radical and enzymatic reactions,
there is a release of oxidized phospholipids.¹⁶ In conjunction with a pattern of hemodynamic flow, these phospholipids induce the overexpression of intercellular adhesion molecules (eg. VCAM-1) in endothelial cells.¹⁹ Overexpression of adhesion proteins produces a steep rise in intimal macrophage count due to the increased rate of diapedesis and maturation of entering monocytes. Once in the vessel, macrophages phagocytose oxidized LDL via their scavenger receptors.¹⁷ Foam cells that are characteristic of atherosclerotic lesions are subsequently formed when enzymatic action fails to digest the internalized LDL as it accumulates in the cytosol.¹¹

As this process continues, a visible collection of intimal foam cells, called a fatty streak, forms. Although often asymptomatic and present universally in the general population after the first decade, fatty streaks precede atheroma formation.¹³ As foam cells accumulate beneath the endothelium, they initiate a cascade of specific cell reactions and release various cytokines, including Tumor necrosis factor alpha (TNF-α) and Interleukins such as (IL-1β), that initiate lesion formation.¹⁸ These cytokines further induce the expression of chemotactic and endothelial cell adhesion molecules which recruit additional monocytes to the forming lesion.¹⁹ Thus, the initiation of atherogenesis produces a cyclical chain of events that furthers growth of the fatty streak.

Atherosclerotic plaque formation caused by vascular smooth muscle cell proliferation and migration, fibrous accumulation, and calcification within the intima distinguishes atherosclerotic lesions from precursor fatty streaks. Various enzymatic products released from foam cells start a cascade of events, beginning with the activation of endothelial growth factor receptor by oxidative stressors.²⁰,²¹ Epidermal growth factor
proceeds to activate specific matrix metalloproteinases (MMP), which cleave the cell to cell adhesion molecule N-cadherin in the extracellular matrix.\textsuperscript{22} This frees VSMCs to migrate freely from the media to the intima along the chemotactic gradient established by cytokines released from the same foam cells. Once in the intima, VSMC cell proliferation, fibrosis, and calcium deposition forms a solid plaque.\textsuperscript{23} As the plaque increases in size, it extends into the arterial lumen and diminishes blood flow.

Occasionally, atherosclerotic plaques may rupture, contributing to coronary thrombosis. Such an event occurs as a direct action of the cytokines, proteases, and free radicals produced from the activated foam cells and T cells.\textsuperscript{24} These molecules, particularly the MMPs, destabilize fibrous plaques through digestion of the collagenous extracellular matrix and the basement membrane of endothelial cells.\textsuperscript{25} Such degeneration of connective tissue support leaves the atheroma weak and increasingly susceptible to physical damage. Upon rupture, the plaque’s fibrous core, which contains tissue factor, becomes exposed to circulating coagulation proteins and initiates thrombus formation.\textsuperscript{10} Formation of intraarterial thrombus may cause acute ischemia to end-organs, distally.\textsuperscript{26,27}

**Atherosclerosis Treatment**

**Pharmacotherapy**

Various noninvasive options exist that are aimed at blocking pro-inflammatory pathways responsible for the progression of the disease. Management of risk factors such as hypertension and diabetes with lifestyle modification and medications remain crucial in the treatment for atherosclerotic disease. Statins and COX-2 inhibitors such as aspirin
are also commonly employed as pharmacologic agents.\textsuperscript{28,29} Other more specific agents that has shown promise against atherogenesis are TNF-alpha inhibitors and vaccination with oxidized LDL or specific heat shock proteins.\textsuperscript{30} However, similar to other agents, these novel approaches are considered preventative and unable to sufficiently limit atherosclerosis pathology following progression of atherogenesis and thrombosis. Despite maximal medical therapy, atherosclerosis remains a progressive disease.

**Interventional**

In cases of severe or symptomatic stenosis, various interventional measures can be taken to alleviate atherosclerosis. These include endovascular techniques such as angioplasty and stent placement as well as surgical procedures such as endarterectomy and bypass grafting. With the increasing prevalence of athereosclerotic disease, such procedures produce a major strain on the healthcare system. In the United States, costs associated with vascular intervention is in excess of $21 billion annually, with a large portion of this burden being from reintervention needed for restenosis.\textsuperscript{31,32}

The problem of restenosis is in large part due to inflammation caused by the procedures themselves. Both endovascular and open surgical interventions cause inflammation to the vessel wall via stretch of the vessel wall from angioplasty, foreign body reaction to intravascular stents or surgical manipulation of the vasculature. This inflammation is initially necessary for vascular healing. However, when persistent or in excess it causes vascular smooth muscle cell migration from the tunica media to the tunica intima followed by proliferation and secretion of extracellular matrix within the
intima. The end-product of this process is neointimal hyperplasia and associated restenosis.

Initiated by biomechanical injury of the endothelium, this process begins when the subendothelium is exposed to blood flow. This results in platelet adhesion and aggregation to the injured segment. Activated upon adhesion, platelets release cytokines, including TNF-α, IL-1β, and MCP-1, which recruit leukocytes to the source of vascular damage. These inflammatory cells produce cellular products that induce further recruitment of leukocytes and initiate the migration and proliferation of VSMCs and adventitial fibroblasts. Within a week to a month following endovascular or surgical intervention, normally inactive and contractile VSMCs become activated and migrate from the medial layer of the artery into the neointima. Proliferation of VSMCs and remodeling of the extracellular matrix in the vessel wall caused by MMP-9 further accelerates this process. Ultimately, the neointimal hyperplasia resulting from the accumulation of VSMCs and extracellular matrix along the intimal wall causes luminal narrowing. Due to diminished blood flow, restenosis limits the long-term benefits of vascular procedures. For example, angioplasty of the femoral artery results in patency rates below 40% after one year. Durability of stenting and bypass is only slightly higher.

Drug eluting stents (DES), when inserted at the sight of intervention, have arisen as a possible tool for limiting vascular inflammation and protecting against neointimal hyperplasia and restenosis. Currently used DES agents, including paclitaxel and sirolimus, have been shown to inhibit VSMC proliferation and extracellular matrix
formation due to their antimitotic actions.\textsuperscript{46,47} However, the non-specific actions of these cytotoxic agents also inhibits reendothelialization by endothelial cells, a normally protective process after injury. The chronically exposed subendothelium perpetuates chronic inflammation and adhesion of platelets and coagulation factors, which can lead to thrombosis and luminal narrowing at any time.\textsuperscript{48–51} Thus, DES insertion may not only fail to regulate vascular inflammation but cause additional damage. Because of these complications, no absolute pharmacotherapeutic options currently exist to improve the results of surgical revascularization procedures in the treatment of atherosclerosis.

**Lipid Mediators and the Resolution of Vascular Inflammation**

**Resolution of Acute Inflammation**

Acute inflammation plays a central role in response to vascular injury and is necessary for tissue repair. However, the resolution of inflammation is just as critical as its initiation to prevent scarring, tissue damage, and predisposition for thrombosis that may result from persistence of leukocytes and the other cells they recruit. In the intima of the vasculature, resolution of inflammation is essential in limiting neointimal hyperplasia and stenosis. It is the failure of resolution that leads to the pathological progression of atherosclerosis and the low patency of interventional treatments.

It is important to note that suppression of inflammation is not the same as resolution. Targeting the very early stages of the inflammatory response is not sufficient for preventing chronic inflammation in vessels already under pathologic distress. In such cases, leukocytes already present in the tissue persist in releasing cytokines involved in
recruitment of other leukocytes, migration of VSMCs, and fibrosis. Furthermore, developing neointimal hyperplasia cannot subside independently, even in the absence of an active inflammatory response. These inflammatory processes must be resolved to allow the restoration of the inflamed vessel to its normal status.

Though until recently considered a passive process, the discovery of specialized pro-resolving lipid mediators derived from polyunsaturated fatty acids (PUFA) has suggested that resolution of acute inflammation may instead be actively regulated. The four classes of SPMs that mediate this process include the lipoxins (LXs), derived from ω-6 PUFA arachidonic acid (AA), and the resolvins (RvDs), protectins (PDs), and maresins (MaRs) derived from the ω-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These molecules were discovered through the biochemical analysis of exudates produced in self-limited models of sterile inflammation. In this model, an air filled cavity was produced under the skin that remained sterile until injection of proinflammatory stimuli that initiated acute inflammation. Inflammation was shown to spontaneously resolve, and analysis of the exudates using a lipidomic approach and liquid chromatography–tandem mass spectrometry (LC-MS/MS) revealed the presence of novel lipid mediators in the resolving exudate.

SPMs, have displayed anti-inflammatory and pro-resolving effects in a variety of animal models, including sepsis, peritonitis, colitis, retinopathy, and periodontal disease. Whereas many molecules exist that are capable of suppressing the inflammatory response, SPMs are unique in their ability to stimulate the resolution stage
involved in returning inflamed tissue to homeostasis. In this respect, SPMs are of particular interest for their pro-resolving affects in a vascular setting.

**Anti-inflammatory and Pro-resolving Actions of SPMs**

Before delving into the unique pro-resolving affects of RvDs and other SPMs in vascular injury, it is important to mention that the initiation of inflammation begins with the production of arachidonic acid derivatives. These pro-inflammatory lipid mediators include prostaglandins such as PGE\textsubscript{2} and PGI\textsubscript{2} involved in vasodilation and leukotrienes such as LTB\textsubscript{4} involved in chemotaxis and adhesion of polymorphonuclear neutrophils.\textsuperscript{63,64} In concert with other cytokines and complement proteins, these molecules set the stage for leukocyte migration into tissue.

The anti-inflammatory affects of SPMs begin through attenuation of this initial leukocyte infiltration by their omega-3 fatty acid precursors. EPA and DHA compete with AA as substrates for the lipoygenases involved in eicosanoid production, limiting production of prostaglandins and leukotrienes and promoting synthesis of SPMs in a process called “class-switching” (Figure 1).\textsuperscript{65} Furthermore, EPA and DHA embedded in the membranes of leukocytes significantly alter membrane phospholipid composition and limit fluidity, negatively altering leukocyte activation, proliferation, and cytokine release.\textsuperscript{66} Thus, these precursors act to block the inflammatory process in its early stages.
Once the inflammatory process progresses, SPMs prevent the infiltration of circulating leukocytes into the vessel wall. In endothelial cells, LXs and RvDs retard the permeability of vessels to neutrophils by down regulating cell adhesion molecules such as VCAM-1. Similarly, RvD1 reduces production of potent inflammatory cytokines such as MCP-1 and PDGF-B. Such affects prevent inflammatory cells from gaining access to and disrupting tissue composition and function. This might be especially important in the context of atherosclerosis, where limiting the recruitment of phlogistic monocytes prevents accumulation of foam cells and the resulting development of plaque.

SPMs also act to clear leukocytes that have already infiltrated vascular tissue. This process begins by signaling for the elimination of granulocytes already at the site of

Figure 1. Temporal progression of lipid mediator class switching. Following pathological insult, eicosanoids are essential in initiating the inflammatory response. Prostaglandins and leukotrienes signal for neutrophil passage into the tissue as part of the standard vascular response. The acute inflammatory response terminates with lipid mediator class switching, as LXs and RvDs recruit nonphlogistic monocytes, which clear apoptotic neutrophils through efferocytosis. Resolution returns inflamed tissue to homeostasis.119
injury through their phagocytosis by macrophages. SPMs stimulate the nonphlogistic recruitment of monocytes, which transform into macrophages programmed to clear apoptotic neutrophils.\textsuperscript{69,70} The clearance of leukocytes indirectly mediates further anti-inflammatory and pro-resolving actions of the inflamed tissue. Uptake of apoptotic neutrophils causes macrophages to release TGF-\(\beta\)1, an anti-inflammatory cytokine that decreases signaling of toll-like receptors on leukocytes, reducing their activity.\textsuperscript{71}

Interestingly, at the site of inflammation, the process of neutrophil apoptosis is itself regulated and may be induced by RvDs and LXs which override the NF-\(\kappa\)B and I\(\kappa\)-B\(\kappa\) inflammatory pathways.\textsuperscript{72} This also results in the inhibition of TNF, IL-1, IL-6, IL-8 and IL-12 production in endothelial cells and monocytes.\textsuperscript{73} Thus, as SPMs initiate resolution by stimulating neutrophil apoptosis and their nonphlogistic clearance, they indirectly prevent further inflammatory infiltration of the inflamed tissue.\textsuperscript{74}

Such combined anti-inflammatory and pro-resolving affects of SPMs are also observed in combatting progression of neointimal hyperplasia. In attenuating proliferation, restricting migration, and preventing the phenotypic switch characterized by loss of contractile protein expression in VSMCs, RvD1 actively regulates intimal thickening and limits stenotic narrowing of the vasculature.\textsuperscript{9,75} In the case of plaque rupture, resolvins also restrict the inflammation driven accumulation of circulating platelets.\textsuperscript{76} This is in part accomplished through SPM regulated phagocytosis of apoptotic cells, which causes release of vascular endothelial growth factor, stimulating the reendothelialization of blood vessels and protection of intima from circulating leukocytes and coagulation factors.\textsuperscript{77} As a result, SPMs appear to limit the progression of two of the
most devastating inflammatory features of vascular injury: neointimal hyperplasia and thrombosis following plaque rupture. Such actions are essential for restoring inflamed vessels to normal conditions.

**DHA Derived SPM Biosynthesis**

Given their beneficial affects in resolving vascular inflammation, SPM pathways have arisen as a potential therapeutic means of addressing cardiovascular disease.\(^7\) SPMs must be produced at adequate levels to effectively manage their pro-resolving roles at the site of inflammation.\(^8\) Thus, understanding how to increase production or availability of SPMs in a vascular setting is of clinical interest. In this context, it is necessary to understand the process of their biosynthesis. Here, we focus on the pathways of SPM production derived from DHA.

As is the case for other biological mediators, SPM production from DHA requires substrate availability as well as proper expression, activity, and cellular localization of the enzymes involved. In humans, the basal serum concentration of unesterified DHA is roughly 2–4µM with an increase of up to 500% following \(\omega\)-3 supplementation of 1.5g/day over six weeks.\(^9\)–\(^12\) Meanwhile, blood levels of RvD1 in healthy individuals are 30.9pg/ml, and other DHA derived resolvins, protectins, and maresins have been measured at varying but comparable amounts.\(^13\) Conversion of DHA to these products via oxygenation by the lipoxygenases has been observed through liquid chromatography/tandem mass spectrometry-based informatics (Figure 2).\(^5\)\(^4\),\(^7\)\(^4\) The final products of these specific enzymatic reactions are stereoselective, with the S
stereoisomers formed through 15-lipoxygenase (15-LO) or 12-lipoxygenase (12-LO) activity and R stereoisomers triggered by aspirin acetylated COX-2.\textsuperscript{59,84}

**Figure 2. SPM biosynthesis from DHA.**
A) DHA is converted into 17-hydroxy-DHA (17-HDHA) by 15-lipoxygenase, which undergoes further transformation into resolvins such as RvD1 by 5-lipoxygenase or into protectins such as PD1 by epoxidation hydrolysis. Meanwhile, 12-lipoxygenase converts DHA into 14-hydroxy-DHA (14-HDHA) and 5-lipoxygenase converts 14-HDHA into maresins such as MaR1.\textsuperscript{85} B) A detailed schematic of resolvin biosynthesis from DHA with a complete illustration of enzymatic oxidation steps, intermediates, and products.\textsuperscript{79}
15-LO and 12-LO oxidation of DHA to 17-hydroxy-DHA (17HDHA) and 14-hydroxy-DHA (14HDHA) appears to be the rate-limiting step in the biosynthesis of RvDs, MaRs, and PDs. 68 15-LO and 12-LO preferentially bind to lipid bilayers, and different isoforms have comparable activity that increase upon action by inflammatory cytokines. 86–88 In macrophages, expression of each enzyme has also been shown to increase under inflammatory conditions in a time dependent manner. 89–91 Amplifying production of SPMs through such increases in activity and expression of these enzymes has proven to protect against vascular injury. 68 However, heightened expression of 15-LO and 12-LO can also be harmful. 92 Because the substrate specificity of both 15-LO and 12-LO are equivalent for AA, EPA, and DHA, increased activity and expression of these enzymes contributes to the increased biosynthesis of pro-inflammatory lipid mediators. 93,94 Though both enzymes have been identified in ECs and VSMCs, little is known about how changes in their expression levels either protect from or contribute to vascular inflammation. 95,96 Therefore, it would be of particular interest to track their expression accompanying SPM biosynthesis in vascular cells.

Similarly, it is unclear how 5-lipoxygenase (5-LO) mediates a pro-resolving response in EC and SMC. Acting further downstream in the pathway of DHA derived SPM biosynthesis, 5-LO also catalyzes synthesis of both pro-inflammatory and pro-resolving lipid mediators in vascular cells. 79,97,98 Consequently, increased activity and expression of 5-LO from inflammatory signaling has both protective and destructive affects, as is the case for the other lipoxygenases. 74,99–101 In fact, inhibition of 5-LO as a means to prevent leukotriene biosynthesis has arisen as a potential treatment for
inflammatory diseases, including atherosclerosis. However, because of its potential involvement in SPM production from DHA, alternatives to 5-LO inhibition are preferable.

Here, the answer may lie in the enzyme’s cellular localization. Localization is determined by phosphorylation of various amino acids in 5-LO, where phosphorylation at Ser-271 promotes and and phosphorylation at Ser-523 suppress nuclear localization. Ser-271 is phosphorylated by a member of the mitogen-activated protein kinase family p38-MAPK, which has been shown to be activated by AA. Meanwhile, phosphorylation of Ser-523 is mediated by protein kinase A (PKA), which is activated by a variety of signals, including RvD1 and LXA4. Unphosphorylated Ser-271 5-LO and Ser-523 phosphorylated 5-LO are inhibited from migrating into the nucleus across the perinuclear membrane, the site of leukotriene synthesis, and remain at a higher concentration in the cytosol where they can interact with COX-2 and the other lipoxygenases to produce SPMs. Thus, in regulating subcellular localization, phosphorylation affects the substrates and co-dependent enzymatic interactions available to 5-LO, potentially influencing the production of SPMs over that of leukotrienes.

Despite the observed expression of 15-LO, 12-LO, and 5-LO in EC and VSMC, it remains unclear whether vascular cells are capable of producing SPMs. Studies in other cell types signify that the presence of this biosynthetic machinery is sufficient for SPM production. Enzymatic transformation to 17S-resolvins has been shown to occur in human whole blood, isolated leukocytes, and glial cells while neuroprotectin is formed from DHA in corneal cells and maresin synthesized in monocytes. Also, cell-cell
interactions between endothelial cells and leukocytes produce resolvin from DHA.\textsuperscript{56} Such involvement of vascular cells confirms that they are at least in part able to produce the mediators necessary for resolution of their own injury. It would be of interest to identify whether vascular cells could individually produce SPMs from DHA as a means for local and efficient treatment of vascular inflammation. Furthermore, tracking how their biosynthesis is enzymatically mediated may serve as an initial investigation for potential pharmaceutical intervention.
Specific Aims

SPMs act to resolve vascular inflammation and may contribute significantly to the prevention of atherosclerotic stenosis and restenosis following vascular procedures. Stimulating synthesis of these mediators directly in vascular cells may increase their local availability, and thus, their role in limiting such conditions. However, the ability of endothelial and vascular smooth muscle cells to produce SPMs from their PUFA precursors remains unknown.

The purpose of this study is to determine whether vascular cells can produce SPMs from DHA and, if they do, how inflammation and mechanical injury of the vasculature alter biosynthesis. Specifically, we aim to:

1) Identify which SPMs are produced in human EC and VSMC as well as in ex vivo isolated blood vessels following treatment with DHA.

2) Investigate dose and time dependent responses of RvD1 production in EC and VSMC after DHA treatment.

3) Characterize changes in RvD1 biosynthesis due to inflammatory and injury related stimuli.

4) Determine the mechanism of DHA and TNF alteration of RvD1 production by investigating patterns of LO expression and localization.

By achieving these aims, we hope to ultimately introduce the foundation for new approaches to avoiding restenosis following vascular intervention.
METHODS

Reagents, Cells, and Treatment Protocol

Primary cultures of human greater saphenous vein ECs and VSMCs were isolated from saphenous vein discarded at the time of bypass operation in a University of California–San Francisco Institutional Review Board-approved protocol as described previously. VSMC were maintained in Dulbecco's Modified Eagle's Medium (DMEM; low glucose; HyClone, Logan, UT) containing 10% FBS (Invitrogen Life Technologies, Grand Island, NY) penicillin/streptomycin/amphotericin B (Lonza 1760) and used between passages 2 and 5. EC (passage 2 to 5) were maintained in Media 199 with Earle's Balanced Salt Solution (Hyclone, Logan, UT) supplemented with 10% FBS, penicillin/streptomycin/amphotericin B (Lonza 1760), ECGS (BD Cat no. 356006) and heparin (17.5 U/ml). DHA (4Z, 7Z, 10Z, 13Z, 16Z, 19Z-docosahexaenoic acid) was obtained from Cayman Chemicals (Ann Arbor, MI). Cells were treated with DHA (50-1000nM) and TNF-α (1ng/ml) at time points of 4, 8, and/or 24 hours.

Sample Extraction and Mediator Lipidomics

Solid-phase extraction columns were used for sample extraction for LC-MS/MS as described previously. 500 pg of deuterium-labeled internal standards d₆-5S-hydroxyeicosatetraenoic acid (HETE), d₄-leukotriene B₄, (LTB₄) d₇-lipoxin A₄ (LXA₄) and d₄-prostaglandin E₂ (PGE₂) were added before extraction to enable the quantification of sample recovery. Extracted samples were analyzed by a LC-UV-MS/MS system, QTrap 6500 (AB Sciex, Framingham, MA, USA) equipped with two Shimadzu LC-
20AD pumps (Shimadzu Corp., Kyoto, Japan). An Agilent Eclipse Plus C18 column (100×4.6 mm×1.8 μm; Agilent Technologies, Santa Clara, CA, USA) was used with a gradient of methanol/water/acetic acid of 60:40:0.01 (v/v/v) to 100:0:0.01 at 0.4 ml/min flow rate. Identification was conducted using previously published criteria using a minimum of 6 diagnostic ions. Quantification was carried out based on the peak area of the multiple reaction monitoring transition and the linear calibration curve for each compound.

RvD1 ELISA

An enzyme-linked immunosorbent competition-based assay (ELISA) was used to detect RvD1 levels in the cell culture supernatant as described by the manufacturer (Cayman Chemical Company, Ann Arbor, MI, USA). This assay is based on the competition between free RvD1 in either the cell culture media or cellular extract and an RvD1 tracer linked to acetylcholinesterase.

Western Blotting

VSMC and EC were lysed in CellLytic M buffer (Sigma, Cat no. C2978) and, after three pulses of low watt sonication on ice, heated at 100°C in Laemlli buffer for 7 min. The lysate (25 μg) was then run on NuPAGE 10% Bis-Tris gel (Invitrogen) and transferred to a PVDF membrane that was probed with anti-5 lipoxygenase antibody (1:500; Novus Biologicals, Littleton, CO, USA), anti-15 lipoxygenase antibody (1:400;
Novus Biologicals) and anti-β-actin (1:4000; Sigma-Aldrich, St. Louis, MO, USA) using a QDot 625 Western blotting kit (Invitrogen).

**Immunofluorescence for 5-LO Localization**

All immunofluorescence staining was completed on 4-well chamber slides (EMS, Hatfield, PA). After treatment, cells were briefly rinsed in PBS and fixed with 4% paraformaldehyde for 20 min at 37°C, followed by permeabilization in ice-cold acetone (10 min at ~20°C) and 1% Triton-X100 (20 min at room temperature). Cells were incubated at 4°C overnight with anti-5-lipoxygenase antibody followed by goat anti-rabbit IgG-conjugated Alexa Fluor 488 (Life Technologies, Carlsbad, CA). Fluorescence imaging was completed with an Olympus BX51 microscope (Olympus America, Center Valley, PA, USA) with an EXFO X-cite 120 system (EXFO Photonic Solutions, Mississauga, ON, Canada), Olympus DP70 digital microscope camera, and DPController software (Olympus). Ten section zones (at ×20 view) were selected randomly on 4 coordinate axes of every well. 5-lipoxygenase localization was quantified through comparison of relative mean cytoplasmic to nuclear intensity with IMAGEJ software.

**Rabbit Aorta Extraction and Endothelial Denudation**

All rabbit specimens were harvested from animals freshly euthanized as part of the IACUC approved UCSF tissue sharing program. Female New Zealand white rabbits (NZWRs; Western Oregon Rabbit Company, Philomath, OR, USA) weighing 3 to 5 kg and maintained on a normal chow diet (LabDiet Rabbit Diet High Fiber; Purina, St.
Louis, MO, USA) were used in all experiments. Aortas were harvested from distal to the left subclavian artery down to the aortic bifurcation with care to avoid excessive stretch or other damage to the vessel. The aorta was then opened en face and divided into 1 cm$^2$ sections. Endothelial cells were denuded via cotton swab for the (-) endothelial cell groups.

Samples were immersed in either DHA free or 1000 nM DHA containing DMEM and incubated at 37°C for 48 hrs. Media was spun in 30 kD centrifugal tubes (Merck Millipore Ltd; Darmstadt, Germany) and assessed for RvD1 production by ELISA. Tissues were collected and immediately snap-frozen for metabololipidomic profiling by LC-MS/MS or ELISA, and cryosections were prepared for immunohistochemistry.

Immunostaining was performed to evaluate the quality of endothelial denudation. Monoclonal antibody against CD31 (1:50, JC70A; Dako Denmark A/S, Glostrup, Denmark) was used for primary antibody, a biotinylated goat-anti mouse for secondary (1:200; Invitrogen) and sections were counterstained with streptavidin-conjugated fluorescin (1:200; Vector, Burlingame, CA, USA).

**Statistical Analysis**

Data are shown as means ± SE. Direct comparisons were made using unpaired or paired Student's t test, and multiple group comparisons were made using 1-way or 2-way ANOVA followed by Bonferroni's post hoc tests where appropriate. In all cases, a level of P < 0.05 was considered significant.
RESULTS

Biosynthesis of Pro-resolving Lipid Mediators from DHA in Vascular Cells

The generation and accumulation of pro-resolving mediators within the vessel wall has been characterized previously. However, the ability of vascular cells to produce SPMs in the absence of leukocytes has not been demonstrated. LC-MS/MS was preformed to identify de novo SPM generation in vitro in EC and VSMC cultures following 24 h treatments of 1000nM DHA (Figure 3). In the collected media, we identified the presence of the following DHA derived precursors and downstream bioactive mediators: 17-HDHA (4500 ± 250 pg/ml in EC; 2300 ± 130 pg/ml in VSMC), 14-HDHA (345 ± 23 pg/ml in EC; 110 ± 25 pg/ml in VSMC), MaR1 (235 ± 28 pg/ml in EC; 245 ± 22 pg/ml in VSMC) and RvD5 (1.6 pg/ml in EC; 3.45 ± 0.35 pg/ml in VSMC). Low levels (0.5 pg/ml) of RvD2 were also detected in both cells. Though not detected in LC-MS/MS analysis, high levels of RvD1 (30 pg/ml) were measured in both EC and VSMC through ELISA.

It should be noted that all experiments were performed in 10% fetal bovine serum, likely containing trace levels of PUFA precursors. Consequently, marginal levels (<0.5 pg/ml) of various AA and EPA derived mediators were observed in all samples. DHA supplementation significantly induced SPM biosynthesis from baseline levels in both EC and VSMC.
Time and Does Dependent Affects on DHA Mediated RvD1 Generation

We next sought to identify a time profile of SPM production to find time points of interest for investigating modulation of SPM biosynthesis. RvD1 was chosen for detailed analysis because of the availability of an ELISA for its detection. Because all experiments were performed in 10% serum, there were low levels of RvD1 released in untreated cells. In EC, increasing DHA doses displayed a similar progression of RvD1.

Figure 3. Local biosynthesis of pro-resolving lipid mediators in vascular cells. Cells were treated with DHA (1000nM) for 24 h. Representative profiles from LC-MS/MS display the generation of pro-resolving lipid mediators in EC and VSMC.
synthesis, with a rapid initial rate of generation that tapered over the course of 24 hours (Figure 4A). In untreated cells, total RvD1 production from baseline was 0.39 ± 0.05 pg in the first 4 hours, 0.52 ± 0.01 pg in the first 8 hours (33% increase from previous 4 hours), and 0.84 ± 0.05 pg in 24 hours (38% increase over previous 16 hours). Increasing substrate availability enhanced the initial rate of RvD1 generation, with 4 h levels at 0.47 ± 0.05 pg for 50nM DHA, 0.83 ± 0.15 pg for 100nM DHA, 1.72 ± 0.08 pg for 300nM DHA, 2.43 ± 0.25 pg with 500nM DHA (p<.01 compared to 4 h ctrl), for 5.19 ± .53 pg with 1000nM DHA (p<.001 compared to 4 h ctrl). Similarly, the rate of biosynthesis from 4 to 8 hours rose slightly following DHA treatment, with increases between 40% and 80% measured for each dose during the time period. RvD1 levels changed minimally thereafter for all doses, with 24 h totals of 1.02 ± 0.03 pg for 50nM DHA, 1.3 ± 0.34 pg for 100nM DHA, 2.89 ± 0.21 pg for 300nM DHA (p<.01 compared to 24 h ctrl), 4.72 ± 0.27 pg for 500nM DHA (p<.001 compared to 24 h ctrl), and 10.1 ± 1.05 pg for 1000nM DHA (p<.001 compared to 24 h ctrl).

Meanwhile, VSMC displayed an analogous time and dose dependent pattern of RvD1 biosynthesis, with all treatment groups producing the majority of total product within 4 hours of treatment (Figure 4B). RvD1 levels at 4 hours were 0.36 ± 0.07 pg for control, 0.35 ± 0.04 pg for 50nM DHA, 1.55 ± 0.48 pg for 100nM DHA, 2.72 ± 0.3 pg 300nM (p<.001 compared to 4 h ctrl), 4.4 ± 0.28 pg for 500nM (p<.001 compared to 4 h ctrl), and 6.82 ± 0.81 pg for 1000nM (p<.001 compared to 4 h ctrl). The rate of production slowed even more considerably than in EC thereafter, such that the total generation of RvD1 plateaued at 24 h at 0.64 ± 0.21 pg for control, 1.29 ± 0.06 pg for 50
nM, 1.65 ± 0.41 for 100nM DHA, 3.03 ± 0.16 pg for 300nM DHA (p<.001 compared to 24 h ctrl), 4.94 ± 0.43 pg for 500nM (p<.001 compared to 24 h ctrl), and 7.43 ± 0.22 pg for 1000nM DHA (p<.001 compared to 24 h ctrl). It is of note that maximum RvD1 production (24 h following 1000nM DHA treatment) was 26% higher in EC than VSMC.

**Enzymatic Regulation of RvD1 Pathway**

A 1000nM dosage of DHA was selected for all subsequent experiments, as this dose gave maximal RvD1 production and is physiologically closest to standard serum concentration for DHA.
With the expectation that 5-LO and 15-LO drive the transformation of DHA to RvD1, we investigated their expression in ECs and VSMCs. Western blot analysis confirmed the presence of 5-LO in EC (Figure 5A) and VSMC (Figure 5C). In EC, 5-LO expression did not change at either 4 or 8 h following 1000nM DHA treatment but increased by 94% (p=.045) at 24 h. In comparison, expression in VSMC remained constant throughout the treatment. Meanwhile, 15-LO expression was also observed in EC (Figure 5B) and VSMC (Figure 5D). Representative bands were noticeably weaker than for 5-LO. 15-LO expression did not vary at any point post-treatment in either cell type.

Figure 5. Expression of 5-LO and 15-LO in vascular cells. A-D) Time-dependent expression of 5-LO and 15-LO in DHA (1000nM) treated EC (A,B) and VSMC (C,D) assessed by western blot analysis. n=3, *P < 0.05 compared to control; unpaired t test.
The effects of DHA treatment (1000nM) on 5-LO cellular localization were also examined (Figure 6). Previous studies have associated increased cytoplasmic presence of 5-LO with SPM biosynthesis. Experiments were with 5 and 24 hours treatments to represent the aforementioned phases of rapid and stalled RvD1 biosynthesis. In both EC and VSMC, DHA caused a significant shift of 5-LO localization from the nucleus to cytoplasm at 5 h (27%, p=.014; 38%, p<.001, respectively). Translocation was not observed at 24 h in either cell, with 5-LO remaining mostly in the nucleus.

![Figure 6. Effect of DHA on sub-cellular distribution of 5-LO.](image)

A-F) Representative immunofluorescence images of 5-LO in EC (A-C) and VSMC (D-F) with IgG control (A, D), 5-LO untreated control (B, E) and 5-LO DHA treated cells (1000nM for 5h; C,F). (G, H) Quantitative analysis of 5-LO translocation (ratio of cytoplasmic to nuclear fluorescence) in EC (G) and VSMC (H). n≥3 where n= each well of a 4-well chamber slide. *P < 0.05 compared to 5-LO control; unpaired t test.

**RvD1 Production is Reduced Following Endothelial Denudation ex vivo**

The relative contribution for RvD1 production of EC versus VSMC in an ex vivo vessel model was investigated. Following extraction from the rabbit, isolated aorta was
promptly denuded. Successful removal of endothelium was confirmed by immunostaining for CD31 (Figure 7B-C). After 48 hour incubation in 1000nM DHA, media was assessed for RvD1 production. Both undenuded (90% increase, p=.02) and denuded (112% increase, p<.001) aorta saw substantial increases in RvD1 production with DHA treatment (Figure 7D). Furthermore, endothelial denudation caused a significant decrease in synthesis relative to the intact vessel (28% decrease, p=.026).

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**Figure 7. DHA induced RvD1 production in intact and endothelium-denuded rabbit aorta.**

A-B) Immunofluorescence images of sections of rabbit aorta stained with endothelial marker CD31. (A) IgG control (B) intact aorta (+EC) and (C) denuded aorta. (D) Rabbit aorta segments (1cm² area) were incubated for 48hrs in media with and without 1000nM DHA and RvD1 production was measured with ELISA. Results are shown as net RvD1 produced per mg vessel tissue. n=3, *P < 0.05 compared to nondenuded and untreated vessel (+EC/-DHA), unpaired t test.
Inflammatory Cytokines Enhance DHA Derived RvD1 Biosynthesis in EC

We monitored the impact of TNF-α on RvD1 production in endothelial cells to assess the impact of inflammatory cytokines on SPM pathways. A TNF-α dose of 1 ng/ml was selected for optimal stimulation of EC. In the absence of substrate, TNF-α did not increase RvD1 production above baseline levels. Meanwhile, DHA treatment resulted in a modest rate of increase in RvD1 following the initial 4 hours (26% increase from 4 to 8 h and 26% from 8 to 24 h). Simultaneous treatment of DHA with TNF-α produced a similar initial trend in RvD1 synthesis (9.47 ± 2.66 pg in first 4 hours with a 15% increase in the next 4 h). Though slightly higher than RvD1 generated by DHA alone, increases were not significant. However, RvD1 production did not taper in the next 16 hours, increasing by 63% to 18.62 ± 1.26 pg at 24 h (p<.001 compared to DHA at 24 h) (Figure 8A).

To explain these effects, we explored TNF-α influence on 5-LO expression and sub-cellular localization. Expression was significantly induced following 24 h treatment (113%, p=.0069) (Figure 8B). Similarly, TNF-α (5 h treatment) caused a significant shift of 5-LO localization from the nucleus to cytoplasm (81%, p<.001; Figure 8C-F).
Figure 6. TNF augments RvD1 biosynthesis in EC through 5-LO expression and cytoplasmic translocation. A) Time-dependent response of TNF on DHA induced RvD1 production. EC were treated with DHA (1000nM) and TNF (1ng/ml) for 4, 8, and 24 h. Media was collected for ELISA. Results are shown as net RvD1 produced in approx 175ul per well of a 24-well plate. n=3, *: P < 0.05 compared to untreated control at respective time points; †: p≤0.05 compared to 4 h treatment; ‡: P<0.05 compared to 8 h treatment; 2-way ANOVA with Bonferroni's post hoc test. #: P < 0.05 compared to DHA; unpaired t test. B) TNF up-regulates 5-LO expression in EC at 24 h. n=3, *: P < 0.05 compared to control; unpaired t test. C-D) Representative immunofluorescence images showing 5-LO cytoplasmic translocation in EC with TNF-α (1ng/ml) at 5 h (D), compared to untreated control (C). E) Quantitative analysis of 5-LO translocation in C-D (ratio of cytoplasmic to nuclear fluorescence) in EC. n≥3 where n= each well of a 4-well chamber slide. *: P < 0.05 compared to control; paired t test.
DISCUSSION

Acute vascular injury resulting from vascular interventions performed for occlusive cardiovascular disease (e.g. angioplasty, stents, bypass grafts, etc.) often results in the development of neointimal hyperplasia and eventual restenosis. Recent evidence indicates that restenosis can be viewed as a resolution deficit within the vessel wall. Thus, the availability of pro-resolving lipid mediators in the vasculature is critical for the resolution of acute inflammation after intervention, halting the underlying cause of restenotic injury. While it is clear that SPMs are present and play an active role in resolution after vascular intervention, the specific ability of endothelial and vascular smooth muscle cells to independently synthesize these mediators was previously unknown. The findings reported in this study indicate that, in the absence of leukocytes and other cells known to produce pro-resolving mediators, cells and tissue of vascular origin can produce SPMs from DHA.

Our LC-MS/MS results revealed 17-HDHA, 14-HDHA, and MaR1 to be the major products of DHA derived SPM biosynthesis, accompanied by minor generation of RvD5 and RvD2. On initial consideration, these findings suggest that SPM pathways in vascular cells are skewed towards the production of MaR1, as it is the only biologically active pro-resolving mediator measured at significant levels. However, the predominance of the resolvin and protectin intermediate product, 17-HDHA, over the maresin intermediate, 14-HDHA, implies that resolvin production pathways are also active. Furthermore, although mass spectrometry did not reveal RvD1 production, high amounts were detected with ELISA. The absence of detected 17-HDHA downstream products in LC-MS/MS
could be attributed to issues with column extraction, specificity of 5-LO for 14-HDHA over 17-HDHA, or relatively high rates of resolvin degradation. Though incomplete, these findings ultimately suggest that in the presence of precursor PUFAs, endogenous pathways of pro-resolving mediator production are biochemically active in vascular cells.

Furthermore, we found that RvD1 biosynthesis is a dynamic process, subject to time and DHA dose dependent rises and mediated by the action of 5-LO and 15-LO. Previous studies had established that these enzymes are expressed in both EC and VSMC.\textsuperscript{95-98} We confirmed this and found that DHA upregulates 5-LO protein expression over time in EC. Although the explanation for this observed increase remains unclear, various possibilities exist that are centered around transcriptional regulation. There is evidence that DHA restricts global DNA methylation and may induce 5-LO expression through demethylation of its promoter sequence in DNA.\textsuperscript{114,115} Meanwhile, DHA may increase activity of NF-\textgreek{kappa}B, a transcription factor for 5-LO, inducing the formation of the transcriptional preinitiation complex.\textsuperscript{116,117} Further investigation of mRNA profiles under experimentally controlled transcriptional regulation is necessary for clarification of the exact mechanism and to explain why no such effects are observed in VSMC.

Despite differences in measured 5-LO expression between EC and VSMC at 24 hours, neither cell type underwent changes in 5-LO nor 15-LO protein levels within the initial 4 hours of treatment, the time frame for the majority of RvD1 formation. This initial rise in RvD1 production may be in part explained by a DHA mediated nuclear to cytoplasmic shift in 5-LO localization in both EC and VSMC, as the cytoplasm is the site of RvD1 synthesis. Such an effect of substrates on 5-LO subcellular position is not
unprecedented, as studies have shown AA induced phosphorylation of Ser-271 in 5-LO to promote nuclear shift of the enzyme.\textsuperscript{105} It is possible that DHA plays a similar role in mediating this process. However, DHA derived RvD1 may also regulate cytoplasmic shift as part of a feed-forward mechanism through phosphorylation of 5-LO Ser-523.\textsuperscript{104} Whether DHA influences 5-LO localization directly, indirectly through RvD1 synthesis, or through a combination of both mechanisms is uncertain, and further studies are necessary to clarify the exact pathway.

Assessment of RvD1 production in isolated rabbit aorta revealed that vessels devoid of endothelial cells generated significantly lower levels of RvD1 than intact aorta, suggesting a critical role for EC in SPM production within the vessel. The normal arterial wall comprises of a single layer of endothelium sitting on top of a thick tunica media that is composed mainly of vascular smooth muscle cells. Thus, it is noteworthy that relatively such few EC produce over a quarter of all RvD1 synthesized in a blood vessel. Similarly, we observed that endothelial cells produced more RvD1 than VSMC in cell culture. These results suggest that the lack of EC may attenuate resolution pathways in the vessel, potentially contributing to the inability of deendothelialized vessels to prevent the progression of neointimal hyperplasia.

One of the prominent findings of this study was that TNF increased RvD1 production in EC, suggesting that endothelial cells may actively participate in a self-resolving acute inflammatory response. The fact that this surge in RvD1 generation does not become significant until after 24 h treatment with TNF is particularly important and indicates the occurrence of lipid mediator class switching in EC. In PMNs and monocytes, acute
inflammatory insult has been shown to produce a temporal regulation of lipid mediator
generation from pro-inflammatory to pro-resolving over time.\textsuperscript{118,119} Our investigation
found that upregulation of 5-LO expression at 24 h coincided with its nuclear to
cytoplasmic shift in the cell, indicating that increases of the enzyme correspond with
RvD1 production over that of leukotrienes. These findings suggest that vascular cells
endogenously respond to inflammatory insult by promoting synthesis of pro-resolving
mediators.

In summary, human vascular cells and rabbit vascular tissue can biosynthesize SPMs
\textit{de novo} from the precursor DHA and may contribute to self-resolution of acute vascular
inflammation associated with neointimal hyperplasia. We hope that these findings initiate
investigation of novel therapeutic options aimed at promoting endogenous biosynthesis of
SPMs in the vasculature to combat the progression of vascular injury.
LIST OF JOURNAL ABBREVIATIONS

FASEB    Federation of American Societies for Experimental Biology
JAMA     Journal of the American Medical Association
JACC     Journal of the American College of Cardiology
PLoS ONE Public Library of Science
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CURRICULUM VITAE

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EDUCATION

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<td>University of California, Berkeley</td>
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Research Experience

**Stanford Stroke Center**, Palo Alto, CA 05/11-08/12

*Research Volunteer*

*• Participated in clinical research focusing on the diagnostic capabilities of medical imaging for cause of stroke.*
*• Analyzed MRI and CT images for investigation of:*
  o coma patient prognostication
  o ischemia surrounding intracerebral hemorrhage

Volunteer Experience

**bWell Center**, Boston, MA 01/14-05/14

*Medical Volunteer in Pediatrics*

*• Provided health resources for patients and their families.*
*• Led “Jump Rope Clinic” to advocate a healthy lifestyle for patients.*
*• Acted as patient liaison:*
  o Directed patients to appointments
  o Entertained kids in waiting room
Armenian Volunteer Corps, Yerevan, Armenia 05/13-07/13  
*Medical Volunteer in Pediatric Orthopedic Department*  
  
- Assisted in clinical practice.  
  - Assisted in applying casts on patients.  
  - Prepared patient rooms for clinical use.  
- Observed surgical procedures ranging from polydactyly adjustment to tendon lengthening.  
- Applied suturing at termination of operations.

John Muir Hospital, Walnut Creek, CA 06/10-06/12  
*ER Volunteer*  
  
- Aided in maintaining patient records.  
- Assisted in preparation of rooms for clinical use.  
- Acted as patient liaison.  
- Observed care of trauma patients.

Leadership Experience

St. Andrew’s Armenian Apostolic Church, Cupertino, CA 10/09-Present  
*Deacon Service*  
  
- Advised members of congregation on spiritual issues.  
- Helped plan and organize church events.  
- Participated in Sunday Service.

UC Berkeley Habitat for Humanity, Berkeley, CA 02/10-05/13  
*Workday Coordinator*  
  
- Planned, organized, and led weekly volunteer outings.  
- Met with various non-profit organizations throughout the Bay Area to discuss our chapter’s potential involvement in their projects.  
- Helped organize fundraisers to fund volunteer excursions.

Homenetmen Scouting, Santa Clara, CA 09/09-06/11  
*Youth Leader and Mentor*  
  
- Involved in program development and institution of troop 300.  
- Met with youth leaders to discuss and advise on current and future directions of chapter.  
- Gave educational lectures and led hikes.
Blood Pressure Thresholds to Predict the Cause of Intracerebral Hemorrhage