APOE, APOER2, AND THE REGULATION OF ENDOTHELIAL CELL FUNCTION

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DEDICATION

This is for my two and four-legged family, here and gone-

I love you all and will never forget what you taught me.

Mom and Dad, thanks for never giving up on me.

You’re my boy, Blue.

APOE, APOER2, AND THE REGULATION OF ENDOTHELIAL CELL FUNCTION

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Cardiovascular disease risk is greater in individuals with the apolipoprotein E (ApoE) allele ApoE4 versus ApoE3, and this is not explained by differences in lipid profiles. A point mutant of the ApoE receptor ApoER2, ApoER2-R952Q, is also associated with greater cardiovascular disease risk, suggesting a role for ApoER2 in vascular health and disease. However, how ApoE, ApoER2, and their variants influence cardiovascular health and disease is poorly understood. We discovered in cultured endothelial cells that ApoE3 activates endothelial NO synthase (eNOS) and thereby stimulates endothelial cell migration, and that both processes require ApoER2. In contrast, ApoE4 is incapable of activating eNOS and in fact blunts ApoE3 activation of eNOS migration. In eNOS-expressing 3T3 cells, we also

found that in contrast to wild-type ApoER2, ApoER2-R952Q is incapable of mediating ApoE3 activation of eNOS. Furthermore, we have determined that $LDLR^{-/-}$;$ApoER2^{-/-}$ mice have markedly worse atherosclerosis than $LDLR^{-/-}$, and in preliminary studies $ApoER2^{-/-}$ have impaired reendothelialization. The **Overall Goal** of this project is to determine how ApoE, ApoER2 and their variants influence endothelial cell function, and the implications of these processes in vascular health and disease. The first aim is to identify the molecular basis for ApoE-ApoER2 function in endothelial cells. The roles of adaptor proteins for ApoER2 and for kinase signaling in ApoE3/ApoER2 activation of eNOS were tested. The basis for impaired signaling by ApoE4 will also be explored. The second aim is to determine how ApoE and ApoER2 influence endothelial cell phenotype in vivo. Carotid artery reendothelialization after perivascular electric injury were compared in wild-type, human ApoE3-expressing, and ApoE4-expressing mice. The third aim is to determine the role of endothelial cell ApoER2 in atheroprotection. We have successfully created floxed ApoER2 mice, and atherosclerosis development will be evaluated in crosses of $ApoER2^{flox/flox}$ with endothelial cell-expressing Cre and $LDLR^{-/-}$ mice in the future. From the unique perspective of apolipoprotein signaling in endothelium, the proposed work will enhance our fundamental understanding of ApoE and ApoER2 in cardiovascular health and disease.

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PRIOR PUBLICATIONS

1. Dubey JP, Jenkins MC, Kwok OC, Zink RL, Michalski ML, Ulrich V, Gill J, Carstensen M, Thulliez P. 2009. Seroprevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in white tailed deer (*odocoileus virginianus*) from Iowa and Minnesota using four serologic tests. Veterinary Parasitology(161)3-4:330-334.

2. Dubey JP, Velmurugan GV, Ulrich V, Gill J, Carstensen M, Sundar N, Kwok OC, Thulliez P, Majumdar D, Su C. 2008. Transplacental Toxoplasmosis in naturally-infected White-Tailed Deer: Isolation and genetic characterization of *Toxoplasma gondii* from fetuses of different gestational ages. International Journal for Parasitology; 38(8-9):1057-63.

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ONGOING STUDIES

LIST OF DEFINITIONS

Aβ ..... Amyloid Beta Peptide

ABCA1 ..... ATP-binding Cassette Transporter 1

AIDS ..... Acquired Immune Deficiency Syndrome

APC ..... Activated Protein C

ApoB ..... Apolipoprotein B

ApoE ..... Apolipoprotein E

ApoER2 ..... Apolipoprotein E Receptor 2

APS ..... Antiphospholipid Syndrome

ARH ..... Autosomal Recessive Hypercholesterolemia

BAEC ..... Bovine Aortic Endothelial Cells

B2GPI ..... Beta-2 Glycoprotein I

CAD ..... Coronary Artery Disease

CAM ..... Cell Adhesion Molecule

Cav-1 ..... Caveolin-1

Cav-3 ..... Caveolin-3

cGMP ..... Cyclic Guanosine Monophosphate

CRP ..... C-reactive Protein

CVD ..... Cardiovascular Disease

DAB1 ..... Disabled-1 protein

DAB2 ..... Disabled-2 protein

DAF2 ..... Diaminofluorescein 2

DMEM ..... Dulbecco's Modified Eagle's Medium

DMSO ..... Dimethyl Sulfoxide

EGF ..... Epidermal Growth Factor

EGM-2 ..... Endothelial Growth Media-2

ELISA ..... Enzyme-linked Immunosorbent Assay

eNOS ..... Endothelial Nitric Oxide Synthase

ERK1/2 ..... Extracellular Signal-Regulated Kinases 1/2

HAEC ..... Human Aortic Endothelial Cells

HDL ..... High Density Lipoprotein

HIV ..... Human Immunodeficiency Virus

HMEC ..... Human Microvascular Endothelial Cells

Hsp90 ..... Heat Shock Protein 90

HUVEC ..... Human Umbilical Vein Endothelial Cells

iNOS ..... Inducible Nitric Oxide Synthase

IVM ..... Intravital Microscopy

JIP-1 ..... JNK-interacting Protein-1

JIP-2 ..... JNK-interacting Protein-2

L-NAME ..... L-NG-Nitroarginine Methyl Ester

LDLR ..... Low Density Lipoprotein Receptor

LPS ..... Lipopolysaccharide

LRP1 ..... Low Density Lipoprotein Receptor-related Protein 1

LRP8 ..... Low Density Lipoprotein Receptor-Related Protein 8

MI .....Myocardial Infarction

NF-κB .....Nuclear Factor-kappa B

NMDAR.....N-Methyl-D-aspartate Receptor

NO .....Nitric Oxide

ox-LDL .....Oxidized Low Density Lipoprotein

PCSK9.....Proprotein Convertase Subtilisin/Kexin Type 9

PI3k .....Phosphatidylinositol 3-kinase

PSD-95.....Postsynaptic Density Protein 95

TLR-4 .....Toll-like Receptor 4

VCAM-1 ..... Vascular Cell Adhesion Molecule-1

VEGF .....Vascular Endothelial Growth Factor

VEGFR2 .....Vascular Endothelial Growth Factor Receptor 2

VLDL ..... Very Low Density Lipoprotein

VLDLR .....Very Low Density Lipoprotein Receptor

VSM .....Vascular Smooth Muscle

CHAPTER ONE
Introduction and Literature Review

CARDIOVASCULAR DISEASE

Prevalence and impact

Cardiovascular Disease (CVD) is the number one cause of death in the world, and one of three Americans and half of Europeans have one or more CVD manifestations such as high blood pressure, stroke, coronary heart disease, arrhythmias, heart failure, congenital heart defects, and peripheral artery disease.(Gonzalez and Selwyn 2003, Mensah, Ryan et al. 2007, Basson 2008, Ton, Martin et al. 2012, Willis, Davies et al. 2012) While the rate of death attributable to CVD in the US has declined, the burden of disease remains high as more than 2200 Americans still die each day from CVD.(Heidenreich, Trogdon et al. 2011) While many different disease manifestations are encompassed by the term CVD, it is well known that atherosclerosis is the initiating factor for most CVD manifestations. Atherosclerosis disease manifestations include coronary artery disease, peripheral artery disease, and ischemic stroke. Atherosclerosis is defined as the hardening of the arteries, but the disease is a complicated one, arising initially from endothelial dysfunction and the accumulation of cholesterol, and the progression reflects the impact of many exogenous and endogenous factors.(Ross 1993, Jousilahti, Rastenyte et al. 1997, Cunningham and Gotlieb 2005, Patti, Melfi et al. 2005, Hui 2008)

Modifiable risk factors

CVD presents as one or more clinical manifestations. A handful of environmental factors exacerbate CVD risk, and these factors can then create secondary modifiable factors, such as that which occurs when poor diet induces diabetes. In fact the majority of external cardiovascular risk factors can be controlled, treated, or modified in order to ameliorate CVD risk (**Fig.1)**. Tobacco smoking is one of the biggest external risk factors for CVD. While quitting smoking literally adds years to one's lifespan, smoking increases the risk of dying from CVD by 50%.(Powell 1998, Dunbar, Gotsis et al. 2012) Smoking is a major risk factor for CVD; however today in the US diet and inactivity have become a massive problem in terms of deteriorating cardiovascular health. Over 30% of American adults are overweight stemming from poor Western diet and/or sedentary lifestyle, and resulting changes in lipid profile account for rising cholesterol levels and circulating fats.(Emery, Schmid et al. 1993, Leung, Yung et al. 2008, Ford and Caspersen 2012, Wilmot, Edwardson et al. 2012) These fats can then help in forming plaques in damaged vessels, eventually blocking off vascular flow and resulting in tissue ischemia.(Reiner and Tedeschi-Reiner 2001, Finn, Nakano et al. 2010) Increases in dietary fat, as well as substantial increases in body weight, also lead to a systemic inflammatory state. This inflammation often affects overall organ function, such as that seen in hypertension or insulin resistance. Indeed systemic inflammation is also a predictor for metabolic diseases, such as diabetes, as blood glucose is often elevated. (Gonzalez and Selwyn 2003, Hsueh and Quinones 2003, Goldstein, Bushnell et al. 2011)

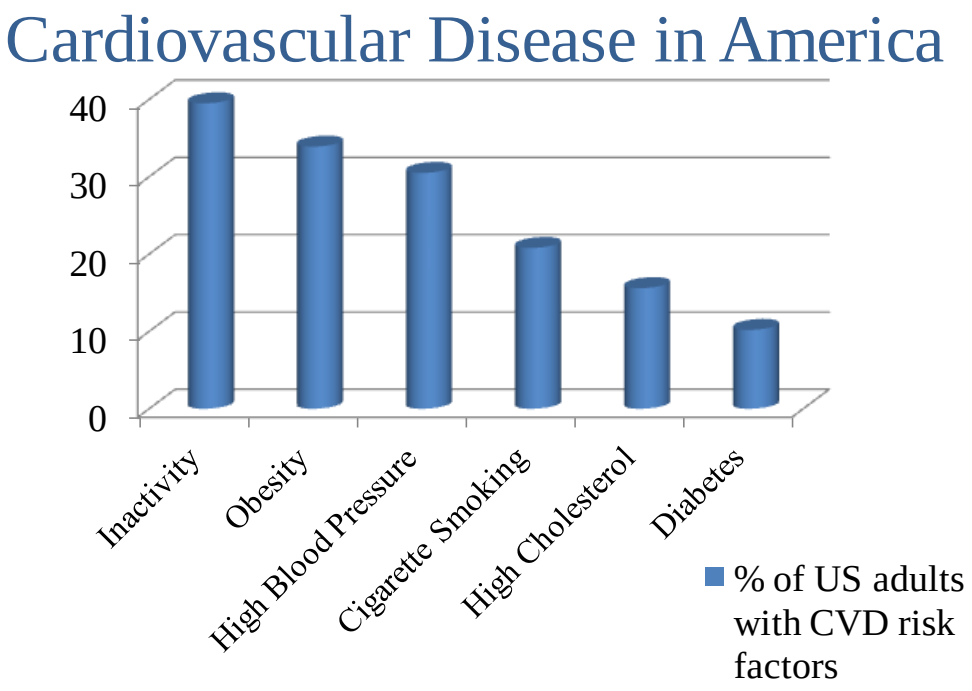


Figure 1: CVD Risk Factors in America.
Modified from *Heart Disease and Stroke Statistics - 2005 Update*: American Heart Association and the American Stroke Association; 2005.

In fact most people who die from CVD have metabolic dysfunction, as well. Therefore while CVD modifiable risk factors provide patients with a means to modify disease risk, patients are often non-compliant with lifestyle changes to ameliorate this risk.(Genest and Cohn 1995, Naruse, Tanabe et al. 2000, Balagopal 2006)

Non-modifiable risk factors

Whereas the majority of CVD risk factors can be controlled, there are some risk factors that cannot be changed. However those possessing these factors can be closely monitored. CVD is an expansive disease with many external causes, but variations within each population do occur depending on age, family history, and gender. Gender is a non-modifiable factor which highlights increased risk for young men. While early cardiac events

are rare between the ages of 33-45 years, 15% of men and 5% of women in this age bracket already show subclinical atherosclerosis in coronary arteries. In older adults, white males rank in the highest percentiles for possessing atherosclerotic vessels. Men are at a 50% greater risk of CVD than a pre-menopausal woman, however after female hormonal descent the CVD risk of men and women equals. While maintaining low risk factor burden from young adulthood through old age improves CVD outcomes overall, there remains a subset of the population that experience onset of symptoms in spite of obvious decreases in disease risk.(Goldstein, Adams et al. 2006, Heidenreich, Trogdon et al. 2011, Ton, Martin et al. 2012, Imes and Lewis 2013)

Furthermore, family history of disease increases CVD risk in offspring. Those with first degree blood relatives who suffer a catastrophic CVD effect before the age of 55 possess increased risk. Finally, while age takes a toll on all body systems, endothelial dysfunction underlies the initiation of CVD, and is thought to occur decades before clinical CVD manifestation. Initiation of atherosclerosis at a young age when risk factors are absent remains a puzzling anomaly, but highlights why current CVD therapeutics are designed to treat after clinical presentation or emergent symptoms, rather than prior to initiation of CVD and endothelial dysfunction.(Glagov, Weisenberg et al. 1987, Genest and Cohn 1995, Luscher and Noll 1995, Cohn 2001, Esper, Nordaby et al. 2006, Karaolis, Moutiris et al. 2010, Imes and Lewis 2013)

Genetic Associations

Despite decades of searching for genes to predispose one to increased CVD risk, there is not one single major genetic risk factor found which could account for all increases in risk assessment. Indeed CVD is a complex disease in which inheritance of risk is not due to disruption of one single gene.(Genest and Cohn 1995, 2010) However, there are many lipoprotein-regulating gene mutations that clearly impact lipid profile. For instance, mutations in the coding sequence of the low density lipoprotein receptor (LDLR) reduce the lipid clearance function of the LDLR, leading to an increase in circulating lipid.(Johnson, Altenburg et al. 2008) In addition a single mutation in Apolipoprotein B (ApoB), a core molecule in lipoprotein particles which binds LDLR, affects lipoprotein binding to LDLR, and thus also affects lipid clearance.(Riches, Watts et al. 1998) Previously considered common mutations in the ATP-binding cassette transporter 1 (ABCA1) have recently been associated with low plasma high-density cholesterol (HDL) levels and therefore increased CVD risk.(Fitz, Cronican et al. 2012) Finally polymorphisms in the apolipoprotein E (ApoE) gene lead to production of three different ApoE isotypes in humans, however changes in lipid levels between these subsets of ApoE are not solely due to changes in lipid profile.(Yamada, Inoue et al. 1992, Shimano, Ohsuga et al. 1995, van Bockxmeer, Mamotte et al. 1995)

APOE

Formation and Structure

Apolipoproteins are transport molecules synthesized mainly in the liver that are important in regulating the composition of circulating lipoproteins by selective binding, thus altering the systemic uptake of lipoproteins in cells. ApoE also serves to act as a stabilizing ligand for cell surface receptors and modulates lipid hepatic clearance. While mainly expressed in the liver, ApoE is also expressed in the brain, adipocytes, macrophages, and steroidogenic cells. ApoE is a surface component of triglyceride-rich lipoproteins, chylomicrons, and HDL, but is critical in forming very low density lipoproteins (VLDL) and chylomicron remnants. In addition, ApoE plays a role in macrophage cholesterol efflux, in the inhibition of T-cell and vascular smooth muscle (VSM) cell proliferation, as well effecting changes to induce neural and cognitive defects.(Basu, Brown et al. 1981, Kayden, Maschio et al. 1985, Newman, Dawson et al. 1985, Eichner, Dunn et al. 2002, Reilly and Rader 2006)

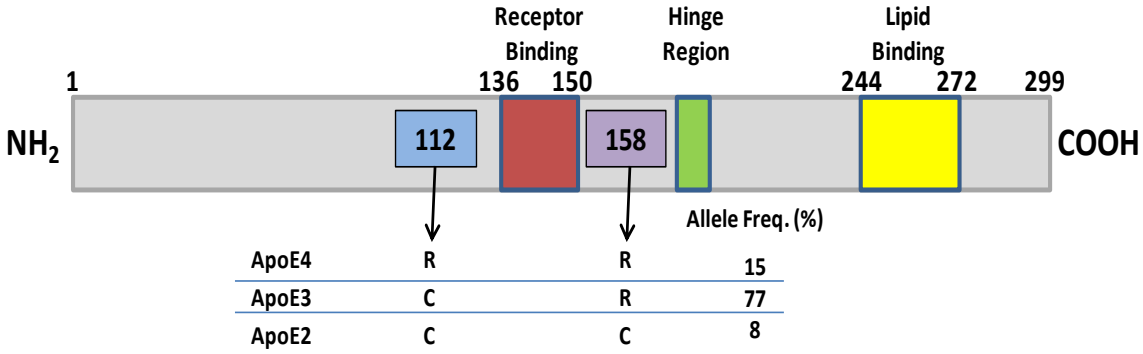


Figure 2: ApoE gene structure and common isoform frequency in general population

ApoE is a 34kD protein encoded by a four exon/three intron gene at chromosome 19q13.2, and is comprised of two structural domains separated by a hinge region (**Fig. 2**). The N-terminal domain is comprised of five alpha helices, which compares similarly in structure to other Apolipoprotein family members.(Mahley, Innerarity et al. 1984) However while ApoE can remain as a monomer, other family members tend to aggregate.(Aleshkov, Abraham et al. 1997) In the lipid bound form, ApoE is able to bind the LDLR with very high affinity, with a K_d approximately $1.2x10_{-10}$ M. Contrastingly free ApoE binds LDLR with 500 times lower affinity than lipid complexed ApoE.(Wilson, Wardell et al. 1991, Dong, Wilson et al. 1994, Morrow, Segall et al. 2000) Furthermore a linker between the N- and C- terminal domains becomes less protease sensitive following lipid binding. While the C-terminal domain of ApoE forms aggregates, residues 1-185 of the N-terminal domain are sufficient for ApoE-bound lipoprotein clearance by the liver.(Wilson, Wardell et al. 1991, Kypreos, Morani et al. 2001)

There exist several cases of humans with ApoE deficiency that exhibit tuberoeruptive xanthomatosis and type III hyperlipoproteinemia, while presenting with elevated cholesterol and normal triglyceride levels.(Ghiselli, Schaefer et al. 1981) However most humans possess one of six phenotypes occurring from the three most common ApoE alleles. ApoE2, ApoE3 and ApoE4 differ by a single amino acid polymorphism at either position 112 or 158 (**Fig.2**). ApoE2 encodes a cysteine at both positions while ApoE4 encodes an arginine, and ApoE3 encodes a cysteine and an arginine at 112/158, respectively.(Weisgraber, Rall et al. 1981)

These point mutations result in conformational changes which must be considered when comparing ApoE3 and ApoE4. In apoE4, Arg-112 orients the side chain of Arg-61 into the aqueous environment where it can interact with glutamate-255, resulting in interaction between the N- and C-terminal domains and a closed confirmation that preferentially binds larger lipoprotein particles. In apoE3, Arg-61 is not available to interact with glutamic acid–255, which allows for an open confirmation and binding to smaller lipoproteins. All animals possess one ApoE isoform, and most express an isoform that contains an arginine at position 112, the same as human ApoE4. However a threonine at position 61 prevents N and C terminal interaction resulting in an open confirmation, as is seen in human ApoE3. (Wilson, Wardell et al. 1991, Dong, Wilson et al. 1994, Weisgraber 1994)(**Fig.3**)

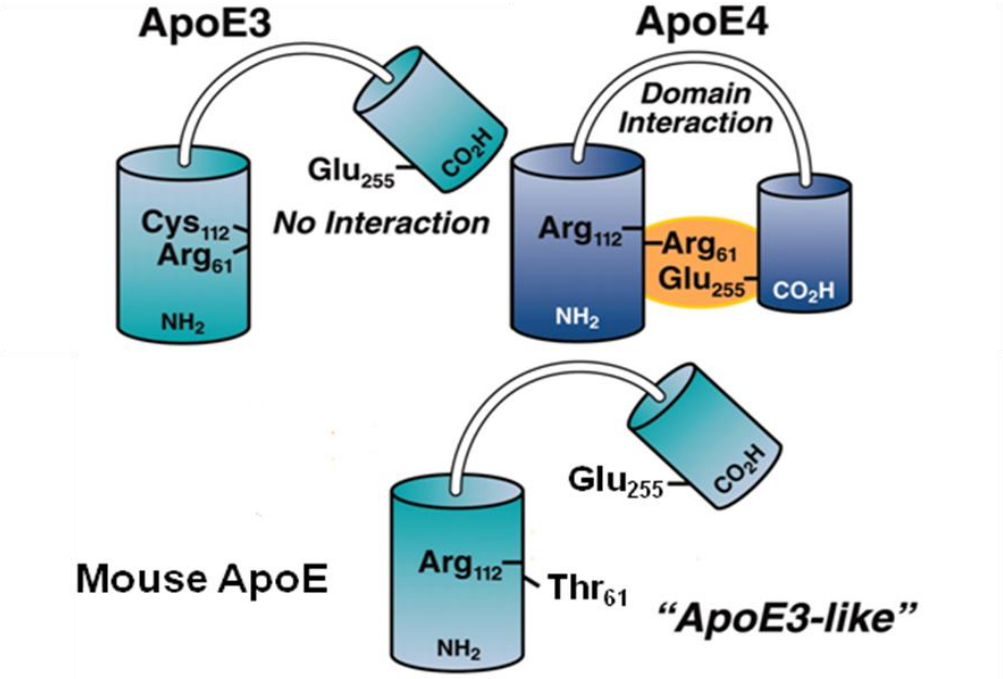


Figure 3: Comparison of human ApoE3, ApoE4, and animal ApoE structures.
Adapted from Mahley RW et al. PNAS 2006; 103: 5644-5651, copyrighted 2006 by National Academy of Sciences.

Non-CVD association and effects of common ApoE isoforms

ApoE4 is most widely known as the major risk factor for Alzheimers disease such that about 70% of all patients possess at least one ApoE4 allele. The depth of ApoE4 research has elucidated many cellular effects underlying the ApoE4 and Alzheimer's correlation. Recent evidence suggests that those with traumatic brain injury are twice as likely to develop Alzheimers, and that ApoE4 is thought to diminish brain recovery following injury. ApoE4 also impairs astrogliosis, a process imperative to modulate a variety of defensive and rescontructive molecular processes in the brain. (Teasdale, Nicoll et al. 1997, Sun, Wu et al. 1998, Friedman, Froom et al. 1999, Ophir, Meilin et al. 2003). ApoE4 is also known to complex with amyloid β (Aβ) peptides, which are known to be toxic to neurons and initiate some of the first damage of Alzheimers, and this significantly increases complement activation. In fact in neurological studies, ApoE4 increases Aβ production, destabilizes neuronal membranes, and increases apoptosis. (Poirier 1994, McGeer, Walker et al. 1997, Ladu, Reardon et al. 2000) Interestingly ApoE4 complexes much more readily with Aβ than does ApoE3, yielding a denser matrix of polymerized monofilbrils.(Sanan, Weisgraber et al. 1994, Aleshkov, Abraham et al. 1997) Furthermore ApoE4 synthesized by neurons undergoes proteolytic cleavage to a much greater extent than apoE3. The resulting ApoE4 fragments with C-terminal truncations then escape the secretory pathway, enter the cytosol, interact with mitochondria, and cause neurotoxicity as mitochondria are important in synaptogenesis.(Chang, Ma et al. 2005, Mahley, Weisgraber et al. 2006) (**Fig.4**)

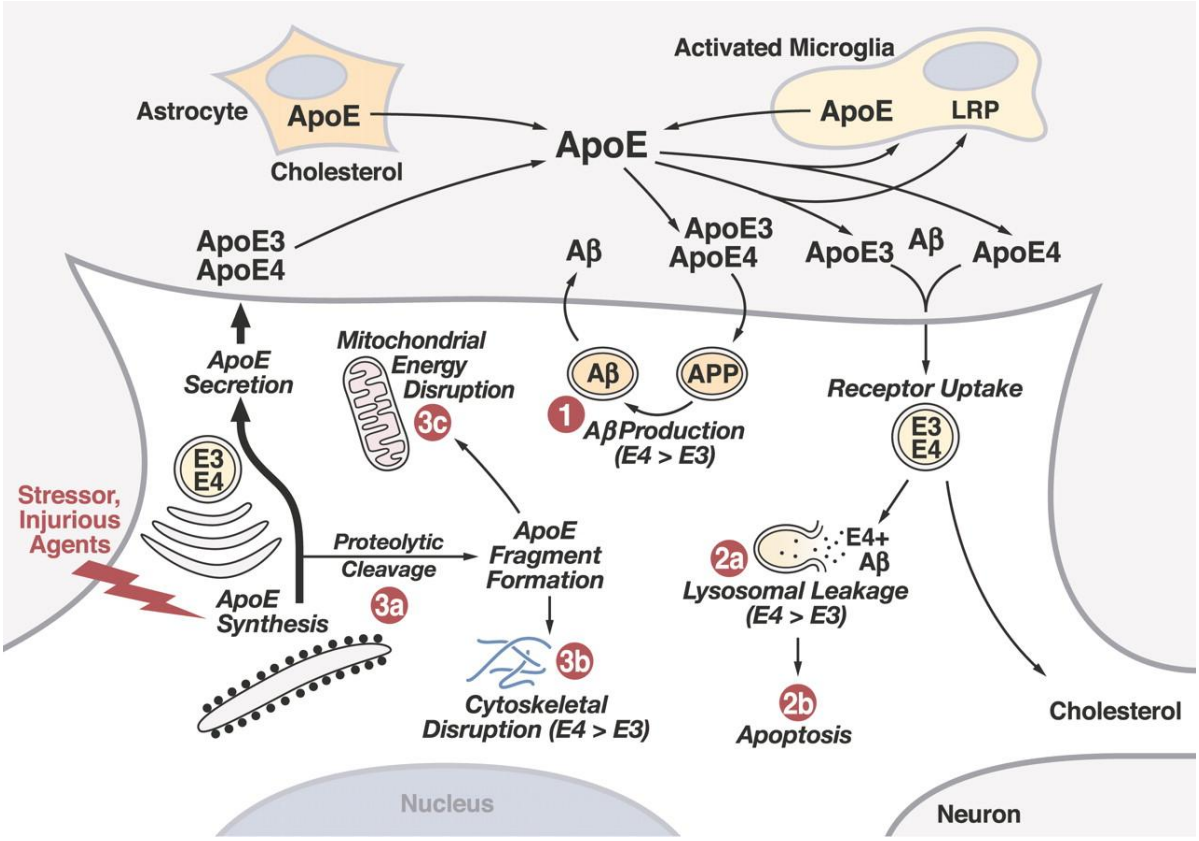


Figure 4: ApoE Effects in Neuropathology.
Mahley R W et al. PNAS 2006;103:5644-5651, © 2006 by National Academy of Sciences

ApoE4 is also associated with a variety of non-Alzheimer's diseases. In healthy, non-demented individuals, carriers of ApoE4 possess memory and an increased rate of hippocampal loss in aging women-which is supported by studies in female humanized ApoE4-expressing mice. (Grootendorst, Bour et al. 2005) ApoE4 is also linked with incidence and progression of many diseases of immune dysfunction, which includes correlation with diseases such as psoriasis, as well as development from human immunodeficiency virus (HIV) to acquired immune deficiency syndrome (AIDs), and progression of cancers in AIDs patients. In multiple sclerosis, ApoE4 is associated with

quicker progression to disease.(Corder, Robertson et al. 1998, Liestol, Kvittingen et al. 2000, Campalani, Allen et al. 2006, Pinholt, Frederiksen et al. 2006) These instances earmark ApoE4 expression with proinflammatory pathology and diseases. Furthermore, while associated with more oxidative damage, ApoE4 smokers are more susceptible to damage from smoking, and they possess higher oxidized LDL (ox-LDL), an inflammatory lipoprotein. (Humphries, Talmud et al. 2001, Manttari, Manninen et al. 2001, Marz, Scharnagl et al. 2004, Talmud, Stephens et al. 2005, Kahri, Soro-Paavonen et al. 2006)

In complex fashion, ApoE4 is not completely predictable as a pathological marker in disease progression. Interestingly, ApoE4 carriers present with lower c-reactive protein (CRP), an indicator/effector of inflammation, levels than both ApoE3 homozygotes and ApoE2 carriers.(Judson, Brain et al. 2004, Marz, Scharnagl et al. 2004, Kahri, Soro-Paavonen et al. 2006, Ukkola, Kunnari et al. 2009) ApoE4 may also aid in HIV entry into cells, and ApoE4 also acts in a contrasting fashion to confer protection against liver damage mitigated by hepatitis C virus.(Wozniak, Itzhaki et al. 2002, Burt, Agan et al. 2008) Interestingly ApoE4 has been shown to offer protection against age-related macular degeneration, a condition of excessive angiogenesis in the choroid and retina. ApoE4 may also shorten or ameliorate symptoms of Giardia and other childhood diarrheal diseases.(Oria, Patrick et al. 2005, Thakkinstian, Bowe et al. 2006, Minihane, Jofre-Monseny et al. 2007, Oria, Patrick et al. 2007)

CVD prevalence of common ApoE isoforms

ApoE3 is most commonly seen in the population at an allelic frequency around 77%, and is considered the most beneficial and neutral isoform. ApoE2 exists to a much lesser degree at an allelic frequency around 8%, and is clinically associated with an increased risk of type III hyperlipoproteinemia, as well as peripheral artery disease. While present at 15% in the population, the majority of ApoE4 literature explores and clarifies the role of ApoE4 in Alzheimer's as the major disease risk factor known to date. However, ApoE4 is also clinically associated with more disseminated atherosclerosis, increased carotid intima-media thickness, left ventricular failure in β-thalassemia, and an 80% increased risk of death from CVD.(Economou-Petersen, Aessopos et al. 1998, Eichner, Dunn et al. 2002, Reilly and Rader 2006) Importantly, in spite of the most studied associations with lipid clearance, these varying cardiovascular implications linked with the common ApoE alleles do not clinically correlate with changes in global cholesterol homeostasis.

Furthermore the prevalence of these isoforms did not evolve from post-reproductive dietary effects, but more likely from effects due to plague-like infection.(van Bockxmeer, Mamotte et al. 1995, Bertram, McQueen et al. 2007, Minihane, Jofre-Monseny et al. 2007, Mahley, Weisgraber et al. 2009) Indeed ApoE4 is considered the ancient allele, and persisted probably because some pathogen resistance was conferred.(Mahley 1988, Finch and Sapolsky 1999, Mahley and Rall 1999, Mahley and Rall 2000, Gerdes 2003) Today ApoE4 exists at higher percentages in high and low latitude populations, most likely to aid those in extreme climates with increased circulating lipids useful in cooling and

thermogenesis. Specifically, ApoE4 is present at higher frequencies in Northern European Caucasians and sub-Saharan Africans versus southern Europeans (**Fig. 5)**.

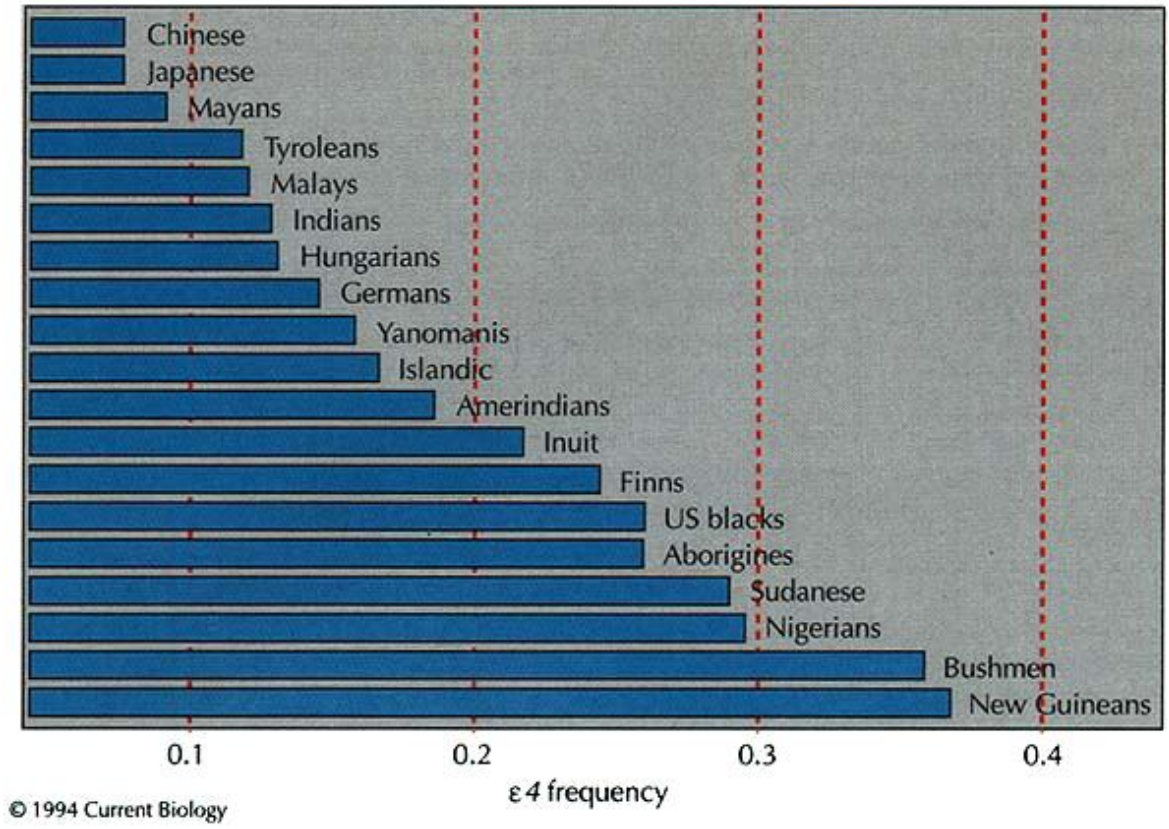


Figure 5: Frequency distribution of the apolipoprotein E allele 4 in the world population.

Adapted from Gerd Utermann, **Alzheimer's Disease: The apolipoprotein E connection,** Current Biology Volume 4, Issue 4, 1994, 362-365. Reprinted with permission license number 3075120932915.

Interestingly ApoE2 is almost non-existent in the Mexican American and American Indian populations. (Corbo and Scacchi 1999, Eichner, Dunn et al. 2002, Singh, Singh et al. 2006)

The most frequent to least common of the six phenotypes in humans are; 3/3, 4/3, 3/2, 4/4/, 4/2, and 2/2. Of interest is that those individuals expressing the 4/3 phenotype possess greater CVD risk than homozygotes expressing 3/3, implicating the interference of ApoE4 on ApoE3-related cardiovascular protective effects.(Eichner, Dunn et al. 2002)

Circulating Lipid Effects

The three ApoE variants bind LDLR family members to clear circulating lipids. While ApoE3 and ApoE4 bind receptors with equal affinity, ApoE2 binds with approximately 2% the binding strength of ApoE3 or ApoE4. In humans the equal binding affinity of ApoE3 and ApoE4 for the LDLR occurs regardless of the lipoprotein particle bound. (Wilson, Wardell et al. 1991, Mahley and Rall 2000)

When compared with ApoE3 carriers, those with ApoE2 have lower cholesterol levels while they possess higher circulating ApoE concentrations, and those with ApoE4 have higher cholesterol levels and lower circulating ApoE concentrations. (Boerwinkle and Utermann 1988) These changes in lipoprotein and lipid profile are due to changes in expression of liver receptors. Specifically, the slower clearance of ApoE2 and higher ApoE levels stimulates the liver to upregulate LDLR lowering cholesterol levels. Conversely ApoE4 is cleared more efficiently from the circulation, thus lowering plasma ApoE4 levels raising cholesterol levels. (Utermann, Pruin et al. 1979, Smit, de Knijff et al. 1988)

Beyond changes in lipid levels, some evidence suggests that different ApoE isoform-bound lipoproteins can affect vascular phenotypes. In fact ApoE-bound lipoproteins affect endothelial apoptosis in an isoform-dependent manner, with ApoE4-lipoproteins associated with higher rates of endothelial cell death.(DeKroon, Mihovilovic et al. 2003) Furthermore, ApoE4 bound to VLDL inhibits the apoptotic properties of HDL by diminishing the phosphorylation of Akt, and important signaling kinase, in endothelial

cells.(DeKroon, Robinette et al. 2006) However studies link carriers of ApoE2 and ApoE4 with differential responses to intake of dietary fat, specifically that ApoE4 carriers exhibited a greater cholesterol response than carriers of ApoE2.(Schaefer, Lamon-Fava et al. 1997, Masson, McNeill et al. 2003, Eberle, Kim et al. 2012)

Genetic Variants and CVD

The risk and severity of cardiovascular disease (CVD) varies in association with different ApoE alleles, which in humans are designated ApoE2, ApoE3 and ApoE4. Compared with ApoE3, ApoE2 and ApoE4 are associated with greater CVD, and are both associated with increased frequency of ischemic cerebrovascular disease. In addition, ApoE2 is associated with greater incidence of hyperlipoproteinemia and peripheral artery disease, and ApoE4 is associated with more disseminated atherosclerotic lesions, more carotid intima-media thickness, and 80% increased risk of death from CVD.(Eichner, Dunn et al. 2002, Reilly and Rader 2006) In atherosclerosis-prone mice on a Western diet expressing ApoE3, atherosclerotic lesions regressed indicating a protective role in the presence of this benign ApoE isoform. (Tsukamoto, Tangirala et al. 1999) In fact macrophages expressing ApoE at lesion sites proved anti-inflammatory as the NO production suppressed cell adhesion molecule (CAM) expression.(Stannard, Riddell et al. 2001) Interestingly heterozygous allelic combinations of the ApoE isoforms can influence CVD risk. For instance E3/E4 heterozygotes exhibit higher CVD risk than E3/E3 homozygotes.(Eichner, Dunn et al. 2002) Relationships between ApoE genotype and CVD in humans appear to be independent of differing influence on lipoprotein clearance and cholesterol or triglyceride levels(van Bockxmeer, Mamotte et al. 1995), and there is also evidence of lipid-lowering-independent

roles of ApoE in vascular protection in mice.(Yamada, Inoue et al. 1992, Shimano, Ohsuga et al. 1995) However the vascular receptor(s) effecting ApoE clinical consequences have not been determined.

APOER2

Expression

Apolipoprotein E Receptor 2 (ApoER2), also called LDL receptor-related protein 8 (LRP8)(Beisiegel, Weber et al. 1989), is mainly expressed in the brain and testes, but also the ovary, placenta, and endothelium. ApoER2 is targeted intracellulary to lipid rafts and caveolae, and between humans and mice there exists approximately a 90% genome homology. ApoER2 is in the LDLR family of type I transmembrane receptors, however since ApoER2 is not abundantly expressed in the liver, its primary function is most likely not related to lipid clearance.(Willnow, Nykjaer et al. 1999)

The receptor is comprised of five structural regions-an extracellular N-terminal ligand-binding domain with cysteine-rich repeats, an epidermal growth factor (EGF) domain, a YWTD β-propeller that binds laminin and other ligands, an O-linked sugar domain, a single transmembrane sequence, and a cytoplasmic domain containing an NPxY motif critical in adaptor protein binding.(Kim, Iijima et al. 1996) ApoER2 also has a ligand binding domain comprised of eight ligand binding repeats, however three of these repeats are often spliced out.(Reddy, Connor et al. 2011) (**Fig.6)** ApoER2 has been studied extensively in neuronal tissue(Willnow, Nykjaer et al. 1999), where it is expressed in the hippocampus,

cerebellum, neocortex, cortical neurons, and olfactory bulb.(Reddy, Connor et al. 2011) The receptor has also been localized to plasma membrane caveolae where it mediates signaling events in cell culture overexpression models.(Riddell, Sun et al. 2001)

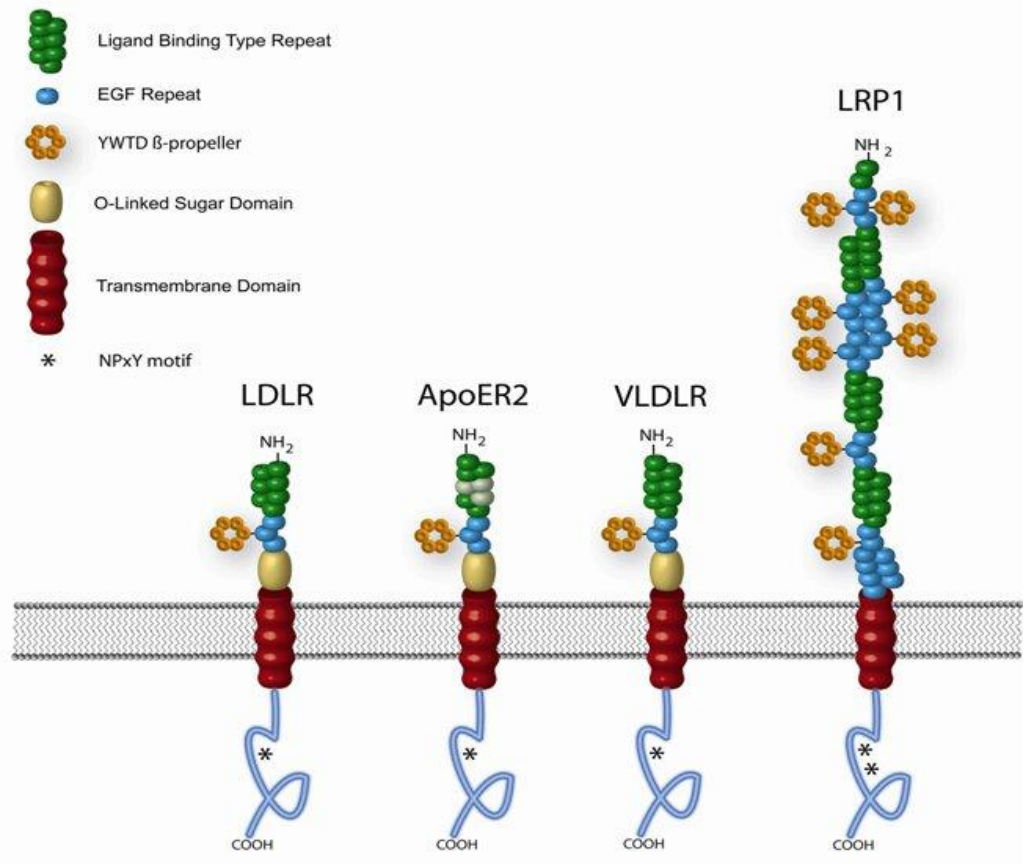


Figure 6: LDL receptor family members.
Adapted from Rebeck, G., LaDu, M., Estus, S., Bu, G., Weeber, E. The generation and function of soluble apoE receptors in the CNS. (2006) *Molecular Degeneration.* Copyrighted 2006 Rebeck et al., licensee BioMed Central Ltd.

Function

In many cell types, ApoER2 acts to endocytose lipoproteins, however the receptor's function extends beyond lipid uptake to participate in crucial neuronal signaling.

In particular, ApoER2 regulates neuronal cell migration during brain development via the regulation of Src family kinases.(Willnow, Nykjaer et al. 1999) These processes are mediated by the intracellular adaptor protein disabled-1 (Dab1) and other adaptor molecules including autosomal recessive hypercholesterolemia (ARH), JNK-interacting protein-1 (JIP-1), JNK-interacting protein-2 (JIP-2), and postsynaptic density protein 95 (PSD-95) that interact with the cytoplasmic domain of the receptor. (Schneider and Nimpf 2003, Stolt and Bock 2006, Masiulis, Quill et al. 2009) In fact the simultaneous interaction of ApoER2 with both Dab1 and postsynaptic scaffolding protein PSD -95 is required for ApoER2 to activate the N-Methyl-D-aspartate receptor (NMDAR) in response to Reelin. Reelin is a glycoprotein critical in stimulating proper migratory function and alignment of neurons to the proper inside-out formation of the neocortex. In order for this developmental process to occur, both ApoER2 and VLDL receptor (VLDLR) are critical for normal growth and development. (Reddy, Connor et al. 2011) In adult neural tissue the primary ApoER2 ligands are ApoE and Reelin which regulate synaptic plasticity. (Strickland, Gonias et al. 2002, Schneider and Nimpf 2003)

ApoER2 signaling has mainly been studied in the brain, but has also been observed in VSM, and it has been demonstrated that a spice variant of ApoER2 in platelets promotes the activation of platelet endothelial nitric oxide synthase (eNOS). (Riddell, Graham et al. 1997) In immune cells ApoER2 propagates activated protein C (APC) signaling via Dab1 in order to confer cytpoprotective and anticoagulant effects.(Yang, Banerjee et al. 2009) However the role of ApoER2 in endothelial cells is not clear.(Korschineck, Ziegler et al. 2001, Sacre, Stannard et al. 2003)

Proteolysis

Ligand binding induces cleavage of ApoER2, especially in the case of Reelin interaction, however even minor cleavage occurs when ApoER2 is unstimulated. Gamma secretase cleaves at the intramembrane region to create intracellular domains, while ApoE binding leads to the release of extracellular domains.(Hoe and Rebeck 2005, Hibi, Mizutani et al. 2009) In addition E3 ubiquitin ligase activates proteosomal degradation of the receptor, except when Reelin is bound which favors lysosomal degradation, while proprotein convertase subtilisin/kexin type 9 (PCSK9) also induces ApoER2 degradation.(Reddy, Connor et al. 2011) However when ApoER2 is bound by a ligand that does not allow for clustering, receptor degradation is diminished. (Reddy, Connor et al. 2011)

Receptor Variants

There are multiple tissue- and species-specific splice variants of ApoER2, which add complexity to their biology in multiple cell types. (Schneider and Nimpf 2003) ApoER2 often undergoes alternative splicing of the ligand binding regions 4-6 in exon 5, and number 8 in exon 7 during development. This splicing alters affinity binding to ligands such as ApoE, as well as changes in exons 9 and 16. Importantly, exon 19 can be alternatively spliced resulting in c-terminal truncation that alters JNK, JIP1/JIP2, and PSD 95 interaction which diminishes Reelin signaling.(Reddy, Connor et al. 2011) One variant of ApoER2, in which an arginine at position 952 is substituted by glutamine (R952Q) in the last c-terminal exon 5' to the upstream intron, has recently been associated with early onset of myocardial infarctions (MI) and coronary artery disease (CAD) in familial and population-based studies. Consistent with having a primary role in signaling and not in lipid clearance, ApoER2 is not

abundant in the liver(Shen, Li et al. 2007), it is targeted to caveolae/lipid rafts(Riddell, Sun et al. 2001), and the increase in early-onset MI and CAD associated with ApoER2-R952Q is not related to differing cholesterol levels or lipoprotein clearance. Compared with wild-type receptor, the ApoER2-R952Q variant displays altered signaling to p38 MAP kinase in Meg-01 cells.(Schneider and Nimpf 2003) Importantly, there is an additive effect of the ApoER2-R952Q variant and the apoE4 allele on cardiovascular disease risk, with the combined genotype of QQ/E4 showing the greatest association with MI (odds ratio 3.88).(Masiulis, Quill et al. 2009) These cumulative observations suggest that there is an important vascular role for ApoER2.

APOE AND APOER2 IN REELIN SIGNALING IN THE BRAIN

Pathway and Functions

While ApoE also binds scavenger receptor type BI and cell glycosaminoglycans, including heparin and heparan sulphate proteoglycans, this interaction occurs in the hepatic clearance of remnant lipoproteins(Mahley and Ji 1999, Libeu, Lund-Katz et al. 2001). However ApoE also binds ApoER2 in platelets to upregulate nitric oxide synthase, but most information known about ApoE binding concerns interaction with ApoER2 and affects signaling in the nervous system. ApoER2 and VLDLR share about 50% DNA sequence homology, and the clustering of these receptors is essential for Reelin signaling during development.(Kim, Iijima et al. 1996) Reelin binds both receptors with similar affinity and induces the ApoER2-VLDLR complex to bind DAB1 at the NPxY motifs

that leads to the activation of Src and subsequent phosphorylation of DAB1 tyrosines.(Benhayon, Magdaleno et al. 2003) ApoER2 then indirectly activates extracellular signal-regulated kinases 1/2 (ERK1/2) by increasing NMDA receptor conductance, as well as increases long term potentiation through exon 19.(Weeber, Beffert et al. 2002, Beffert, Weeber et al. 2005) (**Fig. 7**)

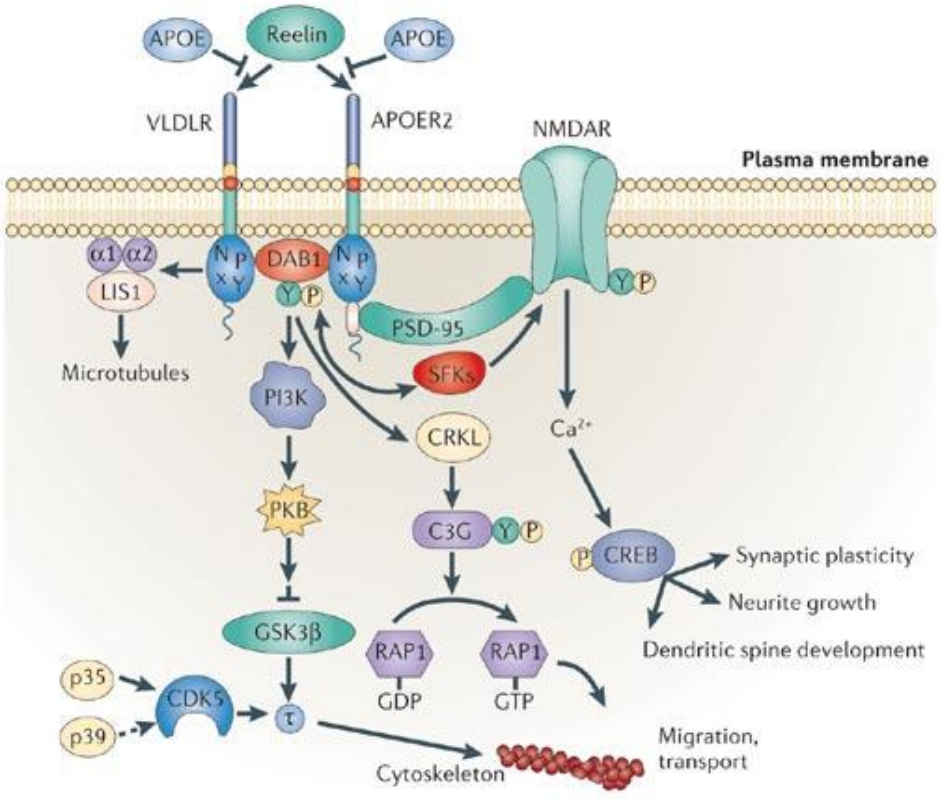


Figure 7: Reelin-Initiated signaling events in neurons.
Adapted from Herz and Chen, *Nature Reviews Neuroscience* **7,** 850-859 (November 2006), reprinted with permission license number 3075111286711.

However, while disabled 2 (DAB2), cannot compensate for loss of Reelin signaling, DAB2 can cause ApoER2 endocytosis in the brain independent of binding to the NPxY region within exon 19.(Yang, Smith et al. 2002, Zhou, Scholes et al. 2003, Cuitino, Matute et al. 2005)

ApoE binds ApoER2 in the brain to affect synaptic plasticity. When Reelin binds ApoER2 and is concurrently bound by ApoE3, this causes receptor endocytosis and eventual recycling of free receptor back to the neuronal cell surface. However when ApoE4 and Reelin concurrently bind ApoER2 to cause clathrin-dependent receptor-ligand endocytosis, receptor recycling back to the cell surface is diminished, further affecting future Reelin signaling to the NDMA receptor, which controls synaptic plasticity and memory.(Chen, Durakoglugil et al. 2010) (Cuitino, Matute et al. 2005)

ENDOTHELIAL DYSFUNCTION IN CVD

Altered Nitric Oxide Production

The endothelial cell monolayer that lines blood vessels and the heart is a multifunctional structure responsible for serving as a barrier and selectively gatekeeping circulating particles from surrounding tissues. The endothelium is a multifunctional structure that regulates blood flow, vascular tone in small and large vessels, coagulation, VSM cell growth, and the adherence and transmigration of inflammatory cells into the vascular wall. (Gonzalez and Selwyn 2003, Mensah, Ryan et al. 2007) One of the primary signaling molecules modulating these processes is nitric oxide (NO), which is mainly produced by

eNOS expressed in endothelial cells, though it can also be generated from dietary anion nitrate as well.(Ataya, Tzeng et al. 2011) NO promotes the growth and migration of endothelial cells, thereby favoring angiogenesis and vascular repair, it attenuates VSM cell growth and migration, and it is antithrombotic and antiadhesive. (Voetsch, Jin et al. 2004)(**Fig. 8)** NO also confers resistance to apoptosis in endothelial cells, through the s-nitrosylation of caspases, thus attenuating their activity. This protective effect is critical to maintain vascular health as apoptosis is evident in both in the endothelial turnover associated with atherosclerosis, as well as in atherosclerosis plaques. (Hansson, Chao et al. 1985) In basal conditions, caveolin-1 and caveolin-3 (cav-1 and cav-3) maintains eNOS in its inactivated state, thereby limiting the production of NO. Increase in intracellular calcium in response to agonist stimulation leads to the disruption of the caveolin/eNOS interaction by calcium-bound calmodulin. Heat shock protein 90 (Hsp90) consecutively binds eNOS and favors the recruitment of Akt, which in turn phosphorylates eNOS on serine-1177. Activation of Akt results from the activation of signaling pathways including the stimulation of phosphatidylinositol 3-kinase (PI3K) in response to a variety of agonists and also statins. The active eNOS dimer is comprised of a calcium/calmodulin, reductase, and heme oxygenase domain which utilizes oxygen and converts L-arginine to produce NO and L-citrulline.(De Caterina, Libby et al. 1995, Dimmeler, Dernbach et al. 2000, Radisavljevic, Avraham et al. 2000, Shiojima and Walsh 2002, Voetsch, Jin et al. 2004, Miao, Fontana et al. 2008)**(Fig.9)** The phosphatase calcineurin is similarly recruited in the eNOS vicinity via hsp90 binding; the subsequent dephosphorylation of eNOS on threonine-495 also contributes

to maintain NO release, independently of any further changes in intracellular calcium.(Sun and Liao 2004, Miao, Fontana et al. 2008)

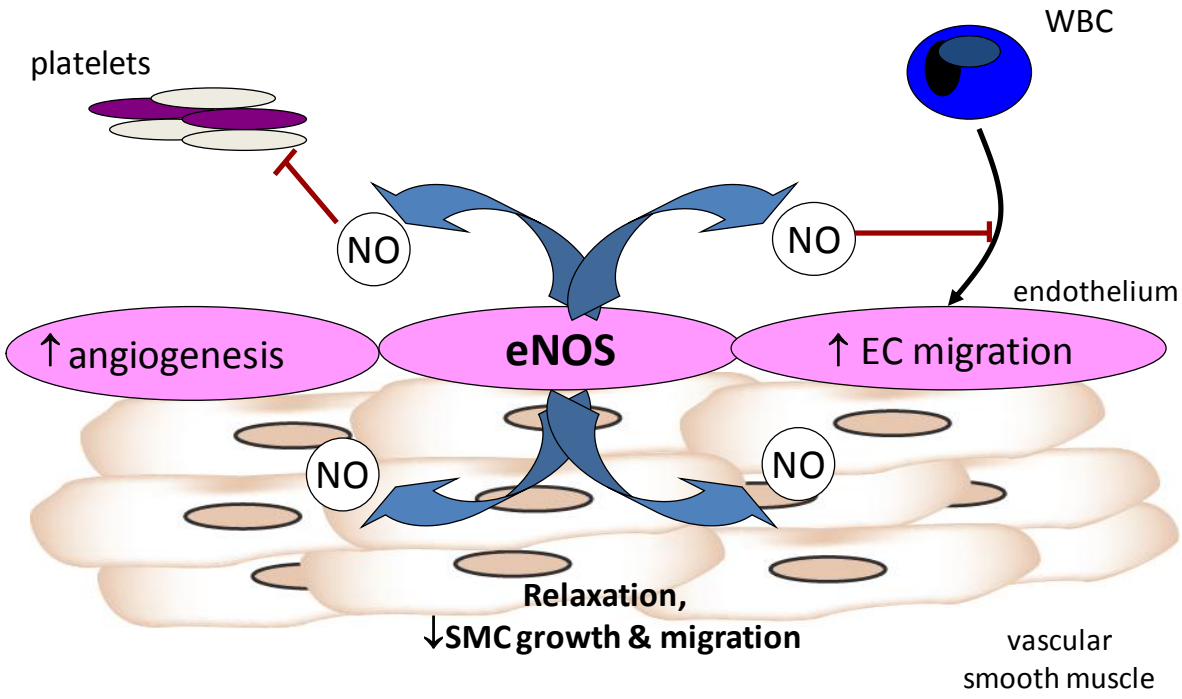


Figure 8: Diverse roles of endothelium-derived nitric oxide. The endothelium serves as a dynamic barrier in the vasculature, producing nitric oxide which prevents adhesion of monocytes and thrombosis, increases angiogenesis, and relaxes and protects the underlying smooth muscle layer.

NO then diffuses into the underlying smooth muscle layer where is utilizes soluble guanyl cyclase to induce VSM relaxation through dephosphorylation of the myosin light chain (Moncada and Higgs 1995, Ataya, Tzeng et al. 2011) Specifically NO utilizes cyclic guanosine monophosphate (cGMP) to activate PI3Kand promote endothelial migration and neovascularization, important processes in angiogenesis and recanalization.(Kawasaki, Smith et al. 2003) NO can also signal to affect the vasculature through oxidation, S-nitrosylation, or ribosylation of proteins.(Ataya, Tzeng et al. 2011) In diseased endothelium, bioavailability

of NO decreases, which leads to upregulation of CAMs increasing monocyte adhesion to the endothelial monolayer. In particular interleukin-1 alpha has been shown to increase vascular cell adhesion molecule -1 (VCAM-1), e-selection, and intracellular adhesion molecule-1 expression enhancing monoctye adhesion,(De Caterina, Libby et al. 1995) and this process is inhibited by NO. Decreased NO also leads to platelet aggregation increasing thrombosis, as well as amplified smooth muscle cell mitogenesis and vasoconstriction, increased microvascular permeability and impaired angiogenesis.(Baldwin, Thurston et al. 1998) Thus, reduction in NO has been shown to lead to hypertension, atherosclerosis, and vasospasm. In fact low NO in endothelial dysfunction leads to arterial stiffness, which is even seen in normotensive patients before the appearance of clinical CVD.(Cohn 2001) While dimished NO can stem from reduction of cofactors, such as the dysregulation of arginine, or endogenous inhibitors, such as asymmetric dimethylarginine,(Vallance, Leone et al. 1992, Cooke 2000) the rate-limiting factor in the reaction is eNOS. (**Fig. 9)** Furthermore in late stage atherosclerosis, eNOS expression is reduced.(Liao, Shin et al. 1995, Oemar, Tschudi et al. 1998) NO is a highly short-lived molecule that is often scavenged through reaction with reactive oxygen species such as superoxide. Overproduction of NO by inhibition of cytochrome-c oxidase or activation of calcium-independent, inducible nitric oxide synthase (iNOS) following inflammatory stimuli also effects vascular damage.(Ataya, Tzeng et al. 2011)

NO in migration

Angiogenesis is the process of forming new blood vessels. Crucial to the initiation of angiogenesis is endothelial cell sprouting and migration.(Carmeliet and Jain 2000)

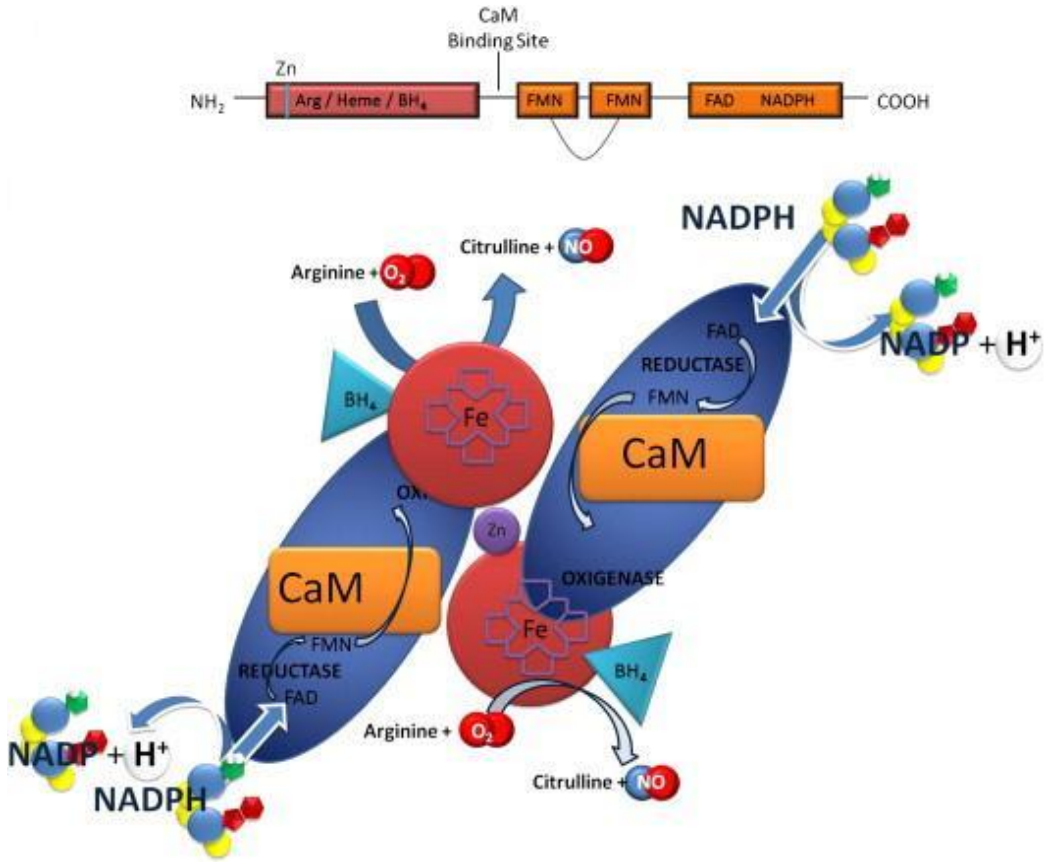


Figure 9: Molecular structure of eNOS monomer and active dimer.
Adapted from Gielis et al., Pathogenetic role of eNOS uncoupling in cardiopulmonary disorders. (1 April 2011), **50,** 7, 765-776. Copyrighted 2010, reprinted with permission license number 3075131411570.

Vascular endothelial growth factor (VEGF) was originally explored as a vascular relaxation factor, capable of dilating blood vessels, as well as increasing vascular permeability. However VEGF is an important stimulator of NO production, signaling mainly though VEGF receptor 2 (VEGFR2) to activate the PI3K-Akt axis to activate eNOS in a parallel pathway to the typical eNOS production of NO.(van der Zee, Murohara et al. 1997, Hood, Meininger et al. 1998, Gingras, Lamy et al. 2000) Furthermore, while VEGF is a potent chemotactic factor to guide vectorial motion of endothelial cells, NO mediates spontaneous podokinesis even in stationary cells. NO-induced endothelial-cell migration is

mediated in part by PKG through the production of matrix metalloproteinase 13 (MMP13). (**Fig. 10)** (Noiri, Lee et al. 1998(Lopez-Rivera, Lizarbe et al. 2005)) NO also enhances the interaction between endothelial cells and extracellular matrix(ECM) by upregulating the expression of αvβ3, which binds ECM. Through a positive feedback loop NO also induces the production and secretion of VEGF.(Murohara, Witzenbichler et al. 1999, Dulak, Jozkowicz et al. 2000) Importantly in a study in an endothelial cell line, different ApoE isoforms were shown to stimulate NO release with differential abilities.(Sacre, Stannard et al. 2003)

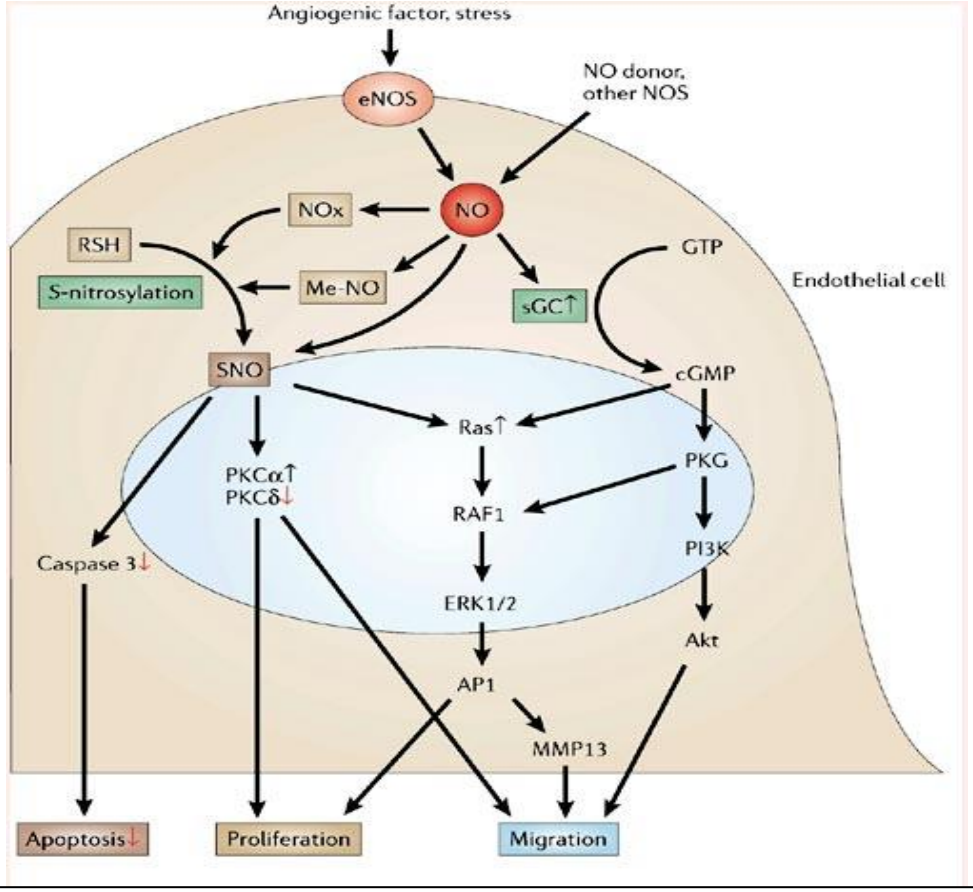


Figure 10: eNOS in Endothelial Cell Migration

Adapted from Dai Fukumura, Satoshi Kashiwagi, Rakesh K. Jain, The role of nitric oxide in tumour progression *Nature Reviews Cancer* 6, 521-534 (July 2006). Reprinted with permission license # 3075141372480.

Role of ApoE/ApoER2 (hypothesis)

As detailed above, there is abundant clinical evidence that the different ApoE alleles and a variant of ApoER2, R952Q, influence the incidence and severity of CVD.(Shen, Li et al. 2007) However, the observations regarding ApoE, ApoER2 and CVD are not explained by alterations in cholesterol or lipoprotein homeostasis, the role of ApoER2 in endothelial cells is poorly understood, and the basis for varying CVD risk related to possessing the ApoE4 allele is entirely unknown. Therefore we hypothesize that variants of ApoE and ApoER2 alter CVD risk by impairing endothelial function.

CHAPTER TWO
Results

APOE3 AND ENDOTHELIUM

Rationale

In order to consider if and how ApoE may affect CVD risk beyond lipid changes, the systemic expression profile of ApoE needs to be considered; primarily, with which tissues this secreted protein would most associate. Since ApoE circulates in the blood, forming a necessary core element of HDL, VLDL, and chylomicron particles, the inner lining of the vasculature would come in constant contact with ApoE.(Mahley 1988, Weisgraber 1994, Mahley and Rall 2000) Furthermore, the initiating step of most cardiovascular disease manifestations is vascular dysfunction, implicating the lumen of vessels as the potential affected partner of ApoE effects.(Patti, Melfi et al. 2005)

The endothelial cell monolayer that lines blood vessels is a multifunctional structure responsible for the regulation of blood flow, vascular tone, coagulation, VSM cell growth, and the adherence and transmigration of inflammatory cells into the vascular wall.(Gonzalez and Selwyn 2003, Mensah, Ryan et al. 2007, Basson 2008) One of the primary signaling molecules modulating these processes is NO, which is normally produced by eNOS expressed in healthy endothelial cells. NO promotes the growth and migration of endothelial cells, thereby favoring angiogenesis and vascular repair, it attenuates VSM cell growth and migration, and it is antithrombotic and antiadhesive.(Moncada, Palmer et al.

1989, Voetsch, Jin et al. 2004) In a diseased endothelium, decreases in bioavailable NO result in many pathological processes detrimental to cardiovascular homeostasis: Monocytes adhere to the luminal layer; platelets are easily activated, aggregate and thrombose; clearance of oxidized LDL is impaired, which leads to increased uptake by macrophages inducing foam cell conversion; endothelial migration and angiogenesis are diminished; and vasoconstriction and mitogenesis of the underlying smooth muscle layer are enhanced.(De Caterina, Libby et al. 1995, Baldwin, Thurston et al. 1998, Ruschitzka, Corti et al. 1999, Schachinger and Zeiher 2000, Taddei, Virdis et al. 2000, Sierra, Coca et al. 2011)

Of the three main ApoE isoforms expressed in humans, ApoE3 is considered the most beneficial variant in terms of associated cardiovascular risk. Initially these differences in risk between isoforms were thought associated with lipid profile changes, however recent studies appear to refute this opinion.(Eichner, Dunn et al. 2002, Song, Stampfer et al. 2004, Reilly and Rader 2006) While recent genome wide association studies imply changes in relationships between ApoE genotype and CVD in humans appear to be independent of differing influence on lipoprotein clearance and cholesterol levels, there is also evidence of lipid-lowering-independent roles of ApoE in vascular protection in mice.(Yamada, Inoue et al. 1992, Shimano, Ohsuga et al. 1995, van Bockxmeer, Mamotte et al. 1995)

ApoE variants may have direct effects on vascular cells since it has been shown that they stimulate eNOS, with ApoE3 having the greatest effect.(Sacre, Stannard et al. 2003) However, the mechanisms of action of the ApoE isoforms in endothelium and their impact on endothelial cell behavior are poorly understood. Since a number of agents that

stimulate endothelial cell migration, such as VEGF, do so via the activation of eNOS and they also promote cell growth(Cines, Pollak et al. 1998), in this chapter we tested the **hypothesis** that ApoE3 promotes endothelial cell growth and migration via the activation of eNOS. Since NO blunts adhesion molecule expression(M, H et al. 1998), we also determined if ApoE3 attenuates endothelial cell-leukocyte adhesion.

Methodology

Primary bovine endothelial cells (BAEC) were harvested within three hours of aorta retention from animals less than 30 months of age from Dallas City Packing (Dallas, TX). Aortas were sectioned horizontally and submerged in 4 degree Celcius RPMI 1640 media plus antibiotics and antimycotics. While carefully keeping aortas submerged in the RPMI media, all vessel branch points were sutured closed with gut 2.0 suture, and clamped with a hemostat on the distal end. The open end of the aortas was then filled with room temperature 2.5 mg/ml collagenase D solution in RPMI media for 8 minutes. Following collagenase digestion of the intimal layer, collagenase solution was carefully removed and seeded on 25cm^2 gelatin-coated cell culture flasks. Cells were then grown to confluency, analyzed and screened for non-endothelial cell contamination, expanded to passage 3, and suspended in fetal bovine serum (Sigma) plus 10% dimethyl sulfoxide (DMSO), and frozen down in cryovials at -80 degrees Celcius. Cells for assays were removed from freeze, and cultured for 3-7 passages using Endothelial Growth Media-2 (EGM-2) (Cambrex Corp.)

The use of human primary endothelial cells, such as human aortic endothelial cells (HAEC) (Cambrex Corp.), human umbilical vein endothelial cells (HUVEC), or human

microvascular endothelial cells (HMEC), would be more physiologically relevant to compare results with those trends seen in human patients. However these cells are expensive and minimally proliferative in culture, thereby making the virtually cost-free and greatly expandable BAEC a more viable source for large scale assays. Many of the following assays were also conducted in human primary cells to ensure that the more physiological response in human cells reflected the results seen in bovine cells. Another cellular source considered is the EA.hy926 partially-transformed human cell line, which is a fusion cell between lung epithelial cells and HUVECs. While this is a cost effective alternative to the human primary cells noted above, the morphology of EA.hy926 vary from classical cobblestone endothelial phenotype, as well as exhibiting slight differences in primary endothelial marker expression and growth characteristics making EA.hy926 a less relevant option to BAEC, in spite of the species relativity of EA.hy926. (Unger, Krump-Konvalinkova et al. 2002)

Human recombinant ApoE3 was expressed in Hek293 cells and purified from the media by heparan-sepharose chromatography.(LaDu, Stine et al. 2006) While the ApoE3 produced is minimally lipidated, this lipidation is not a standardized process, and therefore allows for some variability in particle size as well as lipid concentration beyond what is seen physiologically. While harvest and purification from human plasma would be the ideal source of ApoE3, this presents hurdles due to the human interventions and associated approvals necessary for human experimentation, as well as potential for variations between different patient batches. While a cell-free system which controls lipidation is an option, this creates even more artificial a particle compared to the presently utilized lipidation technique in which ApoE3 binds lipids more naturally in the cell media. While harvesting and

purifying ApoE3 from transgenic expressing ApoE3 mice is an alternative, this is more time consuming and slightly more artificial than the previous two culture methods discussed due to the naturally different HDL-centric lipid profile of mice versus humans.(Hopkins, Huang et al. 2002) In effect any of these methods could have been utilized, but considering the time, monetary, and legal constraints, the Hek293-expression system was the best alternative for this research.

eNOS activation was then assessed in intact cultured BAEC by measuring [^{14}C]L-arginine to [^{14}C]L-citrulline conversion during 15 min incubations with control buffer or varying concentrations of lipid-associated, cell-derived recombinant ApoE3. [**Fig. 1**, n=8, *$p < 0.05$ vs no ApoE3].(Seetharam, Mineo et al. 2006) In order to consider how NO levels may or may not be affected by ApoE3, we chose to use the eNOS activation assay for several reasons. First testing eNOS enzymatic activity in a cell lysate activity assay allows for timely analysis to determine if the eNOS pathway is intact. Second NO is a highly diffusible and short-lived molecule that is rapidly scavenged making direct NO detection extremely difficult due to high variability.(Ignarro 1990) Third, in the chemical reaction in which arginine and oxygen are converted to citrulline and NO, the rate limiting factor is typically availability and expression of the enzyme itself(Wang and Marsden 1995), making an eNOS-centric assay ideal. While diaminofluorescein (DAF-2) is also utilized to detect changes in NO, as well as chemiluminescence,(Tiscornia, Cairoli et al. 2009) again detection of changes in enzyme activity is a more stable readout than detecting transient changes in an unstable gas.

Recognizing the critical role for NO in the promotion of endothelial cell motility (Babaei and Stewart 2002), the effect of ApoE3 on migration was then determined in BAEC. Following growth to near-confluence, the cells were starved overnight in 0% serum Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), and the following day a defined region of cells was removed with a razor blade. The cells were then treated with the conditions shown on the x-axis overnight. The following day the number of cells which migrated past the wound edge was quantified following permeabilization with Triton X-100 (Bio-Rad) , 5% paraformaldehyde (Sigma-Aldrich) fixation and hemotoxylin (Fisher Scientific) staining, and migrating cells were counted under an inverted microscope (Zeiss Axiovert 100M) in a minimum of three high power (100X) fields after 24 h treatment with control conditions (Con), 10% fetal calf serum (serum), or varying concentrations of ApoE3 (**Fig. 2**, n=8, *p< 0.05 vs Con, in all graphs shown the values are mean±SEM). While one may consider if the effects of cells appearing past a wound edge 24 hours after injury may be due to migration or proliferation, this is an understandable concern since both of these processes are increased by expression of NO. However, our lab has previously addressed these concerns utilizing the cell-proliferation inhibitor hydroxyurea in a scratch-wound assay which indicated that most cells that appear past the wound edge in these scratch wound assays migrate versus proliferate (data not shown).(Seetharam 2005) However since both the ability for endothelial cells to migrate and proliferate are indicative of healthy endothelium, both are valuable readouts which could be considered. Furthermore, while a transwell migration assay may be more definitive to compare migratory versus proliferative cells, planar movement of endothelial cells along the surface of a culture dish more closely mimics

the podokinesis required to repair the luminal monolayer or initiate angiogenesis.(Noiri, Lee et al. 1998)

To determine if eNOS activation is required for ApoE3-stimulated BAEC migration, cells were treated with either the control stimulus VEGF (50 ng) or ApoE3 (15 μg), in the absence or presence of the NOS antagonist L-NG-Nitroarginine Methyl Ester (L-NAME) (2 mM) (**Fig. 3**, n=8, *$p<0.05$ vs. control, †$p<0.05$ vs. no L-NAME). While other eNOS inhibitors exist, L-NAME is a good choice as it is an arginine analog, which competes for binding with endogenous arginine, thereby not inferring directly with eNOS expression or structure.(Rees, Palmer et al. 1990)

To assess endothelial cell-leukocyte adhesion(Mineo, Gormley et al. 2005), BAEC were treated for 24h with the x-axis reagent, lipopolysaccharide (LPS) (100ng/ml) in the absence or presence of ApoE3 (15μg/ml) (**Fig. 4**, n=4, *$p<0.05$ vs. control vs. LPS alone). U937 monocytes were then added and incubated with the endothelial cells with mild shaking for 15 min, nonadherent monocytes were removed by washing, and adherent cells per high-power field were counted. LPS was chosen as a classical agonist of monocyte adhesion, as it acts through toll-like receptor 4 (TLR-4) to activate the nuclear factor-kappa B (NF-κB) pathway to upregulate cell adhesion molecules, and this process is abrogated with exogenous NO.(Aljada, Saadeh et al. 2000) The monocyte adhesion method used here is efficient and straightforward, therefore it is ideal for evaluating adhesion in culture. However it should be noted that leukocyte adhesion can and will be measured in vivo using intravital microscopy (IVM). Though this is a much more complex and variable model than

the culture method employed, evaluating adhesion in an animal model using IVM will be more physiologically relevant than conducting monocyte adhesion in cell culture.

In the cell culture studies all findings were replicated in at least 3 independent experiments. Comparisons between 2 groups were done by Student's t tests, and comparisons between 3 or more groups were performed by ANOVA with posthoc Neuman-Keuls testing. A p value <0.05 was considered statistically significant.

Results

ApoE3 and eNOS activation: Using increasing concentrations of human recombinant ApoE3, we evaluated eNOS activation by measuring L-citrulline to L-arginine conversion. At 0.05, 2, and 5 μg/ml of ApoE3 there is an increasing trend of eNOS activation, and at 15μg/ml of ApoE3, eNOS is stimulated. **(Fig.1)** While eNOS assays were conducted utilizing ApoE3 at higher concentrations than 15μg/ml, the increasing trend of eNOS activation in Fig. 1 was not compounded at higher concentrations (data not shown), therefore future assays utilizing ApoE3 were conducted at the 15μg/ml concentration. Furthermore, physiological levels of ApoE in humans and mice are between 5-8 mg/dl, highlighting that the eNOS-stimulating concentration of 15μg/ml used for the cell assays does not exceed physiological range. This assay importantly uncovers ApoE3 as an activator of eNOS in a primary cell culture system, identifying a potential mechanism by which ApoE3 exerts beneficial effects on CVD risk.

ApoE3 and endothelial cell migration: Following incubation with the same increasing concentrations that were tested in the above NOS assay, endothelial migration was explored in the scratch-wound assay using ApoE3 as a potential stimulus. ApoE3 activates eNOS activation to levels that were approximately 70% greater than basal activity, and ApoE3 at 15 μg/ml caused endothelial cell migration several folds higher than non-treated cells. In fact, ApoE3 at 15μg/ml caused marked endothelial cell migration that was comparable in degree to that stimulated by serum **(Fig. 2)**. The scratch-wound assay was then performed in the presence of L-NAME, the eNOS inhibitor, in order to ascertain the relevance of eNOS in ApoE3-induced endothelial migration. Indeed, cells treated with L-NAME and ApoE3 failed to migrate compared to ApoE3-alone controls **(Fig. 3)**, indicating that in order for ApoE3 to induce endothelial migration, eNOS is required.

ApoE3 and monocyte adhesion: ApoE3 ameliorates LPS-induced monocyte adhesion at 15μg/ml, reducing adhesion compared to LPS-alone treated controls. **(Fig.4)** Since LPS potently induces adhesion by upregulating a classical immune pathway; ApoE3 inhibition of this effect by approximately 75% implicates ApoE3 as a non-inflammatory molecule.

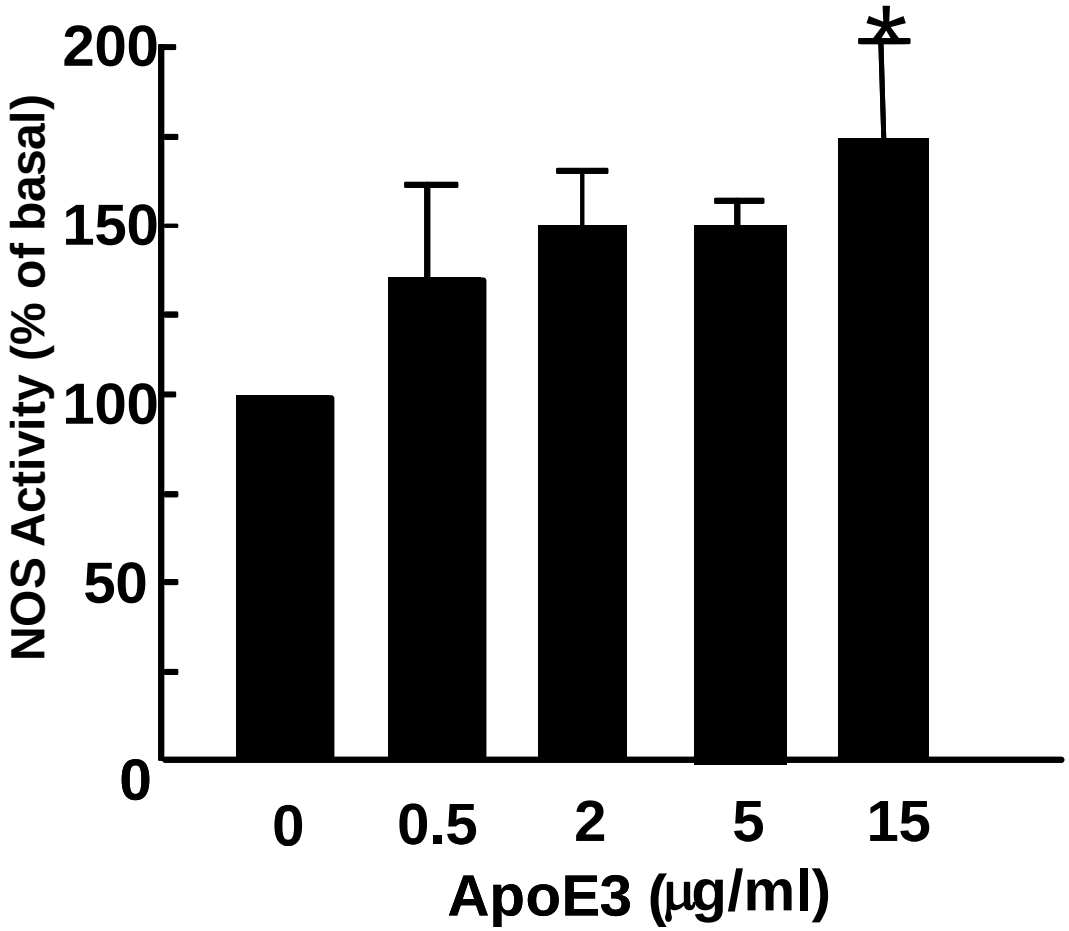


Figure 1: ApoE3 Stimulates eNOS Activity

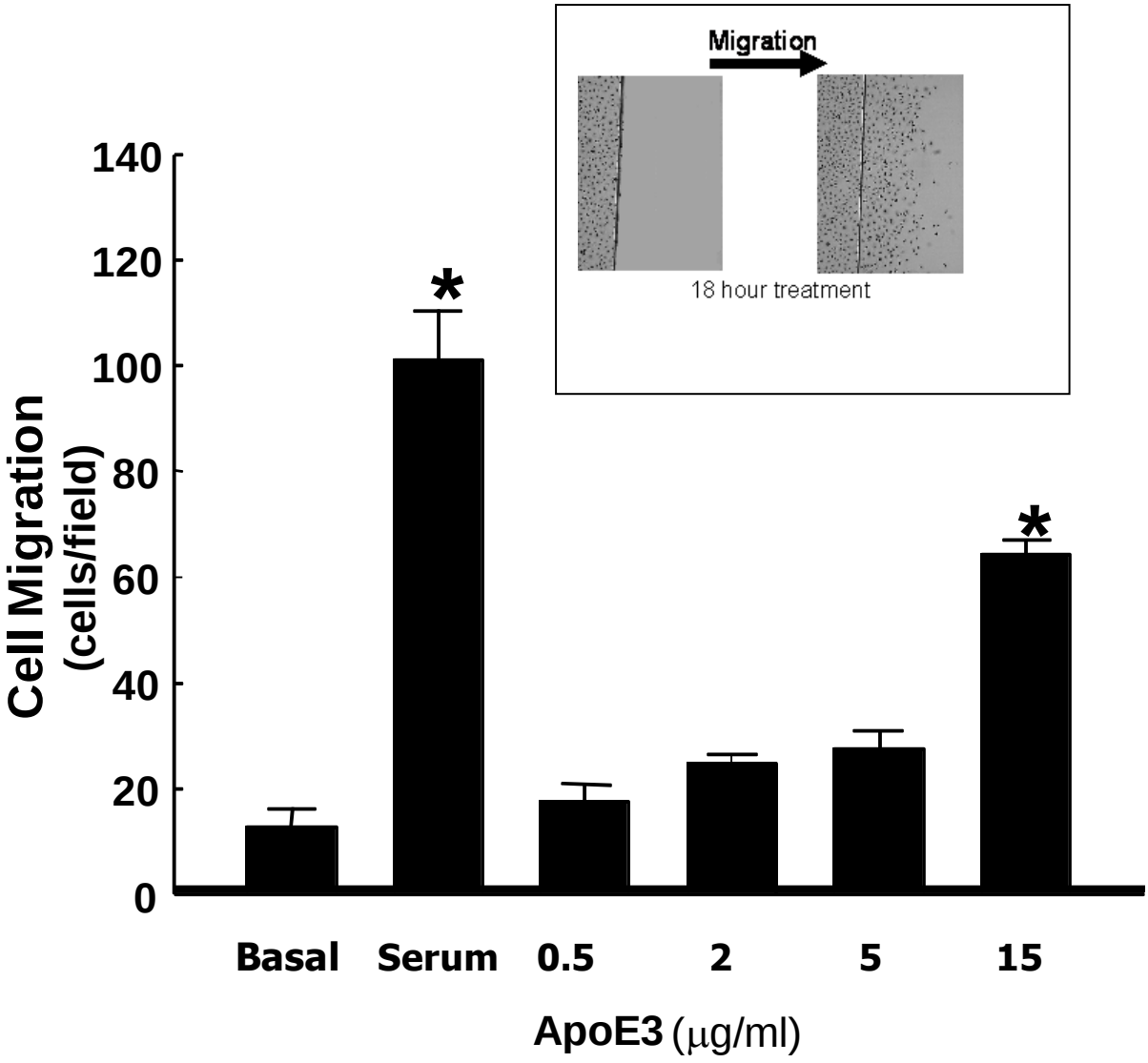


Figure 2: ApoE3 Stimulates Endothelial Cell Migration

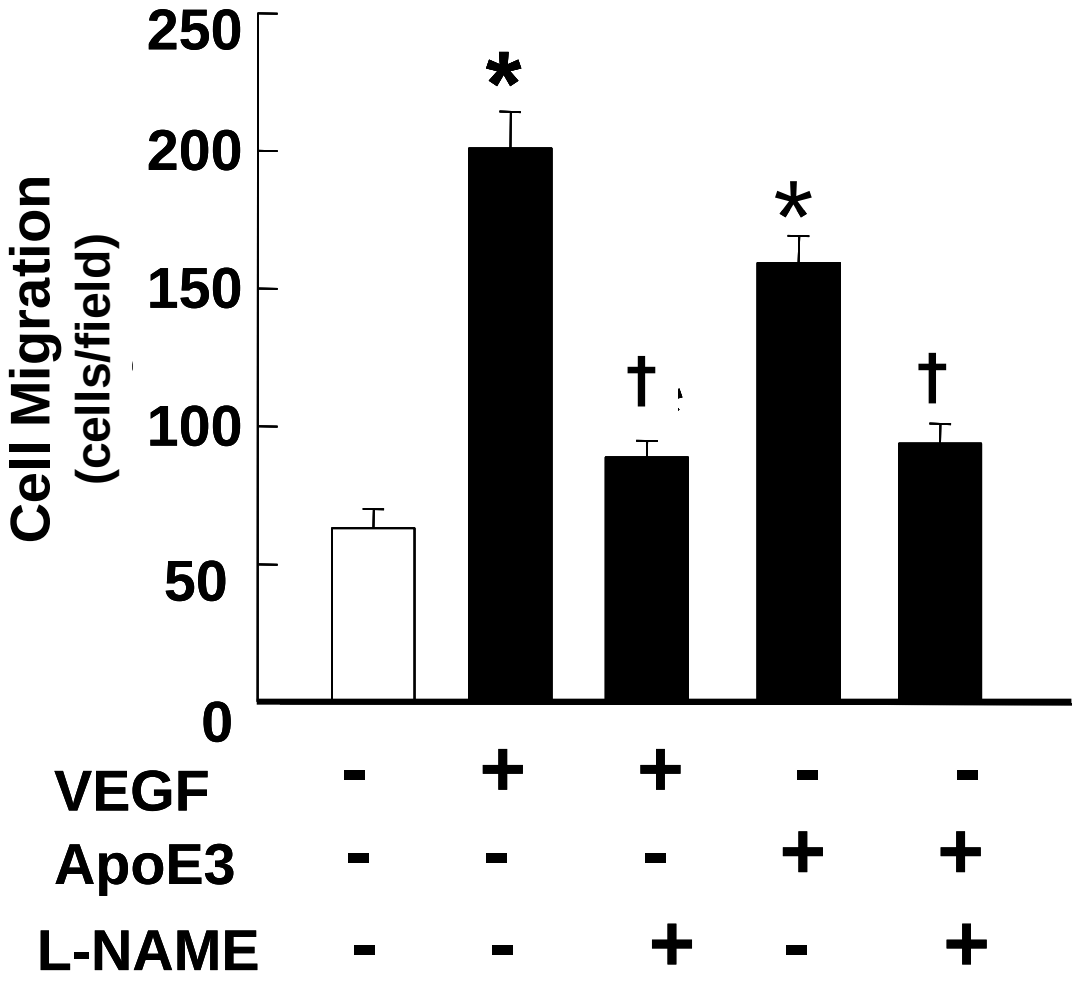
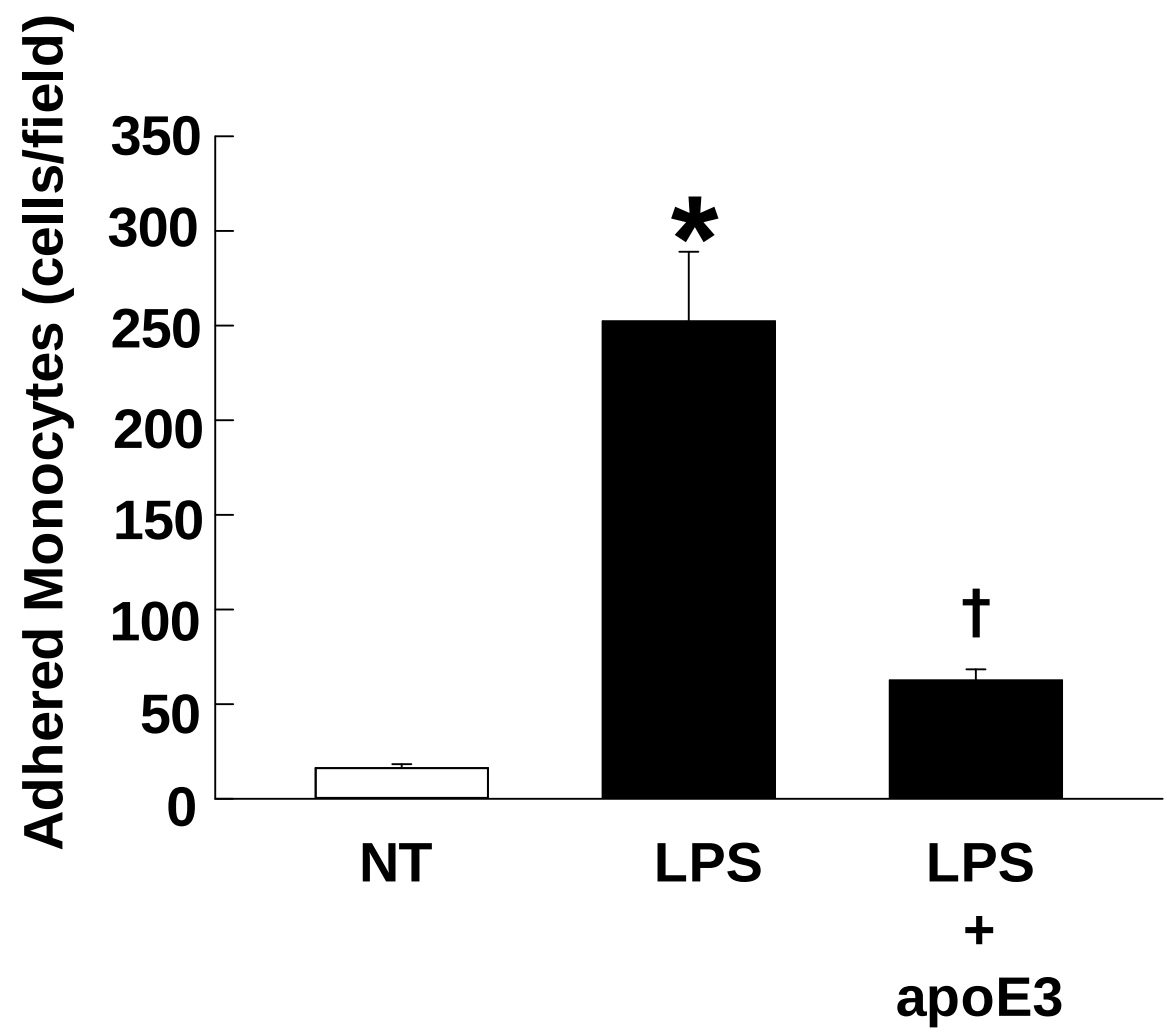


Figure 3: ApoE3 Stimulation of Endothelial Cell Migration requires eNOS

A


Adhered Monocytes (cells/field)
350
300
250
200
150
100
50
0
*
†
NT
LPS
LPS + apoE3

B

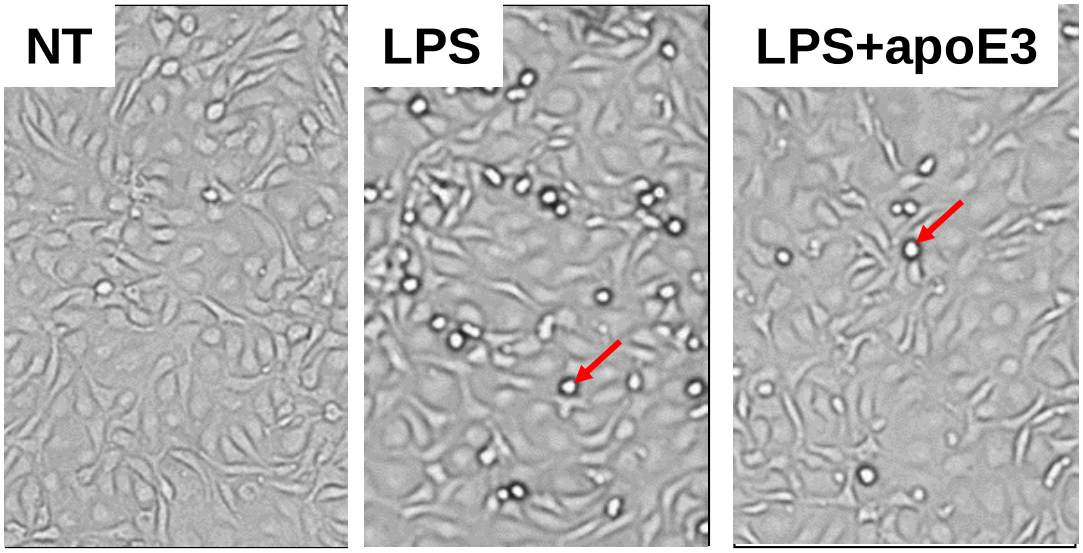


Figure 4: ApoE3 Inhibits Monocyte Adhesion. A: Monocyte adhesion assay in which monocytes adhered to endothelial monolayer were counted and graphed. B: Representative images of groups quantified in A (40X), monoctyes indicated by red arrows.

Discussion

ApoE3, the most common and neutral isoform of ApoE expressed in humans, binds the LDLR family with similar affinity as that exhibited by ApoE4, the ApoE isoform associated with most severe CVD disease risk. While ApoE3 and ApoE4 possess differences in key amino acids that cause opposite conformational changes, as well as bind and clear different lipoproteins preferentially, variance in CVD risk between ApoE3 and ApoE4 carriers is not attributable to changes in global cholesterol homeostasis. However distinction between any ApoE3 and ApoE4 cellular effects have been mainly studied in the brain, leaving questions about CVD linkage unanswered.

While ApoE4 allele is a major risk factor for the development of Alzheimer's disease, the expression of ApoE3 does not carry the same risk. Mechanistically ApoE3 allows for propagation of synaptic activity induced by Reelin, decreases neural apoptosis, and increases dendrite spine density and neuritic growth.(Nathan, Chang et al. 1995) However few studies have explored the effects of ApoE3 in the vasculature in order to ascertain the importance of ApoE3 in CVD protection. In the above results, we have begun to clarify the role of ApoE3 in vascular protection, specifically linking ApoE3 with stimulation of functional readouts of healthy endothelium. We discovered that ApoE3 causes the acute stimulation of eNOS in primary endothelial cells. (Fig. 1) We also demonstrated that ApoE3 potently induces endothelial cell migration (Fig. 2), mirroring the prior findings of Sacre et al in the EA.hy926 endothelial cell line that is a hybrid of primary HUVECs fused with A549 lung epithelial cells(Sacre, Stannard et al. 2003), and that this induction requires eNOS. Finally we showed that ApoE3 abrogates the classical LPS-mitigated monocyte

adhesion effect. To our knowledge these results are the first in primary cell culture that implicate ApoE3 as a positive mediator of endothelial health and homeostasis. These results implicate ApoE3 interaction with endothelium in vascular signaling, which may be one of the main mediators reducing CVD risk in ApoE3 carriers, since the functions shown in the above results are known initiating factors in the progression of CVD.

CHAPTER THREE
Results

APOE3 VERSUS APOE4 IN ENDOTHELIUM

Rationale

In humans there are three common ApoE alleles, ApoE2, ApoE3 and ApoE4, which differ by a single polymorphism at either position 112 or 158.(Weisgraber, Rall et al. 1981) Compared with the common allele ApoE3, ApoE4 is associated with increased frequency of ischemic disease. In addition, ApoE4 is associated with more disseminated atherosclerotic lesions, more carotid intima-media thickness, and 80% increased risk of death from CVD. Though individuals carrying the ApoE4 allele have a greater risk of cardiovascular disease, how the ApoE allelic variants influence cardiovascular health is unknown. Furthermore, individuals who are heterozygous for the ApoE3 and ApoE4 allele possess greater CVD risk compared to ApoE3 homozygotes, indicating a possible dominant negative role of ApoE4 on ApoE3 in the vasculature. (Eichner, Dunn et al. 2002, Reilly and Rader 2006) In this chapter we tested the **hypothesis** that ApoE4 does not activate eNOS, and diminishes ApoE3 activation of eNOS and endothelial migration.

Methodology

In the previous chapter, we showed that ApoE3 induces eNOS activation at 15μg/ml, therefore we utilized the same concentrations of ApoE4 in order to identify any

changes in eNOS activation. This assay was also conducted using higher concentrations of ApoE4, with similar results (data not shown).

ApoE4 was expressed and purified in the same fashion as previously discussed for ApoE3.(LaDu, Stine et al. 2006) Briefly human recombinant ApoE4 was expressed in Hek293 cells and purified via chromatography from the cellular media. Since this initial assay does not account for ApoE4 to be simply nonfunctional versus inhibitory, this possibility will be addressed in the second and third assays.

As ApoE4 appears to interfere in some manner with ApoE3 beneficial actions in ApoE3/E4 heterozygous persons, we evaluated the potential for ApoE4 inhibition of eNOS activation in the presence of ApoE3. The conversion to L-arginine to L-citrulline was measured as discussed earlier, however in this assay both ApoE3 and ApoE4 were incubated with BAEC during the same time frame. However, in order to consider that preincubation with ApoE3 might resist any ApoE4 inhibition; we conducted the eNOS assay in this manner as well. Furthermore, we also preincubated BAEC with ApoE4 to ascertain if ApoE3 added later could overcome any ApoE4 inhibition. Much lower concentrations of ApoE4 were also tested versus ApoE3 in order to determine at how low a level ApoE4 might still impair ApoE3-stimulation of eNOS (results not shown)

As described in detail in the previous chapter, we then utilized the scratch wound assay to determine if ApoE4 could also inhibit ApoE3 in the stimulation of endothelial migration. Contrasting the short timeframe encompassing the eNOS assay, the incubation in the scratch wound assay is overnight. Since it is not known how endothelial cells may process or internalize ApoE3 or ApoE4, ApoE3 and ApoE4 were utilized at equal

concentrations and incubated with cells for the same period of time. Further studies need to be conducted to clarify how exactly ApoE4 is capable of interfering with ApoE3 actions in endothelium, considering effects are seen in both short and extended functional assays.

Results

ApoE4 and eNOS activation: Since both NO production and endothelial migration have a major impact on cardiovascular health, and since both processes are eNOS dependent, eNOS activation by ApoE3 versus ApoE4 was compared in BAEC (**Fig. 5**, n=4, *$p<0.05$ vs basal, †$p<0.05$ vs ApoE3). Whereas there was a doubling of eNOS activity with ApoE3, there was an absolute lack of stimulation by ApoE4.

ApoE4 actions on ApoE3-stimulation of eNOS: ApoE4 completely obliterates ApoE3-activation of eNOS (**Fig. 6**, n=4, *$p<0.05$ vs basal, †$p<0.05$ vs ApoE3 alone). While not indicative of binding patterns of ApoE3 and ApoE4 in endothelial cells, eNOS inhibition by ApoE4 at 15 times less concentration than ApoE3 implies that ApoE4 does not directly interfere with each ApoE3 particle's activation of eNOS. These results might indicate that ApoE4 may induce a general cellular inhibition possible through surface receptor alteration or through modification of intracellular adaptor protein mechanisms. In an attempt to further gather data about ApoE4 versus ApoE3 stoichiometry in eNOS activation, the preincubation conditions mentioned above were also utilized. ApoE4 still blocked ApoE3-activation of eNOS completely, regardless if ApoE3 was added prior to ApoE4, or vice versa (data not shown).

ApoE4 actions on ApoE3-induced endothelial cell migration: ApoE4 blocks ApoE3-induction of endothelial cell migration (**Fig. 7**, n=4, *$p<0.05$ vs basal, †$p<0.05$ vs ApoE3). Interestingly ApoE4 does not block VEGF induced migration as evidenced in the control cells, implicating the inhibitory effect of ApoE4 as ApoE3-specific in endothelial cells. Furthermore, the longer incubation period of this assay identifies ApoE4 as capable of chronically impeding ApoE3 action in endothelial cells.

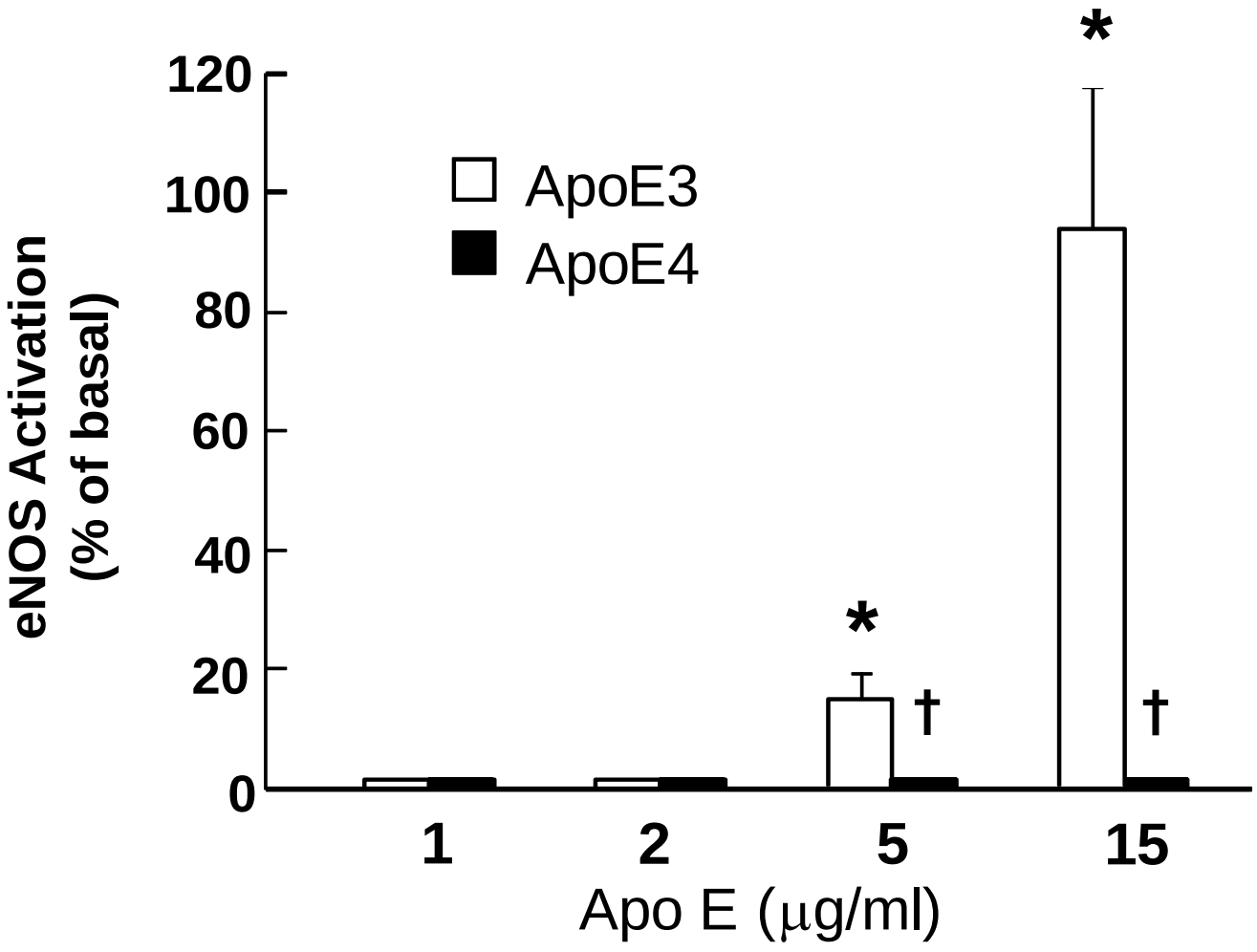


Figure 5: ApoE4 is incapable of activating eNOS

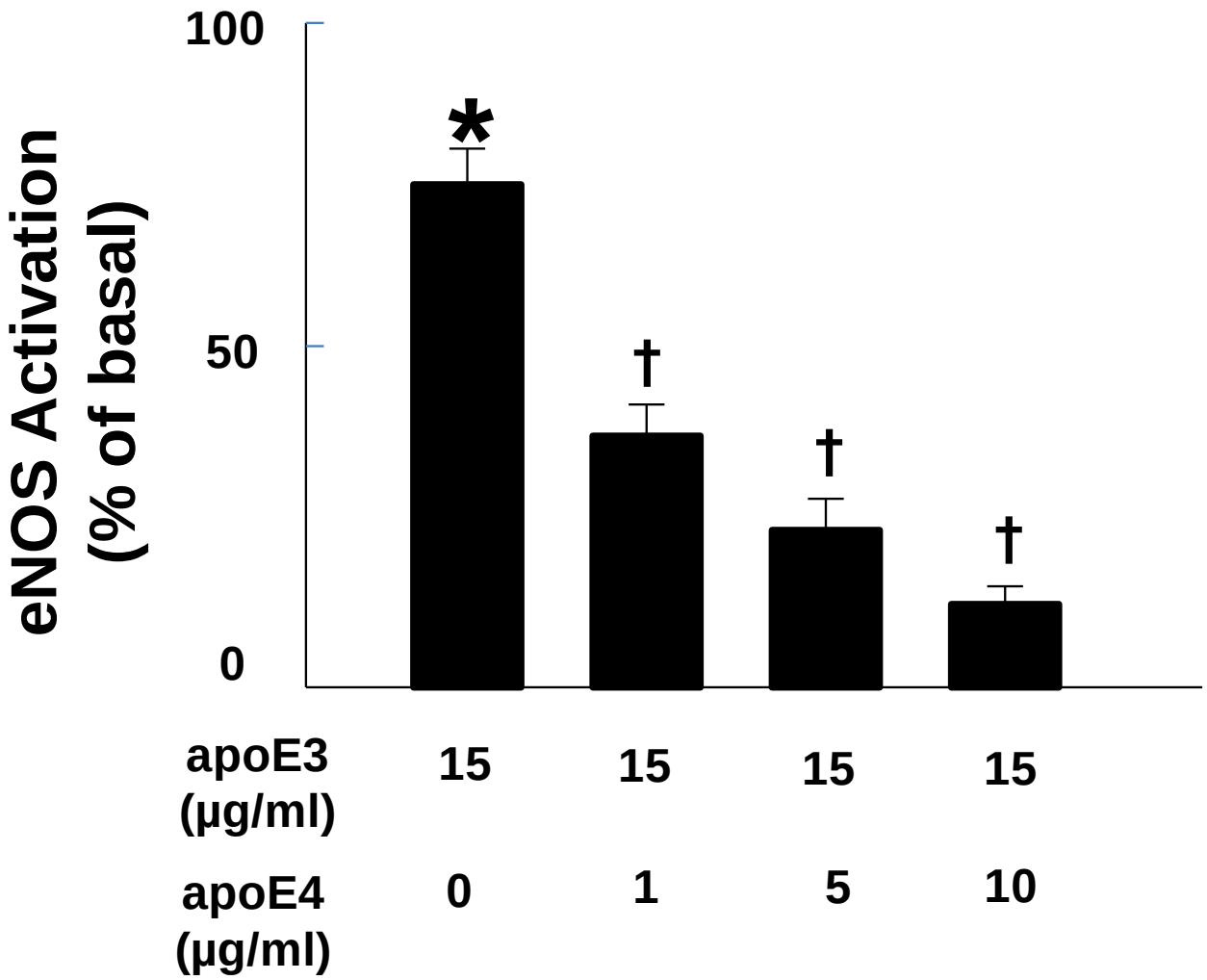


Figure 6: ApoE4 Blunts ApoE3-activation of eNOS

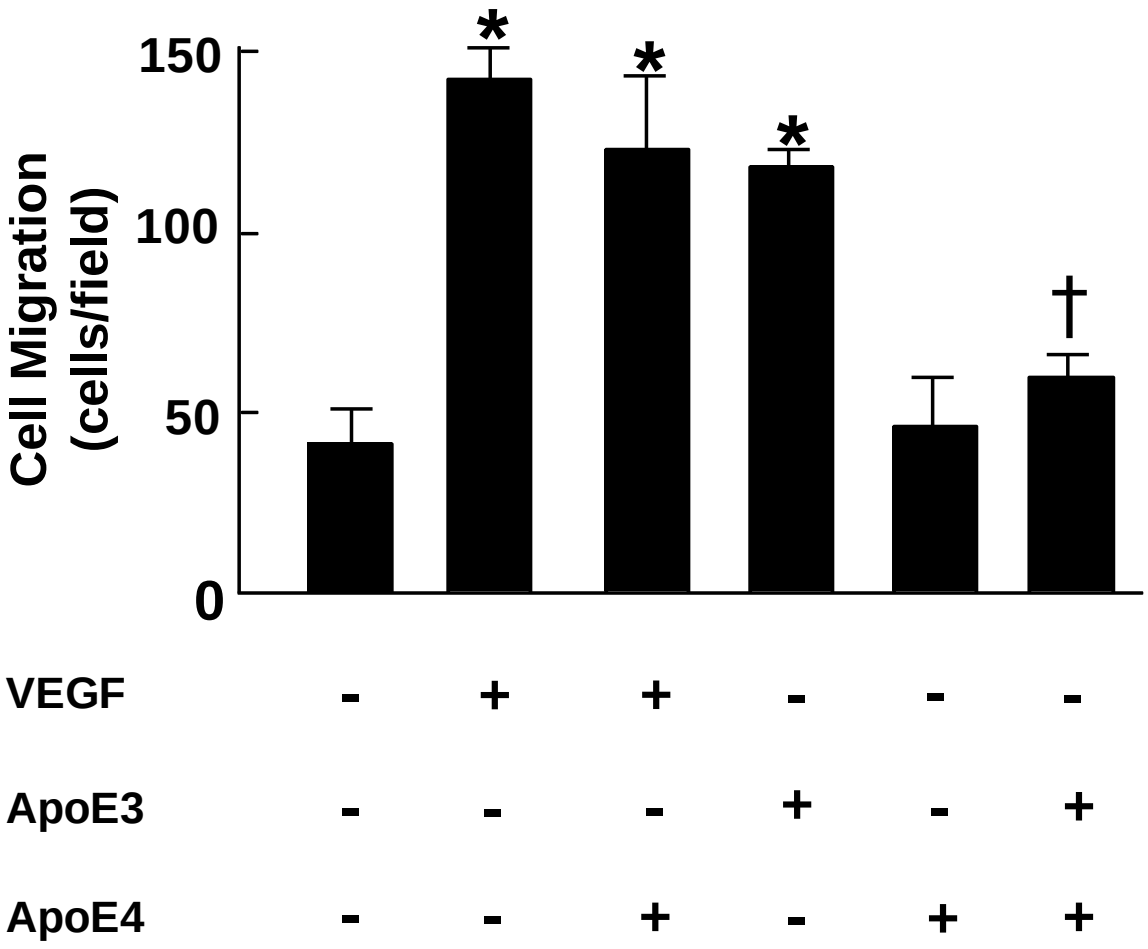


Figure 7: ApoE4 Inhibits ApoE3-induced Endothelial Migration

Discussion

While individuals possessing both an ApoE3 and ApoE4 allele are less in number than ApoE3 homozygotes, ApoE4/3 is the second most common allele expressed in humans.(Eichner, Dunn et al. 2002) This statistic lends credence to pursuit of this chapter's hypothesis. Indeed as shown above, ApoE4 drastically inhibits ApoE3 action in endothelium. These results open further speculation about the actual mechanisms underlying the ApoE4 effects. Specifically, one must consider if ApoE4 inhibits ApoE3 action extracellularly in the lumen, possibly by interfering with ApoE3 receptor binding through ApoE3 displacement from the endothelial receptor, such as through alteration in receptor confirmation. Contrastingly ApoE4 may interfere with the intracellular eNOS pathway, possibly through changing essential adaptor protein interaction with a receptor, or increasing the sequestration of and thus diminishing surface expression of ApoE receptors, as is seen in neurons.(Chen, Durakoglugil et al. 2010) Indeed ApoE4 could accomplish both these feats; therefore both options will need to be pursued since endothelial cells most likely function quite differently when compared to neurons in regard to ApoE3 and ApoE4 actions. Finally, while these studies indicate ApoE4 inhibition of ApoE3 actions in endothelium, hypotheses must also be pursued in the future that address how ApoE4 can so potently block ApoE3 in a very short incubation, while also doing so in a longer incubation. These studies may in fact implicate that ApoE4 diminishing of ApoE3 function might occur in two very distinct manners depending on length of co-exposure, and that patients expressing both alleles may

be subject to multiple pathways of confounding ApoE4 effects in the endothelium, thus increasing endothelial dysfunction and subsequent CVD risk by several means.

CHAPTER FOUR
Results

APOE3 AND APOE4 REGULATION OF ENOS

Rationale

We have shown that ApoE3 stimulates eNOS, and ApoE4 completely abrogates this effect. However, it is not known how ApoE3 or ApoE4 may function in endothelium, especially in the regulation of eNOS. eNOS phosphorylation is a critical posttranslational modification which mediates vascular function.(Poppa, Miyashiro et al. 1998) In transgenic mice expressing a constitutively active eNOS, results show increased vascular reactivity, as well as increased blood flow in a cerebral stroke model, highlighting the importance of eNOS activation in vivo.(Atochin, Wang et al. 2007)

In the classical VEGF pathway of eNOS activation, VEGF binds the endothelial cell surface receptor, most often VEGFR2.(Dellinger and Brekken 2011) This interaction increases Src family kinase activation, which results in PI3k and Akt phosphorylation. Akt is then able to directly phosphorylate eNOS on serine 1177 in humans. This eNOS phosphorylation results in an increase in NO production, often resulting in stimulation of endothelial migration **(Fig. 8)**.(Dimmeler, Dernbach et al. 2000, Dellinger and Brekken 2011)

Clarification of the role of ApoE3, but especially ApoE4, on kinase activation in the eNOS pathway is important to pursue. Initially we considered the role of kinases upstream of eNOS by utilizing pharmacological intervention by kinases inhibitors. Next we

clarified the importance of eNOS phosphorylation itself, as well as that of the direct upstream kinase, Akt. If ApoE4 interferes with ApoE3 stimulation of a kinase in the eNOS pathway, this is essential to understanding ApoE4-induced CVD risk through blunting of eNOS, and identifies new targets for potential therapeutic pharmacological intervention for ApoE4 patients. In this chapter we tested the **hypothesis** that ApoE3 requires Src and PI3k to stimulate eNOS, as well as increases phosphorylation of eNOS and Akt, and that ApoE4 abrogates any induction of ApoE3-induced phosphorylation.

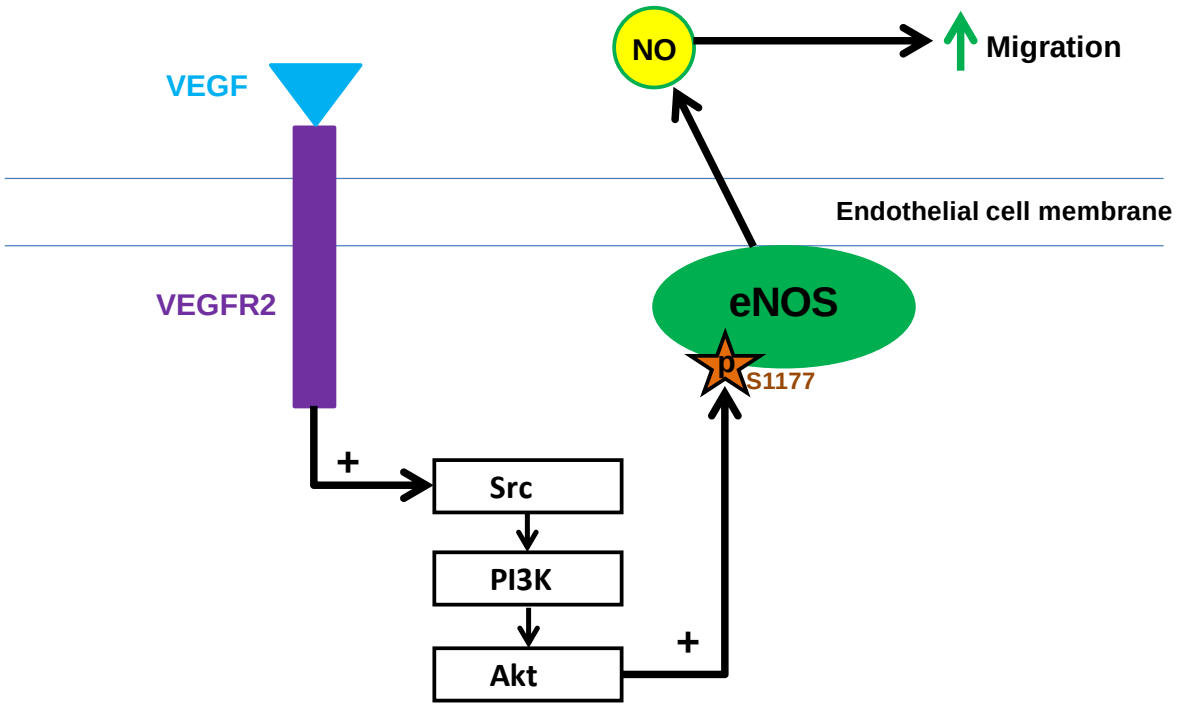


Fig. 8: Classical VEGF-stimulated pathway of eNOS activation: In endothelium, Src kinases mediate the stimulation of eNOS by a variety of agonists, and eNOS stimulation also requires PI3 kinase and Akt.

Methodology

In **Figure 9**, the eNOS assay was utilized to determine the role of Src and PI3k in ApoE3-stimulated eNOS activation. This assay was performed in BAEC, however instead of using siRNA to knockdown protein expression of Src or PI3k in order to diminish kinase activity, frequently-used pharmacological interventions were employed. Specifically I utilized PP2 and LY294002 to inhibit Src family kinases and PI3k in the BAEC, respectively.(Rikitake, Hirata et al. 2002, Steinle, Meininger et al. 2002) Other inhibitors for these kinases exist; however our lab has had much success using the LY294002 and PP2 inhibitors in BAEC, particularly so in eNOS assays without altering the assay itself.(Seetharam, Mineo et al. 2006, Sundgren, Zhu et al. 2011)

Knockdown via siRNA would have also been a viable method for evaluating Src and PI3k importance. However transfection is toxic to cells and requires orchestrating a careful modulation between transfecting efficiently and killing a large majority of cells, and the eNOS assay is most efficient when assaying cells near confluency. The eNOS assay is best optimized when cells are quiescent prior to ApoE3 incubation and transfection distresses cells for 48 hours prior to eNOS assay. Therefore treating cells instead with the aforementioned pharmacological interventions to impede the actions of Src and PI3k just prior to ApoE3 incubation was the less toxic and more time efficient of these inhibitory methods **(Fig. 10)**.

To evaluate eNOS phosphorylation and the activation of Akt in the presence of ApoE3 alone or plus ApoE4, a phospho-assay was performed. BAEC were treated with equal concentrations of ApoE3 +/- ApoE4 for 0, 5, or 10 minutes. Cells were then harvested in SDS-protein sample buffer, boiled, and run on a 10% acrylimide protein gel. Protein was then transferred to membrane and immunoblotted for phosphorylated eNOS or Akt protein, as well as total eNOS and Akt protein as a loading control. Phosphorylated protein was then normalized to total protein loading controls via densitometry, and graphed **(Fig. 11)**. While the effects of ApoE4 on ApoE3-stimulated phosphorylation of eNOS or Akt is important in the understanding of ApoE4 actions, this assay does not differentiate between ApoE4 directly blocking ApoE3-induced phosphorylation, and the potential for ApoE4-induction of phosphatase activity to achieve this effect.

Results

Requirement of Src and PI3k in ApoE3-stimulated eNOS: ApoE3-stimulation of eNOS activation requires Src and PI3k. (**Fig. 9**, n=4, *$p<0.05$ vs basal-data not shown, †$p<0.05$ vs ApoE3 alone). The use of these inhibitors does not diminish basal activity (data not shown), but drastically diminishes ApoE3-induced eNOS activation.

ApoE4 blunts ApoE3-increases in eNOS and Akt phosphorylation: As expected, control cells treated with VEGF (50ng/ml) exhibit drastic increases in phosphorylation of both eNOS (s1179) and Akt (s473). When BAEC were treated with ApoE3 (30μg/ml) alone, eNOS and Akt phosphorylation also increased. However when

cells were co-incubated with equal concentrations of ApoE3 and ApoE4, eNOS and Akt phosphorylation were completely ablated. (**Fig. 10**, n=3, *p<0.05 vs basal-time 0, †p<0.05 vs ApoE3 alone)

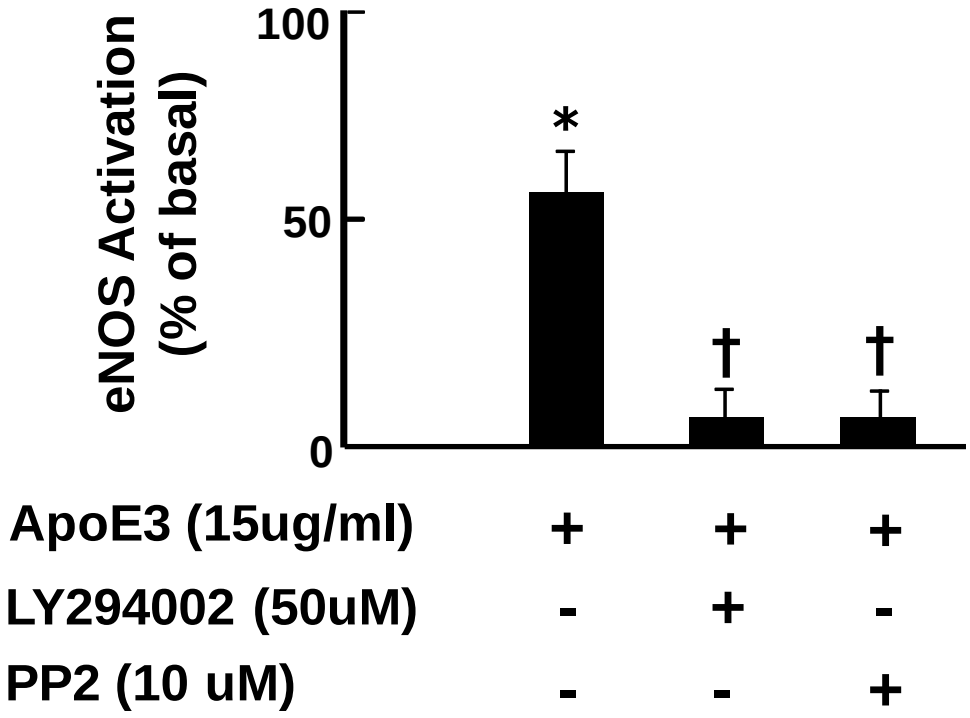


Fig. 9: Src and PI3k are required for ApoE3 activation of eNOS

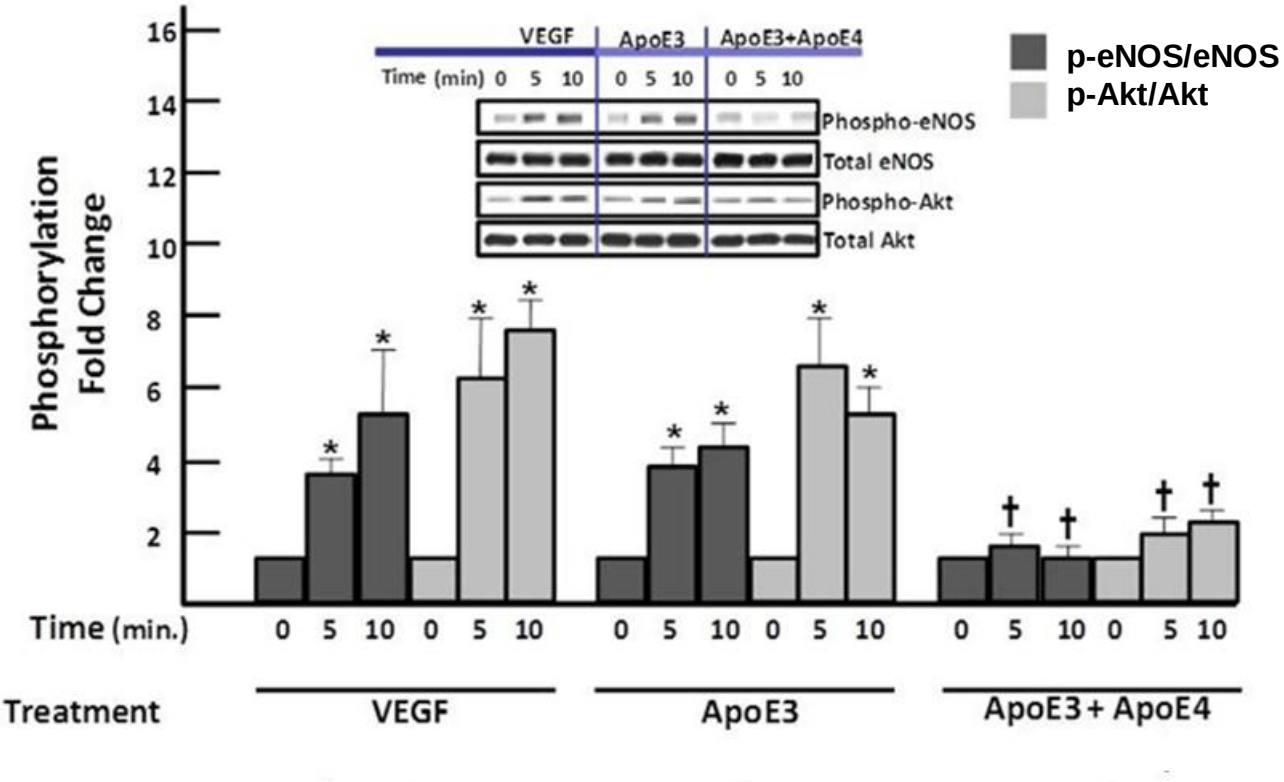


Fig. 10: ApoE4 blunts ApoE3-induced eNOS and Akt phosphorylation

Discussion

eNOS phosphorylation in endothelial cells is crucial for NO production; a vasoreactive molecule critical in the initiation of migration and angiogenesis, smooth muscle cell growth inhibition and relaxation, and prevention of thrombosis and monocyte adhesion. Therefore it was imperative to evaluate the effects of ApoE3, which activates eNOS enzymatic activity, as well as ApoE4, which does not activate eNOS, on changes in the eNOS phosphorylation pathway-the posttranslational modifications essential for NO production.

In order to fully interrogate the intracellular mechanisms and specifically the differences between ApoE3 and ApoE4 effects on the eNOS pathway, we also evaluated changes in Akt phosphorylation, the kinase which directly phosphorylates eNOS, as well as the necessity for upstream kinases Src and PI3k in ApoE3 activation. Interestingly, ApoE3 was capable of inducing increases in Akt and eNOS phosphorylation to the same degree as the classic, very potent agonist of eNOS, VEGF. This may imply that in homozygous patients ApoE3 may function to the same degree or possibly function synergistically with VEGF to activate eNOS and produce more NO, thus lessening CVD risk in this manner compared to ApoE4 carriers.

While this assay implicates ApoE4 impairment of ApoE3-increases in eNOS and Akt phosphorylation, it is not clear if this effect is due to changes in phosphatase

activity, or extracellular effects interfering between ApoE3 and the receptor responsible for transmitting the ApoE3 activation cues on the eNOS pathway. Clarifying the endothelial receptor(s) responsible for ApoE3 and ApoE4 vascular actions will help to elucidate the pathway through which various ApoE isoforms effect CVD risk in humans.

CHAPTER FIVE
Results

APOER2 IN APOE REGULATION OF ENDOTHELIUM

Rationale

The effects of ApoE3 and ApoE4 on eNOS activation in endothelial cells are clear. Assays that are both short, with incubations under 15 minutes, and longer, with overnight incubations, produced similar results implicating ApoE4 as harmful to proper eNOS functioning and pathway conduction, and as being capable of impairing the beneficial effects of ApoE3.

With the aim of further teasing out the mechanisms by which ApoE3 and ApoE4 affect endothelium, a receptor was next implicated in propagation of the ApoE3 beneficial signal. Unlike other LDLR family members, ApoER2 is not abundantly expressed in the liver and therefore does not likely affect global cholesterol homeostasis, indicating a signaling role for this receptor.(Beisiegel, Weber et al. 1989, Willnow, Nykjaer et al. 1999) In the brain, ApoE binds ApoER2, in order to propagate Reelin signaling and stimulate neuronal migration during development, while maintaining synaptic plasticity in adults.(Beffert, Weeber et al. 2005, Petit-Turcotte, Aumont et al. 2005) While extensively studied in the central nervous system, ApoER2 is also expressed in the endothelium and has been localized to caveolae where it modulates signaling events in overexpression assays.(Riddell, Sun et al. 2001, Ramesh, Morrell et al. 2011)

The potential roles of ApoER2 in vascular homeostasis have been minimally addressed, with a few studies acknowledging ApoER2 effects in vascular smooth muscle cells and platelets.(Riddell, Graham et al. 1997, Riddell, Vinogradov et al. 1999, Korschineck, Ziegler et al. 2001, Pennings, Derksen et al. 2007, Urbanus, Pennings et al. 2008) What led us to consider ApoER2 and its potential role in endothelium is that there are multiple tissue- and species-specific splice variants of ApoER2, which add complexity to their biology in multiple cell types.(Schneider and Nimpf 2003) Furthermore, a variant of ApoER2 in which an arginine at position 952 is substituted by glutamine (ApoER2-R952Q) has recently been associated with early onset MI and CAD in familial and population-based studies, identifying a function for ApoER2 in CVD.(Shen, Li et al. 2007) While ApoER2 has been shown to cluster VLDLR in the brain(Hiesberger, Trommsdorff et al. 1999, Drakew, Deller et al. 2002), in this chapter we tested the **hypothesis** that ApoER2 is the main endothelial receptor responsible for propagating ApoE3 effects on eNOS activation, endothelial migration, and monocyte adhesion, and that the ApoER2-R952Q mutant cannot stimulate ApoE3-induced eNOS.

Methodology

To ascertain the role of ApoER2 in actions beneficial to the endothelium as stimulated by ApoE3, a knockdown system was utilized to disrupt the potential eNOS signaling tamdem of ApoE3-ApoER2. BAEC were transfected via LipofectAMINE 2000 (Invitrogen) to target ApoER2 with siRNA. Double-stranded RNA with sequence 5’-

ACUGGAAGCGGAAGAAUAC-3' was designed to target the open reading frame of the bovine ApoER2 (accession number XM_865091), and control dsRNA was purchased from Dharmacon (catalog D-001210-01-20). Forty-eight hours following transfection, ApoER2 protein knockdown was confirmed via immunoblotting (**Fig. 11A**). Control cells versus cells with decreased ApoER2 expression were then treated in the eNOS assay-stimulated with the classical NOS agonist VEGF or ApoE3 to implicate ApoER2 in the activation of eNOS by ApoE3 (**Fig. 11B**). VEGF was chosen as a control agonist since the known receptor that propagates VEGF stimulation of eNOS is VEGFR, and VEGF activation of eNOS should not be affected by knockdown of ApoER2. While viable cell numbers decreased due to the toxicity of transfection, by the time of harvest for immunoblotting or eNOS assay cell confluency was approximately 80-90%, within range for optimal functioning of the eNOS assay. While results indicating loss of ApoE3-stimulated eNOS in the absence of ApoER2 implicates ApoER2 in ApoE3 signaling to eNOS, this assay does not eliminate the possibility that VLDLR or another receptor may dimerize with ApoER2 to propagate ApoE3 signal to eNOS. However knockdown of VLDLR in BAEC does not affect ApoE3-stimulation of eNOS (data not shown). This approach, while greatly diminishing the possibility that VLDLR is involved in any ApoER2-ApoE3-stimulation of eNOS, does not eliminate the potential for the involvement of other LDLR-family members that are expressed in endothelium, such as Megalin and LDLR-related protein 1 (LRP1).(Buc-Caron, Condamine et al. 1987, S.K., Gilemann et al. 1992)

The role of ApoER2 in ApoE-stimulated endothelial cell migration was then addressed utilizing the aforementioned knockdown system in BAEC. Following Sham or

ApoER2 siRNA transfection, cells were used in the scratch-wound assay in the absence or presence of ApoE3, or control agonist VEGF (**Fig. 12**). Again, this assay does not eliminate the possibility that a secondary receptor aids in the ApoE3-ApoER2 stimulation of migration, but determines if ApoER2 is required.

To establish the role of ApoER2 in ApoE3-diminishing of monocyte adhesion, BAEC were transfected to knockdown ApoER2 as previously described (**Fig 13A**). Following monocyte adhesion protocol as previously discussed, monocytes adhering to the endothelial monolayer were quantified and graphed, and representative images of the study groups are shown (**Fig. 13B, C**). As mentioned earlier, this assay does not eliminate a second receptor in the role of ApoE3-ApoER2 abrogation of LPS-induced monocyte adhesion.

The ApoER2-R952Q point variant (**Fig. 14A**), which originally led us to consider ApoER2 as a receptor that might have a role in cardiovascular functions, was preliminarily addressed in eNOS activation. The 3T3 cell line was chosen to ascertain this mutant, as it possesses little endogenous ApoER2, the presence of which would confound the effects of overexpressed mutant receptor. Cells were co-transfected with ApoER2 receptor, specifically sham, wild type, or R952Q variant, as well as eNOS cDNA (**Fig. 14B**). In order to show that all receptor transfected experimental groups were equally capable of enzyme action, basal eNOS activation was quantified and graphed (**Fig. 14C**). The eNOS assay was then performed in these cells (**Fig. 14D**).

An overexpression system has also been utilized to preliminarily evaluate the mutant receptor in BAEC, and while there exists the same trend as seen in the 3T3 cells,

these results are not significant yet (data not shown), most likely due to the presence of endogenous wild-type receptor. Indeed, evaluation of the ApoER2-R952Q assay would be most physiologically relevant if performed in endothelial cells. However using another system other than simple overexpression, such as knockdown of endogenous ApoER2, then reconstitution of sham, wild type, or mutant receptor was not successful in our primary cells, as two transfections within three days, or even four days, proved too cytotoxic (data not shown). Though not as potently as in primary endothelial cells, ApoE3 activates eNOS in the EA.hy926 human umbilical vein endothelial cell-lung epithelial fusion cell line, (data not shown). While these cells maintain high viability under stress, EaHy are only minimally transfectable in our hands (data not shown), therefore manipulating ApoER2 in this fashion is not an option at this time. Furthermore, the fusion that produces these EA.hy926 cells also alters some classic characteristics of endothelial cells, such as morphology and growth factor requirements, indicating divergence from classic endothelial responses, another signal that responses in EaHy are not a truly physiological endothelial response.(Unger, Krump-Konvalinkova et al. 2002)

Results

Requirement of ApoER2 in ApoE3-stimulation of eNOS: Knockdown of ApoER2 in BAEC completely abolished ApoE3-induced eNOS activation, while response due to VEGF agonism was unaffected (**Fig. 11**, n=4, *$p<0.05$ vs basal, †$p<0.05$ vs ApoE3 in control transfected cells).

ApoER2 is essential for ApoE-3stimulated endothelial cell migration: BAEC in which ApoER2 was knocked down failed to activate eNOS in response to ApoE3, while response to VEGF was unaffected (**Fig. 12**, n=4, *p<0.05 vs basal, †p<0.05 vs ApoE3 in control transfected cells).

ApoE3-abrogation of LPS-induced monocyte adhesion involves ApoER2: When ApoER2 is reduced in BAEC, ApoE3 cannot confer the previously shown endothelial protective effect to prevent monocyte adhesion (**Fig. 13**, n=4, *p<0.05 vs basal, **p<0.05 vs LPS, †p<0.05 vs ApoE3 in control transfected cells).

ApoER2-R952Q is incapable of activating eNOS: Cartoon representation of area of R952Q mutation as indicated by red star (**Fig. 14A)**. Equal expression was demonstrated by immunoblotting (**Fig. 14B**). eNOS activation was then measured during 15 min incubations with control buffer or ApoE3 (15 μg) indicating equal basal activation (**Fig. 14C)**. Whereas ApoE3 did not activate eNOS in sham cells, ApoE3 caused a 2-fold increase in eNOS activity in cells expressing WT ApoER2 (**Fig. 14D**, n=7, *p<0.05 vs basal, †p<0.05 vs WT). In contrast, in cells expressing ApoER2-R952Q, eNOS activation was entirely absent.

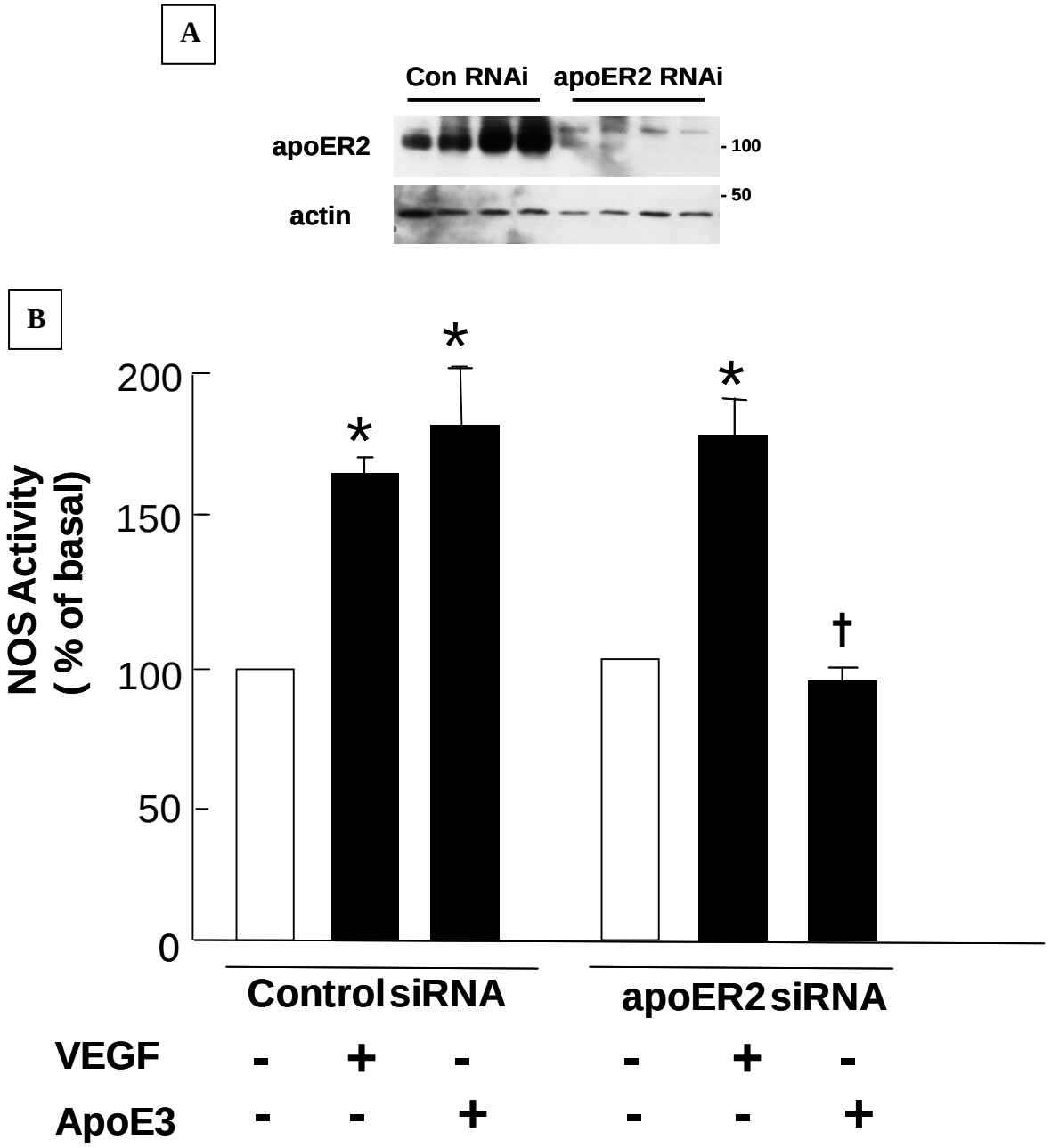


Figure 11: ApoER2 is required for ApoE3 activation of eNOS. A: Immunoblotting of BAEC lysates depicting siRNA-targeting and knockdown of ApoER2 protein. B: Graphic quantification of eNOS assay in BAEC shown in A in which ApoER2 expression is decreased.

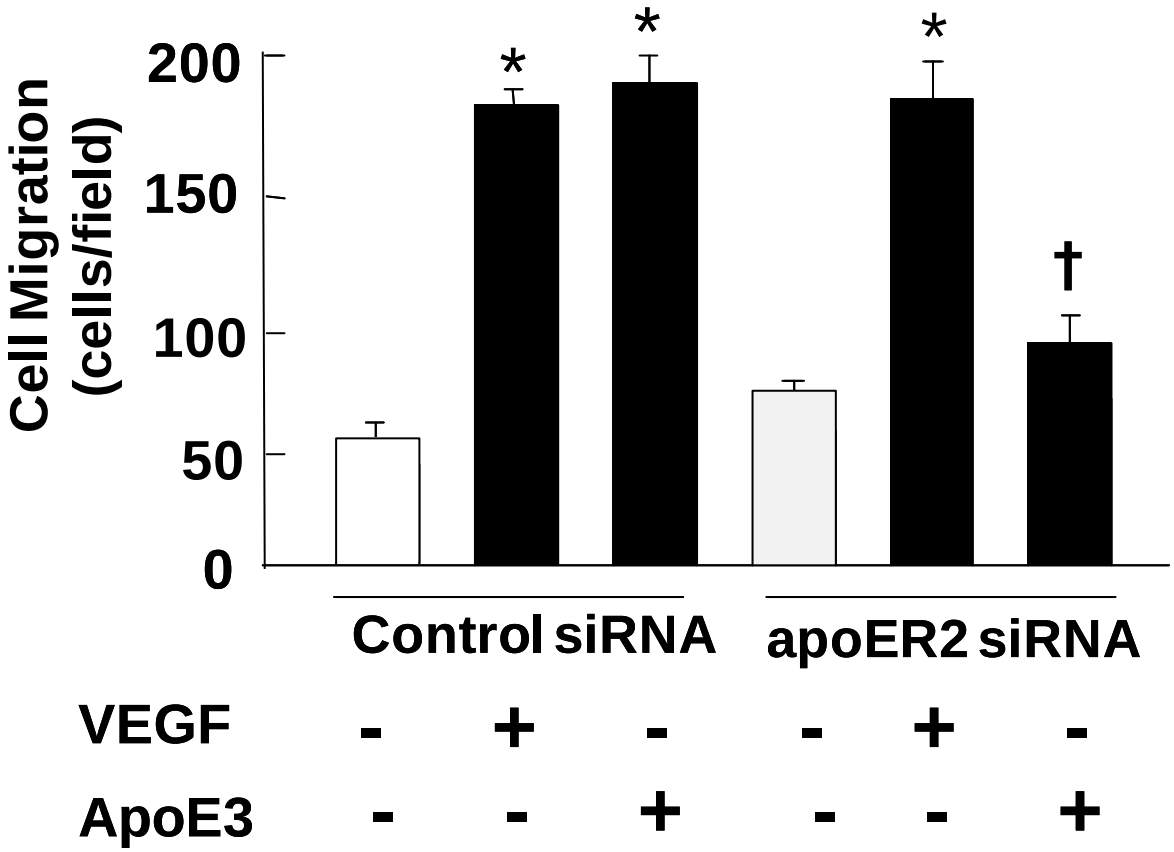
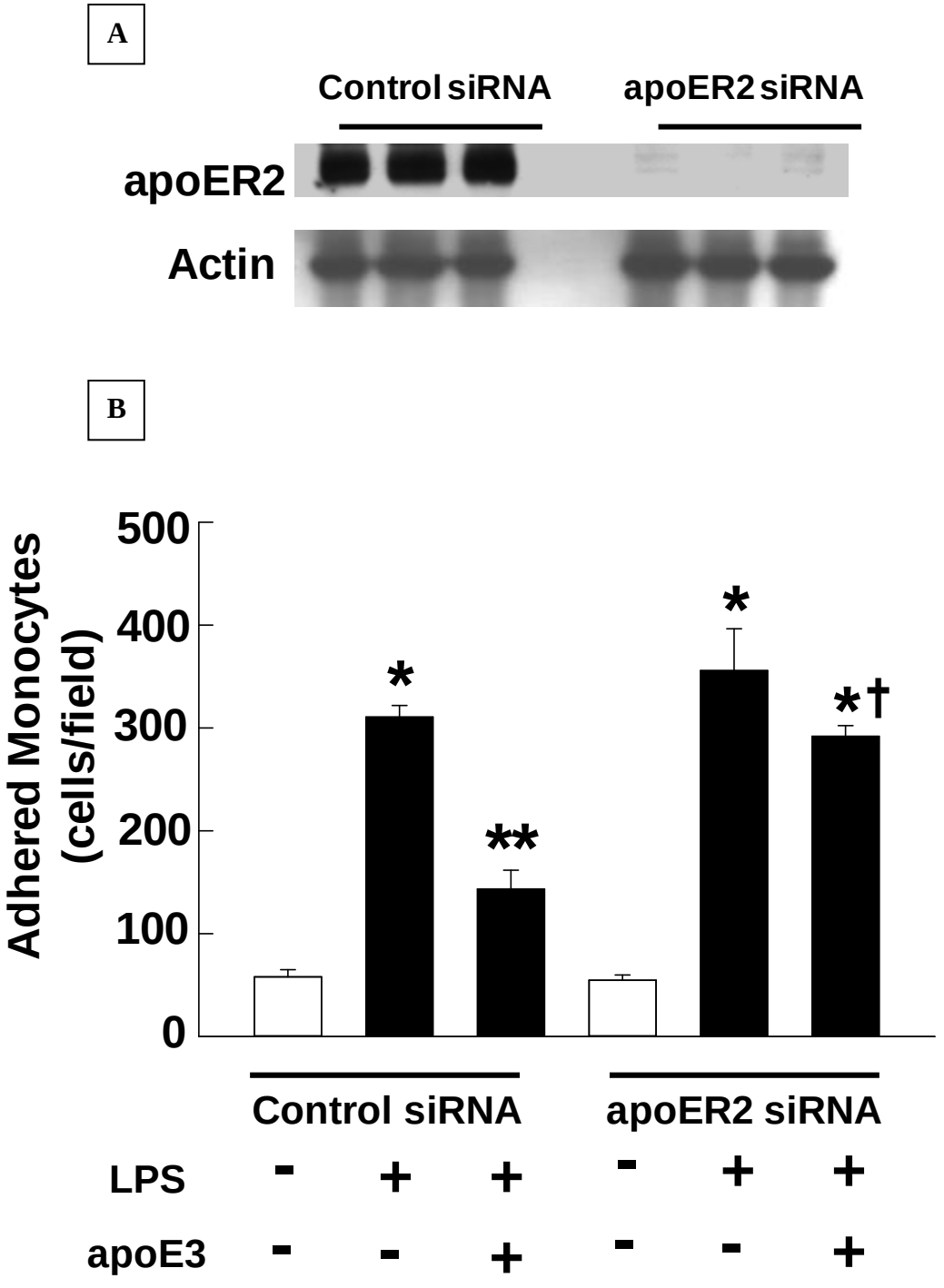


Figure 12: ApoER2 is required for ApoE3-stimulated endothelial cell migration


A
Control siRNA
apoER2 siRNA
apoER2
Actin
B
Adhered Monocytes (cells/field)
500
400
300
200
100
0
*
**
*
*†
Control siRNA
apoER2 siRNA
LPS - + + - + +
apoE3 - - + - - +

C

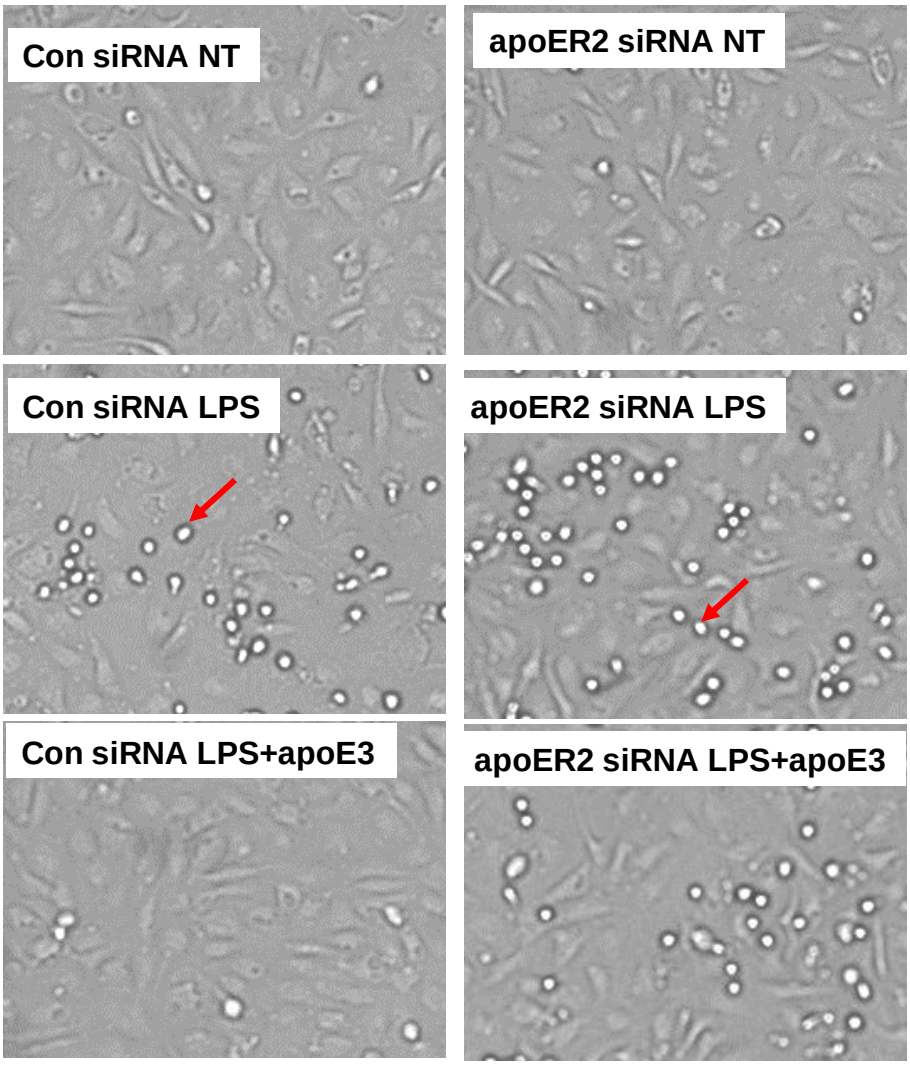
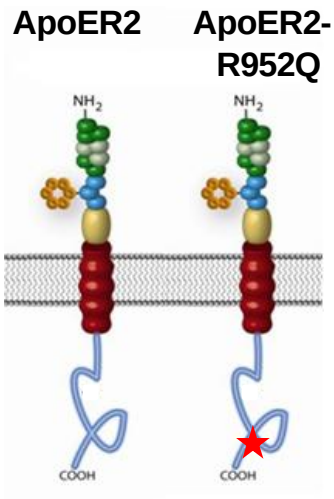
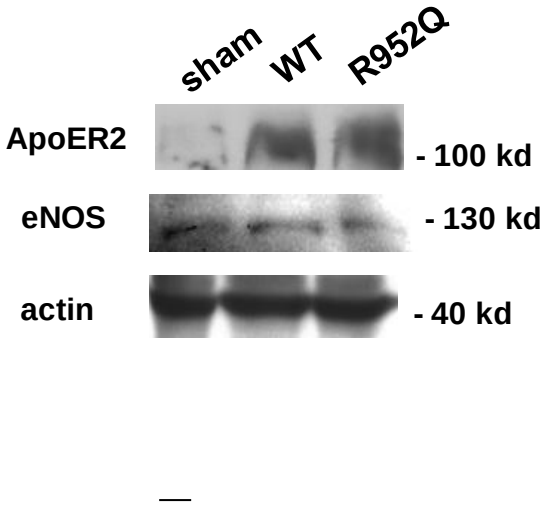


Figure 13: ApoER2 is required for ApoE3-abrogation of LPS-induced monocyte adhesion. A: Immunoblotting of BAEC lysates depicting siRNA-targeting and knockdown of ApoER2 protein. B: Graphic quantification of monocyte adhesion assay in BAEC shown in A in which ApoER2 expression is decreased. C: Representative images of groups quantified in A, B (40X), monoctyes indicated by red arrows.

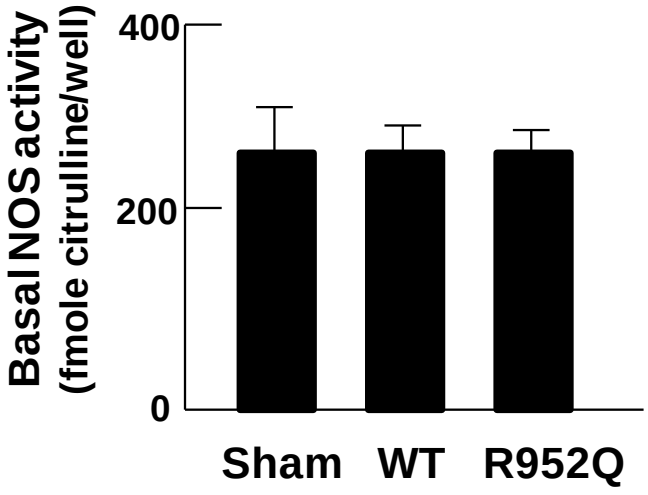
A


ApoER2
ApoER2-
R952Q
NH2
NH2
COOH
COOH

B


sham
WT
R952Q
ApoER2
- 100 kd
eNOS
- 130 kd
actin
- 40 kd

C


Basal NOS activity
(fmole citrulline/well)
400
200
0
Sham
WT
R952Q

D

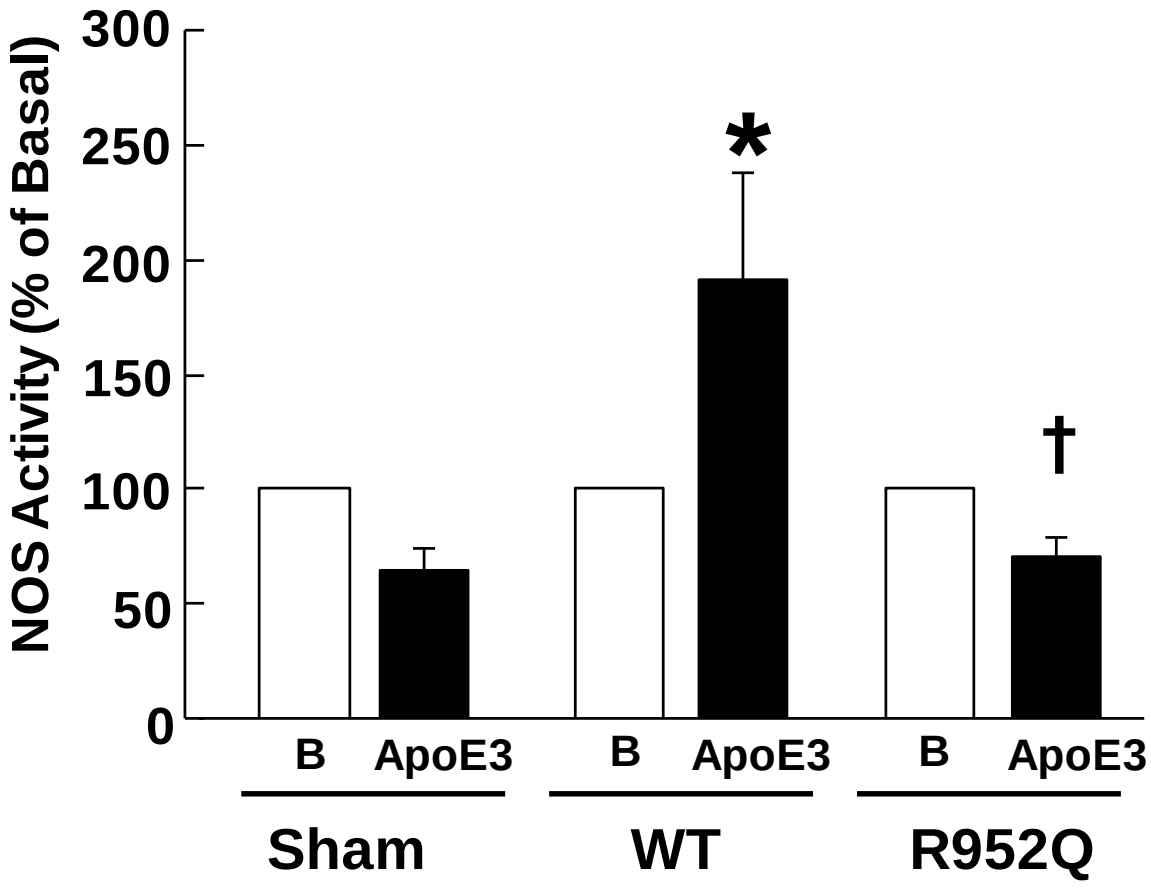


Figure 14: ApoER2-R952Q variant is incapable of activating eNOS. A: Cartoon schematic of point of R952Q mutation in ApoER2 cytoplasmic tail as depicted with red star. B: Immunoblotting of siRNA-directed overexpression of Sham, WT ApoER2, mutant ApoER2-R952Q, and eNOS in 3T3 cell lysate. C: eNOS assay in 3T3 cells indicating equal basal activation of eNOS in all receptor transfected cell groups. D: Graphic representation of eNOS assay in ApoE3-stimulated 3T3 cells in which Sham, WT ApoER2, and mutant ApoER2-R952Q were transfected.

Discussion

ApoER2 is a transmembrane receptor mainly expressed in brain, but also in testes, ovary, placenta, and endothelium; it is also expressed in splice variants on platelets.(Beisiegel, Weber et al. 1989, Willnow, Nykjaer et al. 1999, Pennings, Derksen et al. 2007)

In the brain, ApoER2 clusters with VLDLR to propagate Reelin signaling in development.(Benhayon, Magdaleno et al. 2003, Andrade, Komnenovic et al. 2007) In neurons, Reelin binds ApoER2, and when ApoE3 or ApoE4 concurrently bind, the receptor is endocytosed. When ApoE4 is bound in this process and causes receptor endocytosis, ApoE4 never reaches the cytoplasm, ApoER2 is sequestered in the cell, and surface expression of the receptor is reduced, impairing further Reelin signaling and synaptic plasticity. However when ApoE3 is bound, ApoER2 readily recycles back to the surface, allowing for more signaling.(Lovestone, Anderton et al. 1996, Chen, Durakoglugil et al. 2010) Furthermore ApoER2 deficit in mice has been associated with decreased learning and memory capabilities, indicating importance of the receptor in adult neural processes, as well. (Barr, MacLaurin et al. 2007)

In sperm, ApoER2 is crucial in sperm maturity and motility, inflicting ApoER2 knockout mice as sterile due to reduced phospholipid hydroperoxide glutathione peroxidase. Low levels of this protein mirror most cases of infertility in humans, implicating ApoER2 dysfunction in human sterility as well.(Andersen, Yeung et al. 2003)

In platelets, splice variants of ApoER2 are necessary for disease pathogenesis in the antiphospholipid syndrome (APS) (Pennings, Derksen et al. 2007), a Lupus-like condition where the body makes auto-antibodies to endogenous proteins. These circulating antibodies then bind the circulating protein beta2-glycoprotein I (β2GPI), which then dimerizes with surface ApoER2 on platelets, sensitizing platelets resulting in thromobosis, a key disease manifestation in APS, and this process is similar in endothelium.(Pennings, Derksen et al. 2007, Agar, van Os et al. 2010, Cugno, Borghi et al. 2010, Ramesh, Morrell et al. 2011)

Indeed the presence of ApoER2 is crucial to maintain the homeostasis in many physiological processes; however it has also been linked in disease processes as mentioned above. These complex and contrasting roles for ApoER2, which often occur in the presence of ApoE, made this receptor a valid choice to signal in tandem with ApoE3 in endothelium. As hypothesized, ApoER2 was necessary to modulate ApoE3-induced eNOS activation, endothelial cell migration, and ameliorate monocyte adhesion. Divergently the ApoER2-R952Q point variant was incapable of conducting the ApoE3 benefits of eNOS activation in a cell system, implicating changes induced by the arginine to glutamine mutation in the ApoER2 cytoplasmic tail as necessary for ApoE3 to activate eNOS.

Implicating the role of ApoER2 in the advantageous ApoE3 activities in endothelium lends more clarity to the ApoE3 pathway that increases physiological NO production, and thus associated decreases in CVD risk in patients. These knockdown experiments evaluate and necessitate the role of ApoER2 during ApoE3-induced activities, and similar assays in VLDLR-diminished cells confirm that VLDLR is not required for these

processes. However in order to ensure that no other LDLR family members, such as LRP1 or Megalin, are also associated with these effects, separate targeting assays would need to address these candidates, as well.

CHAPTER SIX
Results

APOE, APOER2, AND REGULATION OF ENDOTHELIAL FUNCTION IN VIVO

Rationale

Disruptions in endothelial cell monolayer integrity contribute to the incidence and severity of vascular diseases including neointima formation and atherosclerosis. (Ross 1993, Cunningham and Gotlieb 2005, Hui 2008) ApoE3 overexpression in mice has been associated with regression of atherosclerosis(Tsukamoto, Tangirala et al. 1999), however the effects of ApoE4 has not been explored in endothelium in vivo. The role of ApoER2 in the endothelium in vivo has been addressed in terms of antiphospholipid syndrome by our lab.(Ramesh, Morrell et al. 2011) In fact the functions of ApoER2 in vivo outside of the brain have just begun to be breached in the literature.

In cultured endothelial cells we discovered that ApoE3 stimulates eNOS via ApoER2 and thereby promotes endothelial migration, whereas ApoE4 displays impaired eNOS activation. Therefore utilizing the information identified in vitro and evaluating the effects of ApoE3, ApoE4, and ApoER2 on reendothelialization and atherosclerosis in vivo will begin to clarify their role in CVD risk.

Finally, there is a region in the c-terminal tail of ApoER2, termed the NPxY motif, which is essential in the brain for adaptor protein interaction following Reelin binding to ApoER2 and VLDLR. This NPxY region is critical for Src activation and resulting DAB1

recruitment and subsequent tyrosine phosphorylation. Mice lacking DAB1 exhibit grossly altered cortical layering and ataxia, which reflects that seen in ApoER2 and VLDLR double knock-outs. Following DAB1 activation, PI3k and Akt are activated which results in actin remodeling.(Drakew, Deller et al. 2002, Papakonstanti and Stournaras 2002, Weiss, Johanssen et al. 2003)

In this chapter we tested the **hypotheses** that ApoE3 promotes reendothelialization, that ApoER2, specifically the NPxY motif, is required for reendothelialization, and that ApoER2 is atheroprotective.

Methodology

In the first experiment, any effects of the presence versus absence of ApoER2 were evaluated. It is important to note that although these studies will occur in mice with endogenous ApoE, which has beneficial characteristics similar to human ApoE3 (Stevenson, Marshall-Neff et al. 1995), the identity of the ApoER2 ligand that is operative in the function of the receptor in the endothelium will not be delineated at this time.

Since dysfunction or destruction of the endothelial layer is often the initiating step of CVD (Ross 1993, Cunningham and Gotlieb 2005, Patti, Melfi et al. 2005), the importance of ApoER2 in the recovery of the endothelial layer was pursued. To determine the role of ApoER2 in the reendothelialization process, a carotid artery perivascular injury model was studied using our established method.(Seetharam, Mineo et al. 2006) Briefly, using bipolar forceps, the exposed carotid artery is subjected to electric current along 4 mm

of artery length (2W for 2 sec for each mm of artery) in 12-16 week old male mice. Mice were anesthetized by intraperitoneal injection of avertin (combination of 40ml 2.5% tribromoethanol and 310 μl tertiary amylalcohol in 39.5 ml water) at 0.02 ml/g body weight prior to the procedure. To determine the area of denudation, the mouse is injected with Evan's blue dye (Sigma-Aldrich) 72 hours following injury, this dye is incorporated into the denuded region, and the area of dye incorporation is quantified by blinded image analysis using Scion Image (free software from NIH). This results in endothelial denudation with minimal effect on the medial layer.(Seetharam, Mineo et al. 2006) Full reendothelialization occurs in 7d in wild-type C57BL/6 male mice (**Fig. 15**). We have evaluated the remaining area of denudation 3d following initial denudation (**Fig. 16, 17, 19**). Ideally this study will be reproduced in our floxed mice in which ApoER2 has been selectively deleted from the endothelium. Results in these tissue specific knockout mice would then be compared to that seen in Fig. 16 to evaluate if the results mirror those seen in global ApoER2 knockout mice. This study could pinpoint endothelial ApoER2 as the location of receptor required for the reendothelialization process.

Most animals, including mice, express a single form of ApoE that is structurally comparable to human ApoE3 similarly maintaining an open conformation.(Wilson, Wardell et al. 1991, Dong, Wilson et al. 1994, Weisgraber 1994) In this regard, animal ApoE exhibits analogous trends in disease risk compared to humans with ApoE3. Altering the closed structure of ApoE4 with a small molecule corrector or Arg-61 mutation prevents the N- and C- terminal domain interaction, and thus decreases disease risk in an ApoE3-like manner and also improves neurotoxic effects. (Raffai, Dong et al. 2001)

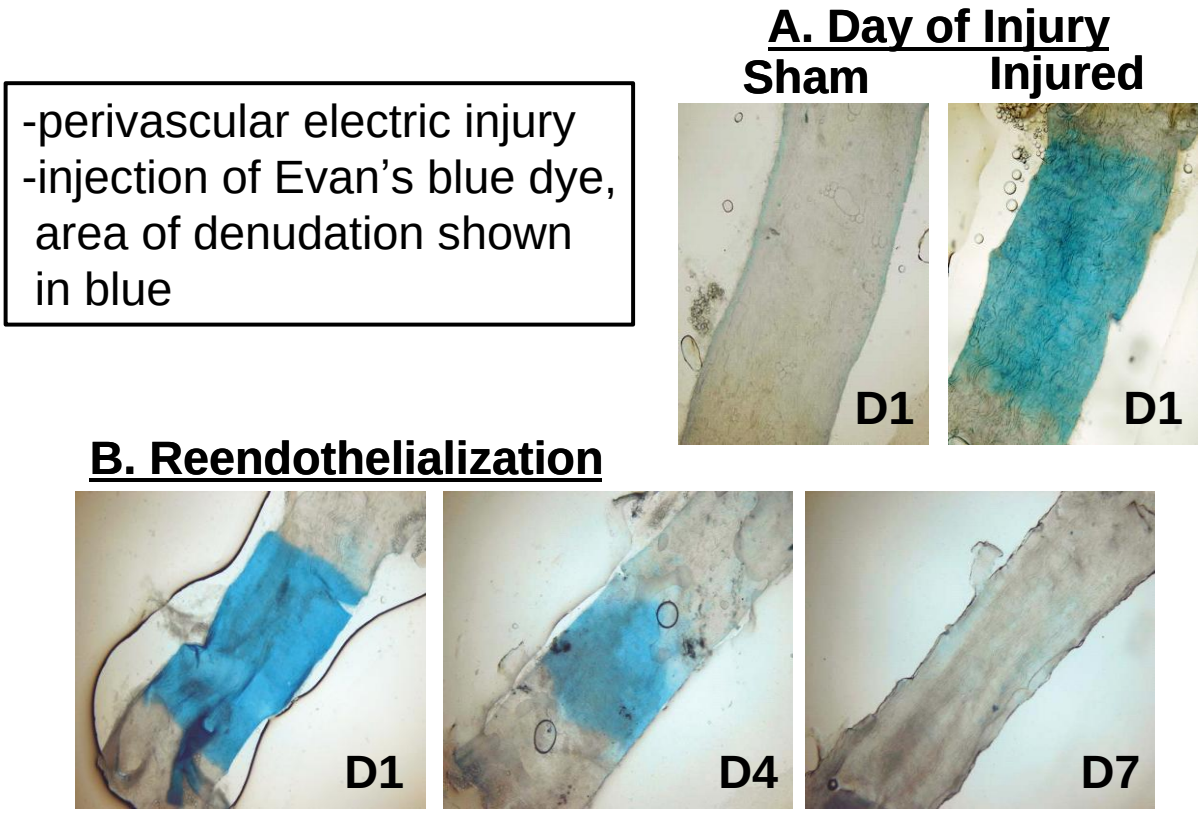


Figure 15: Carotid arteries of 12-16 week old male mice, arteries shown en face. A: Carotid artery injury model on day of injury, area of injury shown in blue. B: Typical reendothelialization process of C57/Bl6 male mice, 50% recovery on day 4, complete recovery by day 7.

In order to evaluate ApoE3 and ApoE4 in mice, several options for expression of the human proteins were considered. Available transgenic mice in which ApoE3 or ApoE4 are overexpressed exist with an ApoE background. While these mice prove a good option, other alternatives in which the human ApoE proteins are expressed closer to physiological levels are more relevant choices. In that regard, we contemplated the use of knock-in mice in which ApoE3 or ApoE4 are inserted in the endogenous ApoE genome.

These mice are indeed a great option, as mouse ApoE promoter elements drive physiological expression of the inserted ApoE3 or ApoE4 genes, and lipid profiles are not skewed.(Sullivan, Mace et al. 2004) While these mice were initially pondered, adenoviruses which express ApoE3 or ApoE4 were instead utilized since initiation of expression could be manipulated, and viral titers could also be controlled to maintain ApoE3 or ApoE4 levels within desired limits. Furthermore employing the adenovirus strategy allows for expression of ApoE3 or ApoE4 in a model in which ApoE3 has already been demonstrated to impact atherosclerosis. (Tsukamoto, Tangirala et al. 1999) Furthermore, the use of the adenoviruses allows for addressing the proposed hypotheses more rapidly than if additional mouse breeding were required to afford expression of human ApoE3 or ApoE4 in the absence versus presence of ApoER2.

As far as background strain was concerned, ApoE null mice were also considered for adenoviral injection to alleviate complications of endogenous ApoE on ApoE3 or ApoE4 effects, however the vastly skewed lipid profile of these mice proved a confounding consequence that would likely overshadow more subtle effects produced by ApoE3 or ApoE4. Therefore ApoE3 or ApoE4 expression by adenoviral vector was utilized to induce production of the human apolipoproteins in the presence of endogenous mouse ApoE. Our laboratory has previously used adenoviral vectors to express human apolipoproteins in mice (Seetharam, Mineo et al. 2006), and the second-generation adenoviral ApoE3 and ApoE4 (abbreviated adenoE3 and adenoE4) to be utilized were kindly provided by Dr. Dan Rader at University of Pennsylvania. His laboratory previously demonstrated that adenoE3 potently blunts atherosclerosis in $LDLR^{-/-}$ mice over a 6 week

period in the absence of changes in lipid profile indicating use of these vectors to improve CVD risk.(Tsukamoto, Tangirala et al. 1999)

Briefly, human ApoE3 and ApoE4 cDNA were subcloned into the SnaBI site of the shuttle plasmid vector pAd-CMV-link, which contains adenoviral replication machinery, and the cytomegalovirus immediate early gene enhancer and promoter, thus producing recombinant adenoviruses. The viruses were expanded in Hek293 cells, purified and titered, and mice were administered 1×10^{10} viral particles via intravenous injection in order to minimize their immune response. Human ApoE expression was evaluated via enzyme-linked immunosorbent assay (ELISA) analysis of mouse plasma at day 7 following injection. Reendothelialization was performed on day 7, with artery harvest on day 10 (**Fig. 17A/B**), which is a timepoint at which the adenoviral ApoE levels are comparable to concentrations found in humans. Furthermore, lipoprotein subclasses in mice injected with adenoE3 or adenoE4 are similar.(Tsukamoto, Hiester et al. 1997)

The third study was done in collaboration with Dr. David Hui at the University of Cincinnati, in which the effect of global ApoER2 deletion on atherogenesis was investigated in crosses of $LDLR^{-/-}$ and $ApoER2^{-/-}$ mice. When 10 weeks old, mice were put on a high fat Western diet (Harlan-Teklad 88137) for 24 weeks, and plasma was drawn for lipid profile. Aortas were then harvested, prepped enface, and stained for lipid deposition with Oil Red O. Lesions in both the thoracic and abdominal aorta were quantified and graphed. Though ApoE knock-out mice could have been utilized as an atherosclerosis-prone model following crossing to ApoER2-deficient mice, LDLR knock-out mice were the better option due to the less skewed lipid profile compared to ApoE knock-out mice on Western

diet. Specifically total cholesterol in ApoE$^{-/-}$ mice on Western diet can be up to four times greater than that produced in LDLR$^{-/-}$.(Daugherty 2002) While these results are striking (**Fig. 18)**, LDLR$^{-/-}$; ApoER2$^{-/-}$ mice will be studied in the same fashion so as to determine if endothelial ApoER2 is in fact the tissue expression required for atheroprotection.

Last, we sought to gain mechanistic information as to how ApoER2 affects vascular functioning in vivo. In the brain Dab1 is crucial for Reelin signaling through ApoER2. In endothelial cells PI3k and Akt activation are also important in ApoE3-stimulation of eNOS, and ApoER2 is also required for this stimulation. I then considered that this upstream adaptor protein Dab1 might be important in ApoER2 actions in the endothelium, as well. ApoER2-EIG mice have been created in which the region directly upstream of the NPxY motif in the cytoplasmic tail of ApoER2 has been altered (NFDNPVY changed to EIGNPVY) which inhibits DAB1 binding and associated downstream activity.(Beffert, Durudas et al. 2006) In order to determine if effects seen in Fig. 16 in the absence of ApoER2 are in fact due to the absence of the NPxY region, I studied ApoER2-EIG mice in the reendothelialization procedure. While effects seen in this EIG mutant mouse would implicate the NPxY region of ApoER2 in vascular effects, and DAB1 interaction with this region is crucial in neuronal function and development, DAB2 also binds this region in other tissues, and thus could be the relevant adaptor molecule in the endothelium, not DAB1.(Cuitino, Matute et al. 2005)

Results

ApoER2 is required in reendothelialization: Carotid artery reendothelialization following perivascular electric injury was compared in ApoER2$^{+/+}$ and ApoER2$^{-/-}$ male mice. Using methods previously reported by our laboratory and others Evans blue dye incorporation was used to assess the relative degree of reendothelialization postinjury as discussed earlier. **Fig. 16** upper panel, shows representative images of the intimal surface of carotid arteries 72h postinjury, with Evans blue dye incorporated in the regions of remaining denudation. There was a trend of greater remaining denudation in ApoER2$^{-/-}$ versus ApoER2$^{+/+}$ mice (**Fig. 16**, lower panel, n=8-9 per group, *$p<0.05$).

ApoE4 impairs normal reendothelialization: Carotid artery reendothelialization following perivascular electric injury was compared in C57/Bl6 WT male mice. Mice expressing human ApoE3 exhibited full recovery similar to uninjected mice. However mice expressing human ApoE4 showed impaired reendothelialization (**Fig. 17B**, n=6 per group, *$p<0.05$ vs LacZ, †$p<0.05$ vs ApoE3).

ApoER2 is athero-protective: LDLR$^{-/-}$;ApoER2$^{-/-}$ mice on a Western diet for 24 weeks had far greater lipid deposition in the aorta than LDLR$^{-/-}$; ApoER2$^{+/+}$ mice on the same diet (**Fig. 18**, n=10, *$p<0.05$ vs. LDLR$^{-/-}$;ApoER2$^{+/+}$). Importantly, these findings were not related to differences in lipoprotein, triglyceride or cholesterol status.

The NPxY Motif is required for reendothelialization: Mixed-background mice in which the NPxY sequence (NFDNPVY changed to EIGNPVY) has been altered to

prevent adaptor protein binding and transduction of Reelin signaling were studied in the carotid artery reendothelialization model. ApoER2-EIG mice displayed significantly more area of injury compared to wild-type mice remaining following 72 hours of recovery. Interestingly ApoER2-EIG mice showed similar area of denudation remaining compared to global ApoER2 knock-out mice (**Fig. 19**, n=10, *$p<0.05$ vs. WT).

Comparisons between 2 groups were done by Student's t tests, and comparisons between 3 or more groups were performed by ANOVA with posthoc Neuman-Keuls testing. A p value <0.05 was considered statistically significant.

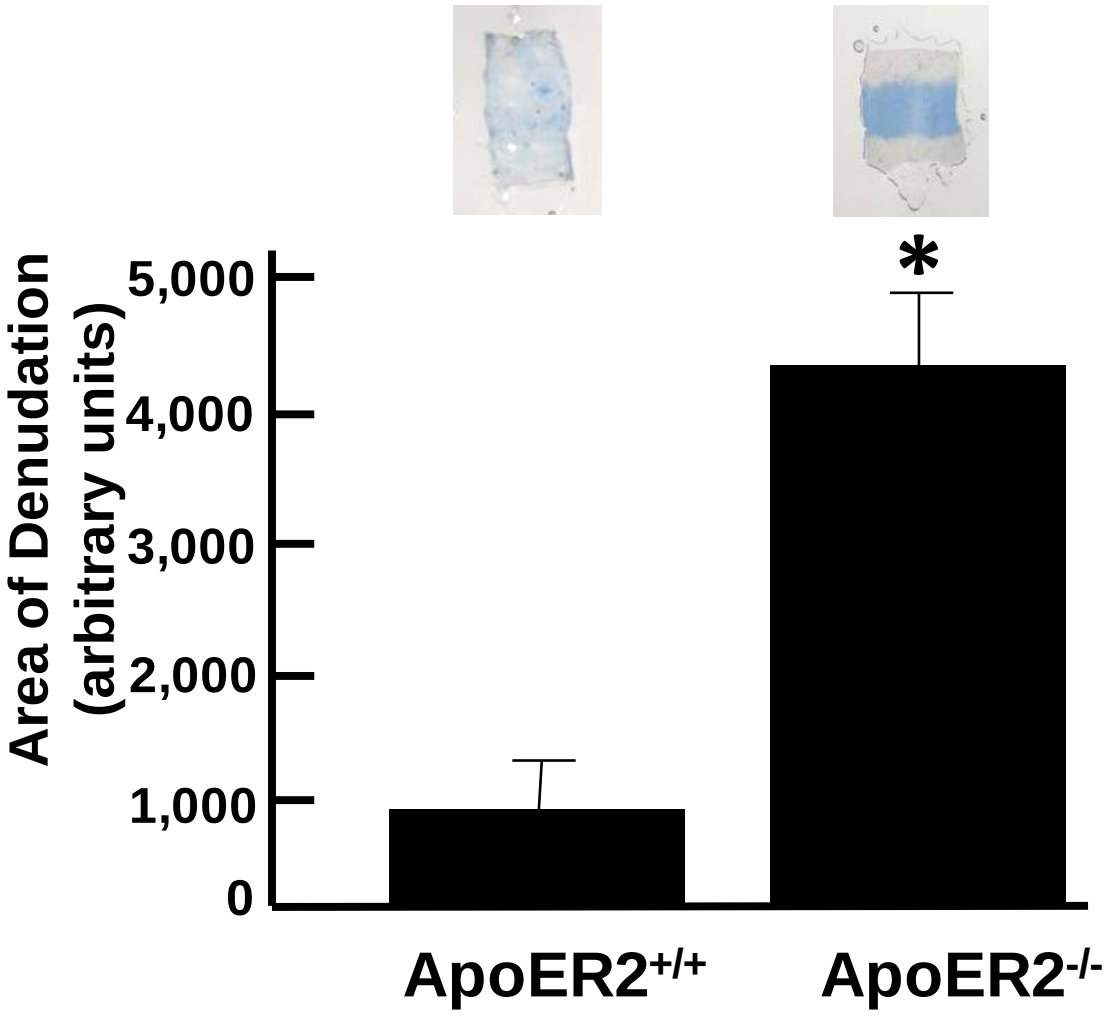


Figure 16: ApoER2 is required for normal reendothelialization in mice

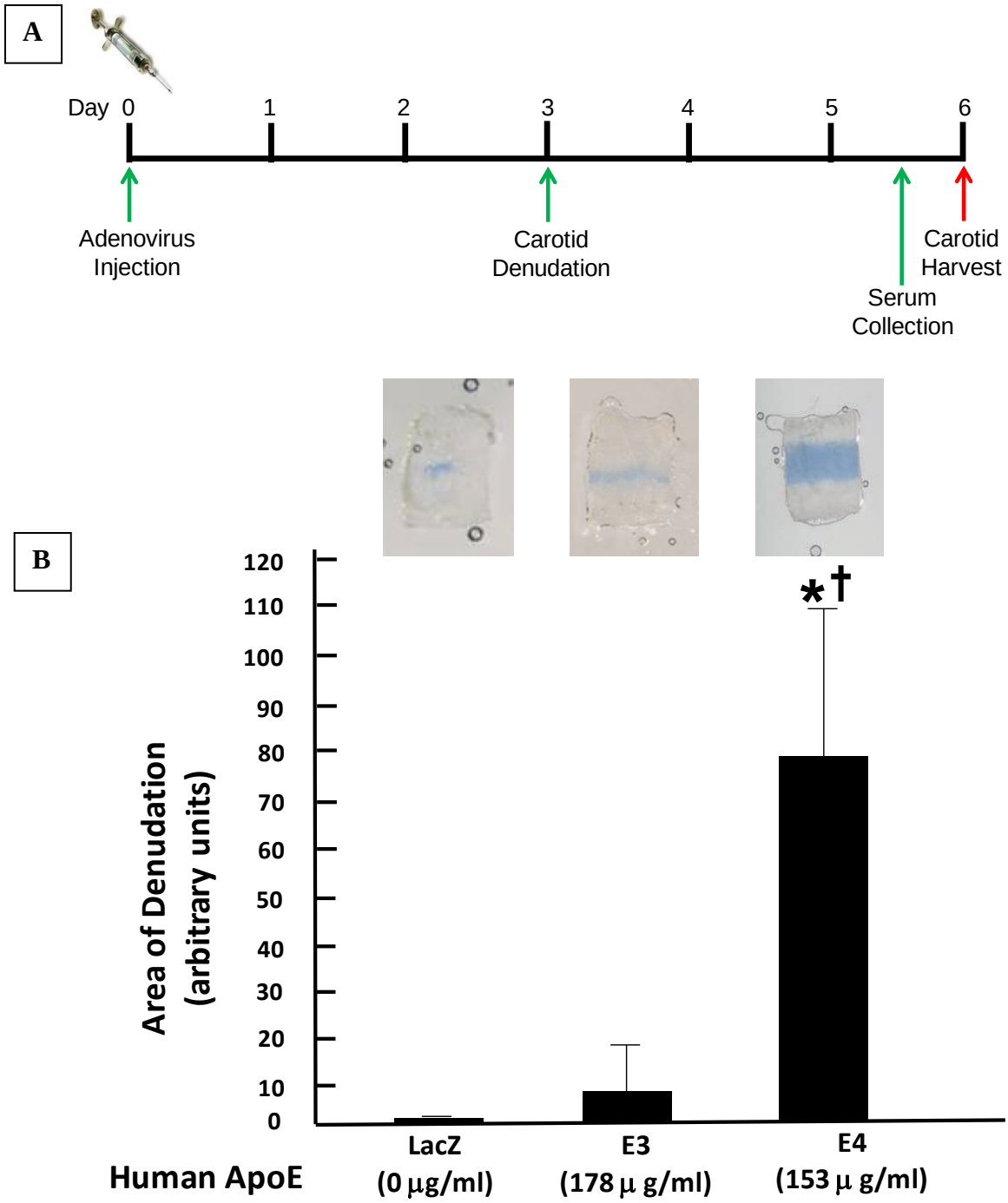
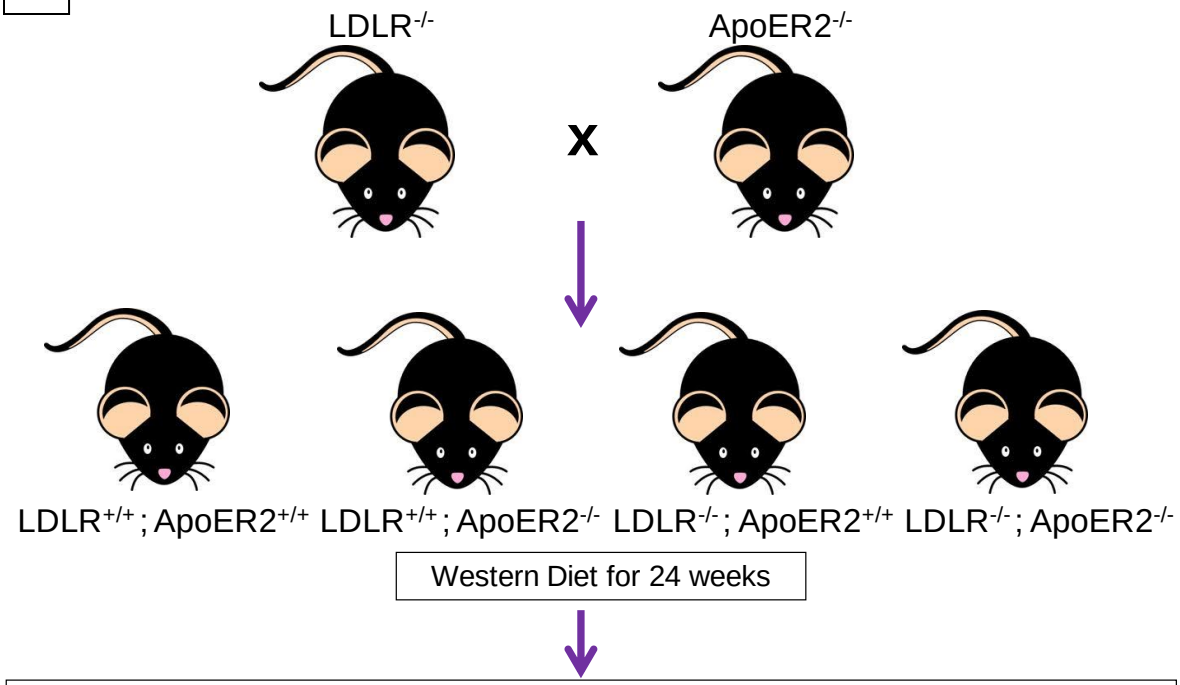


Figure 17: ApoE4 impairs reendothelialization in ApoE$^{+/+}$ mice. A: Experimental timeline. B: Area of denudation reamining 72 hours post injury, human ApoE expression levels prior to harvest shown below graph

A


LDLR-/-
ApoER2-/-
x
LDLR+/+ ; ApoER2+/+
LDLR+/+ ; ApoER2-/-
LDLR-/- ; ApoER2+/+
LDLR-/- ; ApoER2-/-
Western Diet for 24 weeks
Evaluation of Atherosclerosis
Total Chol.
263±23 mg/dl
293±43 mg/dl
1617±94 mg/dl
1672±61 mg/dl

B

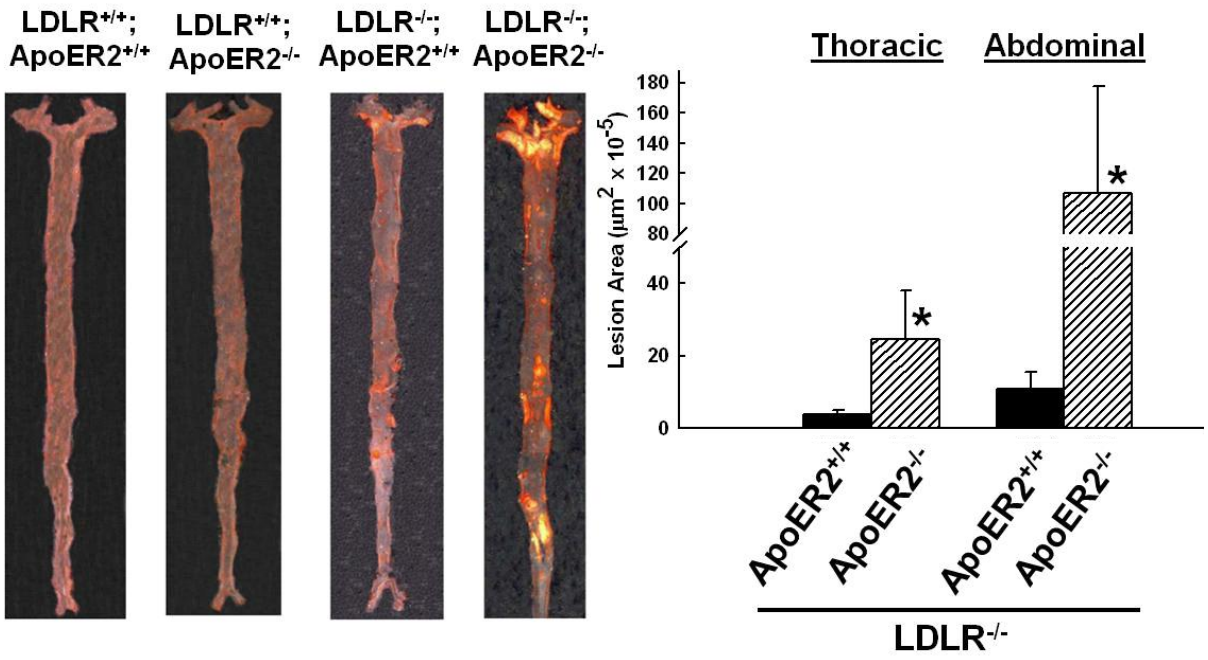


Figure 18: ApoER2 Provides Protection against Atherosclerosis: A: Atherosclerosis study design. B **Left Panel:** Oil Red O staining of lipid deposition in en face preparations of aorta from adult male LDLR$^{+/+}$;ApoER2$^{+/+}$, LDLR$^{+/+}$;ApoER2$^{-/-}$, LDLR$^{-/-}$;ApoER2$^{+/+}$ and ApoER2$^{-/-}$;LDLR$^{-/-}$ mice fed Western diet for 24 weeks. **Right Panel**: Atherosclerotic lesion size in thoracic and abdominal aorta.

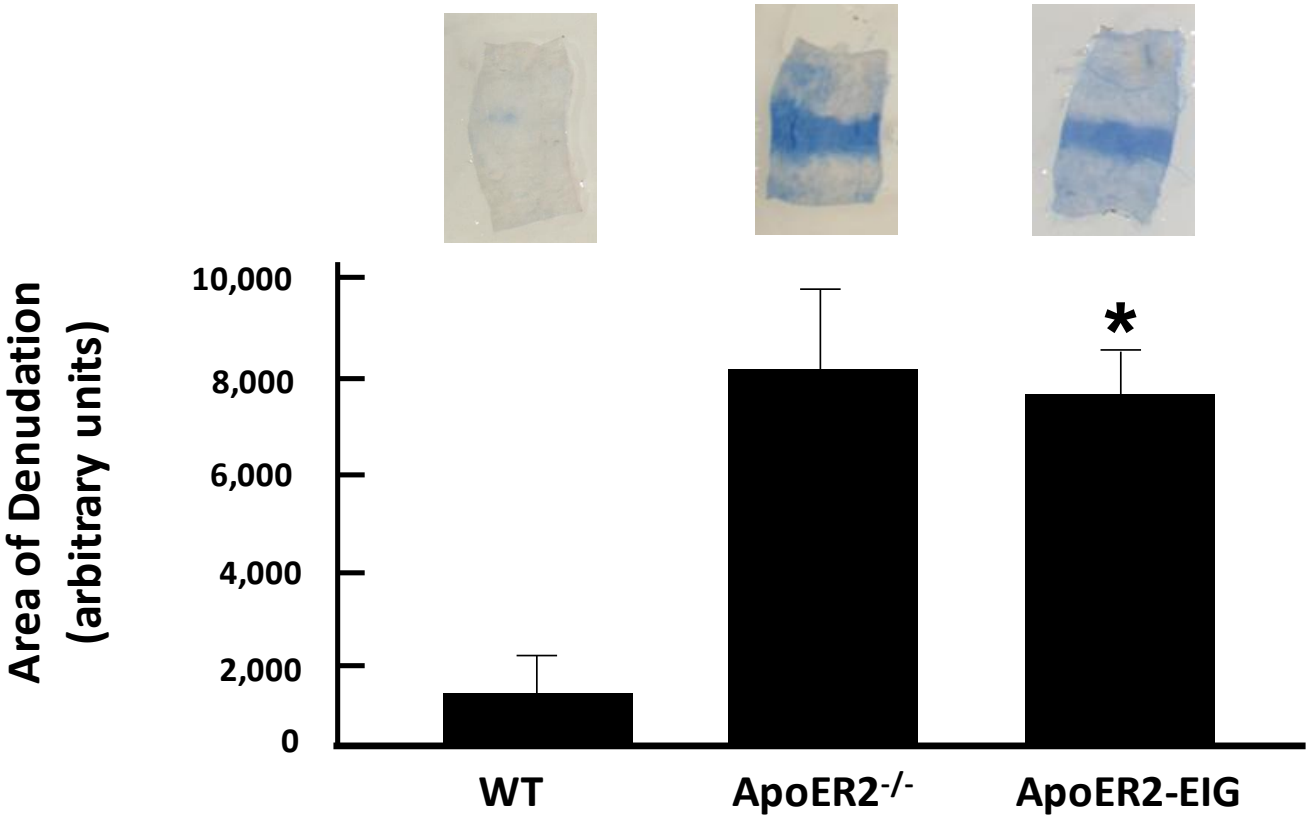


Figure 19: The NPxY Motif of ApoER2 is Required for Reendothelialization

Discussion

Studies discussed earlier pinpoint ApoE3 and ApoER2 as important proteins in vascular homeostasis in terms of protection against endothelial dysfunction. This chapter explores the importance of ApoE3, ApoE4, and ApoER2 in reendothelialization, a process which reflects endothelial migration in vivo and is critical in vascular repair and angiogenesis.

As shown, ApoE3 stimulates eNOS activation through ApoER2. Other studies indicate that production of NO increases expression of VEGF, which acts in a cycle to produce more NO. Furthermore intracellular production or exogenous use of secreted NO triggers signaling through s-nitrosylation or cGMP S-nitrosothiol. S-nitrosylation then results in increases in Ras activation, which increases endothelial migration. NO also activates protein kinase C alpha (PKCα) and inhibits protein kinase C delta (PKCδ) to increase migration. Finally, NO can also act through cGMP-dependent protein kinase to produce matrix metalloproteinase 13 which stimulates endothelial migration. (Hansson, Chao et al. 1985, Shizukuda, Tang et al. 1999, Lopez-Rivera, Lizarbe et al. 2005, Ataya, Tzeng et al. 2011)

While Figure 16 and 17 exhibit beneficial roles for ApoER2 and ApoE3 in reendothelialization, these experiments do not address how the ApoE3-ApoER2-NO pathway specifically induces migration. Thus more cell culture studies need to be undertaken to address the potential targets above such that the migratory pathway of ApoE3-ApoER2-produced NO can be traced to complete mechanistic studies.

The reendothelialization model is not a true disease model, therefore it would be advantageous to address another functional readout of diseased endothelium in vivo to parallel the carotid artery injury studies. Human carriers of ApoE4 exhibit worse atherosclerosis, and ApoE3 expression in mice has been shown to regress atherosclerosis. In Figure 18, $LDLR^{-/-}$ mice deficient in ApoER2 exhibit worsened atherosclerosis. The next key study would be to utilize the adenovirus to express ApoE3 in mice to assay if atherosclerosis could be regressed in ApoER2 knockout mice, and furthermore if atherosclerosis could be regressed in mice deficient in endothelial-expressed ApoER2. However this complicated experimentation would be difficult to analyze since atherosclerosis is such a complex disease. Preliminary studies indicate that ApoE3 expression does not inhibit progression of atherosclerosis in $LDLR^{-/-}$ mice in our hands, nor does ApoE4 increase lesion formation in this same strain of mouse (data not shown). How factors and events differentially prevent atherosclerosis initiation versus regress established disease are not clarified. Studies would need to be modified such that ApoE3 or ApoE4 is given prior to Western diet to better address role of ApoE variants in atherosclerosis initiation, or a less complex disease model could be used to more clearly elucidate the role of ApoE3/ApoE4 in CVD risk. Specifically neointimal formation induced by epon resin probe in endothelial denudation in arteries would lend information about how endothelial denudation would affect a true pathological process relevant to ApoE4 carriers. Thus if ApoE3 expressing mice exhibit less neointimal formation than wild type or ApoE4 expressing mice, this would lend credence to the role of ApoE3 in vascular protection in vivo as shown in the

reendothelialization studies, since destruction of the endothelial layer leads to neointimal formation.

To expand our hypothesis further to consider the role of endothelial ApoER2 is rather straightforward and involves conducting another atherosclerosis progression experiment such as that shown in Fig. 18 and utilize mice without endothelial ApoER2$^{-/-}$ to compare lesions to those in global receptor knock-outs and wild type mice. However pinpointing the ApoE4-ApoER2 role in atherosclerosis is less clear cut. While humans that are homozygous for ApoE4 and express the mutant ApoER2-R952Q receptor (which we have shown displays eNOS-activation deficiencies) exhibit increased CVD risk compared to ApoE4 homozygotes or ApoER2-R952Q carriers alone, this synergistic effect would be difficult to assay in mice since ApoER2$^{-/-}$ mice already show increased atherosclerosis. Therefore greater lesion formation in ApoE4-expressing mice would not indicate if increases were due to ApoE4 expression or receptor deletion. However if ApoE4-expressing mice showed greater lesion formation than that seen in receptor-deficient mice, this might indicate the role of an additional pathway or secondary receptor utilized by ApoE4 in atherosclerosis progression, which seem to be implied by the ApoE4/ApoER2-R952Q disease risk study in humans. While in vitro VLDLR appears to not propagate ApoE3 signaling to activate eNOS, evaluating ApoE3 positive vascular effects in vivo in the presence of the global loss of ApoER2 and VLDLR in a double knockout mouse would be impossible, since these double knockout mice don't live to adulthood and exhibit gross phenotypic effects due to impaired Reelin signaling in utero. However, endothelial specific double knockout mice could be created which would address the role of ApoER2 and VLDLR in vascular homeostasis in the

presence of adenovirally-expressed ApoE3. Others are studying the role of Megalin and LRP1 in atherosclerosis, with which collaboration would be beneficial to evaluate lesion formation or regression in respective receptor knockout mice in the presence of ApoE3. Indeed ApoER2 is a transmembrane receptor known to cluster with other surface receptors, so the potential for assisted signaling in endothelium via ApoE3 stimulation of NO-dependent processes in the vasculature should be thoroughly investigated.

CHAPTER SEVEN

Ongoing Studies

CURRENT DIRECTIONS AND FUTURE HYPOTHESIS

Molecular basis for ApoE-ApoER2 signaling in the endothelium

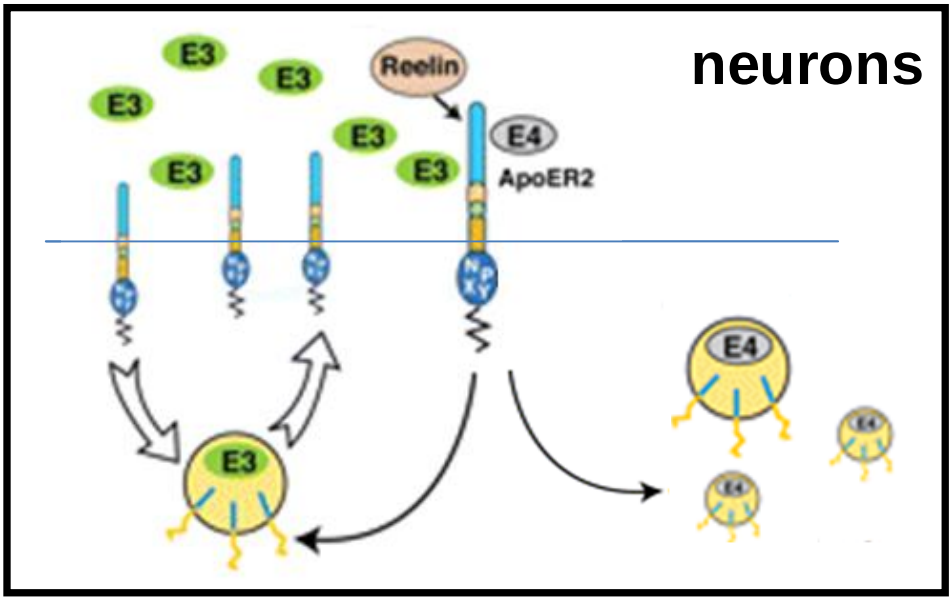
As shown in the earlier results, ApoE3 stimulates eNOS and endothelial migration, and prevents leukocyte adhesion, and this is mediated through ApoER2. Conversely, ApoE4 inhibits ApoE3-activation of eNOS, as well as migration. Furthermore, in mice ApoE4 impairs the normal reendothelialization course. However, the molecular mechanisms by which ApoE4 decreases normal endothelial activity, as well as how it diminishes the beneficial functions of ApoE3, are not known.

Intracellular differences between ApoE3 and ApoE4 signaling in endothelium

In neurons, Herz et al. determined the differential effects of ApoE3 and ApoE4 on ApoER2 surface expression when Reelin is bound to receptor. In biotinylation studies, ApoE4 bound to ApoER2 decreased the surface levels of ApoER2, in contrast to ApoE3 which did not decrease surface levels. In **Figure 20A** modified from the Herz paper, when Reelin binds ApoER2, and ApoE4 is concurrently bound, ApoER2 is endocytosed, and gets sequestered in the cell, unable to recycle back to the neuronal surface to propagate further signaling. Contrastingly, ApoE3 allows for ApoER2 recycling back to the cell surface.(Chen, Durakoglugil et al. 2010) Using this Herz model, we tested this hypothesis in

endothelium, utilizing the same biotinylation and avidin method. While ApoER2 is expressed to a lesser extent in endothelial cells versus neurons, we were able to biotinylate ApoER2 in endothelium (**Fig.20B**).

A



B

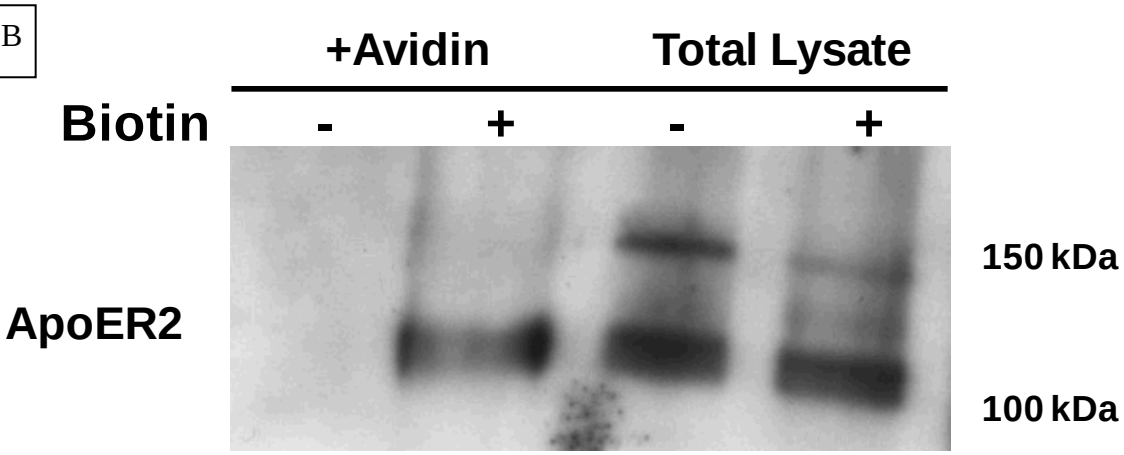


Figure 20: A: Herz model of ApoE3/ApoE4 alteration of ApoER2 surface expression in neurons. B: Biotinylation of surface ApoER2 in endothelial cells

Briefly, we treated cells with equal concentrations of ApoE3 or ApoE4, then incubated cells with biotin. Lysates were then incubated with avidin agarose beads, then protein attached to beads was eluted and immunoblotted for ApoER2. Following results in triplicate, neither ApoE4, nor ApoE3, treatment, altered cell surface expression of ApoER2 in primary endothelial cells (results not shown).

Next, the relevance of known ApoER2 intracellular adaptor proteins is being addressed. The important results that EIG mutant mice exhibit defective reendothelialization following carotid injury led us to consider associated adaptor proteins of the NPxY region in vitro. In the brain when Reelin binds extracellulary Dab1 interaction is induced to bind with the NPxY region in the cytoplasmic tail of ApoER2. However in other tissues, Dab2 is the primary interacting partner of the NPxY region. While Dab1 protein and mRNA detection in EaHy and HAEC have been difficult, as well as siRNA-targeted knockdown and detection of protein changes, modifications to current protocols are ongoing to better ascertain the role of Dab1 in endothelium following treatment with ApoE3, ApoE4, or both. Furthermore, in neurons Src recruitment to the c-terminal tail to ApoER2 activates Dab1, therefore it is of importance to consider changes in Dab1 phosphorylation. Specifically measuring changes in tyrosine phosphorylation of Dab1 are also ongoing following treatment with ApoE3, ApoE4, or both in endothelial cells. Following confirmation or rejection of Dab1 in ApoER2 signaling following ApoE3 treatment, the role of Dab2 in ApoER2 induced signaling will be addressed in endothelium.

Extracellular changes in ApoER2 signaling following ApoE3/ApoE4 treatment

While surface expression of endothelial ApoER2 does not change following ApoE3/Apo4 treatment, there is little known about other effects of ApoE3/ApoE4 extracelllularly besides what is purported based on structural differences between the lipoproteins. ApoE3 maintains an open conformation and preferentially binds HDL, while ApoE4 exhibits a closed conformation and preferentially binds larger lipoproteins like VLDL. However since changes in CVD risk between ApoE3 and ApoE4 carriers are not explained by changes in lipid profiles, this is likely not due to changes in lipoprotein affinity.(Dong, Wilson et al. 1994, Hatters, Peters-Libeu et al. 2006)

However the size of the ApoE3- or ApoE4-lipoprotein particle may interfere with receptor clustering or conformational change. Initially it was considered that ApoE4 effects on ApoE3 in the NOS assay was due to ApoE4 displacement of ApoE3 particles, or aggregation before assay in the media. However considering that ApoE3 and ApoE4 bind LDLR-family receptors with equal affinity, we preincubated cells with ApoE3 then treated with ApoE4. ApoE4 still inhibited ApoE3 activation of eNOS following ApoE3 incubation, lending some credence to the potential hypothesis that ApoE4 does not displace ApoE3 on ApoER2, or preaggregate ApoE3 in the media. However studies looking into the stoichiometry underlying the effect of ApoE4 on ApoE3 will need to be considered in the future.

Another hypothesis that was pursued was that while ApoE3 may utilize ApoER2, ApoE4 exerts actions through another receptor, such as VLDLR. Thus VLDLR

was targeted by siRNA knockdown in endothelial cells treated with ApoE3 and ApoE3 plus ApoE4. In the presence of decreased VLDLR, ApoE3 still activated NOS, indicating that ApoE3 does not require VLDLR for these protective actions. Likewise ApoE4 still inhibits ApoE3-activation of NOS in the presence of decreased VLDLR, indicating that ApoE4 does not require VLDLR to blunt ApoE3 function. To confirm that ApoE3 and ApoE4 only require ApoER2 to exert effects on eNOS in endothelium, soluble extracellular domain of ApoER2 will be utilized in the NOS assay to block ApoE3 and ApoE4 NOS effects.

In APS, ApoER2 binds β2GPI to transmit apl effects in endothelium. To evaluate if any other circulating proteins or cell surface receptors are aiding ApoER2 signaling following ApoE3 or ApoE4 interaction, co-immunoprecipitation of ApoER2 following ApoE3 or ApoE4 treatment will be conducted, followed by analyzation of bound proteins. Should another receptor cluster ApoER2 following ApoE3 treatment, and not ApoE4 treatment, this might infer that the size or structure of ApoE4 prevents close interaction of receptors, or prevents conformational change by ApoER2 necessary for cluster and signaling. Likewise any changes in binding domain on ApoER2 by ApoE3 and ApoE4 will be addressed by utilizing small molecules to block specific domains on ApoER2, as well as via the creation of ligand-binding domain mutants of ApoER2.

Endothelial effects of ApoE and ApoER2 signaling in mice

Mice were created in which exons 1 and 2 of the ApoER2 gene were flanked by loxP sites for recombinase excision (**Fig. 21A**). These floxed homozygous mice possessing the altered ApoER2 gene were then crossed with mice that constitutively express

VE-cadherin cre recombinase (VE-Cad-Cre), which targets excision of loxP-flanked gene regions in endothelium. LoxP sites were tested by crossing ApoER2 flox;flox mice with mice expressing global cre recombinase, and brain samples were immunoblotted for ApoER2 and denominator actin (**Fig.21B**).

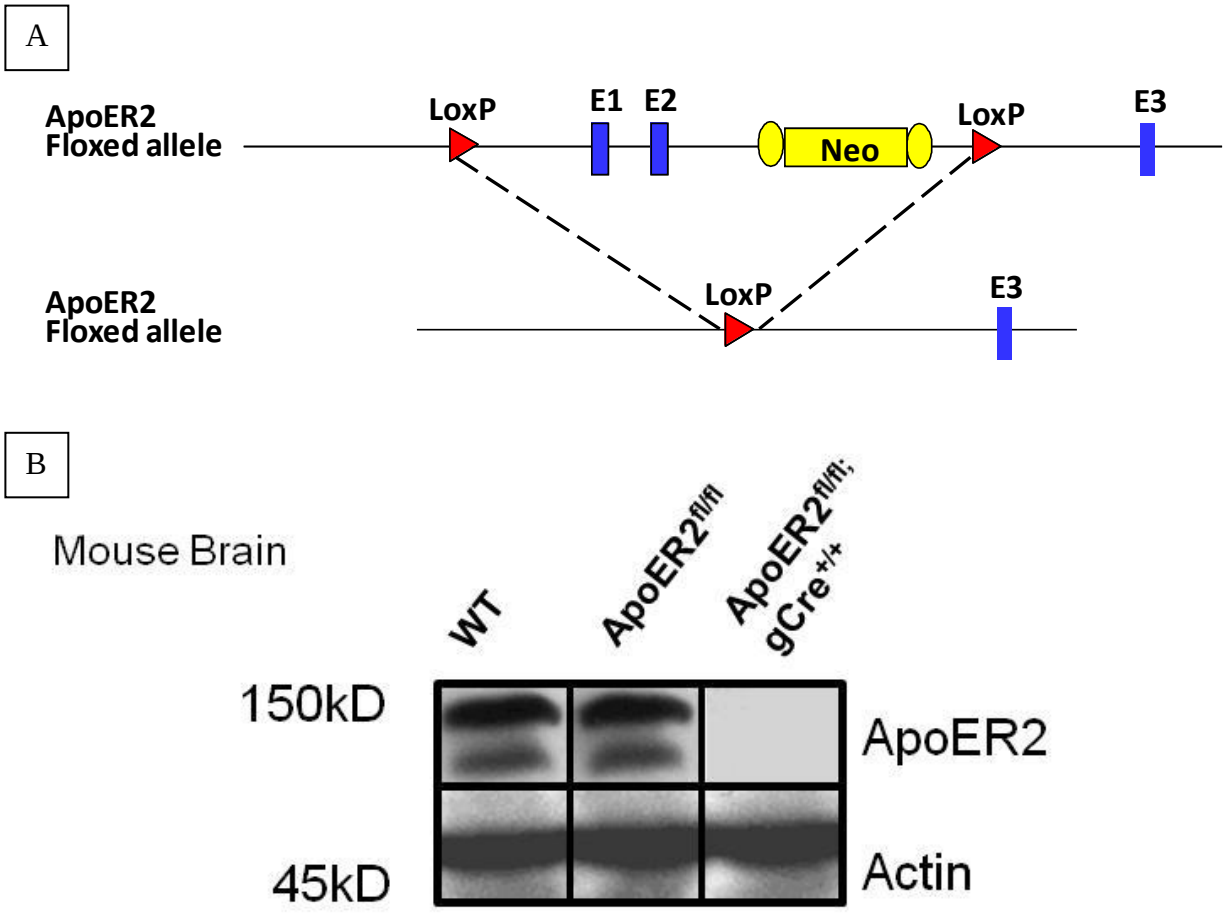


Figure 21: A: Floxed ApoER2 gene schematic before (top) and after (bottom) cre recombinase excision. B: Brain immunoblot analysis of ApoER2 floxed sites following cross with global cre recombinase.

Analysis in **Figure 21B** indicates that deletion of floxed portion does not create a hypomorph, as indicated by no visible protein following global cre excision.

Atherosclerosis will be evaluated these mice deficient in endothelial ApoER2, utilizing the model shown in Figure 18. While global loss of ApoER2 in mice results in increased atherosclerosis on an $LDLR^{-/-}$ background, we hypothesize that in fact endothelial deletion of ApoER2 in this mouse strain will result in similar lesion development, indicating that endothelial ApoER2 is required for lumen homeostasis.

Second, the reendothelialization model should be perform in mice without endothelial ApoER2, and compared to global deletion of ApoER2 as shown in Figure 16. This study would test the hypothesis that endothelial ApoER2 is required for reendothelialization in mice. Next the ApoE3-ApoER2 tandem signaling in endothelium should also be evaluated. Utilizing the adenovirally-expressed ApoE3 in mice deficient in ApoER2 compared to endothelial deletion of ApoER2, then subjecting groups to the carotid injury model, would indicate if in fact ApoE3 requires endothelial ApoER2 to improve monolayer repair.

Following results indicating the importance of endothelial ApoER2 in endothelium, as well as requirement of ApoER2 for ApoE3 signaling in vivo, other relevant vascular dysfunction models should be pursued. Since ApoER2 and ApoE3 have been shown to be important in the recovery of the endothelial monolayer in vivo, and ApoE3 requires ApoER2 to abrogate leukocyte adhesion in vitro, the hypothesis that ApoE3 requires endothelial ApoER2 in vivo to ameliorate leukocyte adhesion in mice should be addressed via intravital microscopy.

The ApoER2-R952Q variant, associated with increased CVD, should also be explored. Specifically, addressing the importance of the mutant receptor in cultured

endothelial cells would be primarily pursued. NOS assays were conducted overexpressing wild type versus mutant receptor, however a significant difference was not seen in NOS activation in the presence of endogenous receptor ($p=0.06$, data not shown). Therefore, the cleanest analysis of mutant ApoER2 function would be achieved by targeting endogenous ApoER2 with siRNA to knockdown expression, then overexpressing wild type or mutant receptor, and evaluating NOS activation and associated upstream kinase activity, or endothelial migration following ApoE3 treatment. This is a difficult proposition as discussed earlier, but is most relevant clinically compared to the more artificial 3T3 system utilized. Ultimately, a knock-in mouse expressing this variant receptor will also be created, in which to evaluate reendothelialization and leukocyte adhesion. To evaluate ApoER2-R952Q endothelial response to ApoE3, the adenoviral expression system could again be used to overexpress ApoE3 in the presence of mutant receptor in order to test the hypothesis that ApoER2-R952Q cannot respond to ApoE3.

In conclusion, this project holds great promise to fully clarify the role of ApoE and ApoER2 in endothelium, as well as the molecular mechanisms involved, in order to explain the changes seen in CVD disease risk in patients expressing ApoE or ApoER2 variants.

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