APOLIPOPROTEIN E IN DIABETIC DYSLIPIDEMIA AND ATHEROSCLEROSIS

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ABSTRACT

Lance A Johnson:

Apolipoprotein E in Diabetic Dyslipidemia and Atherosclerosis

(Under the direction of Nobuyo Maeda, PhD)

Each year, cardiovascular disease (CVD) kills more Americans than any other cause of death. The majority of the diseases contributing to CVD can be traced back to the pathological process of atherosclerosis, in which fatty material collects along the walls of arteries, limiting flexibility and obstructing blood flow. Plasma lipids, particularly in the form of low-density lipoprotein (LDL) cholesterol, contribute significantly to the formation of atherosclerotic plaques and are an important determinant of CVD risk. Thus, it is critical to understand the roles of the crucial components of normal lipoprotein metabolism that regulate plasma lipids, such as apolipoprotein (apo) B, apoE and the LDL receptor. In addition, patients with diabetes are two to four times as likely to develop CVD as non-diabetic patients. One important reason for this discrepancy is the process of diabetic dyslipidemia – a cluster of harmful changes to normal lipoprotein metabolism commonly seen in patients with diabetes. ApoE is the primary ligand for several lipoprotein receptors, making it a crucial component in the clearance of lipid from the circulation and a major determinant of plasma cholesterol and cardiovascular disease risk. The APOE gene is polymorphic, resulting in production of three common isoforms: ApoE2, E3, and E4. In addition to its role in lipoprotein metabolism, recent findings have also suggested a role for ApoE in glucose metabolism. In Chapter 2, I examine the role of apoE3 and apoE4 during the process of diabetic dyslipidemia and atherosclerosis. Similarly, Chapter

3 focuses on the crucial interaction between apoE and the LDLR in the background of diabetes. In Chapter 4, I explore the increased risk of CVD in patients with diabetes in detail, focusing on the various models of diabetic atherosclerosis available to researchers and how they may help understand the cause of this risk. Finally, in Chapter 6 I provide a supplemental examination of the the role of apoB, and in particular the LDLR binding region of apoB100, in the development of hyperlipidemia and atherosclerosis. Together, this work highlights the importance and interconnectedness of glucose and lipid metabolism, and sheds new light on the critical role of apolipoprotein E in the development of dyslipidemia and atherosclerosis during diabetes.

ACKNOWLEDGMENTS

Nobuyo literally has an open door policy. Over the past few years I have walked into her office countless times to ask questions and advice. I usually begin with some variation of the phrase "Nobuyo, can I bother you for a minute?" A credit to her patience and willingness to help, she has never once said "No", "Not right now", or even "Could you come back later?" She has answered every single question I have come up with (although sometimes with another question), and I greatly appreciate the many hours she has spent mentoring me along the way. In addition to their penchant for teaching, both Nobuyo and Oliver easily inspire without words. A graduate student could not ask for a better example of the hard work and dedication that it takes to be a successful scientist. But long hours are not enough, and the passion, the curiosity, and the love of science that Nobuyo and Oliver demonstrate is absolutely infectious.

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outside of it. I also had a great deal of assistance from a bright young group of future scientists. Marcus, Taylor, Sabrina, and Melissa – they made me feel old, but have all helped tremendously.

Most of all, I want to thank my wife, Ryann, who has been there from the beginning of my graduate career, and without whom there may never have been an end. I was also fortunate enough to marry into a generous, intelligent family – and Ryann's parents, Donna and Glynn, have provided incredible support and encouragement during my graduate career. Ryann has always been amazingly supportive, quick to rearrange her schedule when my schedule in the lab needed it – and as Nobuyo would be happy to know, she routinely told me to 'just go to the lab and do some work' on weekends (although it was mostly to get me out of her hair). She has pushed me to work hard, celebrated when I succeed, and picked me up and brushed me off when I haven't. Even when every experiment seems to fail, every hypothesis seems to be wrong, and every reviewer seems to be out to get me, I can still go home at night and feel like the luckiest scientist on Earth.

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LIST OF ABBREVIATIONS

ACE	Angiontensin converting enzyme
AGE	Advanced glycation end product
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoBEC	Apolipoprotein B Editing Complex
ApoE	Apolipoprotein E
AT1	angiotensin II type 1
CRP	C-reactive protein
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DiI	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-perchlorate
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
GF	Perigonadal fat
GK	Glucokinase
GLUT-2	Glucose transporter - 2
GLUT-4	Glucose transporter – 4
GPx-1	glutathione peroxidase-1
H&E	Hematoxylin and eosin
HDL	High-density lipoprotein

HFD	High fat diet
HSPG	Heparan sulftate proteoglycan
I-125	Iodine 125
IF	Inguinal fat
Il-6	Interleukin 6
Ins2	Insulin 2
ITT	Insulin tolerance test
IR	Insulin receptor
IRS-2	Insulin receptor substrate – 2
LA	Lipoic acid
LCMV-GP	lymphocytic choriomeningitis virus glycoprotein
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LDLR-/-	Low-density lipoprotein receptor knock-out
LRP	Low-density lipoprotein receptor related protein
MCP-1	Monocyte chemotaxic protein 1
MetS	Metabolic syndrome
mRNA	Messenger ribonucleic acid
NDST-1	N-deacetylase/N-sulfotransferase 1
NEFA	Non-esterified fatty acid/ Free fatty acid
NO	Nitric Oxide
NOD	Non-obese-diabetic mice
NS	Non-significant

OGTT	Oral glucose tolerance test
PAI-1	plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PPARα	Peroxisome Proleferator Activated Rceeptor – alpha
PPARγ	Peroxisome Proleferator Activated Rceeptor – gamma
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SR-B1	Scavenger receptor B1
SREBP	Steroid receptor element binding protein
STZ	Streptozotocin
TG	Triglyceride
TNF-α	Tumor necrosis factor 1
TP	Thromboxane A2 receptor
UC	Ultracentrifuge
VLDL	Very-low density lipoprotein

Chapter 1

INTRODUCTION

1.1 Cardiovascular Disease

Each year, cardiovascular disease (CVD) kills more Americans than any other cause of death. Diseases of the heart alone caused 30% of all deaths, with other diseases of the cardiovascular system causing substantially more death and disability (1). Worldwide, deaths from vascular disease are estimated to rise from 16.7 million in 2002 to 18.1 million in 2011. In the United States alone, over 81 million people suffer from one or more of the various forms of CVD, including hypertension, coronary heart disease (myocardial infarction and angina pectoris), stroke, and heart failure (2).

The majority of these diseases can be traced back to a single process: atherosclerosis. Atherosclerosis is a condition in which fatty material collects along the walls of arteries, limiting the flexibility of the arteries and obstructing blood flow. This material thickens, hardens (forming cholesterol crystals and calcium deposits), and occludes the vessels. The process is thought to be initiated by an inflammatory injury response – in which monocytes invade the intima of the vessel and differentiate into macrophages (3). Once there, the macrophages begin to phagocytose fat and cholesterol, in particular cholesterol-rich Low Density Lipoprotein (LDL) particles, expanding in size and eventually becoming 'foam cells', named due to their 'foamy' lipid-filled appearance. At late stages of disease, pieces of the atherosclerotic plaque can break off and move through the affected artery to smaller blood vessels, blocking them and causing tissue damage or death (3).

1.2 Lipoprotein Metabolism

All lipoproteins consist of a triglyceride (TG) and cholesterol ester core surrounded by an outer shell composed of free cholesterol (un-esterified), phospholipids and various protein components.

They are organized into various classes as defined by their density. LDL, like other lipoproteins, is a complex of lipid and protein that facilitates the transport of these crucial, but water insoluble, lipids through the circulation. As alluded to above, LDL cholesterol level is an important determinant of CVD risk (4).

Lipoprotein metabolism involves two pathways, exogenous and endogenous, which depend primarily on the source of the circulating lipids; dietary (exogenous) or hepatic (endogenous) origin (5). Following a meal, dietary fat and cholesterol are absorbed and re-packaged by the enterocytes of the small intestine, and secreted into the lymphatics as chylomicrons – large TG-rich lipoprotein particles which eventually enter the bloodstream at the left subclavian vein (Figure 1.1). As they travel through the circulation, fatty acids (FA) are extracted from the chylomicron particles by peripheral cells through the action of Lipoprotein Lipase (LpL), leaving smaller, more dense Chylomicron Remnants that are eventually internalized by the liver (5).

The liver also synthesizes cholesterol and TG and packages them into Very Low Density Lipoprotein (VLDL) particles, which are secreted into the circulation (6). Again, peripheral tissues can take what they require, and VLDLs are remodeled into Intermediate Density Lipoproteins (IDL), and finally the more dense, choelsterol-rich LDL particles (Figure 1.1). The liver, as well as peripheral tissues, can also internalize whole lipoprotein particles, and the apolipoprotein components discussed later in this chapter are important mediators of this clearance. High Density Lipoprotein (HDL) particles mediate a process known as reverse cholesterol transport, in which the smaller, denser HDL particles acquire cholesterol from peripheral tissues as well as from VLDL and LDL and transport them back to the liver (7). Importantly, HDL can mediate this same process in atherosclerotic plaques, removing cholesterol and fat from the vessel wall and returning it to the liver (8).

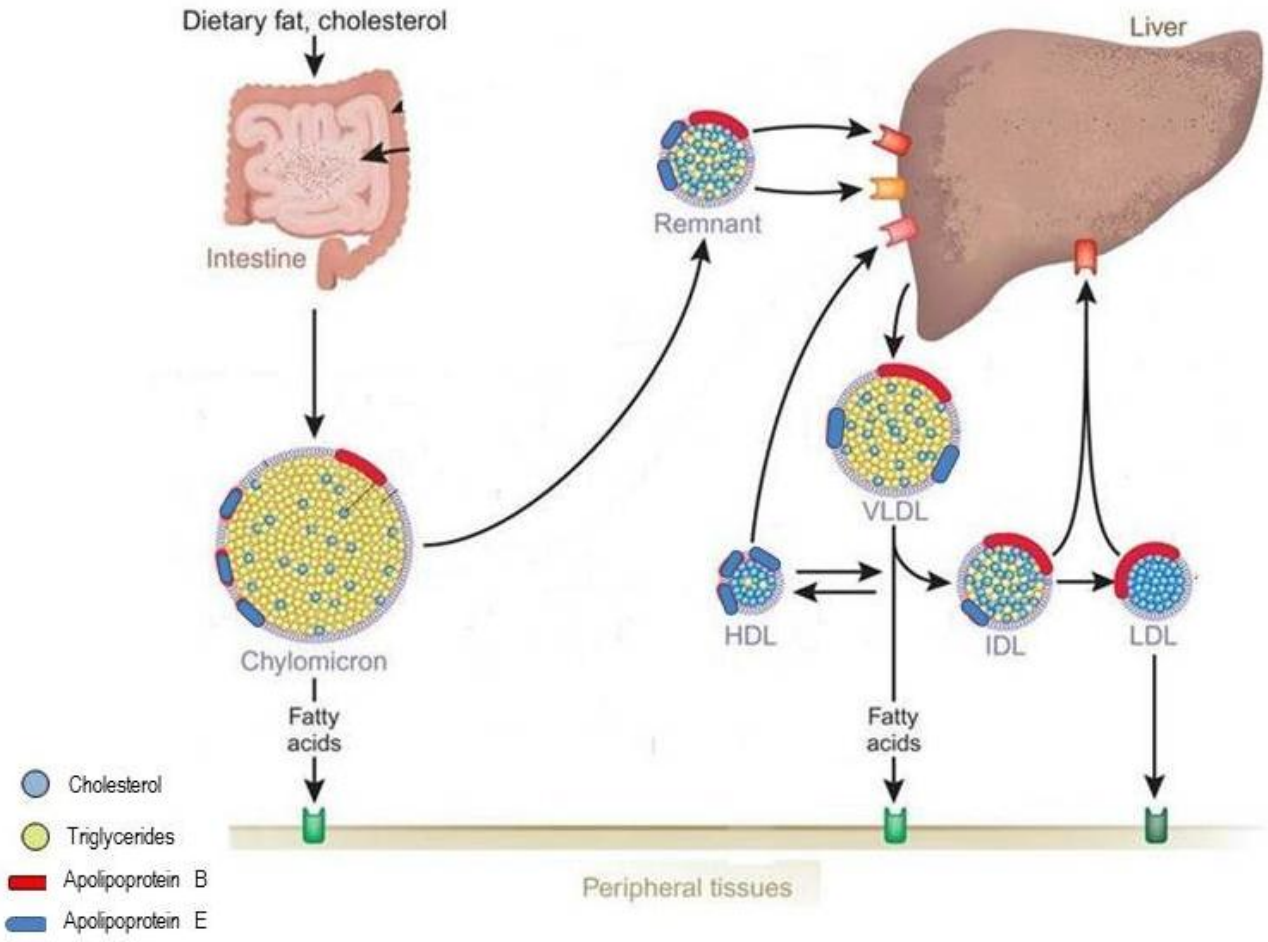

Figure 1.1. **Normal Lipoprotein Metabolism.** Normal lipoprotein metabolism involves uptake of dietary lipid, the secretion of chylomicrons, and their eventual uptake by the liver in the postprandial state. Conversely, during times of fasting, the liver secretes VLDL, which can be converted to IDL and then LDL through lipolysis. Reverse cholesterol transport involves the exchange of cholesterol and apolipoproteins from VLDL and IDL particles to HDL and their return to the liver. Figure adapted from Lusis et al., Nature Genetics 40, 129 - 130 (2008).

1.3 Apolipoprotein B

Apolipoprotein B (apoB) is large (4536 amino acids) structural component of all lipoproteins with the exception of HDL. Each lipoprotein particle contains one molecule of apoB. ApoB is an essential component of chylomicrons, VLDL, IDL and LDL and normally exists in two forms, apoB100 and apoB48 (6, 9). ApoB100 is the full length protein, while apoB48 is a product of posttranslational mRNA editing by the apoB editing complex (apoBEC). ApoBEC introduces a stop codon at 2153, resulting in a truncated version of the apoB protein (6, 9). ApoBEC is highly expressed in the intestine of humans, but it is expressed in both the intestine and liver in mice. Consequently, as dietary lipid is absorbed in the small intestine, it is packaged into apoB48-containing chylomicrons and enters the lymphatic system for eventual secretion into the peripheral circulation (5). In humans, apoB48 is present only on chylomicrons and chylomicron remnants, while all lipoproteins secreted from the liver contain apoB100. However, due to hepatic apoBEC expression in mice, both apoB100 and apoB48 are secreted from the liver (9).

ApoB100 is a critical determinant of LDL cholesterol, as it functions as a ligand for the Low density lipoprotein receptor (LDLR). Because the LDLR binding domain of apoB is in the C-terminal half of the protein, apoB48 does not bind to the LDLR. Instead, apoB48-containing lipoproteins require other apolipoproteins to act as a ligand for receptor binding and hence clearance from the circulation. In Supplemental Chapter 6, I discuss the role of apoB, and in particular the LDLR binding region of apoB100, in the development of hyperlipidemia and atherosclerosis ("Absence of hyperlipidemia in LDL receptor-deficient mice having apolipoprotein B100 without the putative receptor-binding sequences")

1.4 Apolipoprotein E

Apolipoprotein E (apoE) is a small (299 kD) circulating protein associated primarily with VLDL and HDL, and is the primary ligand for several lipoprotein receptors, making it a crucial component in the clearance of lipid from the circulation. Its importance as a major determinant of plasma cholesterol and cardiovascular disease risk is underscored by the spontaneous hyperlipidemia and atherosclerosis in mice lacking apoE (10). Mice lacking apoE (apoE-/-) accumulate cholesterol-rich remnant particles with total plasma cholesterol levels exceeding 400 mg/dl (11). Although diets high in fat and cholesterol accelerate plaque development, apoE-/- mice develop complex fully-formed atherosclerotic lesions even when fed a low fat, low cholesterol diet (12).

In humans the APOE gene is polymorphic, resulting in production of three common isoforms: apoE2, E3, and E4. The apoE isoforms differ from one another by only one or two amino acids at position 112 and 158, with E2 having cysteines at both positions, E3 a cysteine at 112 and arginine at 158, and E4 arginines at both positions (10). ApoE consists of two main structural domains that are connected by a hinge region (Figure 1.2). The N-terminal domain consists of a four helix bundle and contains the receptor binding region (residues 136-150). The C-terminal domain consists of a series of alpha helices and contains the major lipid/lipoprotein binding region (residues 244-272). Despite independently folding, the two domains can influence the properties of one another. The cysteine /arginine residues at positions 112 and 158 influence interactions between the two domains. For instance, when the cysteine at 112 is experimentally replaced by an arginine in an apoE3 moleculue, the positive charge effectively pushes the arginine at position 61 into a position where it forms a salt bridge with glutamine 255 (13). Consequently, the N-terminal domain is pulled close together with the C-terminal domain (Figure 1.3, top). The modified apoE3

molecule demonstrates a lipid binding preference for VLDL similar to that of apoE4. In the absence of this interaction the preference is for high density lipoproteins, as with apoE3 (13). The compact form of ApoE4 also leads to a much lower stability than apoE3. Likewise, apoE3 has a lower stability than apoE2 (14). The interaction of the N- and C-terminal domains in apoE2 resembles that of apoE3. The difference between these two molecules instead arises from the additional cysteine at position 158 in apoE2, which affects the LDL receptor binding region by indirectly generating an additional salt bridge with arginine 150, thereby lowering the overall positive charge and thus the receptor binding potential of the region.

Upon biding to lipid, apoE is undergoes a major conformational change (13-15). The apoE molecule is thought to form a molecular envelope (horseshoe shape) around the surface of the phospholipid outer shell of a lipoprotein particle (Figure 1.3, bottom). Interestingly, it has been demonstrated that artificial lipid complexes created in vitro with apoE4 bind to the LDLR with a similar to slightly higher affinity than lipid complexes made with apoE3. In contrast, particles made with apoE2 demonstrate a dramatically lower LDLR binding affinity (<5% compared to apoE3 and apoE4) (13). When complexed with VLDL however, there is a clear isoform gradient in regards to cell surface receptor binding in the order of: VLDL-4/4 (100%) > VLDL-3/4 (93%) > VLDL-3/3 (82%) > VLDL-4/2 (53%) > VLDL-3/2 (36%) > VLDL-2/2 (30%) (16). In addition, apoE4 has been shown to have a preference to bind to triglyceride-rich VLDL, while apoE3 prefers to bind smaller, denser HDL particles (17).

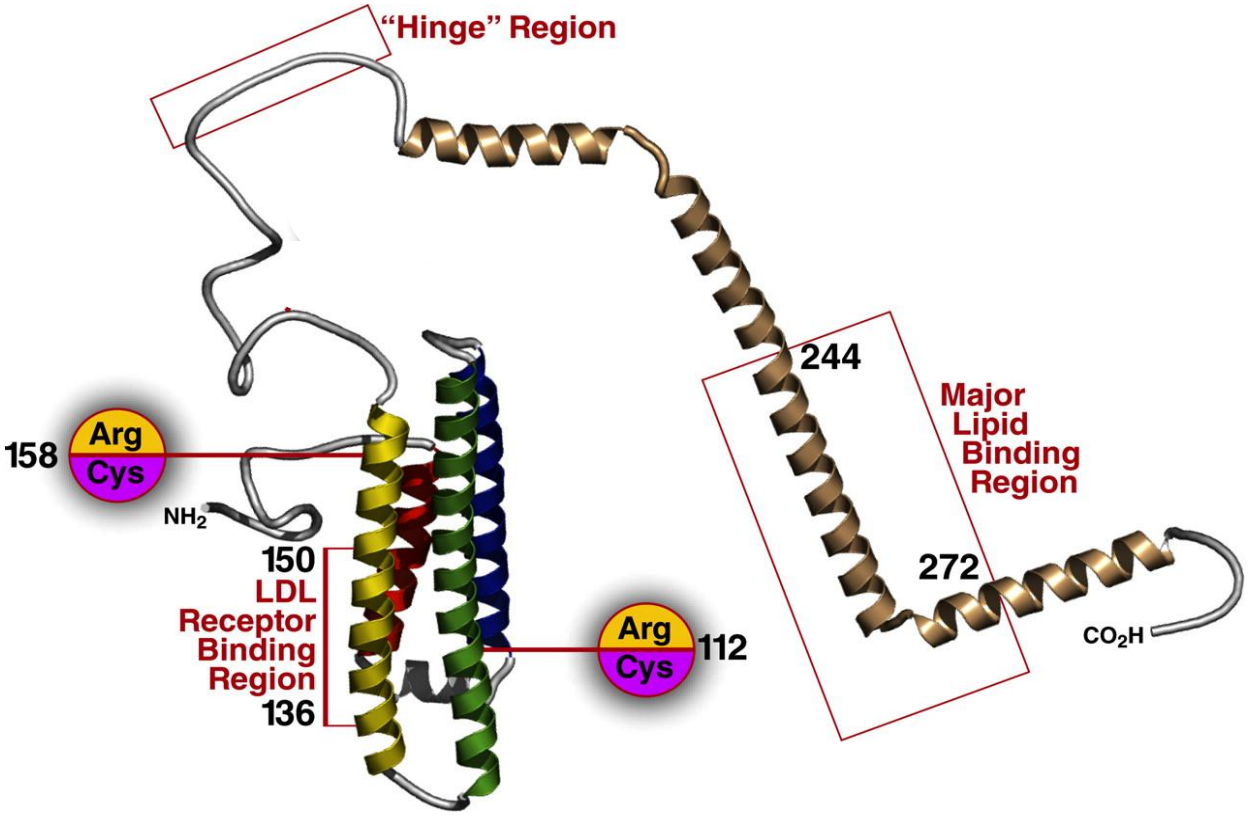

Figure 1.2. **Structure and functional domains of ApoE.** The N-terminal domain of apoE consists of a four-helix bundle (helix 1 = red, helix 2 = blue, helix 3 = green, helix 4 = yellow). The LDL receptor binding domain is located in a region of helix 4. The C-terminal domain of apoE is predicted as a series of α-helices and contains the major lipid/lipoprotein-binding region. Figure adapted from Zhong N , Weisgraber K H. J. Biol. Chem. 2009;284:6027-603.

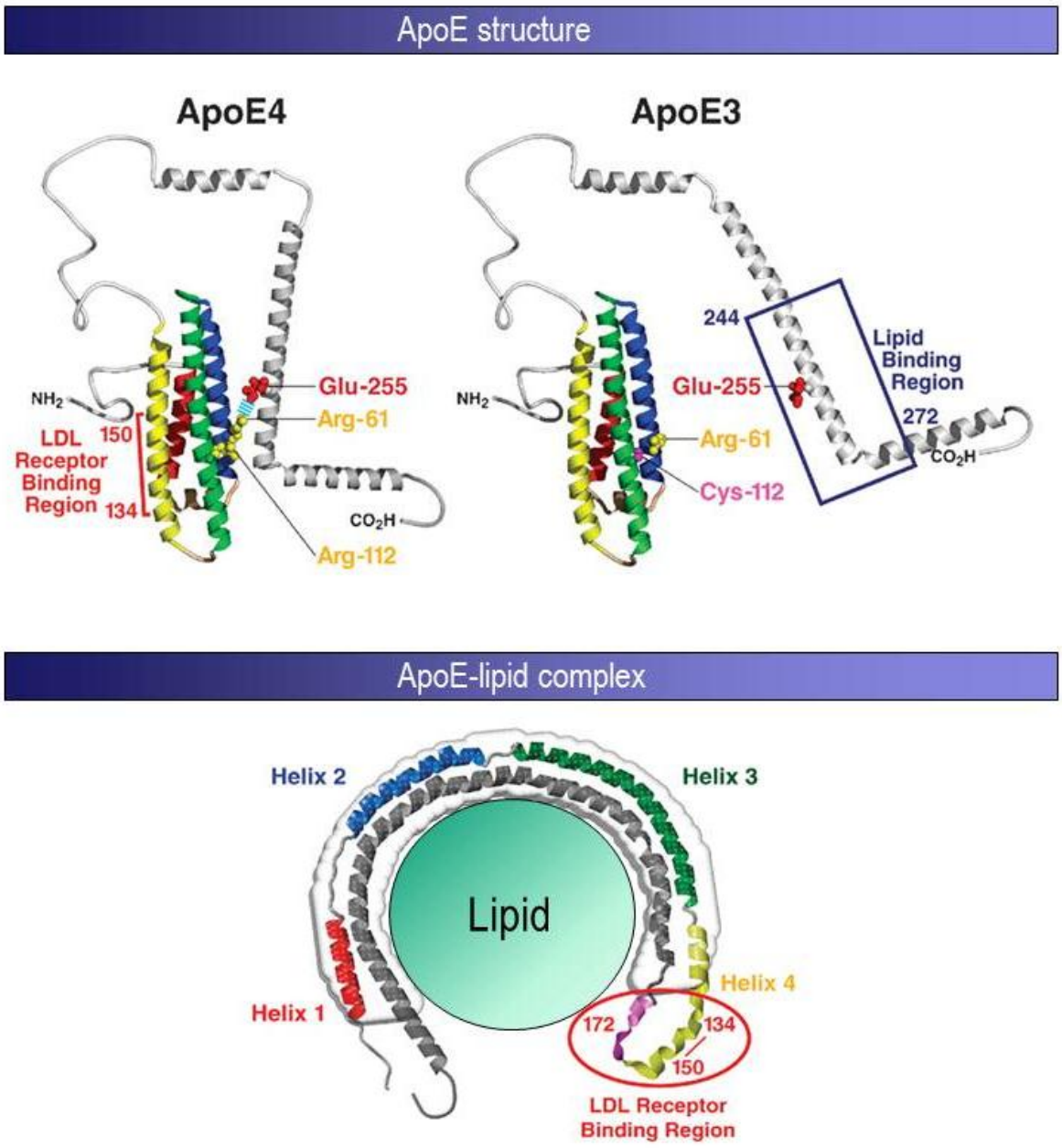

Figure 1.3. **Interaction of apoE N- and C-terminal domains and binding to lipid.** *Top:* The substitution of arginine for cysteine at position 112 induces structural changes in apoE3 and apoE4. Arg 112 in apoE4 pushes Arg 61 into a position to form a salt bridge with Glu 255. This Arg 61 – Glu 255 salt bridge pulls the N- and C-terminal domains together in the apoE4 molecule. *Bottom:* Upon biding to lipid, apoE undergoes a major conformational change, forming a circular molecular envelope around a phospholipid core and exposing the receptor binding region. Figure adapted from Mahley RW, Weisgraber KH, Huang Y. Apolipoprotein E: structure determines function, from atherosclerosis to Alzheimer's disease to AIDS. J Lipid Res. 2009 Apr;50 Suppl:S183-8. Epub 2008 Dec 22.

Even with varying distributions among different ethnic groups, E3 is overwhelmingly the most common isoform, with approximately 93% of the world's population inheriting at least one copy (Table 1.1). The E4 isoform occurs in 24% of the population, while the E2 allele is carried by less than 14% (10). *APOE* genotype has been shown to affect the relative risk for several important diseases (Figure 1.4). For example, patients with one or two copies of APOE4 have 10-15 mg/dl higher LDL cholesterol and increased rates of CVD. Conversely, patients with a copy of APOE2 have lower LDL, higher HDL and a decreased rate of cardiovascular events when compared to individuals homozygous for APOE3 (10, 15). APOE genotype is also strongly associated with risk and age of diagnosis for late-onset Alzheimer's disease in the order of E4>E3>E2 (14). More recent findings have pointed towards an involvement of apoE in glucose metabolism. For example, several data suggest that in certain populations, APOE genotype is associated with plasma glucose and insulin levels (18-19) and postprandial glucose response (20). APOE genotype also appears to play a role in determining the risk, progression and/or severity of Metabolic Syndrome (MetS) (21-22). Studies have also suggested a role for ApoE in various diabetic pathologies. For instance, an isoform-specific association of apoE in risk of diabetic nephropathy and retinopathy has been demonstrated in several cases (23). Most importantly in regards to CVD, diabetic apoE4 carriers have been shown to have increased carotid atherosclerosis (24), and elderly diabetic apoE4 carriers have an increased risk of CVD-associated death (25). Human epidemiological studies, however, are difficult to conduct. Because the frequency of APOE2 and APOE4 homozygotes are low, most studies have been carried out on heterozygous carriers in comparison to APOE3 homozygotes. Consequently, the phenotypic differences observed are small and easily influenced by other concomitant factors, both genetic and environmental.

	apoE2		apoE3	apoE4	
Genotype:	**2/2**	**2/3**	**3/3**	**3/4**	**4/4**
Population (%)	1%	12%	60%	21%	2%
Position 112	cys		cys	arg	
Position 158	cys		arg	arg	
LDLr affinity	< 5%		100%	≥100%	
LDL Cholesterol	decreased		normal	increased	

Table 1.1. **ApoE isoforms.** The three apoE isoforms have varying population distributions. The isoforms differ by only one or two amino acids (position 112 and 158). However, these small changes result in differing affinity for the LDLr and subsequently in plasma LDL cholesterol (10).

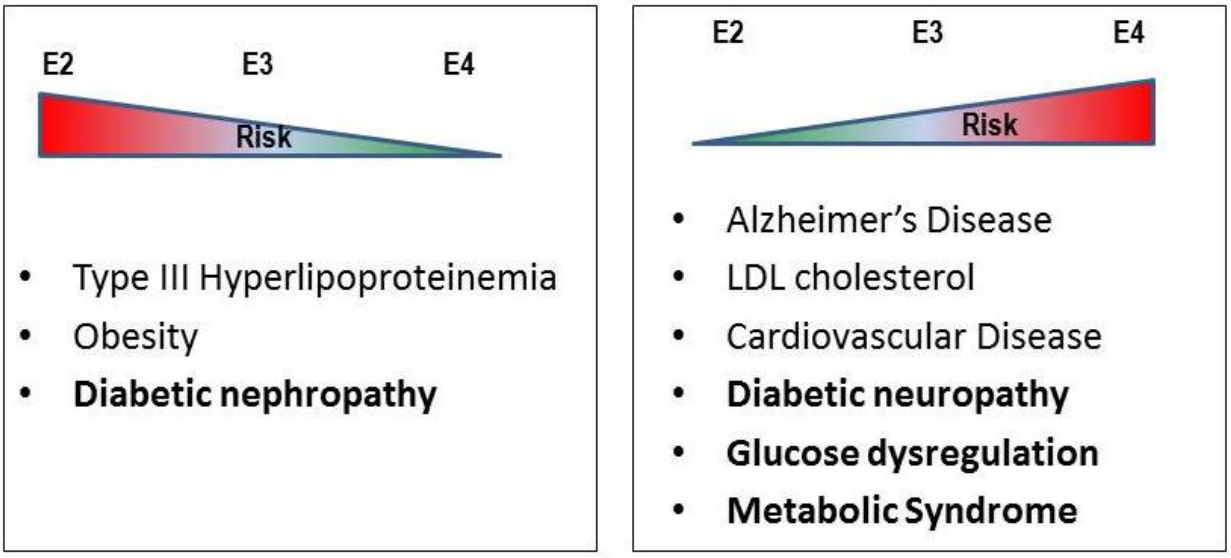

Figure 1.4. **ApoE isoforms, risk factors for disease**. Many different diseases have been shown to be affected by *APOE* genotype. For most disease states, the E4 allele confers risk (Right panel). However, for some diseases, the E2 allele confers additional risk (Left panel) (18-25).

1.5 Mouse Models of Human ApoE

Given the strong apoE isoform effect on the regulation of lipoprotein metabolism and the etiology of cardiovascular disease, it is essential for researchers to have access to translatable mouse models in which to study these effects. To this point, our laboratory has previously generated mice that express human apoE2, apoE3 and apoE4 using targeted gene replacement techniques (26). These mice have had the coding sequences for mouse apoE carefully replaced with conding sequences for the various human apoE isoforms. The replaced genomic fragment contains the 3′ part of intron 1, exons 2–4 of the human APOE gene, and 1.5 kb of 3′-flanking DNA, while the 5' and 3' portions of the mouse gene are intact. Thus, the target replacement mice retain their endogenous mouse ApoE promoter and all cis elements that are known to be important for the regulation of the ApoE gene. Mice targeted in this manner generate human apoE mRNA with similar tissue distribution and at an indistinguishable level as mouse apoE mRNA is expressed in wild type mice (27).

Mice that express human apoE2 (E2) have severely elevated plasma lipids closely resembling the human lipoprotein disorder known as type III hyperlipidemia. Type III hyperlipidemia is characterized by high chylomicrons and VDL and the most common causal factor is the homozygosity for APOE2 (28). Yet only less than 5% of homozygous APOE2 individuals develop type III hyperlipidemia. In addition, its onsent is about 30 years of age in men, while post-menopausal in women (28). In contrast to humans, both young male and female E2 mice have plasma cholesterol and triglyceride levels 2-3 times higher than wild type mice and mice with apoE3 (Figure 1.5). Consequently, the E2 mice develop atherosclerosis even on a low fat diet (26). As expected, mice expressing E2 show a significant reduction in their clearance of circulating

VLDL. Therefore, the severe hyperlipidemia observed in the E2 mice correctly reflects the low affinity of E2 for the LDLR.

The replacement of mouse apoE with human apoE3 or human apoE4 leads to total plasma lipids and lipoprotein profiles similar to mice with wild type mouse apoE (Figure 1.5) (26-27). E3 and E4 mice do not differ significantly in total plasma lipid or apolipoprotein levels. No atherosclerosis develops in these mice, even when a Western type diet was administered. However, when challenged with a high fat diet containing 15% fat, 1.25% cholesterol and 0.5% sodium cholate, both E3 and E4 mice had 3-4 times higher total plasma cholesterol and significantly larger atherosclerotic plaques compared to wild type mice (26-27). However, E4 mice accumulated more cholesterol-rich VLDL compared to mice with E3. In addition, E4 expressing mice tended to have larger atherosclerotic plaques on the atherogenic diet (26-27). In both Chapter 2 and Chapter 3, I have employed these useful humanized mouse models in order to exmaine the previously uninvestigated role of apoE in the setting of diabetes.

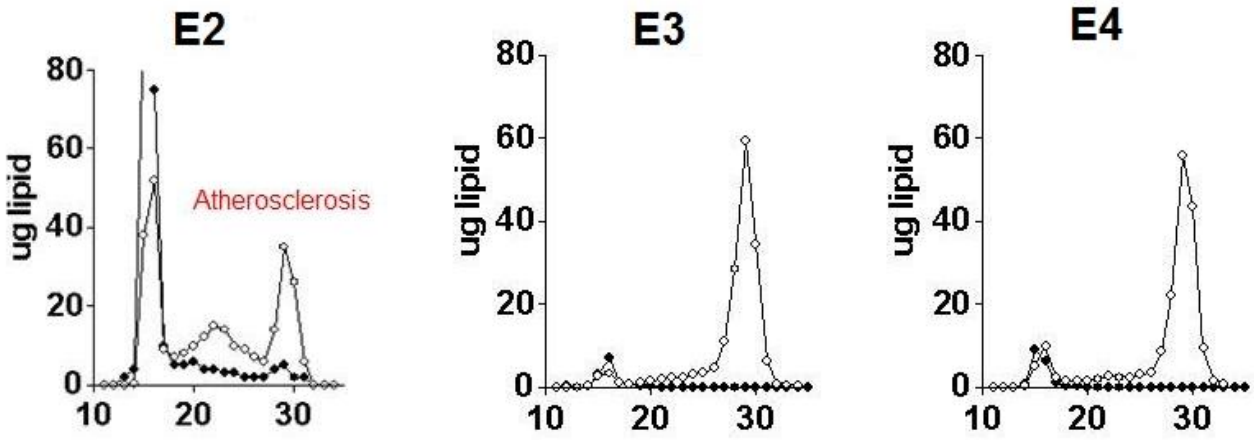

Figure 1.5. **Lipoprotein profiles in human apoE mice.** Mice homozygous for APOE2, E3, or E4 were fed a Western type diet containing 21% fat and 0.15% cholesterol. Cholesterol (white chircles) and triglyceride (black circles) distribution among various lipoprotein fractions in human apoE mice. Lipoprotein fractions were separated by fast performance liquid chromatography (FPLC). Fractions 12-17 correspond to VLDL, 19-25 to LDL, and 28-32 to HDL. E2 mice have 2-3 times higher total plasma cholesterol and triglycerides due to a defect in the clearance of VLDL and develop atherosclerosis even on a low fat diet. Mice with E3 and E4 have very similar lipid distribution patterns, with the majority of plasma cholesterol residing in the HDL fractions. (26-27, 29)

1.6 Low Density Lipoprotein Receptor

The major receptor through which apoE mediates lipoprotein clearance is the LDLR, and the apoE isoforms exhibit differential LDLR binding affinities (15). The LDLR is an 840 amino acid transmembrane receptor belonging to the Low density lipoprotein receptor gene family. LDLR protein synthesis is regulated by the level of free cholesterol within the cell. High intracellular cholesterol results in an inhibition of LDLR expression while depleted intracellular cholesterol levels stimulate transcription (25). LDLR complexes are found at the cell surface in clathrin-coated pits. The LDLR recognizes both apoE and full length apoB100, binding and then internalizing the apoE and apoB100 containing lipoprotein particles within a clathrin-coated vesicle (30). The LDLR contains a series of seven sequence repeats of negative charge at the N-terminus known as the class A domain. Binding of apoB requires class A repeats 2-7 while binding of apoE requires only repeat 5 (31). As mentioned earlier, the apoE isoforms demonstrate a binding affinity to the LDLR in the order of E2 << E3 ≤ E4 (13). Lipoprotein size also determines apoE binding epitope availability and even though the binding repeats of apoE are exposed during its lipid-free state, high affinity binding to the LDLR does not occur until apoE is complexed with lipid (31-33). Following endocytosis, the vesicle fuses with a late endosome and the acidic change in pH results in a conformational change to the receptor that releases the bound lipoprotein particle. The LDLR, as well as apoE, is then either degraded or recycled back to the cell surface where the neutral pH results in a change back to native conformation (Figure 1.6) (23, 34-35).

Defects in the LDLR in humans result in familial hypercholesterolemia (FH), a genetic disorder characterized by abnormally high plasma lipids (36). Due to the LDLR's important role in clearing LDL from the circulation, these patients suffer from chronic elevations in plasma

cholesterol and premature atherosclerosis. Mice deficient in the LDLR have lower total plasma cholesterol levels (~200 mg/dl) than their apoE-/- counterparts (>400 mg/dl). However, the majority of this cholesterol is in the form of the highly atherogenic LDL (37). Despite the differences in lipoprotein distribution between the two models, the pathology of atherosclerosis in the both the apoE-/- and Ldlr-/- mice is very similar to that seen in humans. Beginning as small foam cell formations deposited along the vessel wall, the lesions progress to large mature plaques containing a fibrous cap, necrotic core with cholesterol deposits and calcifications and thickening of the medial and adventitial tissue (11-12). The capability to model human-like atherosclerotic plaques in these mice has provided scientists with an experimental system in which to study multiple facets of disease progression as well as investigate potential therapies. In Chapter 2, I used mice deficient in LDLR in order to examine the effect of the human apoE isoforms on the progression of diabetic atherosclerosis.

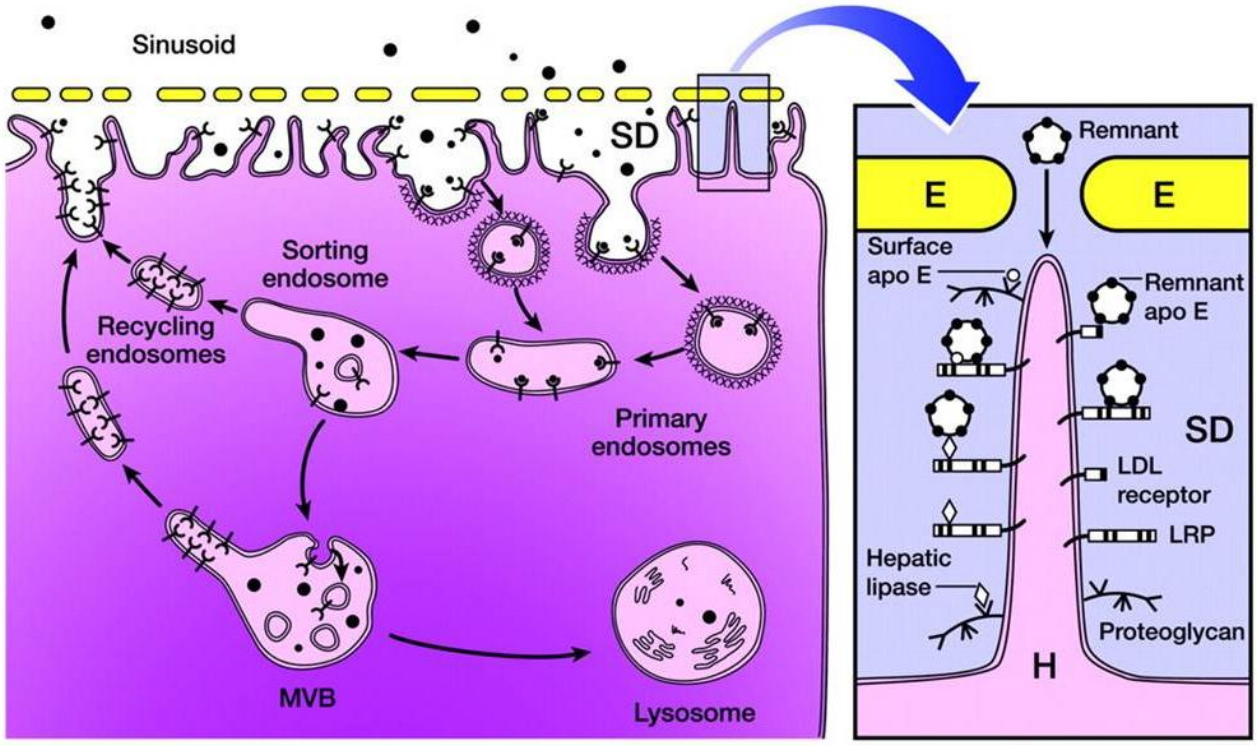

Figure 1.6. **Receptor-mediated uptake and processing of lipoproteins in the liver.** *Left:* Lipoproteins (small black circles) enter the space of Disse (SD) where they bind to cell surface lipoprotein receptors such as the LDLR (Y shape). The receptors are then internalized with their lipoprotein cargo in clathrin-coated pits, forming primary endosomes. The acidic pH of the sorting endosome stimulates the receptors to release their lipoproteins and dissociate from the clathrin coat. The lipoproteins are then transported to multivesicular bodies (MVB), or late endosomes, and eventually make their way to the lysosome. Recycling endosomes carry the receptors back to the cell surface. *Right:* Enlargement of a hepatic microvillus (H) and its adjacent endothelial cells (E). VLDL remnants pass through the endothelial cell fenestrae to the SD where they bind via apoE to LDLR or other members of the receptor family such as the LDLR related protein (LRP) and proteoglycans. Hepatic lipase bound to proteoglycans hydrolyzes lipids from the remnant particles. Figure adapted from Havel RJ, Hamilton RL. Hepatic catabolism of remnant lipoproteins: where the action is. Arterioscler Thromb Vasc Biol. 2004 Feb;24(2):213-5.

1.7 Overexpression of the LDLR

According to conventional wisdom, increasing expression of the LDLR is considered beneficial for reducing plasma cholesterol and atherosclerosis, as the LDLR works to clear atherogenic lipoprotein particles from the circulation. The high affinity of apoE4 for the LDLR has been proposed to lead to increased apoE-mediated cholesterol uptake, higher intracellular cholesterol and thus a subsequent down-regulation of LDLR expression (38). Down-regulated LDLR levels would then lead to a reduction in apoB100-mediated LDL clearance and thus high levels of circulating LDL cholesterol and an increase in atherosclerosis. This hypothesized downregulation of the LDLR by apoE4 has been thought to explain the association between apoE4 and increased risk of CVD (38).

Contrary to this hypothesis, our laboratory has previously shown that increasing Ldlr expression caused severe atherosclerosis in mice with human apoE4, but not apoE3, when the animals were fed a high fat diet (38). The mice used in these studies are heterozygous for targeted replacement of the mouse Ldlr gene with a human LDLR minigene ($Ldlr^{h/+}$) that produces stabilized mRNA with a longer than normal half-life. As a result, carriers of the stabilized human LDLR minigene have steady state LDLR expression levels 2-3 times higher than wild type mice (38). To examine the interaction between the human apoE isoforms and LDLR, human apoE mice were crossed with $Ldlr^{h/+}$ mice (E2h, E3h and E4h). Overexpression of the LDLR ameliorates the hyperlipidemia observed in the E2 mice and rendered them resistant to diet-induced atherosclerosis (Figure 1.7) (39). Increasing LDLR expression in apoE3 expressing mice resulted in a reduction in HDL cholesterol, but had no significant effect on non-HDL cholesterol or the development of atherosclerosis. However, increasing LDLR expression in apoE4 expressing mice resulted in the marked accumulation of cholesterol-rich, apoE-poor VLDL remnant particles and the development

of distinct atherosclerotic plaques when the mice were fed a high fat diet (Figure 1.7). Thus, the human apoE mice expressing increased LDLR exhibit lipoprotein distributions more similar to those associated with APOE genotype in humans. We believe this is a result of apoE "trapping", whereby the LDLR binds and traps apoE to the point where the supply available for lipoprotein binding becomes inadequate (38). ApoE exchange onto lipoproteins is required for efficient internalization by the LDLR, and without adequate apoE to mediate this process, these lipoproteins can become stuck in the circulation. We propose that apoE4 may be particularly sensitive to this process of trapping due to its strong affinity for the LDLR. In Chapter 3, I use this same model to examine the effects of the apoE-LDLR interaction in the background of diabetes.

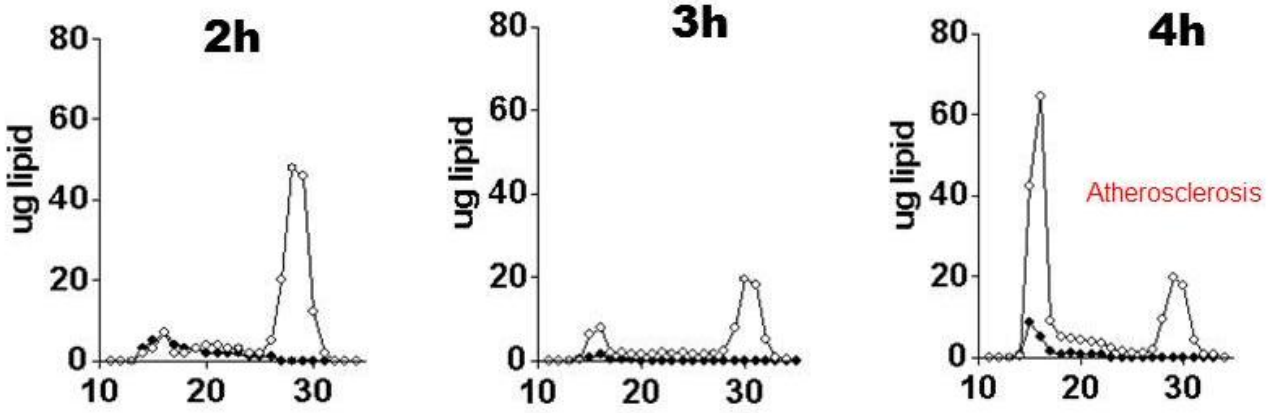

Figure 1.7. **Lipoprotein profiles of human apoE mice overexpressing the LDLR**. Cholesterol (white chircles) and triglyceride (black circles) distribution among various lipoprotein fractions in human apoE mice overexpressing the LDLR and challenged with a Western type diet. E2h and E3h mice carry the majority of their cholesterol within the HDL fractions. However, overexpression of the LDLR in the presence of E4 results in a harmful increase in VLDL and VLDL remnant cholesterol in addition to reduced HDL cholesterol, leading to the development of atherosclerosis. Figure adapted from Malloy SI, Altenburg MK, Knouff C, Lanningham-Foster L, Parks JS, Maeda N. Harmful effects of increased LDLR expression in mice with human APOE*4 but not APOE*3. Arterioscler Thromb Vasc Biol. 2004 Jan;24(1):91-7.

1.8 Diabetes

In addition to the heavy financial and health burden of CVD, over 7% of the United States population has been diagnosed with diabetes (40-43). Diabetes mellitus is a disorder of glucose metabolism, defined as fasting plasma glucose level ≥ 7.0 mmol/L (126 mg/dL) or plasma glucose ≥ 11.1 mmol/L (200 mg/dL) two hours following an oral glucose challenge (75 gram load) during a glucose tolerance test (44). Diabetes is traditionally divided into two subsets, type 1 or type 2 diabetes. Type 1 diabetes, formerly referred to as as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes, results from pancreatic failure to produce insulin. Type 2 diabetes, formerly referred to as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, is a result of insulin resistance, a condition in which cells fail to efficiently respond to insulin, and can be accompanied by insulin deficiency at late stages of the disease (44). Several debilitating complications are associated with the progression of diabetes. These include hypertension, retinopathy, neuropathy, nephropathy, and infection (45).

The damage done by diabetes extends far beyond the typical complications described above, as diabetics are four to five times more likely to develop CVD than non-diabetic patients (40-43). Cardiovascular disease caused by atherosclerosis is an important complication of diabetes, and is the leading cause of mortality among patients with diabetes, accounting for 65% of all deaths (40-43). The epidemic of obesity and Metabolic Syndrome (MetS) means millions of additional Americans will soon find themselves with a severely increased risk of developing CVD (42). Chapter 4 discusses the increased risk of CVD in patients with diabetes in detail, and explores the plethora of mouse models of diabetic atherosclerosis available to researchers aiming to unravel the source of this risk ("Macrovascualar complications of diabetes in atherosclerosis-prone mice").

The increased risk of CVD in patients with diabetes can be attributed to several factors, including the following. Hyperglycemia can lead to endothelial dysfunction, as the chronic elevation of blood glucose level can lead to angiopathy (46). The endothelial cells lining the blood vessels take in more glucose than normal, as they rely on an insulin-independent method of glucose uptake. These cells then form more surface glycoproteins than normal, and cause the basement membrane to grow thicker and weaker (46). Diabetes is also commonly accompanied by an increase in inflammation and a pro-thrombotic state (47). Elevated concentrations of C-reactive protein (CRP), an acute phase reactant, has been associated with diabetes, and an increase in fat mass, as often seen in MetS and type 2 diabetes, can lead to release of adipose tissue specific inflammatory molecules, or adipokines. Patients with insulin resistance also frequently manifest several alterations in coagulation mechanisms. These alterations include increased fibrinogen levels, increased plasminogen activator inhibitor-1 (PAI1), and various platelet abnormalities (47). Hypertension often occurs in obese, MetS and type 2 diabetes patients, and has been shown to be postively correlated with insulin resistance (48). Hypertension nearly doubles the risk for CVD, and compared to the other modifiable risk factors described above, high blood pressure has the largest impact on CVD mortality (48-50).

1.9 Models of Diabetes

Numerous models of diabetes have been employed by researchers to date, including chemical, diet-induced, and genetic models of both type 1 and type 2 diabetes. These models, as well as the common models of atherosclerosis introduced earlier, are described in detail in Chapter 4. Due to their simplicity, mouse models of type 1 diabetes are the most commonly used animal models of

diabetes, despite representing only 10% of patients with diabetes (51-52). The most commonly used model of type 1 diabetes is chemically induced by the drug streptozotocin (STZ). STZ is a DNA alkylating agent that is particularly toxic to the pancreas as its uptake is mediated through glucose transporter 2 (Glut2) receptors, which are abundant in pancreatic β-cells (53). Repeated, low dose injection of STZ results in insulin deficiency and hyperglycemica with minimal toxic side effects to other cell types (54). In Chapter 2, I used STZ to induce diabetes in the well-established LDLR deficient model of atherosclerosis.

Genetic models of type 1 diabetes are also available to researchers, including non-obese diabetic (NOD) mice. Mirroring the islet cell autoimmunity thought to contribute to type 1 diabetes in humans, NOD mice begin to develop diabetes at an early age due to immune cell infiltration of the pancreatic islets (55). The studies described in Chapter 3 employ another such genetic models of type 1 diabetes, the "Akita" mutation. Akita mice spontaneously develop diabetes due to a missense mutation (C96Y) in the insulin 2 (Ins2) gene that disrupts a disulfide bond between A and B chains of insulin and leads to improper folding of insulin and eventual pancreatic β-cell loss (56). The non-obese, insulin deficient Akita mice develop progressive hyperglycemia, and are an established model in which to study diabetic complications such as retinopathy and nephropathy (57-58), as well as diabetic atherosclerosis (59). Male mice heterozygous for the Ins2 Akita mutation (Ins2+/-) develop diabetes as early as 2 months of age, making them an attractive genetic model of type 1 diabetes.

The metabolic syndrome (MetS) is a cluster of metabolic disturbances that include insulin resistance, hypertension, obesity and dyslipidemia (48). These metabolic disturbances predispose patients to the development of type 2 diabetes. Consequently, the vast majority of mouse models of type 2 diabetes lean heavily on one or more of these disturbances as a model system. Frequently

employed methods to induce type 2 diabetes in the mouse rely on insulin resistance and/or obesity brought about by genetic or dietary means (51). The most common obesity-induced models of type 2 diabetes are the leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice. Deficiencies in the leptin signaling pathway in these mice lead to hyperphagia, obesity and consequent insulin resistance (60). Other genetic models, such as the insulin receptor substrate protein 2 (IRS2) deficient mice, introduce partial or complete deficiencies in insulin receptors to mimic the pathophysiology of insulin resistance.

1.10 Diabetic Dyslipidemia

An important potential explanation for the increased CVD risk associated with diabetes is diabetic dyslipidemia, a common cluster of harmful changes in lipid metabolism frequently noted in patients with diabetes (61). Both poorly managed type 1 diabetes and type 2 diabetes lead to harmful modifications of lipoprotein metabolism. Specifically, there are four distinct changes noted in patients with diabetes: 1) postprandial lipemia, 2) elevated very low density lipoprotein (VLDL) triglycerides, 3) a reduction of high density lipoprotein (HDL) cholesterol, and 4) smaller average LDL particle size (Figure 1.8, orange arrows).

The rate of clearance of chylomicrons from the bloodstream is often slower in patients with untreated type 1 or type 2 diabetes compared with normal subjects. The liver is the primary site of remnant clearance, and alterations in various LDL receptor family members during diabetes, such as LDLR and LRP, may contribute to this slower postprandial lipid clearance (61).

There is an increase in the production and secretion of VLDL by the liver in patients with diabetes, particularly in those with type 2 diabetes (61-64). Thus, patients with diabetes are often

hypertriglyceridemic. Insulin down-regulates the process of VLDL secretion, and reduces plasma fatty acid levels – a determinant of hepatic VLDL flux. The increase in VLDL production in patients with diabetes is alleviated by the administration of insulin (64).

HDL cholesterol is reduced during diabetes, possibly as a result of the increase in VLDL TG described above. Lipids are frequently exchanged between lipoproteins in circulation, and an increase in plasma concentrations of TG-rich VLDL could lead to decreased HDL cholesterol as VLDL TG is exchanged for HDL cholesterol esters (65).

Lastly, while total LDL is not usually increased during diabetes, a decrease in the size and an increase in density of LDL are often noted (61, 66). Smaller, more dense LDL is more likely to undergo detrimental modifications such as oxidation and glycation, making it preferentially taken up by foam cells, leading to atherosclerosis (61).

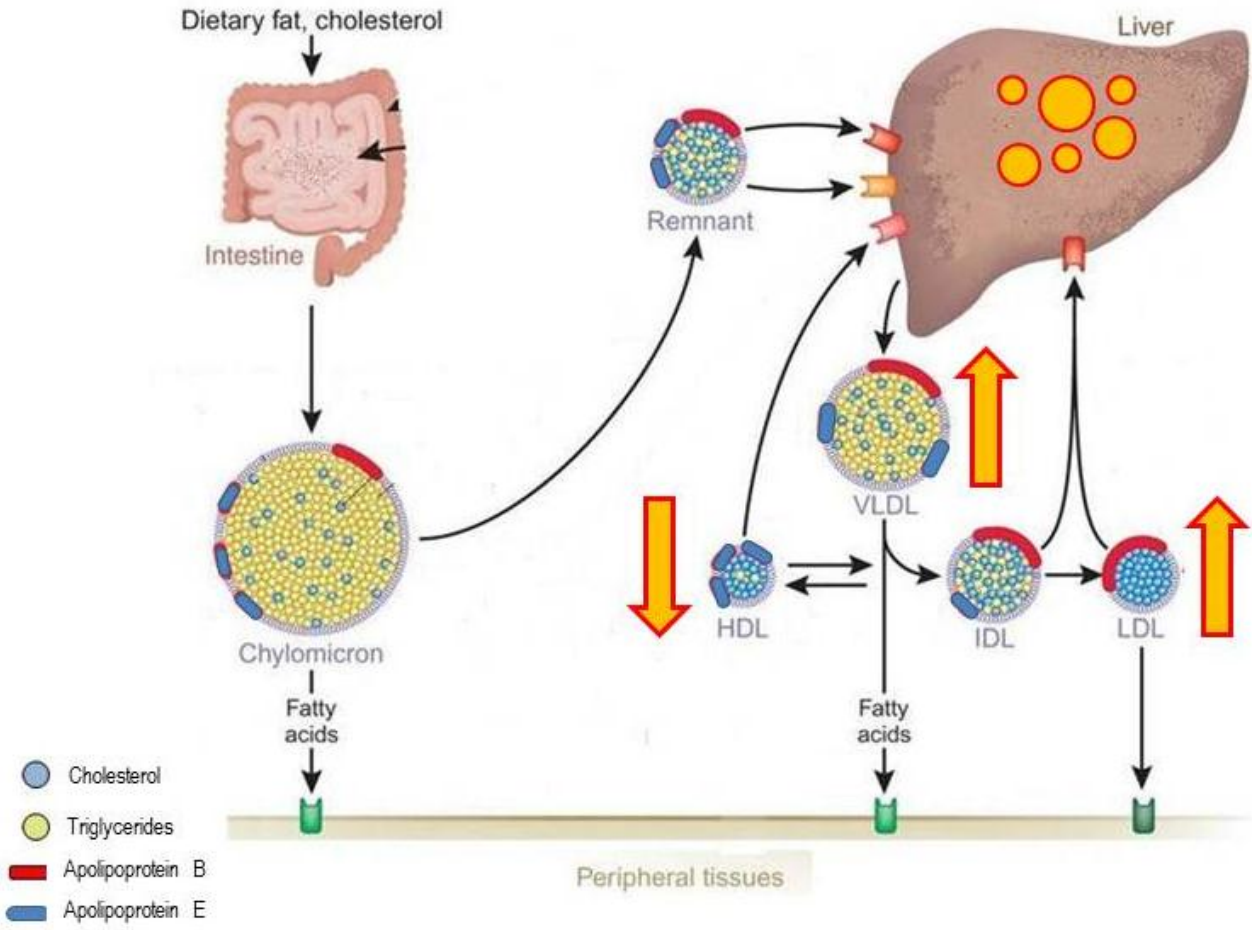

Figure 1.8. **Diabetic Dyslipidemia.** The orange arrows signify the three common changes that occur in diabetic dyslipidemia: 1) an increase in VLDL triglycerides, 2)an increase in small, dense LDL, and 3) a decrease in HDL cholesterol. In many cases, diabetic dyslipidemia is associated with hepatic dysfunction, including increased VLDL secretion and an increase in hepatic lipid content (orange circles). Figure adapted from Lusis et al., Nature Genetics 40, 129 - 130 (2008).

1.11 ApoE in Diabetes

An essential mediator of lipid metabolism in normolipidemic patients, I hypothesize that ApoE plays a major role in diabetic dyslipidemia as well (Figure 1.9). Increases in VLDL triglycerides, decreases in HDL, the accumulation smaller/denser LDL, slower clearance of post-prandial chylomicrons, a decrease in lipoprotein lipase (LPL) activity, and a decrease in LDLR expression, are all commonly noted phenotypes associated with both type 1 (insulin dependent) and type 2 (non-insulin dependent) diabetes (61). Interestingly, all of these components of dyslipidemia are areas of lipid metabolism in which ApoE has previously been shown to play a direct role. In Chapter 2, I examine the role of the human apoE isoforms in the regulation of dyslipidemia and atherosclerosis during diabetes ("Human apolipoprotein E4 exaggerates diabetic dyslipidemia and atherosclerosis in mice lacking the LDL receptor").

While the process of dyslipidemia is a critical determinant of cardiovascular disease during diabetes, there are many other potential contributing factors, as described in Section 1.5 and in detail in Chapter 3. Separating these lipid-independent effects of diabetes on cardiovascular disease from the effects of diabetes-induced hyperlipidemia has long been a problem for researchers. To date, all current mouse models in which diabetes exacerbates atherosclerosis suffer from severe diabetes-induced hyperlipidemia, making it incredibly difficult to separate the lipid dependent and independent effects of diabetes (51-52, 61).

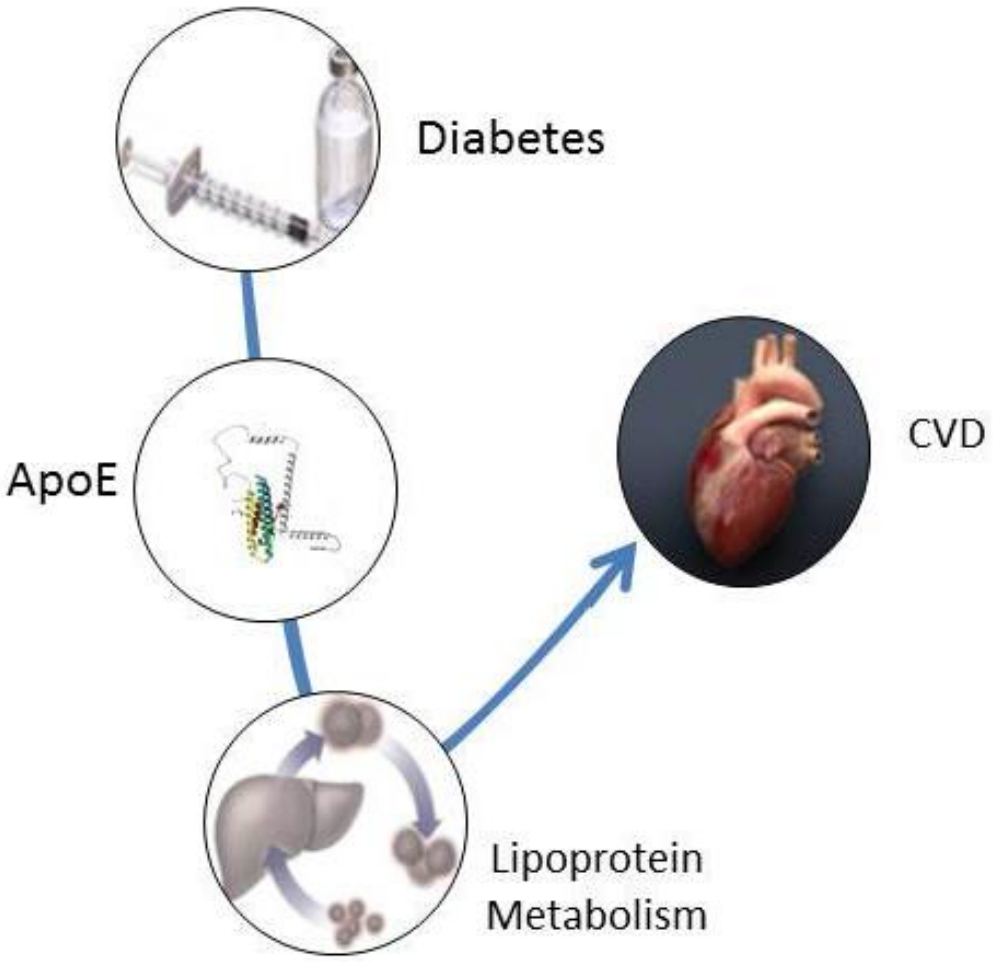

Figure 1.9. **Central hypothesis.** ApoE plays an essential role in the modulation of diabetic dyslipidemia, serving as a critical link between the processes of glucose and lipoprotein metabolism.

Along these lines, there may be additional roles for apoE during diabetes outside its effects on the regulation of dyslipidemia. For example, the apoE4 isoform has been shown to be a less efficient inhibitor of glycation and oxidation compared to the E3 and particularly the E2 isoform. Modifications such as glycation and oxidation to the cells of the vessel wall, and especially to LDL particles themselves, are highly atherogenic and occur more frequently in the diabetic setting (67-69). In addition, our laboratory previously demonstrated that in macrophages, the apoE-LDLR dynamic changes dramatically depending on apoE isoform, resulting in significant alterations in atherosclerotic plaque development without affecting plasma lipids. For example, in mice expressing E3, the introduction of LDLR-overexpressing macrophages by bone marrow transplant had no effect on atherogenesis. However, in mice expressing E4, the transplant of LDLR-overexpressing macrophages led to a significant increase in atherosclerotic plaque size (70). Plasma lipid-independent mechanisms such as the ones noted above, in which apoE has been shown to have a strong effect in normal settings, may also play an important role in the background of diabetes. In Chapter 3, I explore these potential effects of apoE by describing a novel model of diabetic atherosclerosis, one in which apoE4 lends atherosclerotic potential in the absence of diabetes-induced hyperlipidemia.

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Chapter 2

APOLIPOPROTEIN E4 EXAGGERATES DIABETIC DYSLIPIDEMIA AND ATHEROSCLEROSIS IN MICE LACKING THE LDL RECEPTOR

(This chapter consists of material from a manuscript reprinted with permission from *Diabetes*; 2011; titled "*Human Apolipoprotein E4 exaggerates diabetic dyslipidemia and atherosclerosis in mice independently of the LDL receptor*" by **Lance A Johnson**, Jose Arbones-Mainar, Raymond G Fox, Hyung-Suk Kim, Avani Pendse, Michael K Altenburg, Hyung-Suk Kim and Nobuyo Maeda)

2.1 Summary

Objective - We investigated the differential roles of apolipoprotein E (apoE) isoforms in modulating diabetic dyslipidemia – a potential cause of the increased cardiovascular disease risk of patients with diabetes. Research Design and Methods – Diabetes was induced using streptozotocin (STZ) in human apoE3 (E3) or human apoE4 (E4) mice deficient in the low density lipoprotein receptor (LDLR-/-). Results - Diabetic E3LDLR-/- and E4LDLR-/- mice have indistinguishable levels of plasma glucose and insulin. Despite this, diabetes increased VLDL triglycerides and LDL cholesterol in E4LDLR-/- mice twice as much as in E3LDLR-/- mice. Diabetic E4LDLR-/- mice had similar lipoprotein fractional catabolic rates compared to diabetic E3LDLR-/- mice, but had larger hepatic fat stores and increased VLDL secretion. Diabetic E4LDLR-/- mice demonstrated a decreased reliance on lipid as an energy source based on indirect calorimetry. Lower phosphorylated Acetyl-CoA Carboxylase content and higher gene expression of fatty acid synthase in the liver indicated reduced fatty acid oxidation and increased fatty acid synthesis. E4LDLR-/- primary hepatocytes cultured in high glucose accumulated more intracellular lipid than E3LDLR-/- hepatocytes concomitant with a 60% reduction in fatty acid oxidation. Finally, the exaggerated dyslipidemia in diabetic E4LDLR-/- mice was accompanied by a dramatic increase in atherosclerosis. Conclusions – ApoE4 causes severe dyslipidemia and atherosclerosis independent of its interaction with LDLR in a model of STZ-induced diabetes. ApoE4 expressing livers have reduced fatty acid oxidation, which contributes to the accumulation of tissue and plasma lipids.

2.2 Introduction

Cardiovascular disease (CVD) caused by a worsening of atherosclerosis is an important complication of diabetes and is the leading cause of mortality among patients with diabetes (1). Patients with poorly managed type 1 diabetes or type 2 diabetes commonly have elevated very low density lipoprotein (VLDL) triglycerides, a reduction of high density lipoprotein (HDL) cholesterol, and smaller, dense LDL. This common cluster of harmful changes in lipid metabolism is referred to as diabetic dyslipidemia (2).

Apolipoprotein E (apoE) is a small circulating protein associated predominantly with VLDL and HDL. It is the primary ligand for several lipoprotein receptors, making it a crucial component in the clearance of lipid from the circulation and a major determinant of plasma cholesterol and CVD risk (3). In humans, the APOE gene is polymorphic, resulting in production of three common isoforms: apoE2, apoE3, and apoE4. The apoE4 isoform is carried by over a quarter (28%) of the US population, and is associated with higher LDL cholesterol and an increased risk of CVD (3). In addition to its well-established role in CVD, recent findings have implicated a role for apoE in glucose metabolism as well. Epidemiological studies have suggested that in certain populations APOE genotype may influence plasma glucose and insulin levels (4, 5), postprandial glucose response (6), the development of Metabolic Syndrome (7, 8), and a myriad of diabetic complications (9). In addition, apoE4 carriers with diabetes have been shown to have increased carotid atherosclerosis (10), and elderly apoE4 carriers with diabetes have an increased risk of CVD-associated death (11).

Increases in VLDL triglyceride, decreases in HDL, the accumulation of smaller more dense LDL, slower clearance of post-prandial chylomicrons and a decrease in LDL receptor (LDLR)

expression are all noted phenotypes associated with both type 1 and type 2 diabetes (2). All of these components of diabetic dyslipidemia are areas of normal lipid metabolism in which apoE has previously been shown to play a direct role. The major receptor through which apoE mediates lipoprotein clearance is the LDLR, and the apoE isoforms exhibit differential LDLR binding affinities (12). Therefore, we sought whether apoE isoforms retain differential roles in diabetic dyslipidemia and atherosclerosis in the absence of the LDLR by employing a mouse model of diabetes induced by streptozotocin (STZ).

In this study, we show that dyslipidemia and atherosclerosis are greatly exaggerated in diabetic LDLR-/- mice expressing human apoE4 (E4LDLR-/-) compared to those with human apoE3 (E3LDLR-/-), despite a similar degree of hyperglycemia. This E4-specific aggravation of diabetic dyslipidemia is central to the liver, and is associated with a reduction in hepatic lipid oxidation, an accumulation of liver triglycerides, and increased rates of VLDL secretion.

2.3 Methods

Mice and induction of diabetes

Mice homozygous for replacement of the endogenous *Apoe* gene with the human *APOE*3* (E3) or *APOE*4* (E4) allele (13,14) were crossed with mice deficient in the LDLR (15). All mice were on C57BL/6 backgrounds. Male mice were fed normal chow diet *ad libitum* (5.3% fat and 0.02% cholesterol) (Prolab IsoPro RMH 3000; Agway Inc). Diabetes was induced at 2 months of age by peritoneal injections of streptozotocin (STZ) for 5 consecutive days (0.05 mg/g body weight in 0.05 M citrate buffer, pH 4.5). Mice maintaining glucose levels >300 mg/dl throughout the

course of the study are considered “Diabetic”. “Non-diabetic” control mice were injected with vehicle citrate buffer. Biochemical analyses were carried out at 1 month post-STZ unless otherwise stated. Animals were handled under protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina.

Biochemical assays

After a 4 hour fast, animals were anesthetized with 2,2,2-tribromoethanol and blood was collected. Plasma glucose, cholesterol, phospholipids, free fatty acids and ketone bodies were measured using commercial kits (Wako). Triglycerides and insulin were determined using commercial kits from Stanbio and Crystal Chem Inc., respectively. Liver triglycerides were extracted as described (16). Lipoprotein distribution and composition was determined with pooled (n=6-8) plasma samples (100 □l) fractionated by fast protein liquid chromatography (FPLC) using a Superose 6 HR10/30 column (GE Healthcare). Pooled plasma (800 µl) was separated by sequential density ultracentrifugation into density fractions from <1.006 g/ml (VLDL) to >1.21 g/ml (HDL) and subjected to electrophoresis in a 4-20% denaturing SDS-polyacrylamide gel (17). Carboxylmethyl lysine (CML) advanced glycation end products (AGEs) were measured using an ELISA with antibodies specific for CML-AGEs (CycLex, Nagano, Japan). Apolipoprotein E and apolipoprotein CIII were measured using an ELISA with antibodies specific for apoE (Calbiochem) and apoCIII (Abcam). Protein expression by western blot was determined using antibodies against AMPKα, phosphorylated (Thr172) AMPKα, ACC, phosphorylated (Ser79) ACC, and β-actin (Cell Signaling). Lipid tolerance test was performed by gavaging 10 ml/kg olive oil following an overnight fast. For VLDL secretion, plasma TG was measured following injection of Tyloxapol (Triton WR-1339; Sigma) via tail vein (0.7 mg/g BW) after a 4 hour fast (18).

VLDL lipolysis was estimated by incubating VLDL (25□g TG in 60 □l PBS) at 37° C with15U of bovine lipoprotein lipase (Sigma). The reaction was stopped by adding 3 µl of 5M NaCl, and fatty acid release (FAtimepoint - FA0) was measured as above.

Lipoprotein clearance

VLDL and LDL were labeled with 1,1' - Dioctadecyl - 3,3,3',3' - tetramethylindocarbocyanine iodide (DiI) or I^{125} as previously described and injected to recipient mice via the tail vein (100µg DiI-VLDL or 5 x 10^5 counts I^{125} lipoproteins, diluted in 200 µl of PBS) (18). Plasma fluorescence was measured using an Olympus FV500 (Texas Red filter) with a SPOT 2 digital camera. Radioactivity was counted on a Wallac 1470 Wizard Gamma Counter (EG&G Wallac).

Lipid and glucose uptake, DNL and oxidation

Primary hepatocytes were isolated from 3 month old mice as described (19). Yield ranged from 3 to 6 x 10^6 cells/g liver, and viability (assessed by Trypan blue staining was >90%. Following overnight culture in hepatocyte media (Xenotech), cells (100,000/well, 24-well plate) were washed and cultured 72 hours in DMEM supplemented with 5% fetal bovine serum (v/v) with 5 mM (Low) or 25 mM (High) glucose. Prior to dilution, [1-^{14}C]-Palmitic Acid was re-suspended in 1% BSA solution. For oxidation measurements, cells were incubated for 2 hours with 1 µCi/ml D-[1-^{14}C]-Glucose or 1 µCi/ml [1-^{14}C]-Palmitic Acid (Perkin Elmer) while CO_2 was trapped using a customized 48-well NaOH trap (20). For 2-Deoxyglucose uptake, hepatocytes were starved in serum-free medium containing 135 mmol/l NaCl, 5.4 mmol/l KCl, 1.4 mmol/lCaCl2, 1.4 mmol/l MgSO4, and 10 mM Na4P2O7 for 30 min and then incubated for 10

min with 1 μCi/ml 2-Deoxy-D[1-^{3}H]glucose (Perkin-Elmer). For fatty acid uptake and 2-Deoxyglucose uptake, cells were washed 3x with ice-cold HBSS, lysed in 1 ml of 1% SDS and radiation counted. To estimate *de novo* lipogenesis, cells were incubated with 1 μCi/ml D-[1-^{14}C]-Glucose for 24 hours, washed 3x with PBS, scraped in 1 ml of methanol:PBS (2:3) and freeze thawed in liquid nitrogen. The lipid layer was then extracted using a chloroform:methanol (2:1) extraction. Radiation was counted in 5 ml SX18-4 Scinteverse BD scintillation fluid (Fisher) using a LKB Wallac 1214 RackBeta liquid scintillation counter (Spectrofuge)

Indirect Calorimetry

Mice were placed in a calorimetry system (LabMaster; TSE Systems) for 48 hours and monitored for O_2 consumption, CO_2 production, and RQ (RQ = VCO_2 / VO_2, where V is volume). Activity was determined by counting the number of breaks in light barriers on the X, Y, and Z axis.

Atherosclerosis

After 3 months of diabetes, mice were euthanized with a lethal dose of 2,2,2-tribromoethanol and perfused at physiological pressure with 4% phosphate buffered paraformaldehyde (pH 7.4). Morphometric analysis of plaque size at the aortic root was performed as described (19). Apoptotic cells were detected in 8 μm frozen sections of the aortic root with a kit that detect DNA fragmentation (Chemicon). Macrophages were detected with a 1:500 dilution of MOMA-2 (Abcam) and a 1:2000 dilution of Goat polyclonal Secondary Antibody to Rat IgG - H&L Cy5 (Abcam).

2.4 Results

Induction of diabetes

E3LDLR-/- and E4LDLR-/- mice had indistinguishable plasma glucose levels at the beginning and at the end of the study (Figure 2.1A, Table 2.1). They showed a similar response rate to STZ, with 86.4 and 84.6% of injected E3LDLR-/- and E4LDLR-/- mice becoming diabetic. One month after STZ injection, average plasma glucose levels of E3LDLR-/- and E4LDLR-/- mice reached 424 ± 30 and 407 ± 44 mg/dl, respectively, and remained >400 mg/dl for the 3 month study period (Figure 2.1A, Table 2.1). Plasma insulin levels in both genotypes dropped severely (Figure 2.1B). The severity of diabetes was also comparable between the two diabetic groups as estimated by plasma levels of ketone bodies, daily food intake and urine excretion (Table 4.1).

Diabetic dyslipidemia

Non-diabetic E3LDLR-/- and E4LDLR-/- mice had similar total plasma cholesterol and triglycerides (Figure 2.2A-B) as well as similar lipoprotein distribution profiles (Figure 2.2C-D). While the induction of diabetes led to increases in total cholesterol and total triglycerides in both E3LDLR-/- and E4LDLR-/- mice as early as 1 month after STZ treatment, the increase in plasma cholesterol was significant only in E4LDLR-/- mice when compared to their non-diabetic counterparts. Most importantly, plasma cholesterol and triglycerides in the diabetic E4LDLR-/- mice were significantly higher than those in diabetic E3LDLR-/- mice at 1 and 3 months of diabetes (Figure 2.2A-B).

VLDL and LDL cholesterol, as well as VLDL triglycerides, increased in both genotypes after STZ treatment (Figure 2.2E-F). However, VLDL triglycerides and LDL cholesterol were both

two-fold higher in E4LDLR-/- mice than in E3LDLR-/- mice (Figure 2.2E-F). Consequently, the LDL to HDL ratio, an established risk factor for atherosclerosis, was 2.2-fold higher in diabetic E4LDLR-/- mice than in diabetic E3LDLR-/- mice. Given that the diet provided was low in fat and cholesterol, and food consumption did not differ between the two diabetic groups (Table 2.1), the enhanced hyperlipidemia in E4LDLR-/- is independent of dietary lipid intake.

VLDL fractionated from plasma by sequential ultracentrifugation had a similar composition of cholesterol, triglycerides, and phospholipids (Figure 2.3A). Diabetes induced a 5 and 26% increase in apoB100 and a 10 and 57% increase in apoB48 in E3LDLR-/- and E4LDLR-/- mice, respectively. Although total amounts of apoB100 and apoB48 are approximately 30% more in the diabetic E4LDLR-/- mice compared to diabetic E3LDLR-/- mice, their distributions among different classes of lipoproteins were not significantly altered (Figure 2.3B-E). Similarly, the amount of total plasma ApoA1 did not differ by apoE genotype (Figure 2.3F-G). Diabetic E3LDLR-/- and diabetic E4LDLR-/- mice had similar lipoprotein distributions of apoE (Figure 2.4A-B) and apolipoprotein CIII to apoE ratios, an important marker of lipolysis and uptake (Figure 2.4C). Rates of lipolysis were also similar between VLDL isolated from diabetic E3LDLR-/- or diabetic E4LDLR-/- mice (Figure 2.4D), suggesting similar rates of VLDL conversion between the two diabetic groups. In addition, the degree of glycation in the VLDL-LDL lipoprotein fractions was similar in all groups (Table 2.1). In summary, diabetic E4LDLR-/- mice develop a far more deleterious plasma lipid and lipoprotein distribution profile than diabetic E3LDLR-/- mice, characterized by a substantial increase in the total amount of circulating lipoproteins, although not in the composition of the lipoprotein particles themselves.

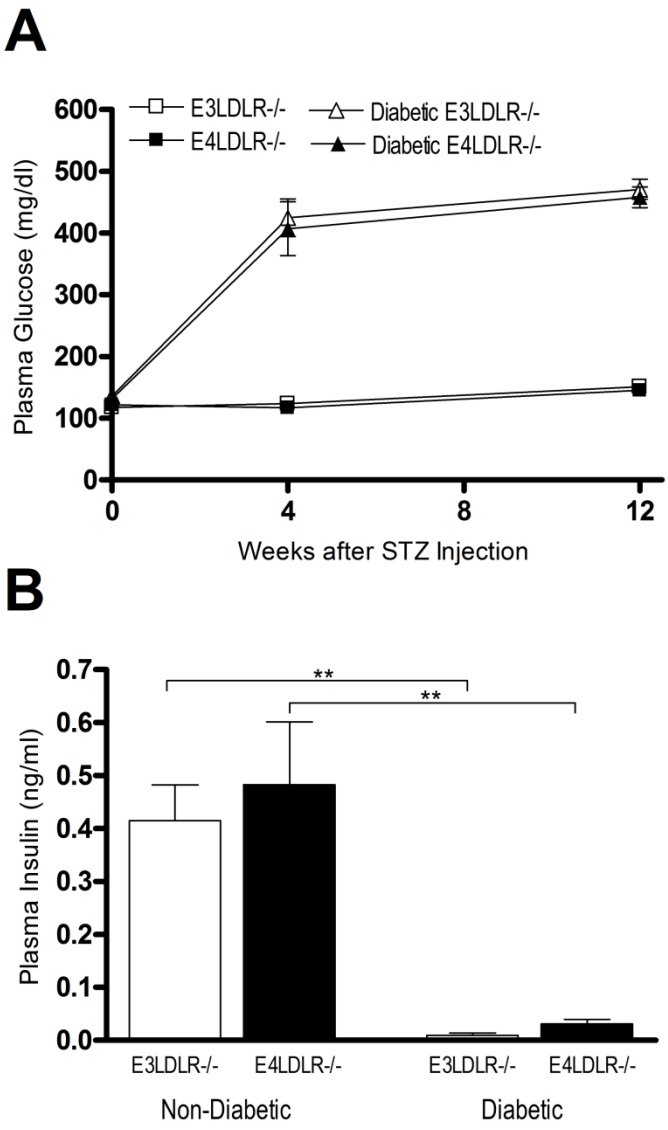

Figure 2.1. Induction of diabetes. *A:* Fasting plasma glucose of non-diabetic E3LDLR-/- (white squares) and non-diabetic E4LDLR-/- (black squares) mice, and following injection of STZ in diabetic E3LDLR-/- (white triangles) and diabetic E4LDLR-/- (black triangles) mice. $n =$ 8-12 per group. *B:* Fasting plasma insulin, as determined by ELISA. (* $P < 0.05$, ** $P < 0.01$)

	E3LDLR-/-	E4LDLR-/-	Diabetic E3LDLR-/-	Diabetic E4LDLR-/-
Body Weight (g)	29.5 ± 0.9	30.2 ± 1.3	23.9 ± 0.9 †	24.6 ± 1.0 †
Total Cholesterol (mg/dl)	338.5 ± 14.4	372.4 ± 42.7	449.7 ± 46.7	795.9 ± 56.4 * †
Total Triglycerides (mg/dl)	296.9 ± 24.7	311.2 ± 26.5	431.8 ± 56.3	624.8 ± 48.1 * †
Free Fatty Acids (mmol)	0.92 ± 0.09	0.91 ± 0.10	1.16 ± 0.08	1.47 ± 0.05 * †
Glucose (mg/dl)	146.3 ± 6.1	151.3 ± 4.9	470.8 ± 16.3 †	479.3 ± 21.4 †
Insulin (ng/ml)	0.42 ± 0.07	0.48 ± 0.12	0.01 ± 0.01 †	0.03 ± 0.01 †
Ketone Bodies (µmol/L)	87.8 ± 27.4	122.5 ± 16.7	231.0 ± 43.9 †	244.5 ± 49.7 †
Lipoprotein AGEs (µg/ml)	2.02	1.95	2.30	2.38
Food Consumed (g/day) #	4.8 ± 0.7	5.7 ± 1.1	11.9 ± 1.3 †	12.6 ± 1.9 †
Urine Excretion (ml/day) #	2.0 ± 0.2	1.7 ± 0.2	13.8 ± 0.5 †	13.6 ± 0.3 †

Table 2.1. Metabolic parameters of LDLR-/- mice expressing human apoE3 or apoE4. For the "Diabetic" groups, measurements were taken 3 months after STZ treatment. Data are means ± SD. * $P > 0.05$, genotype effect. † $P > 0.05$ treatment effect. n = 8-18 per group. # n = 4-5 per group.

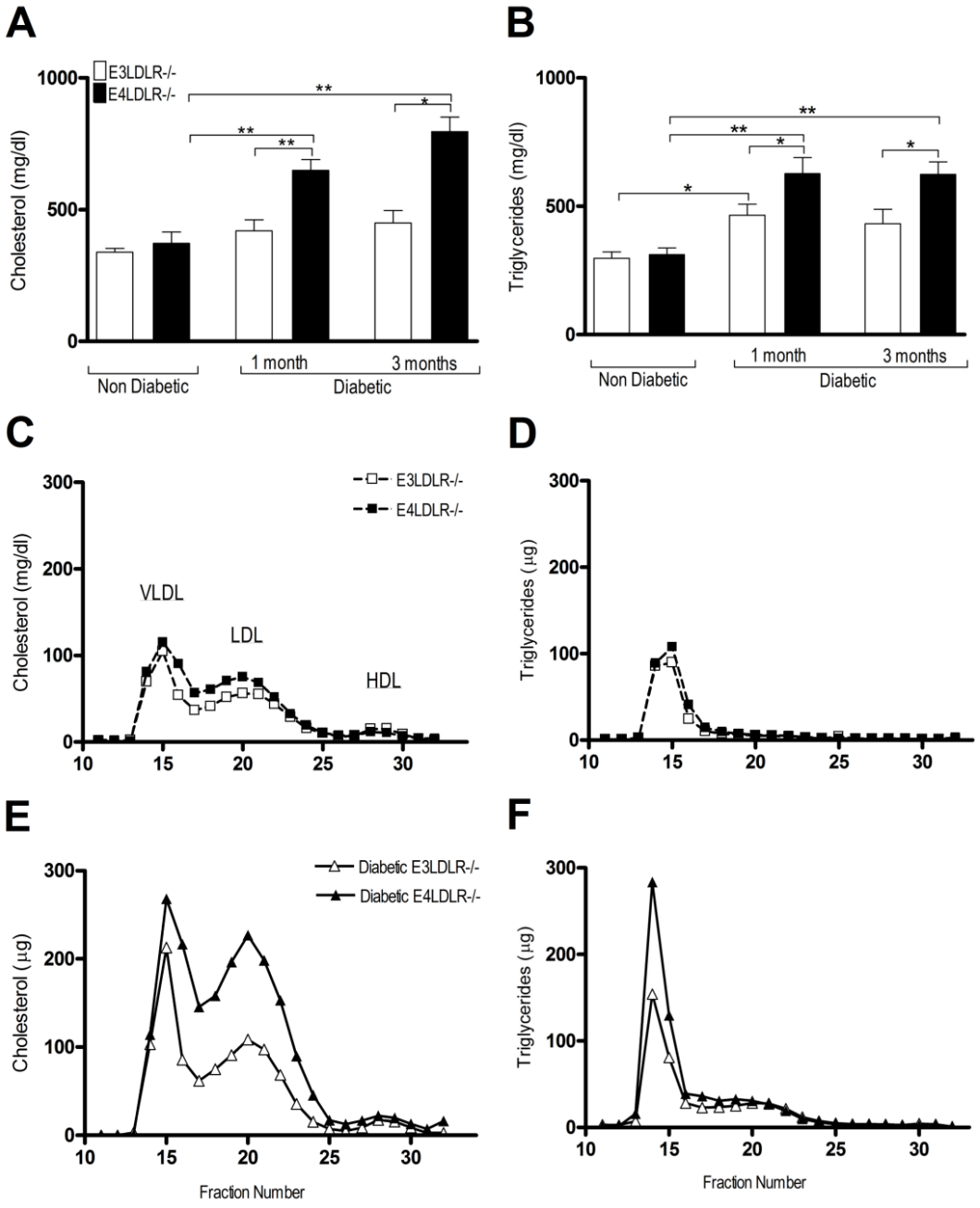

Figure 2.2. Plasma lipids and lipoprotein profiles. *A-B:* Total plasma cholesterol (A) and triglycerides (B) following a 4 hour fast. n = 8-18. *C-D:* Pooled (n = 6-8) plasma samples were separated into lipoprotein fractions by FPLC. Lipoprotein cholesterol (C) and triglyceride (D) profiles of non-diabetic E3LDLR-/- (white squares) and non-diabetic E4LDLR-/- mice (black squares). *E-F:* Lipoprotein cholesterol (E) and triglyceride (F) profiles of diabetic E3LDLR-/- (white triangles) and diabetic E4LDLR-/- mice (black triangles). (* $P < 0.05$, ** $P < 0.01$)

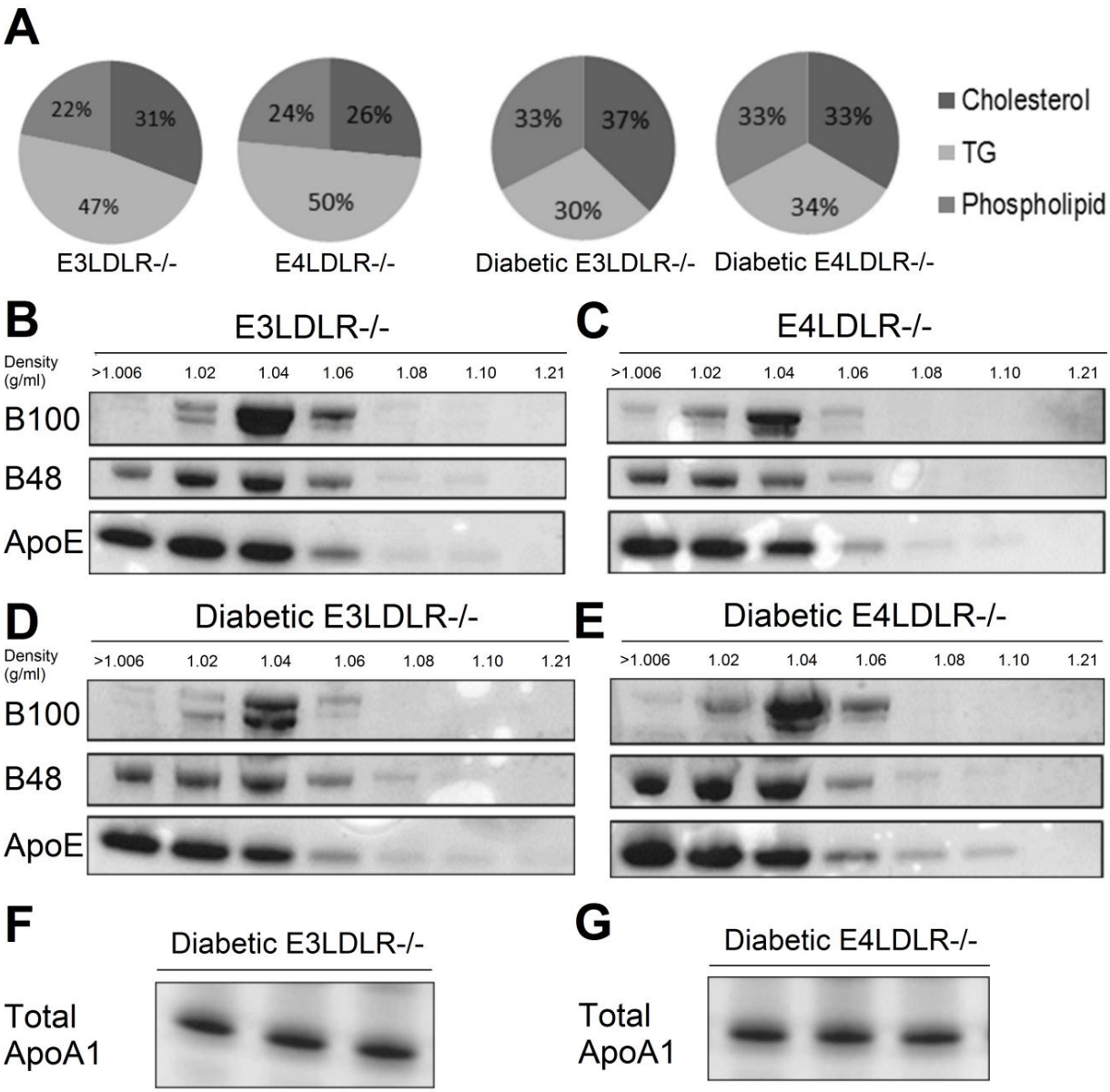

Figure 2.3. Lipoprotein lipid composition and apolipoprotein distribution. A: VLDL distribution of cholesterol, triglycerides, and phospholipids. B-E: Lipoprotein fractions were separated by sequential ultracentrifugation from 800 µl pooled (n = 6) plasma. F-G: Total plasma ApoA1 from individual diabetic E3LDLR-/- (F) and diabetic E4LDLR-/- (G) mice. Apolipoproteins were analyzed by 4-20% SDS-PAGE.

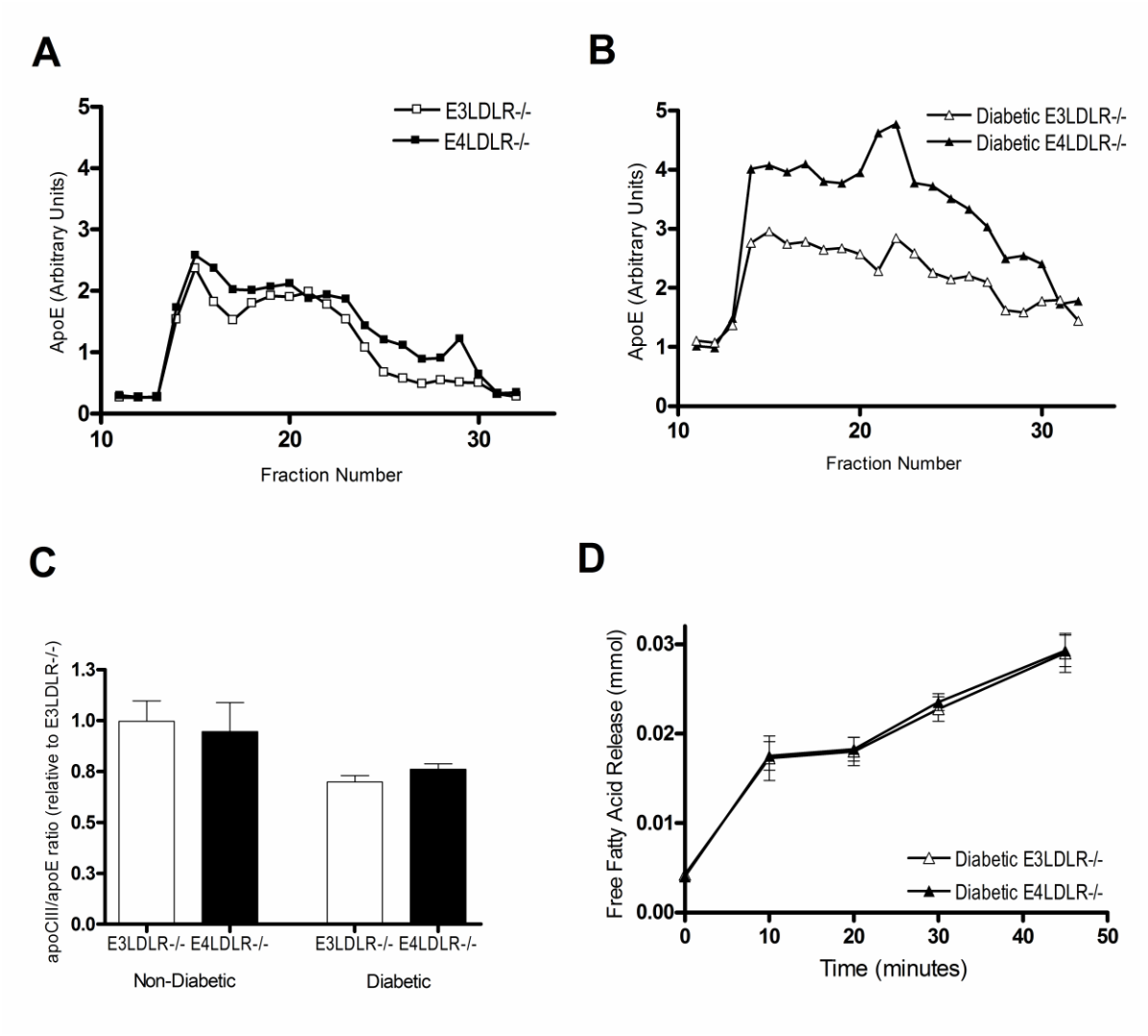

Figure 2.4. Apolipoprotein E distribution, ApoCIII/ApoE ratio and VLDL lipolysis. A-B: ApoE protein was determined by ELISA in lipoprotein fractions separated by FPLC from the pooled plasma (n = 6) of diabetic E3LDLR-/- (A) and diabetic E4LDLR-/- (B) mice. C: Plasma Apolipoprotein CIII and Apolipoprotein E protein was measured by ELISA. n = 4-8. Data is expressed as the ratio of ApoCIII/ApoE relative to the mean of non-diabetic E3LDLR-/- mice as 1.0. D: VLDL was isolated from diabetic E3LDLR-/- (white triangles) or diabetic E4LDLR-/- (black triangles) mice and incubated at 37°C with bovine lipoprotein lipase. The reaction was stopped at timepoints indicated by adding 5M NaCl, and FFA release (FAtimepoint - FA0) was measured. n = 4-6.

Lipoprotein clearance and post-prandial fat tolerance

As the employed model lacks the LDLR, we measured the expression of several other genes involved in lipoprotein uptake. The expression of apoE, LRP1 (LDLR related protein 1), SR-B1 (scavenger receptor B type 1) and NDST1 (N-deacetylase/N-sulfotransferase 1) did not differ significantly between the two diabetic groups, nor by induction of diabetes (Figure 2.5A). Gene expression of VLDLR was increased approximately 8-fold by STZ treatment, but the difference between diabetic E3LDLR-/- and E4LDLR-/- mice was not significant.

We next measured the efficiency of VLDL clearance in diabetic E3LDLR-/- and diabetic E4LDLR-/- mice by injecting VLDL isolated from ApoE deficient mice and labeled with DiI. There was no difference in the fractional catabolism of VLDL between the two diabetic groups (Figure 2.5B). Similarly, there was no difference in the clearance of LDL particles isolated from non-diabetic LDLR-/- mice (Figure 2.5C). We also examined whether or not the lipoprotein particles from diabetic mice with apoE3 or apoE4 are cleared differently by injecting non-diabetic LDLR-/- recipients of ^{125}I-labeled VLDL or LDL isolated from diabetic E3LDLR-/- or diabetic E4LDLR-/- mice. There was no difference in their clearance (Figure 2.6A and B). These data demonstrate that the clearance of VLDL remnants and LDL are not the major cause of the enhanced diabetic dislipidemia observed in E4LDLR-/- mice compared to E3LDLR-/- mice.

Plasma triglyceride levels in the diabetic E4LDLR-/- mice dropped to similar levels in E3LDLR-/- mice after an overnight fast. Following administration of an oral gavage of olive oil to the diabetic mice, however, plasma triglyceride levels of the E4LDLR-/- mice quickly re-established a level two-fold higher than E3LDLR-/- mice and remained high at all points post-gavage (Figure 2.5D). Except for the initial increase, clearance appeared to be equally impaired in the two groups suggesting that diabetes is affecting postprandial lipid clearance equally. Intestinal

absorption assessed by olive oil gavage after Tyloxapol injection did not differ by apoE genotype (Figure 2.7).

VLDL secretion

Altered hepatic VLDL secretion is a possible contributing factor to the elevated plasma lipids in the diabetic E4LDLR-/- mice. Thus, we measured plasma accumulation of triglycerides as a marker of VLDL secretion. Following inhibition of VLDL uptake and lipolysis by injection of the detergent Tyloxapol, the triglyceride secretion rate in non-diabetic E3LDLR-/- and E4LDLR-/- mice was similar, averaging 3.2 ± 0.5 and 4.0 ± 0.4 mg/dl/min triglycerides, respectively (Figure 2.5E). The secretion rate in the diabetic E3LDLR-/- mice did not differ significantly from those of non-diabetic animals, averaging 3.5 ± 0.6 mg/dl/min triglycerides. In contrast, the secretion rate in the diabetic E4LDLR-/- mice was significantly elevated; averaging 7.0 ± 0.5 mg/dl/min, reaching a mean plasma triglyceride level of 1431.3 ± 49.3 mg/dl two hours after Tyloxapol injection (Figure 2.5E).

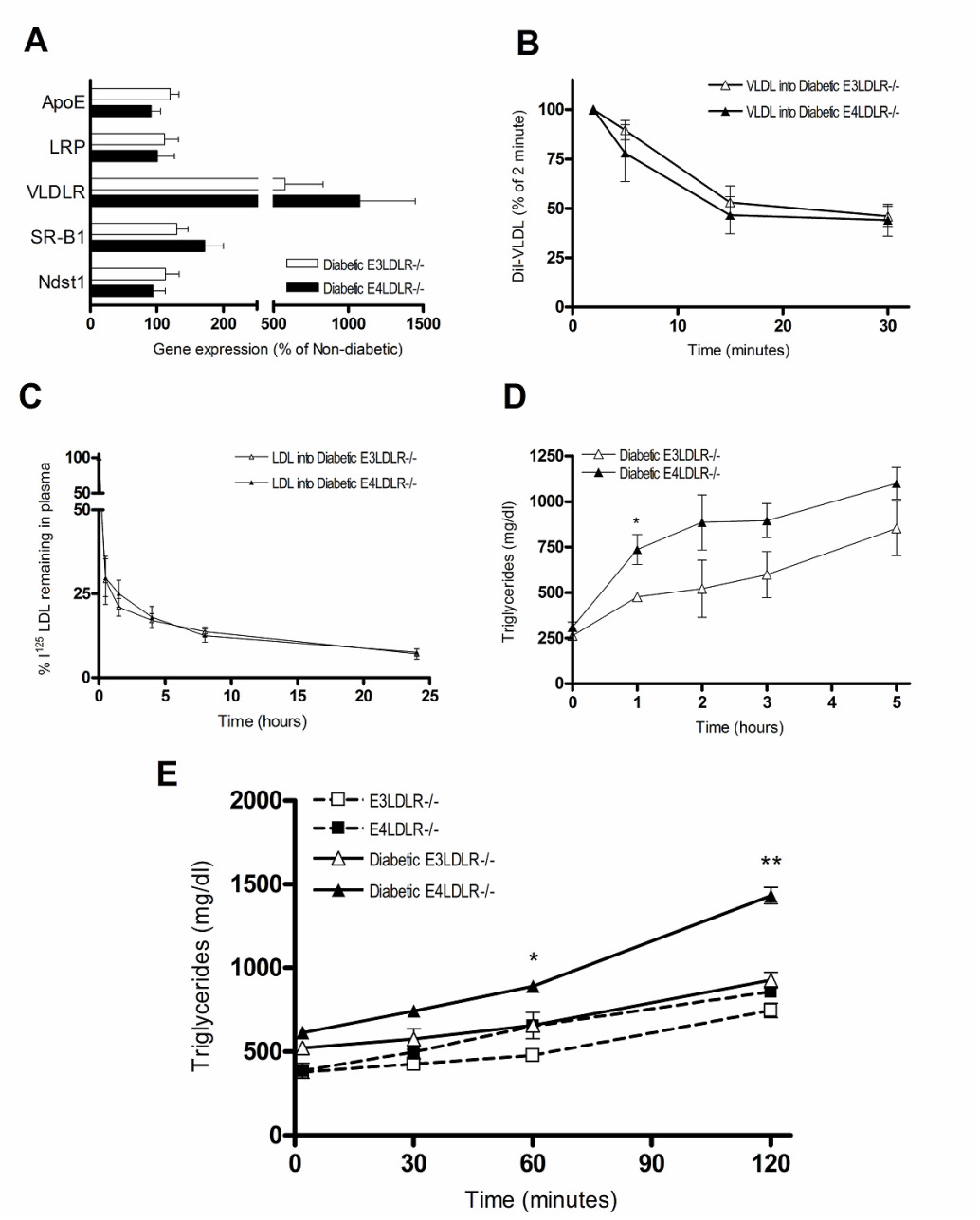

Figure 2.5. Lipoprotein clearance and secretion. *A:* Liver mRNA levels of ApoE, LRP, VLDLR, SR-B1, and NDST1. Data are expressed relative to non-diabetic mice. n = 6-7. *B-C:* Lipoprotein clearance. *B:* VLDL and *C:* LDL clearance. VLDL and LDL were isolated from plasma of APOE-/- mice and LDLR-/- mice, respectively, labeled with DiI and injected into diabetic E3LDLR-/- (white triangles) and diabetic E4LDLR-/- (black triangles) mice after a 4 hour fast. *D:* Post-prandial lipid tolerance. Plasma TG was measured in diabetic E3LDLR-/- (white triangles) and diabetic E4LDLR-/- mice (black traingles) following an oral gavage of olive oil after overnight fast. n = 6-8. *E:* VLDL secretion. Plasma triglycerides of non-diabetic E3LDLR-/- (white squares), non-diabetic E4LDLR-/- (black squares), diabetic E3LDLR-/- (white triangles) and diabetic E4LDLR-/- mice (black traingles) following injection of Tyloxapol. n = 4-6 per group. (* $P < 0.05$, ** $P < 0.01$)

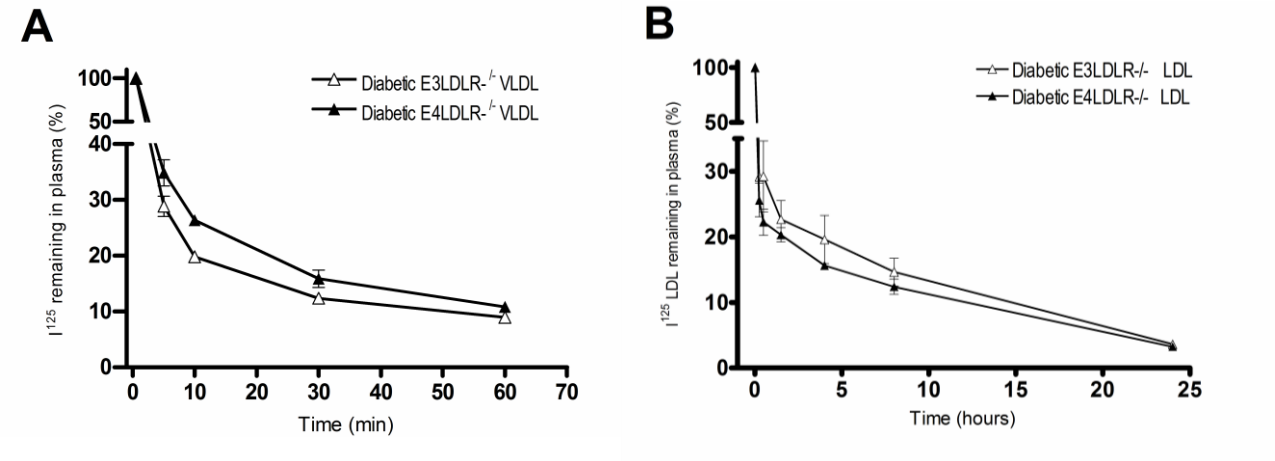

Figure 2.6. Lipoprotein clearance. A-B: VLDL (A) or LDL (B) isolated from diabetic E3LDLR-/- or diabetic E4LDLR-/- mice was labeled with 125I and re-injected via tail vein into non-diabetic LDLR-/- recipients. Plasma was collected at the listed timepoints and measured for radioactivity. All values are normalized to values 2 minutes post-injection.

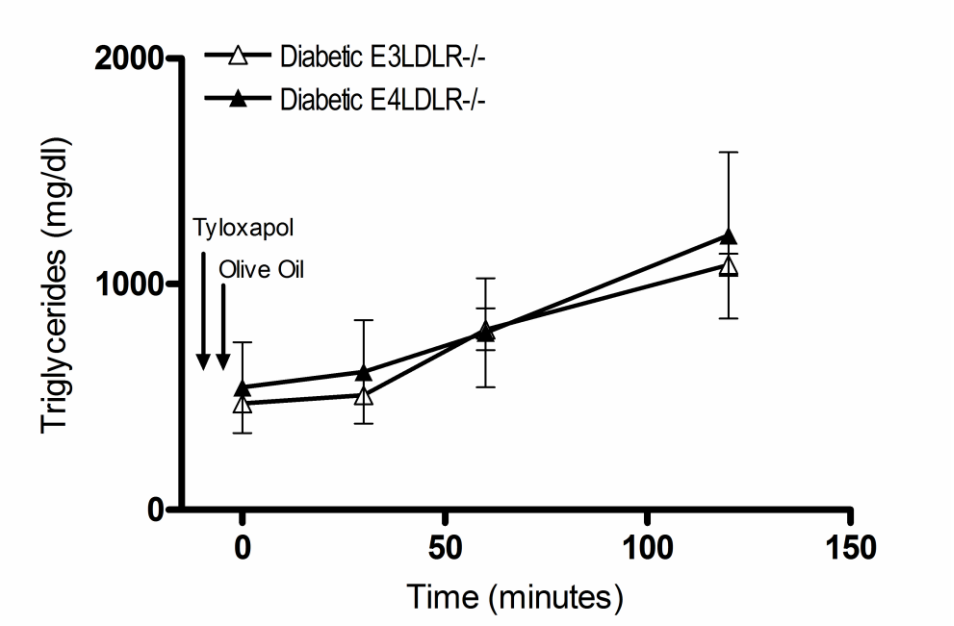

Figure 2.7. Dietary lipid absorption. Diabetic E3LDLR-/- (white triangles) and diabetic E4LDLR-/- (black triangles) mice were given an oral gavage of olive oil (200 µl) 5 minutes after tail vein injection of Tyloxapol (0.7 mg/g BW). Plasma triglycerides were measured 0, 30, 60, and 120 minutes post-gavage. n = 3-4.

Energy usage, hepatic lipid stores and fatty acid metabolism

To assess global energy metabolism in the diabetic E3LDLR-/- and E4LDLR-/- mice, indirect calorimetry analysis was performed. Diabetic E4LDLR-/- mice had significantly higher daily Respiratory Exchange Ratios (VCO_2/VO_2), particularly during the light cycle, than E3LDLR-/- mice (Figure 2.8A-B). This difference in RER was not due to altered activity (data not shown). A higher average RER in the diabetic LDLR-/- mice with E4 (0.915 ± 0.003) compared to those with E3 (0.896 ± 0.004, $p<0.01$) during the light cycle indicates that that the presence of apoE4 results in a lower fractional reliance on lipid as an energy source.

Non-diabetic E3LDLR-/- and E4LDLR-/- mice had similar triglyceride stores in the liver. Interestingly, diabetes induced a significant accumulation of hepatic triglycerides only in E4LDLR-/- mice, as they store twice as much as diabetic E3LDLR-/- mice (Figure 2.8C). This increase was not sufficient to cause overt steatosis and there were no notable histological abnormalities in the livers of the diabetic mice (data not shown). The increase in hepatic TG storage in E4LDLR-/- mice during diabetes was also associated with an increase in plasma free fatty acids (FFA) (Table 2.1).

Diabetes increased hepatic gene expression of SREBP1c and FOXO1, regulators of lipogenesis and VLDL secretion, respectively. Both tended to be higher in diabetic E4LDLR-/- mice compared to diabetic E3LDLR-/- mice, but the increase did not reach significance (Figure 2.8D). Expression of genes for SREBP2, ChREBP, PPARα and CPT1 did not alter significantly between the two genotypes. Importantly, expression of fatty acid synthase (FASN) decreased significantly in E3LDLR-/- livers following STZ treatment, while remaining elevated in diabetic E4LDLR-/- livers (Figure 2.8D). Although protein levels of AMP-activated protein kinase (AMPK) and phosphorylated AMPK (pAMPK) were similar, a significantly lower phosphorylated

Acetyl-CoA Carboxylase (pACC) / ACC ratio was noted in livers isolated from diabetic E4LDLR-/- mice compared to E3LDLR-/- livers (Figure 2.8E-F). Total ACC also tended to be lower, but did not reach significance. A lower pACC/ACC ratio is indicative of decreased fatty acid oxidation and increased fatty acid synthesis. Together, these data suggest that increased fatty acid synthesis and reduced fatty acid oxidation underlie an accumulation of lipid stores in the livers of diabetic E4LDLR-/- mice.

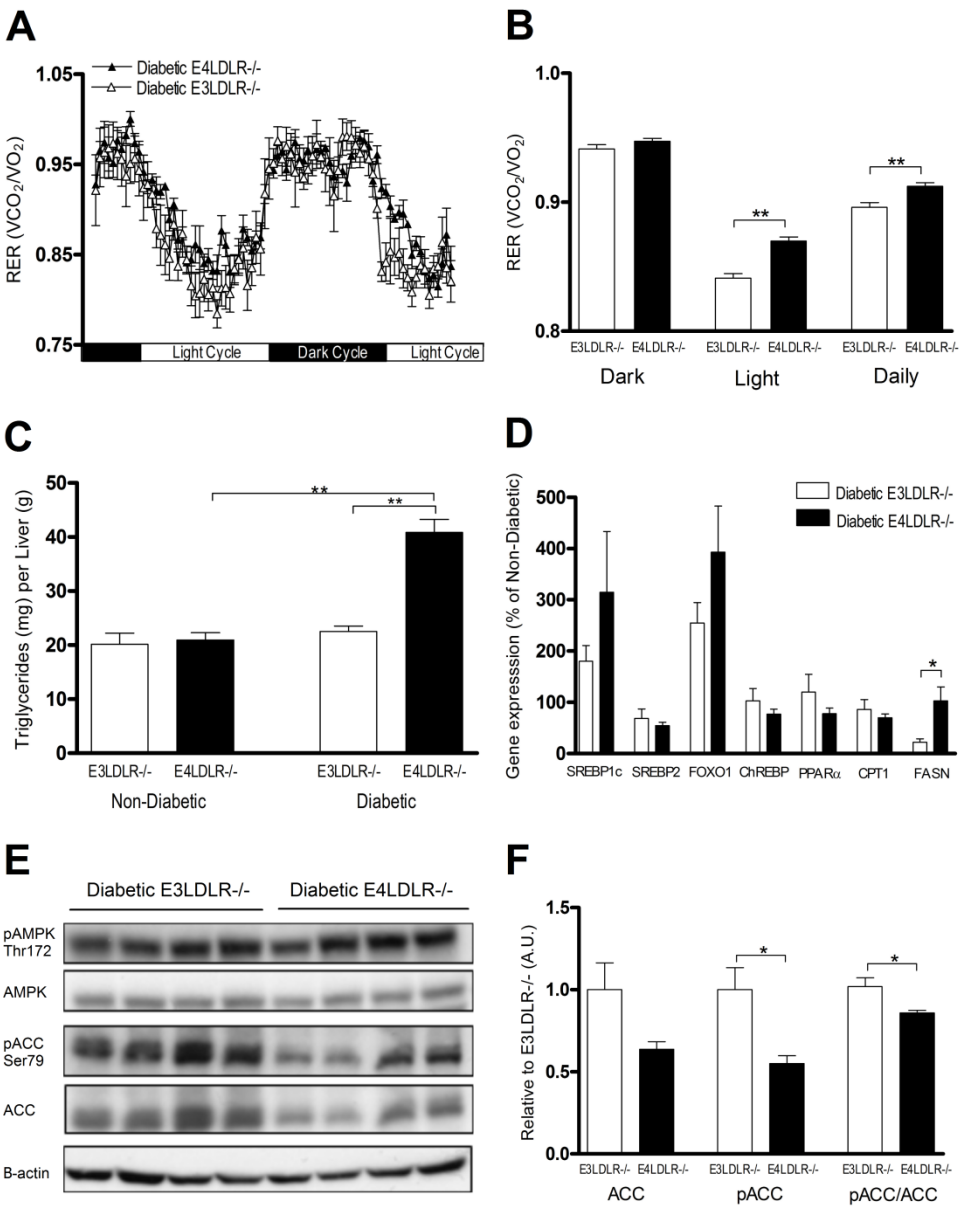

Figure 2.8 . Calorimetry, hepatic TG storage and regulation of FA metabolism. *A-B:* Respiratory exchange ratio (VCO2/VO2) was calculated over 48 hours in diabetic E3LDLR-/- and diabetic E4LDLR-/- mice using indirect calorimetry. $n = 4$. *C*: Hepatic TG content. Liver TG was measured following homogenization and lipid extraction. $n = 6$-8. *D:* Gene expression of SREBP1c, SREBP2, FOXO1, ChREBP, PPARα, CPT1 and FASN in the livers of diabetic E3LDLR-/- and diabetic E4LDLR-/- mice. Data are expressed relative to non-diabetic mice. $n = 8$-14. *E:* Livers from diabetic E3LDLR-/- and diabetic E4LDLR-/- mice were homogenized and protein expression of phosphorylated AMP kinase (pAMPK), total AMPK, phosphorylated acetyl-CoA carboxylase (pACC) and total ACC was determined by Western blot analysis. $n = 4$. *F*: Band intensity from (E) was quantitated using Image J software. Data is expressed relative to diabetic E3LDLR-/- in Arbitrary Units (A.U.).

Glucose and lipid metabolism in primary hepatocytes

To verify a decrease in fatty acid oxidation in the livers of diabetic E4LDLR-/- mice, primary hepatocytes were isolated and cultured for 72 hours in high (25 mM) glucose medium to mimic the hyperglycemic environment of diabetes. Control cells were incubated in low glucose (5 mM) for the same period. Similar to the triglyceride accumulation noted in the liver *in vivo*, primary hepatocytes harvested from E4LDLR-/- mice and cultured in high glucose accumulated more lipid than E3LDLR-/- cells (Figure 2.9A-B). The total area of lipid per cell was 2-fold higher in cells expressing E4 than E3 (16.6 ± 1.9 and 7.7 ± 0.6 μm^2 / cell, respectively) (Figure 2.9B).

FFA uptake, measured using ^{14}C-Palmitic acid, increased in hepatocytes cultured in the high glucose medium, but was not affected by ApoE isoform (Figure 2.9C). Similarly, there was no ApoE isoform effect on the cellular uptake of glucose (Figure 2.9D). The rate of *de novo* lipogenesis (DNL), defined here as ^{14}C incorporation into the lipid layer in cells cultured in the presence of ^{14}C-Glucose, was higher in hepatocytes cultured in high glucose medium than those cultured in low glucose medium. However, hepatocytes isolated from E3LDLR-/-and E4LDLR-/- mice did not differ in their rate of DNL in either the low or high glucose environment (Figure 2.9E).

We next examined the rates of glucose and lipid oxidation in the E3LDLR-/- and E4LDLR-/- hepatocytes. Three days in high glucose medium decreased rates of ^{14}C-glucose oxidation in both E3LDLR-/- and E4LDLR-/- hepatocytes. However, there was no significant difference in glucose utilization between them (Figure 2.9F). Finally, we measured lipid oxidation in primary hepatocytes cultured in high or low glucose using ^{14}C-Palmitic acid. E3LDLR-/- and E4LDLR-/- hepatocytes cultured in the low glucose media demonstrated similar rates of lipid oxidation. When

cultured in the high glucose environment, however, there was a striking down-regulation of lipid oxidation only in E4LDLR-/- hepatocytes (Figure 2.9G). After 72 hours in the high glucose condition, the E4LDLR-/- hepatocytes had lipid oxidation rates approximately 40% of those in the E3LDLR-/- hepatocytes.

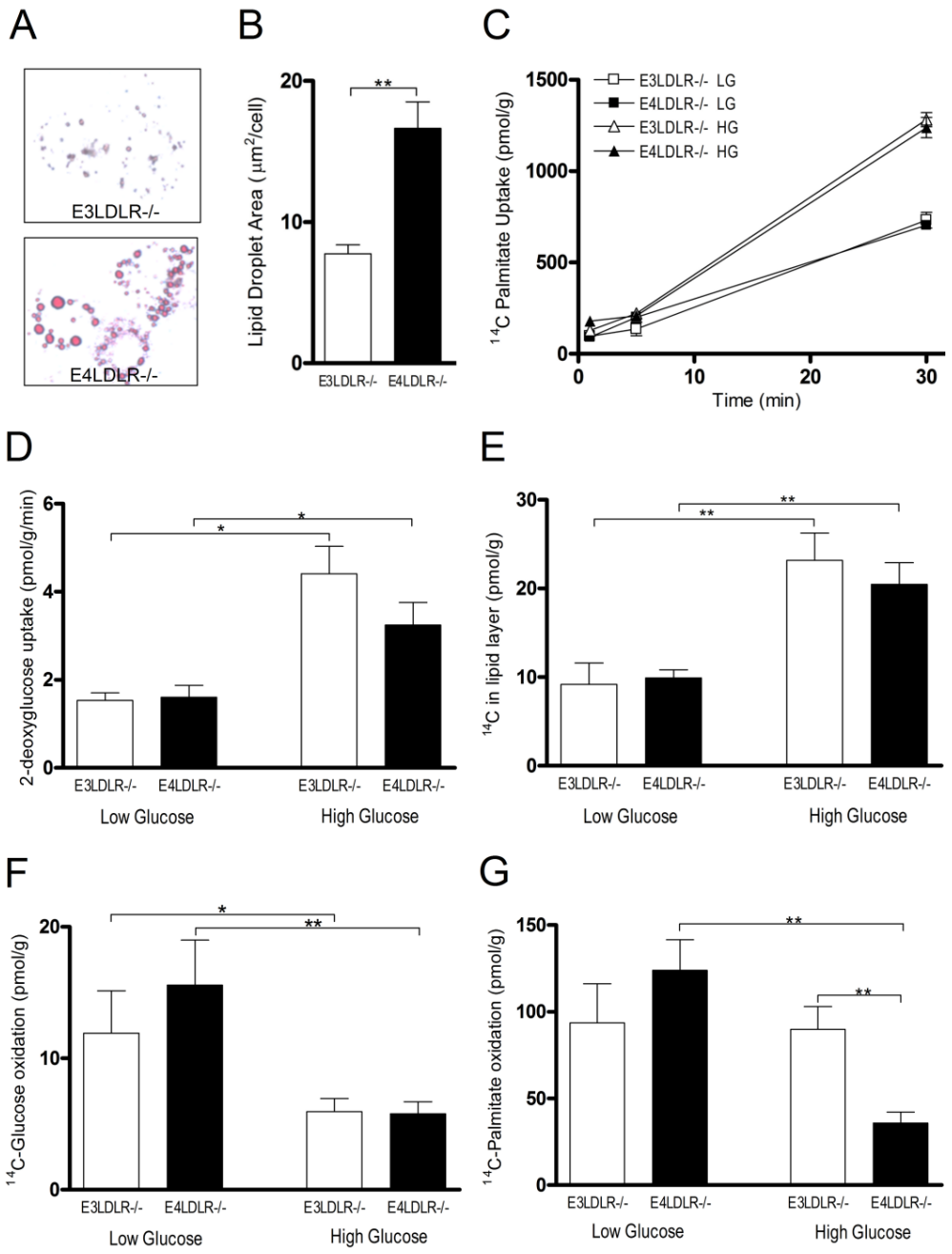

Figure 2.9. Metabolic Analyses of Primary Hepatocytes. *A:* Primary hepatocytes from E3LDLR-/- or E4LDLR-/- mice were cultured for 72 hours in high glucose (HG, 25 mM) media and stained with Oil Red O to highlight lipid droplets. *B:* Total lipid droplet area per cell was quantified by measuring 50 randomly chosen cells from 4 separate cultures per group. *C:* Fatty acid uptake was estimated by counting intracellular radiation after incubating hepatocytes for 1 min with 2 μCi/ml ^{14}C-Palmitate. *D:* Glucose uptake was measured following a 10 min incubation with 1 μCi/ml ^{3}H-2-deoxyglucose after starving cells for 2 hours. *E: de novo* lipogenesis was measured by counting radiation in the lipid layer after 24 hour incubation with ^{14}C-Glucose. *F-G:* Oxidation of ^{14}C-Glucose (G) and ^{14}C-Palmitate (H) was measured by trapping $^{14}CO_2$ during a 3 hour incubation using a customized self-contained CO_2 trap. n = 4-12 wells per trial, 3 trials per group. (* $P < 0.05$, ** $P < 0.01$)

Diabetic atherosclerosis

Reflecting their severe dyslipidemia, diabetic LDLR-/- mice carrying the E4 allele had aortic plaques on average 3-fold greater in area than those with E3 (Figure 2.10). The size of the atherosclerotic plaques in diabetic E4LDLR-/- mice (141,000 ± 19,000 μm^2) was significantly greater when compared to diabetic E3LDLR-/- mice (48,000 ± 9,000 μm^2) as well as to both non-diabetic E3LDLR-/- and non-diabetic E4LDLR-/- mice (32,000 ± 8,000 and 40,000 ± 12,000 μm^2) (Figure 2.10). While diabetic E4LDLR-/- mice had larger and more complex atherosclerotic lesions than diabetic E3LDLR-/- mice, the number of macrophages (Figure 2.11A-B) and apoptotic cells (Figure 2.11C-D) per lesion area were similar between the two groups.

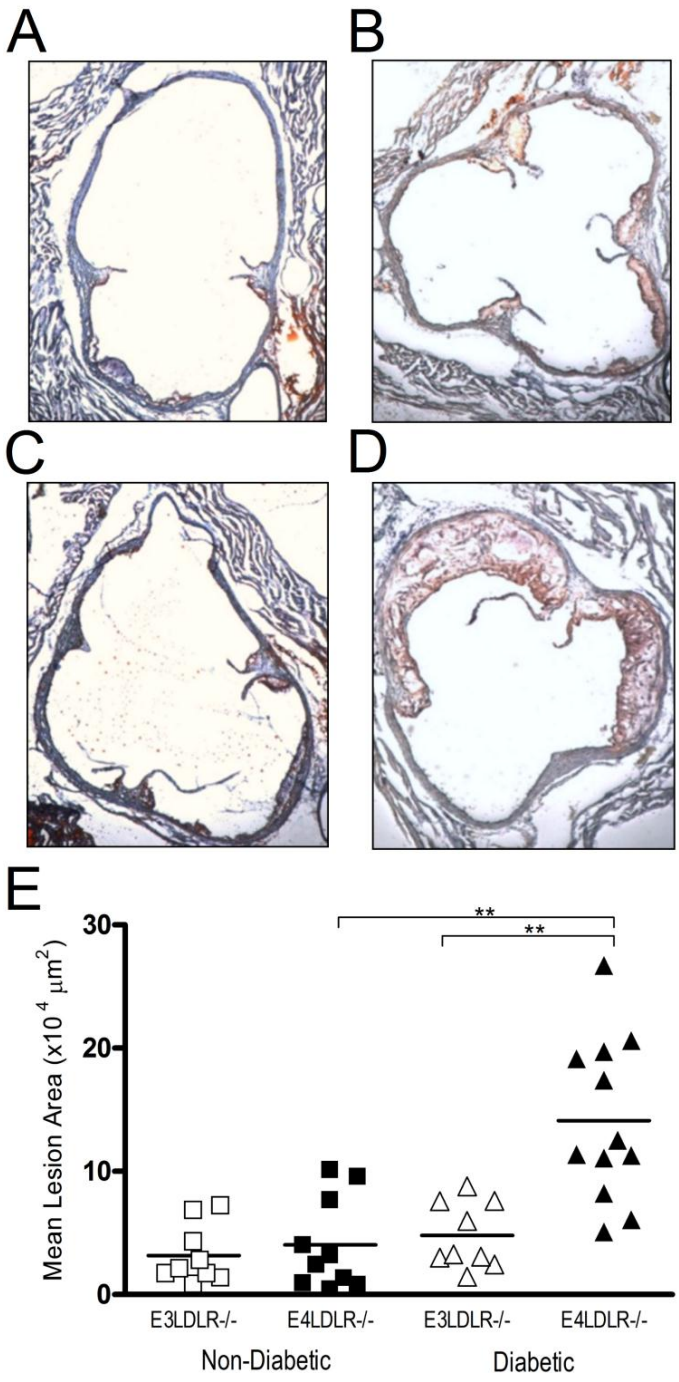

Figure 2.10. Atherosclerosis at the aortic root. *A-D:* Cross sections of the aortic root were stained with H&E and Oil Red O. Photos are representative of the mean plaque size of non-diabetic E3LDLR-/- (A), non-diabetic E4LDLR-/- (B), diabetic E3LDLR-/- (C), and diabetic E4LDLR-/- mice (D). *E:* Data points represent the average lesion area of four distinct histological sections of the aortic root. n = 9-12 per group. (* $P < 0.05$, ** $P < 0.01$)

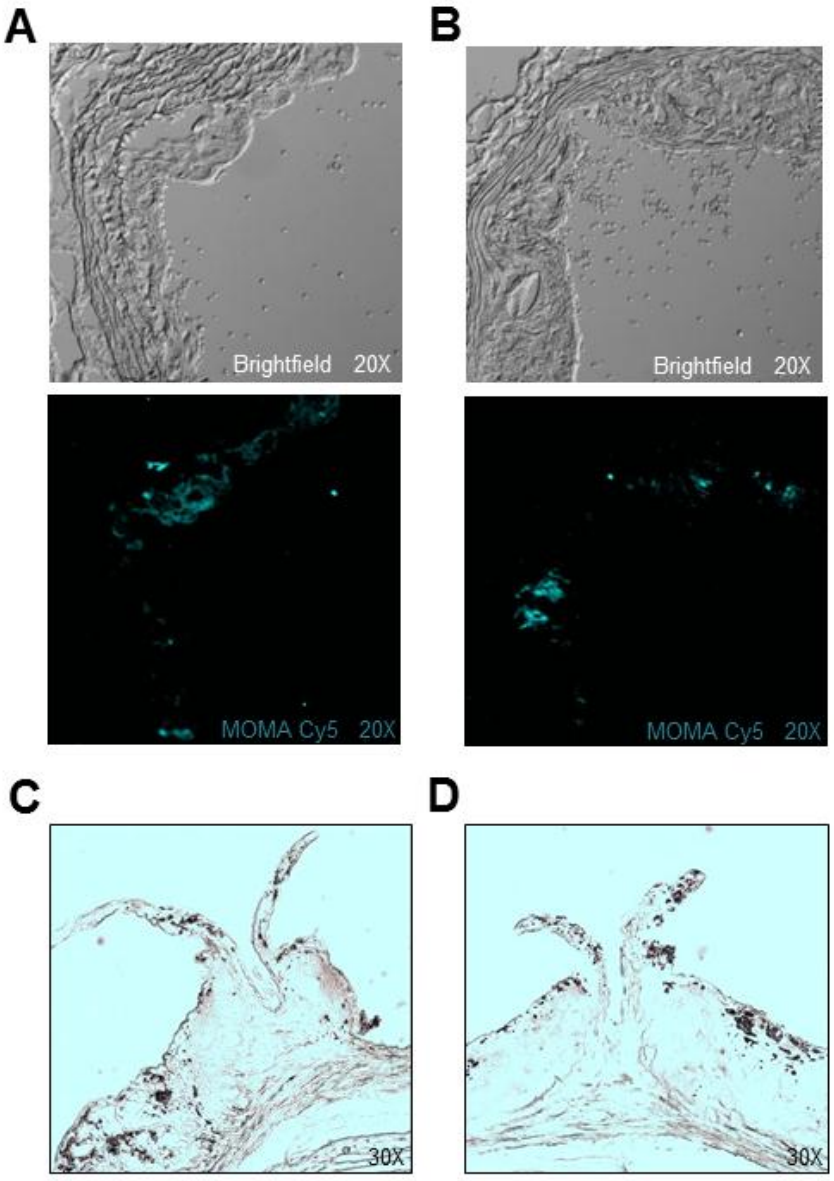

Figure 2.11. Apoptosis and macrophage infiltration in the atherosclerotic plaque. A-B: Macrophages were detected using MOMA-2 antibodies followed by a secondary conjugated to Cy5. Plaques from diabetic E3LDLR-/- (A) and diabetic E4LDLR-/- (B) mice are shown in Brightfield (upper image) and Cy5 (blue, lower image). Magnifications are 20X. C-D: DNA fragmentation was detected in 8 μm frozen sections of the aortic root using ApopTag Fluorescein in situ staining. Pictured are plaques bordering the aortic valves (V-shaped structures at the center of the image) of diabetic E3LDLR-/- (C) and diabetic E4LDLR-/- (D) mice. A 1% methyl-green solution was used to delineate ultrastructure. Magnifications are 30x.

2.5 Discussion

In the present work, we explored the isoform specific role of apoE in diabetic dyslipidemia utilizing diabetic LDLR-/- mice with human apoE4 or apoE3. We found that: 1) Non-diabetic E3LDLR-/- and E4LDLR-/- mice are indistinguishable in all parameters of glucose and lipid metabolism measured in this study. 2) After induction of diabetes, E4LDLR-/- mice, but not E3LDLR-/-, develop enhanced dyslipidemia, characterized by elevated VLDL TG and LDL cholesterol, delayed clearance of post-prandial triglycerides, and an increased rate of VLDL secretion. 3) The severe dyslipidemia in diabetic E4LDLR-/- mice is associated with a larger hepatic lipid stores and a calorimetric profile suggestive of lower lipid utilization. 4) Primary hepatocytes isolated from E4LDLR-/- mice and cultured in high glucose accumulated more intracellular lipid concomitant with a reduction in fatty acid oxidation. 5) Finally, these metabolic disturbances during diabetes culminated in exaggerated atherosclerosis only in E4LDLR-/- mice.

Diabetic dyslipidemia in poorly treated type 1 diabetes and type 2 diabetes share many features (2). In many patients with diabetes, the levels of circulating lipoproteins during fasting are relatively normal (21). Instead, the major impairments to lipoprotein metabolism occur with their ability to clear post-prandial lipoproteins (2). The prolonged elevation of plasma triglycerides following a fatty meal has been demonstrated in individuals carrying the E4 allele (6), and our previous work demonstrated post-prandial lipemia in LDLR over-expressing mice with human apoE4 (22). Higher post-prandial triglycerides in the diabetic E4LDLR-/- mice suggest an interaction between apoE4 and hyperglycemia that elicits a delay in the clearance of chylomicron remnants through non-LDLR mediated pathways. Previously, Goldberg et al showed that STZ-induced diabetic LDLR-/- mice accumulated a “subclass of lipoproteins” that normally are quickly removed from the circulation (23). Our experiments confirmed that postprandial lipid clearance is

impaired in diabetic LDLR-/- mice. However, except for initial increase in plasma triglyceride in E4LDLR-/- mice, the postprandial clearance rate was not different in two groups. In addition, we found no APOE genotype effect on the fractional catabolism of either VLDL or LDL.

VLDL production is increased in type 2 diabetes and in uncontrolled type 1 diabetes (2). In insulin-resistant patients, this increased VLDL production is oftentimes part of a dyslipidemic cycle, in which larger adipose tissue stores are associated with increased FFA release (24). In turn, an increased flux of FFA to the liver stimulates VLDL secretion (25). The FFA-stimulated VLDL production is compounded in an insulin-resistant or insulin-deficient state (2). Along these lines, the increased plasma VLDL in the diabetic E4LDLR-/- mice is accompanied by a 2-fold increase in TG secretion. In comparison, TG secretion in the diabetic E3LDLR-/- mice was not different from that in the non-diabetic controls. Similarly, LDLR-/- mice expressing murine apoE do not have increased rates of TG production following STZ-induced diabetes (23). We note that increased hepatic secretion of TG-rich particles is most likely responsible for the majority of LDL-sized particles in E4LDLR-/- mice, since absence of LDLR has been shown to result in overproduction of TG-rich particles smaller than normal VLDL (26,27). Thus, the dyslipidemic cycle commonly seen in patients with insulin-resistance is reflected by the insulin-deficient E4LDLR-/- mice employed in this study, which have higher circulating FFA, larger hepatic TG stores and higher rates of TG secretion than diabetic E3LDLR-/- mice.

Indirect calorimetry analysis revealed a clear apoE genotype effect on energy usage during diabetes at the whole body level. Diabetic E4LDLR-/- mice had significantly higher RER, demonstrating a lower ratio of lipid to carbohydrate oxidation compared to diabetic E3LDLR-/- mice during the light cycle. This reduced reliance on lipid oxidation appears to be central to the liver and directed by ACC signaling. When active, ACC catalyzes the production of malonyl-

CoA, thereby stimulating lipogenesis and inhibiting the beta-oxidation of fatty acids (28). A significant reduction in the pACC/ACC ratio in the livers of diabetic E4LDLR-/- mice indicates an increase in lipogenesis and decrease in fatty acid oxidation. In addition, STZ treatment decreased FASN gene expression in the E3LDLR-/- mice, as previously reported in wild type mice (29). However, this diabetes-induced reduction did not occur in E4LDLR-/- mice, suggesting a higher rate of fatty acid synthesis in these mice. Higher expression of FASN was not due to stimulation by ChREBP. Expression of SREBP1c, an important regulator of FASN-directed lipogenesis, on the other hand, tended to be higher in the diabetic E4LDLR-/- livers but did not reach significance. Regardless, these data point toward two distinct means (metabolically linked by ACC signaling) by which lipid accumulates in the E4LDLR-/- livers during diabetes – an increase in fatty acid synthesis and a reduction in fatty acid oxidation. Consistent with this data, apoE4 expressing LDLR-/- hepatocytes cultured in high glucose reduced their rate of lipid oxidation to levels approximately 40% of that in E3LDLR-/- hepatocytes and amassed more than twice as much lipid.

LDLR-/- mice are an established model for a high fat diet induced obesity and insulin resistance, and we have previously shown that mice expressing apoE4 are more susceptible to diet-induced glucose intolerance than those expressing apoE3 (30). This propensity of apoE4 expressing mice to develop glucose intolerance likely contributes to the impaired lipid metabolism in the insulin deficient state. In this context, we note that VLDLR mRNA was significantly increased after the STZ treatment and the mRNA levels tended to be higher in the diabetic E4LDLR-/- livers than in the diabetic E3LDLR-/- livers. While the contribution of VLDLR in hepatic remnant clearance during diabetes is not clear, fasting hypertriglyceridemia in VLDLR-/- mice has been previously noted (31) and when over-expressed in the liver, VLDLR appears to function similarly to LDLR and LRP (32). The VLDLR plays an important role in adipocyte TG

accumulation (33), and could be a potential player in the process of hepatic lipid accumulation during diabetes.

Our results support the possibility that apoE functions as a metabolic signaling molecule outside its established role in lipoprotein clearance. For example, a role for apoE as a signaling molecule has been shown previously in the context of Alzheimer's disease (34), where it has been shown to affect activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) in an apoE isoform specific manner (35). Similar apoE4-specific signaling could account for some of the metabolic abnormalities during diabetes highlighted in this study. Alternatively, apoE has been shown to enhance VLDL secretion (36), as well as play a role in the accumulation of lipid in early and intermediary secretory compartments of hepatocytes (37). Thus, inefficient recycling and re-secretion of apoE4 (38), could potentially affect the availability, packaging, and/or transport of TG from the hepatic lipid pool. Irrespective of the precise mode of action, our results demonstrate a possible chain of events in diabetic E4LDLR-/- livers: a down-regulation of lipid oxidation and up-regulation of fatty acid synthesis lead to larger hepatic triglyceride stores which in turn drive an increase in VLDL secretion. Combined with marked diabetic effects which impair VLDL and post-prandial TG clearance, these processes are ultimately responsible for the severe dyslipidemia observed in the diabetic E4LDLR-/- mice.

Importantly, the diabetes-induced acceleration of atherosclerosis occurs only in LDLR-/- mice expressing apoE4. Previous studies of the STZ-induced diabetes in LDLR-/- mice have produced variable results, with some groups showing an increase in atherosclerosis (39, 40), while others show no change (41, 42). Although the reasons for these mixed results are unknown, varying experimental conditions, such as diet, could contribute (43). The E4-specific acceleration of atherosclerosis is likely a direct result of the severe dyslipidemia brought about by STZ-induced

diabetes in these mice, although contributions of other factors, such as apoE4 specific effects on macrophages, cannot be excluded (44). Regardless, our data clearly demonstrates the presence of E4 effects that are independent of the LDLR. Moreover, the exaggerated dyslipidemia and atherosclerosis observed in the diabetic E4LDLR-/- mice extends beyond the scope of type 1 diabetes and has implications for the growing number of apoE4 carriers with insulin resistance and type 2 diabetes. In conclusion, the apoE4-specific atherosclerosis and diabetic dyslipidemia illustrated in this study may also play an important role in patients with diabetes who carry the apoE4 isoform.

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Chapter 3

A NOVEL, NORMOLIPODEMIC MOUSE MODEL OF DIABETIC ATHEROSCLEROSIS: DIABETIC 'AKITA' MICE EXPRESSING HUMAN APOE4 AND INCREASED LDLR DEVELOP ATHEROSCLEROSIS WITHOUT SEVERE HYPERCHOLESTEROLEMIA

3.1 Introduction

The leading cause of mortality among patients with diabetes is cardiovascular disease (CVD), including enhanced atherosclerosis (1-4). Cardiovascular related mortality in patients with diabetes is two to four times higher than normoglycemic patients and consequently, diabetes is regarded as a coronary heart disease (CHD) risk equivalent (5-6). Current estimates by the Center for Disease Control indicate that approximately 11% of the US adult population has diabetes (6).

Diabetic dyslipidemia, a common cluster of harmful changes to lipoprotein metabolism, is an important contributor to the progression of atherosclerosis (7) and lowering cholesterol levels in patients with diabetes reduces CHD risk (5). However, even with a reduction in CHD risk achieved with robust cholesterol-lowering, the absolute CHD rates of patients with diabetes is still significantly higher than low-risk populations (5). This suggests that cholesterol-independent effects of diabetes, such as inflammation and endothelial dysfunction are equally important.

However, past research has failed to adequately separate the effects of diabetes-induced dyslipidemia from the lipid-independent effects alluded to above, particularly in regards to animal models of disease (8-10). This is because current mouse models of diabetic atherosclerosis all employ severely hyperlipidemic models, most in which hyperlipidemia becomes more severe upon induction of diabetes. For instance, in human patients total serum cholesterol values <200 mg/dl are considered "Desirable" and >240 mg/dl are considered "High" under current clinical guidelines (5). Even the 95th percentile of patients aged 20 or older have average cholesterol levels of 318 mg/dl for men and 273 mg/dl for women. In contrast, mouse models of atherosclerosis such as apolipoprotein E (apoE) deficient mice, typically have cholesterol values ranging from 400-1000 mg/dl (11-21), and can go as high as 1715 mg/dl during diabetes (22). In these diabetic models, the consequent hyperlipidemia, rather than the diabetes itself, is likely the major cause of increased atherosclerosis (7-10).

Apolipoprotein E (apoE) is a small circulating protein associated predominantly with VLDL and HDL, and is the primary ligand for the LDLR, making it a crucial component in the clearance of lipid from the circulation and a major determinant of plasma cholesterol and CVD risk (23). The APOE gene is polymorphic, producing three common isoforms: apoE2, apoE3, and apoE4. The apoE4 isoform is associated with higher LDL cholesterol and an increased risk of CVD (23).

In this study, we employ "E4h" mice that are homozygous for replacement of the endogenous mouse APOE gene with the human APOE*4 (E4) allele and heterozygous for an allele that overexpresses the human LDL receptor (h). When fed a high fat/high cholesterol diet, E4h mice accumulate cholesterol-rich VLDL remnants, have reduced HDL, and develop atherosclerosis (24). Here, we describe the generation of distinct atherosclerotic plaques when E4h mice are made diabetic by the $Ins2^{+/-}$ "Akita" mutation, a genetic model of type 1 diabetes (25). Diabetic E4h mice develop atherosclerosis despite plasma cholesterol within a normal range (<175 mg/dl).

To our knowledge, 4hAkita mice represent the first mouse model in which: 1) diabetes is required for atherosclerosis development, 2) atherosclerosis occurs spontaneously on normal chow diet low in cholesterol, 3) no important components of lipoprotein metabolism such as apoE or LDLr are missing, and most importantly 4) atherosclerotic plaques develop in the absence of severe hyperlipidemia. This unique model highlights the importance of distinct lipoprotein profiles rather than only total plasma lipids, and will enable future research address more accurately address the patient-research model discrepancies in the background of diabetes.

3.2 Methods

Mice and induction of diabetes. Mice homozygous for replacement of the endogenous Apoe gene with the human APOE*3 (E3) or human APOE*4 (E4) allele (26-27) were crossed with mice heterozygous for the

$Ins2^{+/-}$ "Akita" mutation (25) and/or with mice heterozygous for a targeted replacement of the mouse Ldlr gene with the stabilized human Ldlr minigene ($Ldlr^{h/+}$) (24, 28). All mice were on C57BL/6 backgrounds. Male mice were fed normal chow diet ad libitum (5.3% fat and 0.02% cholesterol) (Prolab IsoPro RMH 3000; Agway Inc). Biochemical analyses were carried out at 6 months of age unless otherwise stated. Animals were handled under protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina.

Biochemical assays. After a 4 hour fast, animals were anesthetized with 2,2,2-tribromoethanol and blood was collected. Plasma glucose, cholesterol, *phospholipids and ketone bodies* were measured using commercial kits (Wako). Triglycerides and insulin were determined using commercial kits from Stanbio and Crystal Chem Inc., respectively. Lipoprotein distribution and composition was determined with pooled (n=5-6) plasma samples (100 µl) fractionated by fast protein liquid chromatography (FPLC) using a Superose 6 HR10/30 column (GE Healthcare). Pooled plasma (250 µl) was separated by sequential density ultracentrifugation (UC) into density fractions ranging from <1.006 g/ml (VLDL) to >1.21 g/ml (HDL) and subjected to electrophoresis in a 4-20% denaturing SDS-polyacrylamide gel (29). For VLDL secretion, plasma TG was measured following injection of Tyloxapol (Triton WR-1339; Sigma) via tail vein (0.7 mg/g BW) after a 4 hour fast (johnson). ApoB content was determined in pooled (n = 6) plasma samples following UC at <1.006 g/ml by western blot using 1:2000 dilution of α-apoB primary antibody (Abcam) and 1:10,000 dilution of α-Rb HRP-conjugated secondary antibody (Cell Signaling).

Peritoneal macrophage isolation. Macrophages were isolated from the peritoneal cavity 4 days after intraperitoneal injection of 0.5 ml of 4% (w/v) thioglycolate (BD Biosciences). Cells used for gene expression analysis were collected directly and stored at -20C until measurement. Macrophages employed for in vitro analyses were washed with Ham's nutrient mixture F-10 medium, spun at 1000 x *g* for 5 minutes, plated in 12-well plates at a density of 5×10^5 cells/well, and cultured in 5 mM (Low) or 25 mM

(High) glucose media supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were washed 2 hours later to remove non-adherent cells.

Cholesterol uptake. LDL was isolated from human plasma and labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine percholorate (DiI; Molecular Probes, Inc.) as previously described (30). Prior to labeling with DiI, a portion of LDL was oxidized (oxLDL) as previously described (31). To determine LDL uptake, macrophages were cultured with 1 μg/ml DiI-labeled LDL for 4 hours following a 48 hour conditioning in Low or High glucose media. After 4 hours with the DiI-labeled LDL, cells were washed 2X with fresh medium. Lipid was extracted by adding 0.5 ml isopropanol/well, scraping the cells, and shaking on an orbital shaker overnight. Fluorescence was then measured using an IX70 inverted microscope (Olympus) equipped with a filter set for Texas red (exciter 560/55, emitter 645/75; Chroma Technology Corp.) and adjusted to total protein/well as measured by BCA assay (Thermo Scientific).

Atherosclerosis. After 3 months of diabetes, mice were euthanized with a lethal dose of 2,2,2-tribromoethanol and perfused at physiological pressure with 4% phosphate buffered paraformaldehyde (pH 7.4). Morphometric analysis of plaque size at the aortic root was performed as described (32).

3.3 Results

Plasma glucose and lipids

While there was no APOE genotype effect on fasting plasma glucose in the non-diabetic groups, there was a significant reduction in fasting plasma glucose in mice overexpressing LDLR (Figure 4A, left side). All four diabetic groups, regardless of LDLR expression or APOE genotype, had fasting plasma glucose exceeding 400 mg/dl (Figure 1A, right side). Diabetic mice expressing E4 have higher fasting plasma

glucose than diabetic mice with E3, and glucose was higher in diabetic E4h mice compared to diabetic E4 mice, but neither trend reached statistical significance. Conversely, fasting plasma glucose was significantly higher in diabetic E4h mice compared to diabetic E3h mice (Figure 1A, far right).

There were no significant differences in fasting plasma triglycerides between any of the diabetic or non-diabetic groups. There was also no effect of APOE genotype, diabetes or LDLR expression on fasting plasma free fatty acids in any experimental group (Table 1).

Increased expression of the LDLR led to decreased plasma total cholesterol in the non-diabetic mice regardless of APOE genotype (Figure 1B, left side). In contrast, the effect of increased LDLR expression was not present when mice were diabetic. In addition, there was no significant change in total plasma cholesterol upon induction of diabetes in mice expressing E3 (Figure 1B, white bars). However, when mice expressing E4 were made diabetic there was a significant increase in total plasma choleseterol regardless of LDLR expression (Figure 1B, black bars). Taken together, this data demonstrates that diabetic mice expressing human apoE4 have higher plasma glucose and total cholesterol than non-diabetic mice as well as diabetic mice expressing human apoE3.

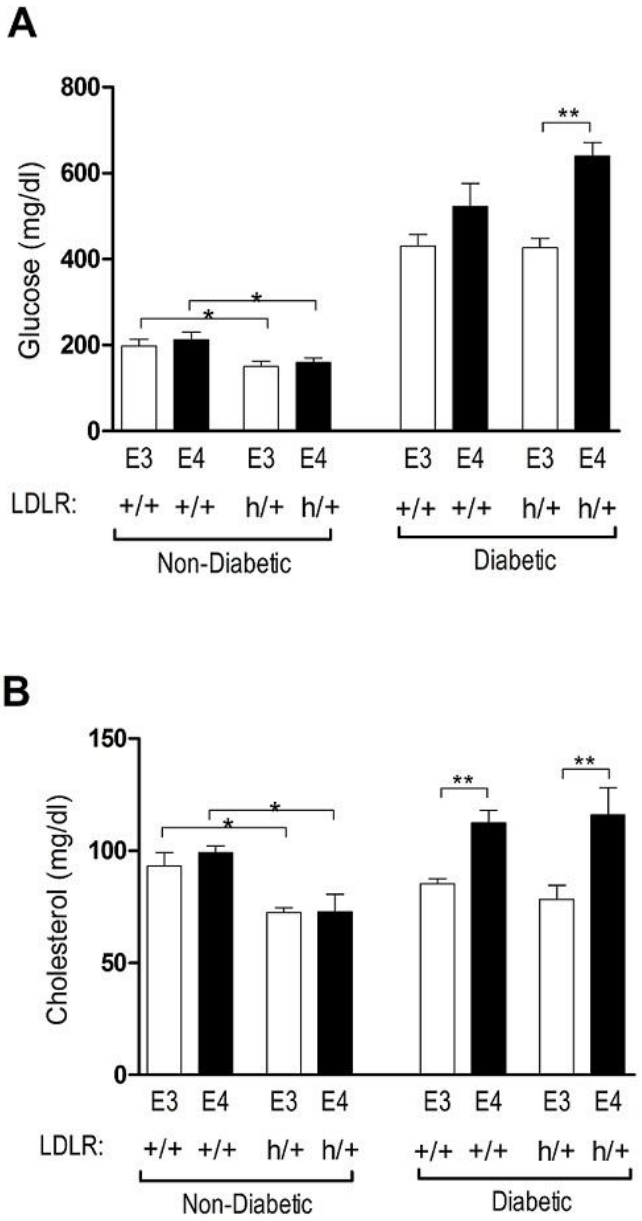

Figure 3.1. **Plasma glucose and cholesterol.** Plasma glucose (A) and total cholesterol (B) was measured in 4 month old non-diabetic and diabetic mice following a 4 hour fast. * $p<0.05$, ** $p<0.001$. n = 5-10.

	Non-diabetic				Diabetic			
	E3	E4	E3h	E4h	E3	E4	E3h	E4h
Triglycerides (mg/dl)	64.6 ± 3.7	67.6 ± 3.1	67.6 ± 9.8	55.6 ± 6.4	70.8 ± 5.3	75.2 ± 4.9	65.6 ± 7.4	58.2 ± 7.5
Free Fatty Acids (mmol)	0.71 ± 0.05	0.74 ± 0.08	0.66 ± 0.05	0.70 ± 0.14	0.69 ± 0.05	0.84 ± 0.11	0.74 ± 0.06	0.70 ± 0.12
Insulin (pg/ml)	n.d.	960.0 ± 93.2	n.d.	974.8 ± 27.3	n.d.	128.2 ± 90.8	n.d.	123.6 ± 46.3
Non-HDL / HDL ratio (cholesterol)	0.71	0.45	1.35	0.78	1.10	0.71	1.82	3.05

Table 3.1. **Metabolic paramaters of E3, E4, E3h and E4h mice.** Fasting plasma triglycerides, free fatty acids, insulin, and non-HDL to HDL cholesterol ratio.

Lipoprotein distribution

We next examined the distribution of cholesterol among various lipoprotein fractions. Separating lipoprotein classes by size-exclusion using Fast Performance Lilquid Chromatography (FPLC) revealed that non-diabetic E3 and E4 mice carry the majority of their cholesterol in the form of HDL and that increasing LDLR levels resulted in a slight reduction in HDL cholesterol (Figure 2A-B).

With the exception of a small decrease in diabetic E3 mice compared to their non-diabetic counterparts, diabetes did not change the levels of HDL cholesterol. However, VLDL and VLDL remnants were increased in all groups. Interestingly, this increase was magnified in the diabetic E4h mice. Thus, although plasma total cholesterol between diabetic E4 and E4h mice was indistinguishable, there is a dramatic shift in the lipoprotein profile. Cholesterol in diabetic E4 mice was primarily in HDL fractions while cholesterol in diabetic E4h mice is primarily in VLDL and VLDL remnant fractions (Figure 2D).

Consequently, the ratio of non-HDL cholesterol to HDL cholesterol, an atherogenic risk factor, was much higher in diabetic E4h mice than in the other groups (Table 1). The shift towards an atherogenic pattern of cholesterol distribution in diabetic E4h mice was mirrored in density fractions separated by ultracentrifugation (Figure 3A-B). The majority of cholesterol in the non-diabetic and diabetic E4 mice was concentrated in HDL density fractions of 1.10 and >1.21 mg/dl (Figure 4A-B, white bars). Conversely, the majority of plasma cholesterol in diabetic E4h mice was found in the VLDL and IDL fractions of <1.006 and 1.02 mg/dl (Figure 3B, black bars).

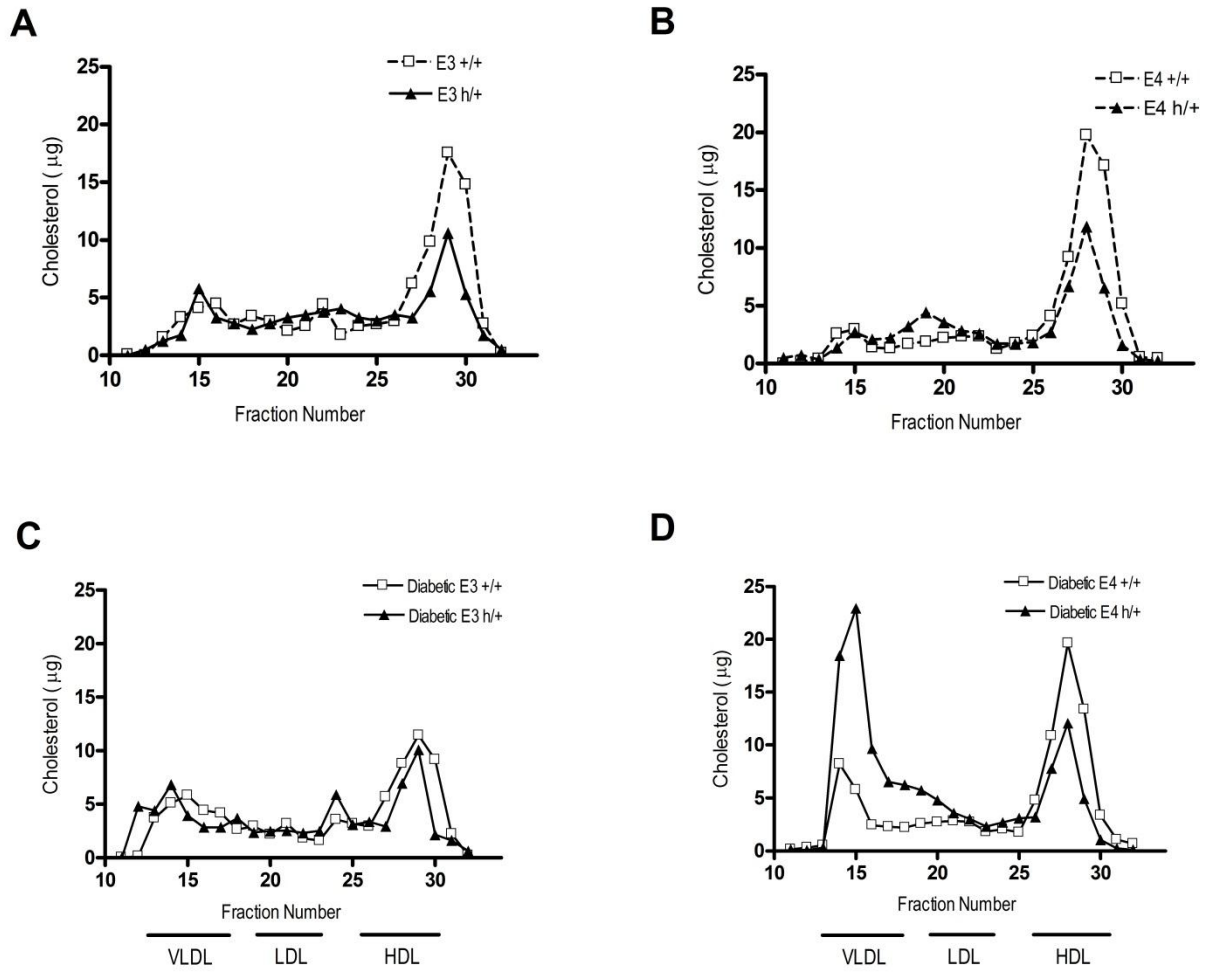

Figure 3.2. **Lipoprotein profiles.** Pooled plasma (100 µl) from non-diabetic E3 and E3h (A), non-diabetic E4 and E4h (B), diabetic E3 and E3h (C), and diabetic E4 and E4h (D) mice was separated on a Fast Performance Liquid Chromatography (FPLC) column and fractions were analyzed for cholesterol. n = 6-8, pooled.

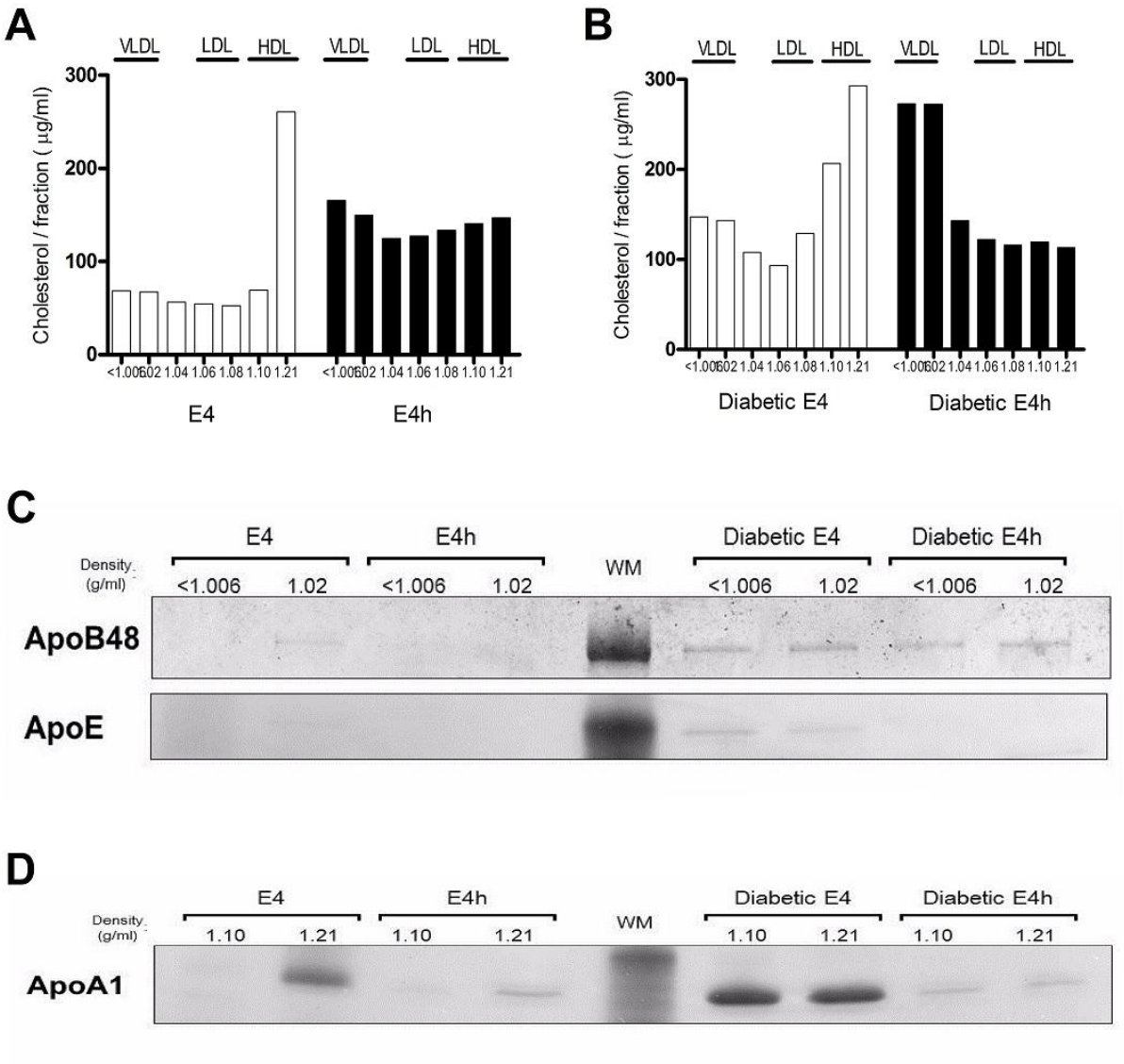

Figure 3.3. **Cholesterol and apolipoprotein distribution**. Pooled plasma (800 ul, n = 6) was separated into density fractions ranging from <1.006 to >1.21 mg/dl by ultracentrifugation. *A:* Cholesterol distribution among the various density fractions in plasma from E4 (white bars) and E4h (black bars). *B:* Cholesterol distribution in diabetic E4 (white bars) and diabetic E4h (black bars) mice. *C-D:* ApoB48 and apoE in the VLDL and IDL fractions of <1.006 and 1.02 mg/dl (C) and apoA1 in the HDL fractions of 1.10 and >1.21 mg/dl (D) was determined by SDS PAGE analysis.

Apolipoprotein distribution

In order to determine the apolipoprotein composition of the diabetic E4 and diabetic E4h mice, ultracentrifugation density fractions were submitted to SDS PAGE analysis. Fasting plasma protein levels of apoB100 were undetectable in all groups, and little to no apoB48 was observed in the VLDL/IDL fractions of <1.006 and 1.02 mg/dl in the non-diabetic E4 and E4h mice. There was a similar amount of apoB48 between the diabetic E4 and diabetic E4h mice, indicating that the number of VLDL remnants in these mice is similar (Figure 3C). However, there was a clear reduction in apoE in these same fractions (Figure 3C). This result indicates that the VLDL remnants in diabetic E4h mice are not only cholesterol enriched, but are also apoE poor. In addition, in the density fractions of 1.10 and >1.21 mg/dl, there was a dramatic decrease in the HDL associated protein apoA1 in the diabetic E4h mice (Figure 3D).

VLDL Secretion

Reduced insulin is associated with increased VLDL secretion (33). The rate of hepatic VLDL secretion was estimated by injecting diabetic E4 and diabetic E4h mice with the detergent Tyloxapol which inhibits particle uptake and lipolysis. The rate of TG accumulation in the plasma following Tyloxapol injection was similar between the two groups for the first 30 minutes post-injection, but slowed significantly in diabetic E4h mice compared to diabetic E4 mice at 1 and 2 hours (Figure 4A). However, the accumulation of plasma cholesterol following injection was identical in both groups (Figure 4B).

To estimate the number of lipoprotein particles being secreted, we measured the amount of apoB in pooled samples (>1.006 mg/dl fraction) at 2, 60 and 120 minutes post-tyloxapol injection. The secretion of total apoB protein was similar between diabetic E4 and diabetic E4h mice, and the amount of B48 secreted relative to B100 was higher in the diabetic E4h mice compared to the diabetic E4 mice (Figure 4C-E). Together, these data suggest that during times of fasting, the diabetic E4h mice secrete primarily B48-containing, cholesterol-rich VLDL.

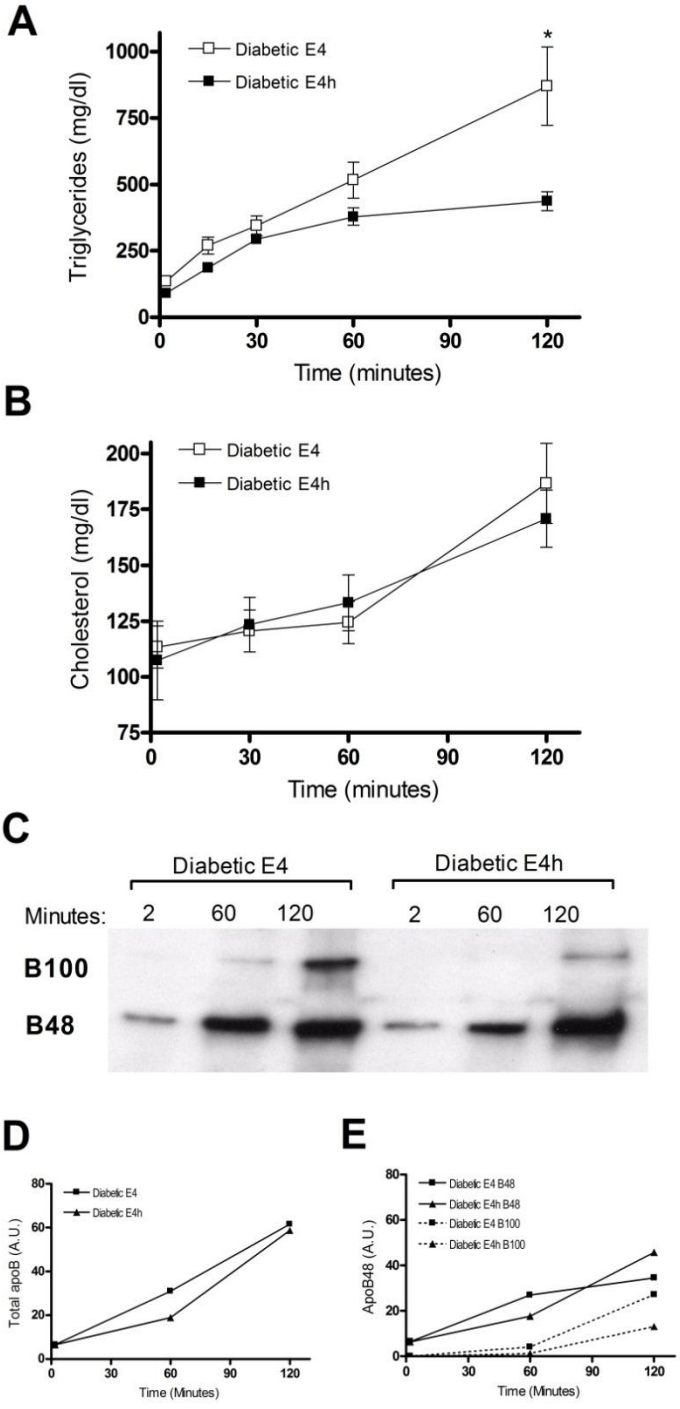

Figure 3.4. **VLDL secretion**. *A-B:* Hepatic secretion of diabetic E4 (white squares) and diabetic E4h (black squares) mice was estimated by measuring the accumulation of triglycerides (A) and cholesterol (B) following injection of the detergent Tyloxapol via the tail vein. *C:* ApoB was measured by western blot in pooled samples of VLDL (>1.006 g/ml density fractions) at 2, 60 and 120 minutes post-injection. *D-E:* The amount of total apoB (A) and apoB48 and apoB100 (B) was quantified from (C) using Image J software.

Inflammation and macrophage LDL uptake

To determine the contribution of an inflammatory state on atherosclerosis development, several plasma markers of inflammation were measured in diabetic E4 and diabetic E4h mice. Measurable Interleukin 6 (IL-6) protein concentrations in the plasma of 6 month old was severely elevated in 3/11 diabetic E4h mice but was only modestly detectable in 1/8 diabetic E4 mice (Figure 5A, left side). Similarly, measurable monocyte chemotaxic protein 1 (MCP-1) concentrations was detected in 5/11 diabetic E4h mice, compared to only 1/7 mice in the diabetic E4 mice (Figure 5B, right side). Concentrations of circulating TNF-α were below the detectable range (<9 pg/ml) for all samples measured (data not shown).

We next examined whether apoE4 and the LDLR interact in macrophages to effect LDL uptake, and thus foam cell and atherosclerotic plaque formation. DiI-labeled LDL uptake was measured *in vitro* using peritoneal macrophages isolated from non-diabetic E4 and E4h mice. We examined the effect of acute exposure to a hyperglycemic environment by incubating E4 and E4h macrophages in high glucose media for 48 hours. When cultured in low glucose, E4h macrophages took up more oxidized LDL compared to E4 macrophages, but the increase did not reach significance. However, when cultured in high glucose media for 48 hours, E4h macrophages took up significantly more oxidized LDL compared to E4 macrophages (Figure 5C). Together, these data suggests that in addition to diabetes-induced lipoprotein disturbances, increased inflammation and macrophage activity may contribute to the atherogenesis noted in the diabetic E4h mice.

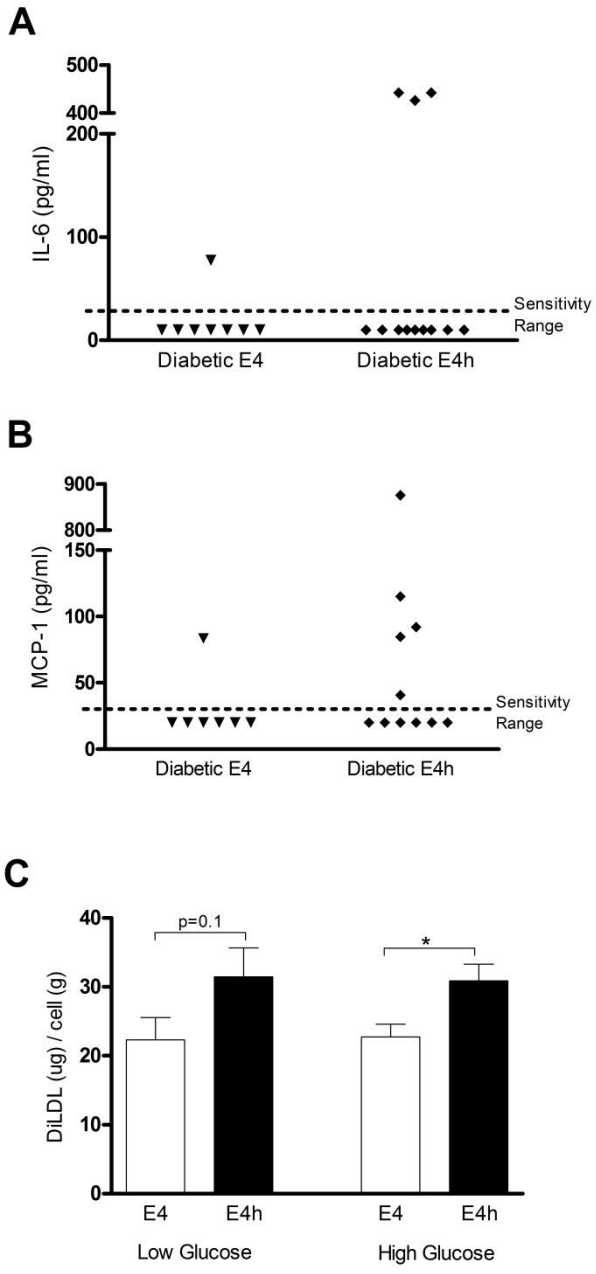

Figure 3.5. **Inflammation and macrophage LDL uptake.** *A-B:* Markers of inflammation. Plasma protein concentrations of IL-6 (A) and MCP-1 (B) were measured using a Luminex Milliplex Analyst system. *C:* Peritoneal macrophages isolated from E4 (white bars) or E4h (black bars) mice were cultured in high glucose media (25 mM) for 48 hours before a 4 hour incubation with DiI-labeled oxidized LDL.

Diabetes-Induced Atherosclerosis in E4h mice

No vessel damage or atherosclerosis was noted in either of the non-diabetic experimental groups at 6 months of age. Likewise, none of the diabetic E3, diabetic E3h or diabetic E4 mice analyzed showed any visible signs of atherosclerosis or foam cell formation. In marked contrast, of the eight experimental groups analyzed in this study, only the diabetic E4h mice developed atherosclerotic pathology. In spite of very low total plasma cholesterol (<175 mg/dl) and despite similar total plasma cholesterol as diabetic E4 mice, all seven diabetic E4h mice that were analyzed at 6 months of age showed clear fatty depositions within sub-endothelial areas (Figure 6). Foam cells were not only present on the luminal surface but were also present in medial smooth muscle layers in two of the seven mice (Figure 6C,E). There was no correlation between total plasma cholesterol or glucose and atherosclerotic plaque size when individual diabetic E4h mice were compared (data not shown).

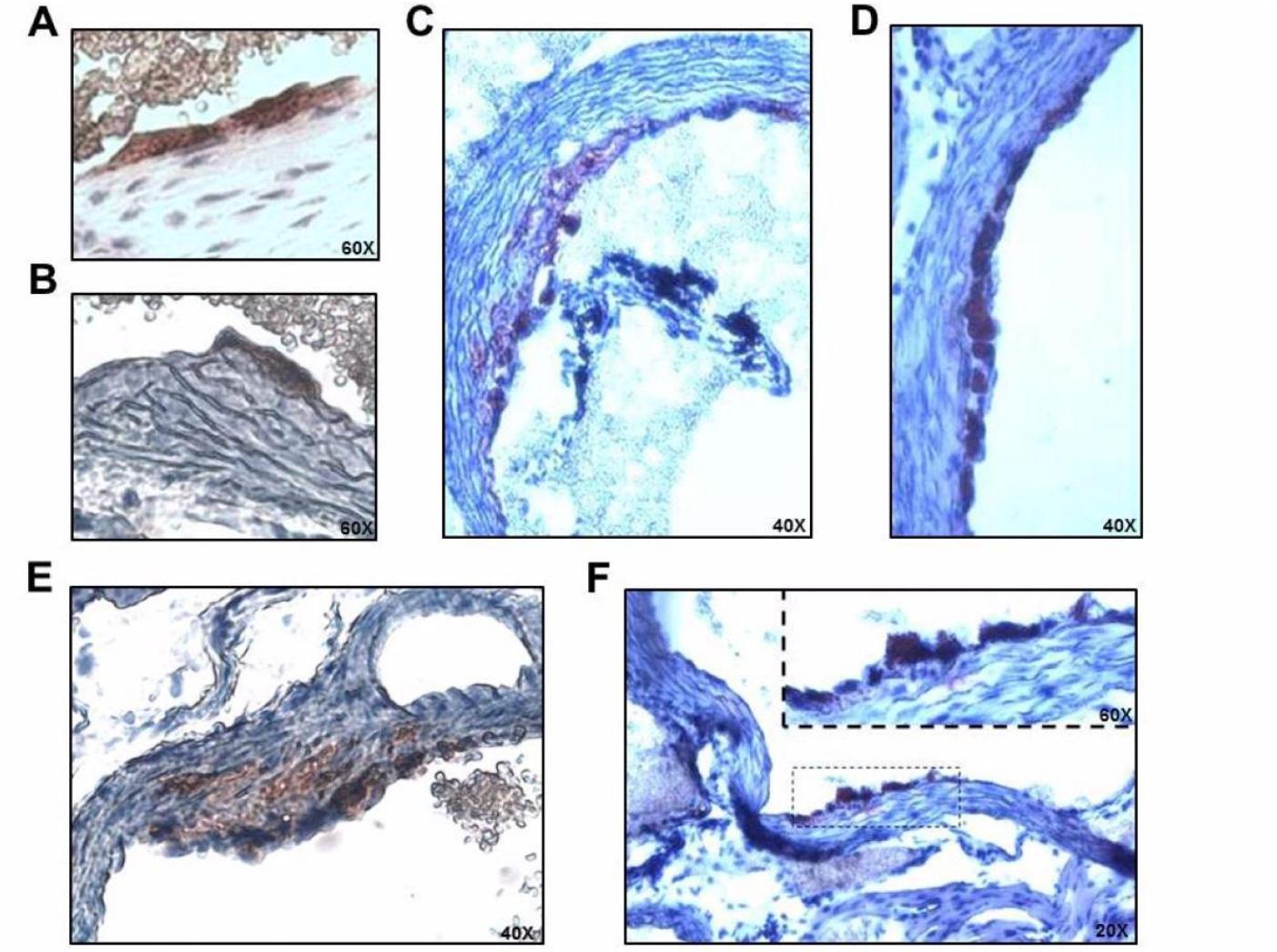

Figure 3.6. **Athersoclerosis.** Atherosclerotic lesions at the aortic root. *A-F:* At 6 months of age, diabetic E4h mice were sacrificed, perfused with 4% PFA and 8 μm sections of the aortic root were sectioned. Sections were stained with H & E and Oil Red O to highlight lipid. Fatty intimal depositions, foam cell formations and atherosclerotic plaques from individual diabetic E4h mice (A-F). Magnification is listed in the bottom right hand corner of each image. *G-H:* Correlation between atherosclerotic lesion area and plasma glucose (G) or total cholesterol (H).

Discussion

Diabetes is a major, independent risk factor for several forms of CVD (5). The increased CVD risk for patients with diabetes is a result of several interconnected factors, including inflammation, endothelial dysfunction, hypertension, and a pro-thrombotic state (34). Unfortunately, past research has often failed to adequately dissociate the atherogenic potential of these factors from the common process of diabetic dyslipidemia (9). Diabetic dyslipidemia is a cluster of harmful changes to lipoprotein metabolism, including an increase in VLDL and a decrease in HDL cholesterol, which is commonly seen in patients with diabetes (7).

Many studies employing mouse models have been unable to demonstrate a diabetes-induced increase in atherosclerosis, including those using apoE-/- (35), Ldlr-/- (12), Ldlr +/- with high cholesterol (13), apoB tg (15), CETP tg (36), and apoA1-deficient mice (14). Although some mouse models have shown a diabetes-induced acceleration of atherosclerosis, it has been difficult to distinguish between effects due to exaggeration of plasma lipids from those of diabetes itself (9). Furthermore, the vast majority of current mouse models of atherosclerosis have plasma cholesterol levels far higher than even the most extreme human patients. For example, men deemed at risk for CVD have cholesterol levels >240 mg/dl, while those in the highest 5% of the population have and average cholesterol of 318 mg/dl (5). In comparison, common mouse models have normal cholesterol levels between 500-1000 mg/dl and have been shown to reach as high as 1715 mg/dl when diabetic (22). In addition, clinical trials have clearly shown that while aggressive cholesterol lowering therapies result in a reduction of CVD risk, even when plasma cholesterol is lowered to an equivalent "non-diabetic level", a patient with diabetes still has a significantly higher CVD risk (5). This strongly suggests that other factors, such as the ones mentioned above, must be considered. Thus, generating a mouse model of atherosclerosis in which the atherogenic effect of diabetes is not clouded by severe hyperlipidemia has long been a goal for researchers.

Here, we describe a novel model of diabetic atherosclerosis – the diabetic E4h mouse – that develops spontaneous atherosclerotic plaques despite total cholesterol levels <175 mg/dl. The major advantages of this model include: 1) No genetic deficiencies; the important components of lipoprotein metabolism that are deficient in common models of atherosclerosis are present, and moreover in human form (apoE and LDLR), 2) Genetic, gradual, inobtrusive model of type 1 diabetes ('Akita' mutation); no toxic effects of diabetes inducers such as STZ or alloxan, 3) No high cholesterol, high fat, or cholate diet is necessary to induce atherosclerosis, and 4) Most importantly, atherosclerosis occurs in the absence of severe hyperlipidemia. To our knowledge, the diabetic 4h mice represent the first normolipidemic mouse model in which diabetes is required for atherosclerosis development.

To induce diabetes, we employed the Akita mutation, a genetic model of type 1 diabetes. The spontaneous Akita mutation, in the Ins2 gene, leads to improper folding of proinsulin and eventual β-cell death (25). As a consequence, male Akita mice develop hyopinsulinemia and severe hyperglycemia beginning around 1 month of age (25). In the hyperlipidemic apoE-/- model of atherosclerosis, introduction of the Akita mutation resulted in a twofold increase in non-HDL cholesterol and a threefold increase in atherosclerosis (37). In this study, we aimed to examine the APOE isoform effect on diabetic atherosclerosis in the setting of a more physiologically relevant range of plasma lipids.

Total plasma cholesterol levels remain quite low in the diabetic E4h mice compared to other models of diabetic atherosclerosis, where it usually exceeds 500 mg/dl (11-22). Thus, the fact that the diabetic E4h mice still develop distinct atherosclerotic plaques is a testament to the importance of APOE isoform and its strong effect on the types of lipoproteins contributing to the total plasma cholesterol levels. HDL cholesterol has been demonstrated to have the strongest effect on CVD risk in humans, and even when in the presence of high LDL, can significantly decrease the risk of CHD (38). Despite similar total cholesterol, the low HDL and high VLDL cholesterol in the diabetic E4h mice likely contributes to their atherogenesis, while the comparatively high HDL and low VLDL in the diabetic E4 mice is atheroprotective.

The LDLR has been shown to regulate hepatic VLDL production, with overexpression of LDLR leading to a decrease in apoB100 secretion (39). Likewise, insulin has a well-established role as an inhibitor of hepatic VLDL secretion (33, 40). In our study, the combination of hypoinsulinemia and overexpression of the LDLR in diabetic E4h mice led to a decrease in apoB100 secretion and the release of cholesterol-rich particles. The similarity of the composition of lipoproteins that accumulate in the plasma with those that were characterized following tyloxapol injection, suggests that hepatic secretion of cholesterol-rich VLDL directly contributes to the atherogenic lipoprotein profile observed in these mice. This accumulation of this cholesterol-rich VLDL under normal conditions may be a direct result of the decrease in available apoE in the diabetic E4h mice.

Additionally, an inflammatory modulating role for apoE has been previously suggested, with data demonstrating an increase in inflammation or a decrease in anti-inflammatory protection in the presence of apoE4 (41). We have also previously shown that increasing LDLR expression in macrophages results in higher LDL uptake in the presence of apoE4 (30). In addition, macrophages expressing apoE4 are less able to promote cholesterol efflux compared to those with apoE2 or apoE3 (30). In our model of global LDLR overexpression, we noted a similar phenomenon in macrophages cultured in a high glucose environment, however only in the uptake of oxidized LDL. Interestingly, this suggests a potential role for the LDLR in modulating the uptake of modified particles previously thought to be limited to scavenger receptors such as CD-36 (42).

In summary, the diabetic E4h model demonstrates an initiation of atherosclerosis in the absence of severe diabetes-induced hyperlipidemia (cholesterol levels <125 mg/dl). With total cholesterol remaining <175 mg/dl, the diabetic E4h mice highlight the importance of lipoprotein cholesterol distribution, inflammation and macrophage activity on the development of atherosclerosis. Plasma lipids in this model are within a physiological range that current mouse models fall far outside of, and imperatively, are reflective of the many diabetic patients who have relatively low plasma cholesterol, but still develop cardiovascular disease. Thus, these mice may provide researchers with a model of diabetic atherosclerosis

in which we can begin to separate and objectively examine many of the lipid-independent effects of diabetes on cardiovascular health. Further research employing this model of diabetic atherosclerosis will greatly aid in the dissection of lipid-independent mechanisms by which diabetes confers its atherogenic effects.

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Chapter 4

MACROVASCUALAR COMPLICATIONS OF DIABETES IN ATHEROSCLEROSIS-PRONE MICE

(This chapter consists of material from a manuscript reprinted with permission from *Expert Reviews in Endocrinology and Metabolism*; 2010 5(1), 89-98 (2010); titled "*Macrovascular complications of diabetes in atherosclerosis-prone mice*" by **Lance A Johnson** and Nobuyo Maeda)

4.1 Summary

The well-established relationship between diabetes and cardiovascular complications, combined with the rapidly increasing prevalence of diabetes, has created a pressing need for better understanding of the mechanisms of diabetic atherosclerosis. Multiple metabolic and diabetes-specific factors have been associated with accelerated atherosclerosis, including dyslipidemia, oxidative stress, inflammation, vascular cell dysfunction, and coagulopathy. This discussion highlights selected studies in which researchers have employed mouse models of diabetic atherosclerosis in an attempt to examine these mechanisms and to test potential therapeutic and preventative measures.

4.2 Introduction

Each year, cardiovascular disease kills more Americans than any other cause of death (1). Diseases of the heart alone caused 30% of all deaths, with other diseases of the cardiovascular system causing substantially more death and disability (1). Increasing this severe health burden is the rapidly increasing prevalence of diabetes. The damage done by diabetes extends far beyond the typical microvascular complications of the disease, such as retinopathy and neuropathy. Diabetics are 2 to 8 times more likely to develop CVD than non-diabetic patients, with up to 80% of patients with type 2 diabetes developing macrovascular disease (2-8). Cardiovascular disease caused by atherosclerosis is the most significant complication of diabetes, as it is the leading cause of mortality among diabetic patients (6).

Several complex and interdependent metabolic conditions affect the pathogenesis of atherosclerosis in diabetes, including hyperglycemia, hyperinsulinemia and dyslipidemia. These

metabolic disturbances are associated with increases in oxidative stress and glycation, endothelial dysfunction, inflammation, and a prothrombotic state that can influence all stages of atherogenesis (1, 8). Despite the extensive clinical data linking diabetes and cardiovascular disease, many questions remain concerning the exact molecular mechanisms by which hyperglycemia and defective insulin signaling actions lead to macrovascular dysfunction.

Animal models have been invaluable in dissecting the various factors involved in the pathogenesis of diabetic macrovascular disease. In recent years, more attention has been focused on mouse models to examine the factors that modulate diabetic atherosclerosis (9-10). The mouse model is cost effective because of the small size of the animals and short generation time, and allows for well-controlled manipulations of diet, drugs, and other treatments. Above all, the mouse is currently the only species in which specific genetic mutations can be introduced into the genome with relative ease. Admittedly, mouse models are not able to completely mimic human diseases. Nevertheless, development of human-like atherosclerotic plaques in the aorta of hyperlipidemic mice, such as apolipoprotein E deficient (apoE-/-) mice and the low-density lipoprotein receptor deficient (Ldlr-/-) mice, has facilitated the investigation of macrovascular complications of diabetes using these models. In this discussion, we review some of the recent studies of diabetic cardiovascular disease by highlighting the contributions of various atherosclerosis prone mouse models of diabetes.

4.3 STZ- Induced Diabetes and Exacerbation of Atherosclerosis

Despite representing less than 10% of human diabetics, mouse models of type1 diabetes are used more often, in part due to their simplicity and ease of diabetes induction. Chemicals such as alloxan and streptozotocin (STZ) have long been used to induce diabetes in rodents (11). STZ is

an alkylating agent that causes DNA damage. Since cellular uptake of STZ is mediated through glucose transporter 2 (Glut2), STZ is particularly toxic to the β-cells of the pancreas where Glut 2 is highly expressed (12, 13). A repeated administration of STZ at a low dose renders mice hyperglycemic with minimal toxic effects to other cells. Although only males develop significant diabetes, thus limiting the scope of most studies, STZ treatment is rapid and effective.

Multiple studies have used STZ to induce diabetes in atherosclerosis-prone mice and confirmed that diabetes enhances atherosclerotic plaque development (Figure 4.1). The extent of enhancement as measured by the size of plaques in the cross section of aortic sinus, and/or by the plaque areas covering the aortic trees (*en face*), differ from experiment to experiment. In general, plaque sizes in diabetic ApoE-/- mice average from 1.5 - 2 fold larger at the aortic sinus and 4 fold larger in aortic surface area compared to those seen in non-diabetic animals (Figure 4.1A). The degree of atherosclerotic enhancement appears to strongly relate to the age of the animal at the time of onset, the duration of diabetes, and the type of diet on which the diabetic mice are maintained. Thus, larger plaque enhancements are generally seen when apoE-/- mice are treated with STZ at younger ages when compared to the more modest effects of diabetes on the development of pre-existing plaques found in older mice. Similarly, plaque-covered areas in the entire aorta appear to increase more during diabetes relative to the plaques in the aortic sinus alone, where plaques begins to develop at earlier age than other parts of aortic tree. These phenomena suggest that diabetes accelerates initiation and growth during the early stages of plaque development, possibly through pathways of the inflammatory response. Also important to note is that although lesions in diabetic animals are consistently larger than non-diabetic controls and therefore more advanced, plaques in diabetic mice are histologically no more complex than those of similar size seen in non-diabetic mice.

Two to three fold increases in atherosclerosis have also been observed in LDLR-/- mice made diabetic by STZ treatment (Figure 4.1B). In some studies, however, no increase in atherosclerosis was observed (14,16). For example, Reaven et al reported that atherosclerosis in the LDLR-/- mice made diabetic with a single high dose of STZ and maintained on high fat diet for 6 months did not differ from those in the control, non-diabetic, LDLR-/- mice (14). The reason for this is not clear, but the plasma glucose levels in these diabetic mice were not as high as in other experiments because they were maintained on a low dose insulin infusion. In addition, the plasma cholesterol levels were not significantly different between diabetic and non-diabetic mice.

In general, STZ-induced diabetes enhances plaque development in atherosclerosis prone mouse models, providing a suitable model system to evaluate perturbation or acceleration of the disease process through the manipulation of relevant factors.

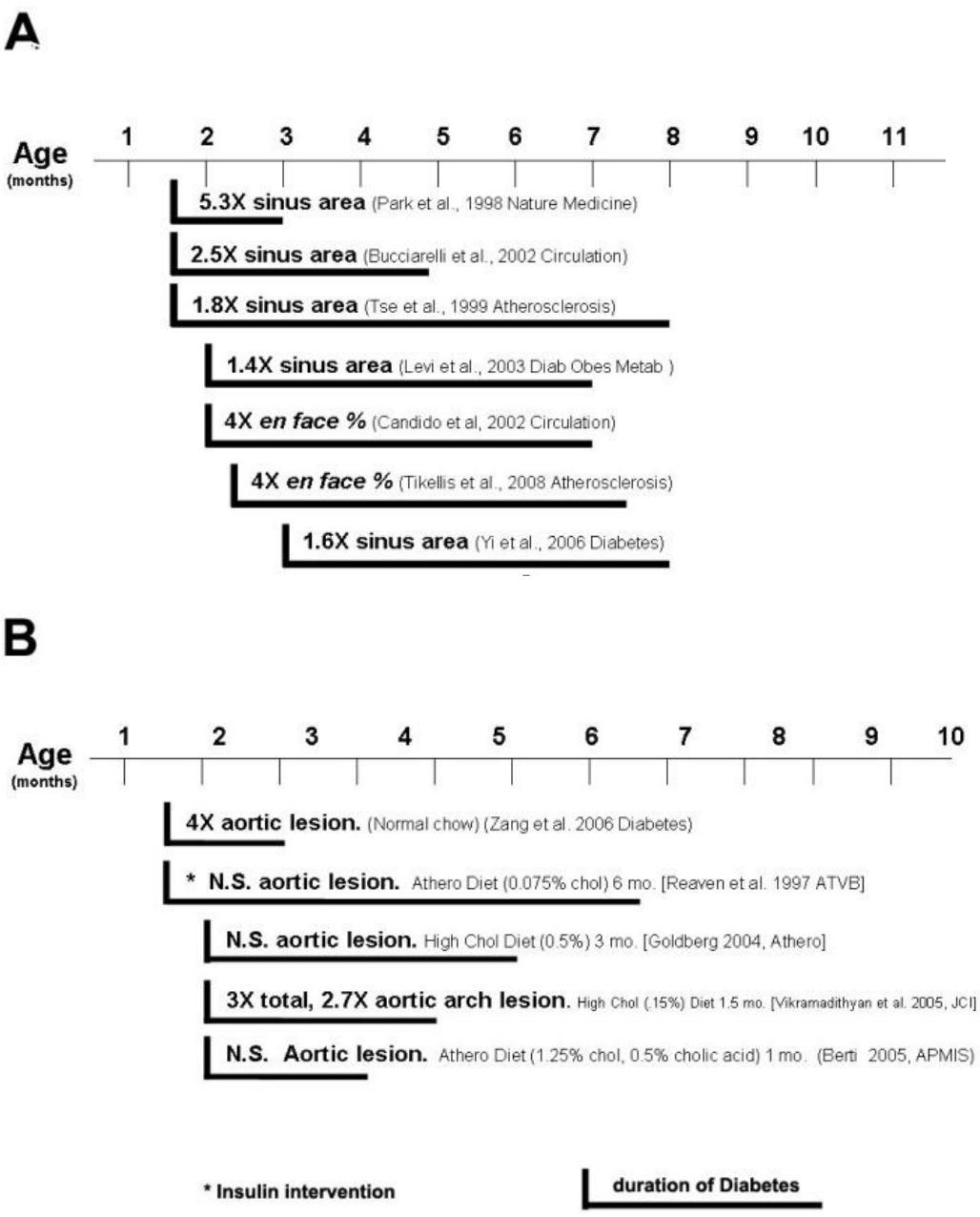

Figure 4.1. **Effect of STZ-induced diabetes on atherosclerotic plaque size.** Change in atherosclerotic plaque area in STZ treated (A) ApoE-/- and in (B) LDLR-/- mice compared to non-diabetic controls (selected studies). The duration of diabetes prior to atherosclerosis evaluation is represented by the horizontal length of the black bar. Age at onset of diabetes is marked by the vertical portion of the black bar.

4.4 Genetic Models of Type1 Diabetes and Atherosclerosis

Genetic models of type1 diabetes are also available. Akita ($Ins2^{C97Y/+}$) mice develop type 1 diabetes due to a cysteine to tyrosine substitution at the amino acid position 97 in the insulin 2 gene (Ins2). This mutation results in misfolding of preproinsulin, and triggers subsequent ER stress, β-cell apoptosis, and insulin deficiency (17). Similar to chemically induced models, diabetic phenotypes of Akita mice are also more severe in males than in females (18). Studies of atherosclerosis prone mice in Akita background have yet to be reported.

The major cause of type1 diabetes in humans is not a direct loss of β cells as occurs in the mouse models discussed above, but instead appears to involve autoimmune responses and inflammatory processes which are triggered by injury and/or infection (19, 20). Non-obese-diabetic (NOD) mice spontaneously develop diabetes beginning at around five weeks of age due to lymphocytic infiltration of the pancreatic islets. Similar to humans, their diabetes involves autoimmunity to islet cells as well as insulitis, but the genetics behind diabetes in NOD mice appears to be complex. NOD mice are highly resistant to diet induced atherosclerosis development and neither diabetic nor non-diabetic NOD mice developed fatty streaks in their aortas when fed high fat, high cholesterol diets (21). Introduction of proatherogenic mutations onto NOD background would be a worthwhile task.

An attractive diabetic model also involving autoimmune response was recently developed by Renard et al who created transgenic mice expressing the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) under control of the rat insulin promoter. The GP mice express viral glycoprotein specifically in pancreatic β-cells and, after infection with LCMV, an immune response specifically destroys the β-cells, resulting in rapid development of type1 diabetes (22). When this GP transgene is placed onto LDLR-/- background, diabetic female mice developed 2.2

and 2.5 fold larger plaques compared to non-diabetic mice when they were fed 0.12% and 0.5% cholesterol diets, respectively (23). Most strikingly, when mice with lesions of the same cross sectional area were compared, 60% of the plaques in diabetic mice fed high cholesterol diet (1.25%) compared to 12% of those in non-diabetic mice contained intralesional hemorrhage. Thus, diabetes in combination with a high cholesterol diet severely decreased plaque stability in GP-LDLR-/- mice.

These genetic models of type 1 diabetes are often not as simple as chemically induced diabetic models. However, they do avoid the potential toxicity caused by chemical treatments, which can alter the pathogenesis of diabetic complications. Together, both chemical and genetic models of type1 diabetes provide researchers with several unique and viable options for the induction of an insulin deficient state.

4.5 Atherosclerosis in Models of Type2 Diabetes

An alarming health issue in recent years is the increase of metabolic syndrome, a cluster of metabolic-related abnormalities that predispose individuals to development of type 2 diabetes (24, 25). The characteristics present in metabolic syndrome include insulin resistance, hypertension, obesity and dyslipidemia: traditional risk factors for atherosclerosis (24). The etiology of both the metabolic syndrome and type 2 diabetes are highly complex. Thus, many studies employing models of type 2 diabetes have focused on investigating the pathophysiological links between specific aspects of the disease and atherosclerosis.

LDLR and ApoE deficient mice have been combined with leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice to produce obesity-induced diabetic models of atherosclerosis (26-29). Both backgrounds markedly increase plasma cholesterol and triglyceride

levels when compared to non-diabetic littermates. However, accelerated atherosclerosis is observed in some, but not all, experiments (26, 27). The reasons for these discrepancies are not clear but may be due to the differences in conditions such as diets used in each experiment. Because atherosclerosis-prone mice such as ApoE-/- and LDLR-/- mice have disturbed lipoprotein metabolism, dietary fat intake has profound effects on their overall energy metabolism. Therefore, it is important to consider fundamental differences in these two models in terms of metabolic consequences. For example, careful observations by Schreyer et al have shown that apoE-/- and LDLR-/- mice respond differently to diets high in fat and sucrose (30). LDLR-/- mice exhibited increased susceptibility to diet-induced obesity, hypertriglyceridemia, leptin resistance, and impaired glucose metabolism. In contrast, apoE-/- mice were resistant to diet-induced hypertriglideridemia or hyperglycemia despite of significant weight gain (30). The absence of apoE in mice is also reported to reduce some of the metabolic complications associated with obesity (31).

Impaired insulin signaling also affects atherosclerosis in mice, and several studies have used partial or complete deficiencies in insulin receptors to re-create the effects of insulin resistance. For example, apoE-/- mice completely lacking insulin receptor substrate protein 2 (IRS2) are hyperinsulinemic and develop significantly increased atherosclerosis despite having comparable glucose and cholesterol levels to apoE-/- mice with wild type IRS2 (32). Notably, a partial loss of IRS2 (IRS2+/-) also results in an acceleration of atherosclerosis in apoE-/- mice, despite indistinguishable levels of plasma lipids compared to control apoE-/- mice with full expression of IRS2 (33). The authors suggest increased macrophage stimulation as a possible explanation for the plasma lipid-independent acceleration of atherosclerosis due to disrupted IRS2 signaling. Similarly, Biddinger et al showed that liver-specific ablation of insulin receptor (IR)

made mice susceptible to diet-induced plaque development (34). However, Ldlr-/- mice lacking insulin receptor in peripheral tissues, but expressing low levels (less than 10 %) in the liver, had improved plasma lipid profiles and reduced atherosclerosis (35). This study stresses the importance of hepatic insulin resistance on the regulation of lipoprotein metabolism and subsequently on the development of atherosclerosis (35). Effects on atherosclerosis due to specific insulin defects in macrophages are controversial. Han et al. showed that high fat fed Ldlr-/- mice transplanted with insulin receptor deficient bone marrow developed larger, more complex atherosclerotic plaques than those mice receiving wild type bone marrow (36). In contrast, myeloid lineage cell-restricted ablation of IRS2 protected apoE-/- mice against atherosclerosis (37). These different outcomes may be explained in part by the varied metabolisms of the two models, and in part by the intricate and multifaceted actions of insulin signaling.

Comparisons of the above studies highlight the metabolic differences often noted between insulin-deficient models of type 1 diabetes versus obesity-induced or insulin-signaling deficient models of type 2 diabetes. Due to the complexity of most models of type 2 diabetes, caution is necessary when interpreting experimental outcomes. However, when taken together, the studies employing various mouse models of type 2 diabetes demonstrate that both obesity and insulin resistance result in adverse metabolic conditions that increase atherogenesis in hyperlipidemic animals.

4.6 Diabetic Dyslipidemia and Atherosclerosis

Type 2 diabetes, as well as poorly managed type1 diabetes, often leads to a cluster of harmful modifications to lipoproteins known as diabetic dyslipidemia (38, 39). Specifically, diabetic patients have elevated very low density lipoprotein (VLDL) triglycerides, a reduction of

high density lipoprotein (HDL) cholesterol, and smaller denser LDL particles (40). Since this lipoprotein profile is a risk factor for atherosclerosis, it has long been debated whether the enhanced atherosclerosis in diabetic mice may largely be a consequence of diabetes-induced hyperlipidemia (41, 42).

In our own experiment, we monitored the plasma lipid levels of diabetic apoE-/- mice fed a diet high in fat but low in cholesterol for 5 months after STZ treatment (43). ApoE-/- mice have a large accumulation of cholesterol-rich VLDL particles in plasma. We found that STZ-induced hyperglycemia did not significantly alter the plasma cholesterol and triglyceride levels of the mice during the first four months following the onset of diabetes. However, plasma cholesterol levels in some of the animals began to rise after four months, and these animals became severely hyperlipidemic. Plasma cholesterol levels in these animals are correlated with the extent of liver damage characteristic of diabetes-associated steatohepatitis. More importantly, the atherosclerotic lesion size in the individual diabetic apoE-/- mice, when evaluated at five months after the onset of diabetes, was not correlated with total plasma cholesterol or glucose levels (43).

Differing from the apoE-/- mice, LDLR-/- mice accumulate LDL-sized particles rather than VLDL particles in the plasma. The plasma total cholesterol levels in the chow-fed LDLR-/- mice are about 200mg/dl, approximately half of those in apoE-/- mice, and they develop atherosclerosis much slower than apoE-/- mice. Consequently, researchers have fed diets containing high fat and modestly increased in cholesterol to LDLR-/- mice in order to accelerate the plaque development. (For example, a diet containing 21% fat and 0.15% cholesterol compared to 4.5% fat and 0.022% cholesterol in normal mouse chow). With this type of diet, Goldberg et al. showed that plasma cholesterol levels in the LDLR-/- mice doubled compared to non-diabetic controls immediately after they were treated with STZ (44). The livers of diabetic LDLR-/- animals demonstrated a

reduced capacity to clear LDL and VLDL particles compared to the livers of non-diabetic animals, demonstrating the impairment of non-LDL receptor mediated clearance of apoB-containing lipoproteins during diabetes. The authors suggest that the accumulation of a subclass of lipoproteins during diabetes, a group that is normally cleared efficiently, may explain the marked elevation of plasma cholesterol noted in STZ-induced diabetic LDLR-/- mice (44).

The differential effects of hyperglycemia and hyperlipidemia on atherosclerosis were examined by Renard et al., in type1 diabetic GP, LDLR-/- mice (23). They observed that when mice were on regular chow diet, although plasma cholesterol levels remained similar to non-diabetic mice, diabetes accelerated arterial macrophage accumulation and the initiation of atherosclerosis. When fed diets containing increased amounts of cholesterol (0.12 and 0.5% cholesterol), however, mice with similar plasma cholesterol levels, whether diabetic or not, developed similar degrees of atherosclerosis (23). The authors suggest that the development of large, advanced atherosclerotic lesions was dependent on dyslipidemia, which diabetic mice were far more susceptible to develop than non-diabetic mice. Similarly, Reaven et al showed that STZ-induced diabetic LDLR-/- mice maintained with low dose insulin had similar plasma cholesterol levels with non-diabetic mice and had similar lesion sizes. These mice were fed a high fat / high cholesterol diet, and all had severe hyperlipidemia (14). Unlike in the type 1 diabetic models described above, lesion size in a model of type 2 diabetic atherosclerosis (ob/ob;LDLR-/-) did not correlate with plasma total cholesterol or triglyceride levels (29).

Mouse models of type 2 diabetes generally develop increased plasma triglycerides and non-esterified fatty acids (NEFA) as discussed above. In addition to its glucose regulatory effects, insulin normally acts to suppress hepatic VLDL secretion from the liver. Hepatic insulin resistance is sufficient to cause dyslipidemia and enhanced atherogenesis through increased and/or

unregulated VLDL secretion, as well as decreased clearance of apoB-containing lipoproteins (34, 35). Thus, mice lacking insulin receptor (IR) in the liver show reduced HDL and increased VLDL and LDL (without affecting plasma total cholesterol), but reduced triglycerides, as a consequence of an increased *Pgc1β* expression and decreased expression of *Ldlr*, *Srebp1c* and *Srbp2* genes (34). After 3 months on an atherogenic diet (15% fat, 1.25% cholesterol and 0.5% sodium cholate) the mice with hepatic insulin resistance raised their plasma total cholesterol levels to about 750mg/dl, and induced substantial plaques in their aorta. As described earlier, Han et al. observed that Ldlr-/- mice expressing low levels of insulin receptor in the liver, but lacking it in peripheral tissues, had reduced VLDL and LDL levels, and reduced atherosclerosis. The authors suggest that a 50% reduction in VLDL and LDL is sufficient to limit atherogenesis, overcoming the pro-atherogenic effects of peripheral insulin resistance in vascular cells (35).

The research highlighted above demonstrates that hyperlipidemia is an essential component of diabetes-induced atherosclerosis. However, exaggerated hyperlipidemia following the induction of diabetes may mask effects specific to hyperglycemia and/or insulin resistance, as well as overwhelm potential therapeutics or preventative measures. Thus, researchers must be cautious when choosing the specific type of diet to be administered to diabetic mice in any given experiment. While it is clear that diabetes-induced hyperlipidemia is a principal contributor to the development of cardiovascular disease, it is not the only contributor. There are also many significant lipid-independent effects on diabetes-induced plaque development, as discussed below.

4.7 Antioxidant Defense in Diabetic Atherosclerosis

Diabetes is usually accompanied by increased production of reactive oxygen species (ROS), free radicals and/or by impaired antioxidant defenses. Thus, increased oxidative stress is

widely accepted as an important player in the development and progression of diabetes and its complications (45). A decrease in intracellular antioxidants such as nitric oxide (NO) and various antioxidant enzymes may also affect direct trapping of free radicals, with a consequent increase in ROS and oxidative stress. Strong evidence also exists that oxidation of lipids, in particular LDL, is one of the most important factors for the initiation of atherosclerosis (46). Furthermore, systemic oxidative stress can lead to acute phase responses including hypercoagulability and plaque rupture. These considerations have provided the rationale for the use of antioxidants to prevent lipid peroxidation and the early onset of atherosclerosis, and as therapies for coronary artery disease (47). However, evidence linking antioxidant vitamins to diabetic complications in humans is still largely circumstantial. For example, although treatment with a high-dose of vitamin E (1800 IU / day) appeared to be effective in normalizing retinal hemodynamic abnormalities and improving renal function in type 1 diabetes in a small randomized trial (48), vitamin C at a dose of 400IU/day had no effect on cardiovascular outcomes or nephropathy in high-risk patients with diabetes in the HOPE study and MICRO-HOPE sub-study (49).

Dietary supplementation of vitamin E, a natural anti-oxidant, decreased the amount of macrophages and vascular fatty streaks, as well as lowered the mortality rate, in STZ-induced diabetic Balb/c mice fed an atherogenic diet (50). However, Balb/c mice are resistant to diet-induced atherosclerosis (51), and protective effects of vitamin on diabetes-induced fully developed have not been examined. However, Hasty et al. showed that this protective effect was absent when observing the obese, hyperlipidemic LDLR-/-;ob/ob mouse model, as dietary supplementation of Vitamin E failed to reduce oxidative stress or atherosclerotic lesion area (26).

Reduced plasma vitamin C levels have been associated with diabetes in humans (52-54). However, mice, unlike humans, synthesize ascorbic acid and hence are not dependent on dietary

vitamin C. To examine the effects of sub-optimal vitamin C intake, our lab created apoE-/- mice that are deficient of L-glonolactone synthase, the key enzyme for ascorbic acid synthesis, and tested the effect of reduced plasma vitamin C (ascorbic acid) levels on plaque development (55). Mice were made diabetic and maintained on low (66mg/l) or high (660mg/l) levels of ascorbic acid supplemented in their drinking water. We found that vitamin C levels in both plasma and liver of mice supplemented with low vitamin C were less than 20% of those in diabetic mice given high vitamin C supplementation (as well wild type mice). However, the atherosclerotic plaque size did not differ between the two groups. Thus, it appears that the level of Vitamin C reduction does not have any effects on diabetes-induced atherosclerosis.

In contrast, a dietary supplementation of α-lipoic acid (LA), another natural antioxidant, completely prevented the diabetes-associated increase of the atherosclerotic lesion size in diabetic apoE-/- mice fed a high fat – low cholesterol diet (43). LA markedly decreased systemic oxidative stress, suggesting that a protection of lipoprotein from oxidative modification by LA probably is an important factor for reduced atherosclerotic lesion development. However, LA treated diabetic mice also had lesser diabetes-induced body weight reduction, milder dehydration, protection from hepatitis, and an accelerated recovery of pancreatic β-cells which lead to a small, but significant, reduction of hyperglycemia over time compared to non-treated diabetic mice. Thus, an improvement in general health likely contributes to the protection from atherosclerosis enhancement in LA-treated diabetic mice. Of note, we as well as others, observed that dietary α-lipoic acid also decreases atherosclerotic lesions in non-diabetic apoE mice without affecting their plasma cholesterol levels (56). The reduction (10-20%) is small, but suggests that α-lipoic acid may have additional athero-protective roles beyond the reduction of hyperglycemia-induced ROS.

The role played by genetically determined differences in the endogenous antioxidant enzymes in affecting oxidative stress and the development of complications in diabetes was also addressed by de Haan and his colleagues. The authors showed that a deficiency of glutathione peroxidase-1 (GPx-1) caused a 2 fold increase in aortic lesions in diabetic apoE-/- mice (57). This atherosclerotic effect occurred despite the fact that lack of Gpx-1 did not affect plasma lipid profiles. The study demonstrates that an intracellular ROS-removing antioxidant enzyme GPx1 plays important roles in regulating atherogenic processes within a diabetic milieu. Recently, the group also showed that oral administration of ebselen, a GPx1-mimetic and antioxidant, significantly reduced aortic lesions in diabetic apoE-/- mice (58). The reduction was site-specific, and the plaque size in the aortic root was not changed. Experiments using cultured endothelial cells suggest that one of the key mechanisms whereby ebselen confers its antiatherogenic effect is through modulation of inflammatory factors by inhibiting the activation of nuclear factor kappa B (NF-κB).

Aldose reductase (AR) catalyzes the reduction of glucose to sorbitol, one of several pathways thought to accelerate diabetic complications via production of excess ROS. This enzyme is expressed at much lower levels in mice compared to humans. To test the hypothesis that greater expression of a gene involved in the toxic metabolism of glucose would enhance diabetic induced atherosclerosis, Vikramadithyan et al made diabetic LDLR-/- mice that over-express human AR (hAR) (59). They found that the over-expression of AR increased aortic lesion size in diabetic LDLR-/- mice without affecting the plasma cholesterol levels. Accumulation of modified lipoproteins was increased in macrophages of the hAR-expressing mice, and expression of enzymes that regulate regeneration of glutathione was reduced in the aortas. The authors have

suggested that the inhibitors of AR could, therefore, be useful in the treatment of diabetic atherosclerosis.

These studies demonstrate that reducing the excess ROS production caused by diabetes has a significant protective effect on the progression of atherosclerosis. However, effects of individual antioxidants vary greatly, and suggest that targeting specific cellular oxidative pathways may be more effective than a global supplement-based reduction in oxidative stress.

4.8 Other Modulations of Diabetic Atherosclerosis

Important by-products of hyperglycemia are advanced glycation end products (AGEs), which result from the nonezymatic glycation of circulating proteins. Binding of AGEs to their receptor (RAGE) induces several inflammatory markers and accelerates vascular lesion development (60). Park et al. showed the therapeutic potential of soluble RAGE (sRAGE) to inhibit the activation of RAGE pathway in diabetic apoE-/- mice, where sRAGE administration completely prevented diabetes-associated increase in atherosclerosis independent of both plasma glucose and lipids (61). In addition to preventing the early stage acceleration of atherosclerosis as a result of diabetes, RAGE suppression also worked to stabilize existing plaques by lowering inflammatory markers and decreasing macrophage and smooth muscle cell (SMC) activation in existing atherosclerotic lesions (62). These reductions in diabetes-associated atherosclerosis highlight the beneficial effects of attenuating AGE and RAGE accumulation, and subsequent inflammatory response, in the atherosclerotic plaque development.

In addition to its vasoconstrictive effects, enhanced activity of the renin-angiontensin system (RAS) may be a detrimental link between several atherosclerotic risk factors, contributing to an increased production of ROS, insulin resistance, and further endothelial dysfunction.

Blockade of the RAS by angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) has been tested in mouse models of diabetic atherosclerosis. For example, Candido et al. showed that the induction of diabetes triggers an increase in the expression of angiotensin-converting enzyme (ACE) in the aorta of STZ treated apoE-/- mice. The treatment with an ACE inhibitor, perindopril, inhibited this diabetes-induced increase of aortic ACE expression and completely inhibited atherosclerotic lesion development, despite only modestly decreasing systolic blood pressure (SBP) (7-8 mm Hg) (63). A similar result was noted when diabetic mice were treated with the angiotensin II type 1 (AT1) receptor blocker, Irbesartan, which decreased collagen content, cellular proliferation, macrophage infiltration, lowered expression of several markers of inflammation, and attenuated atherosclerotic plaque development. Despite lowering the SBP of diabetic mice to a similar degree as Irbestan (7 and 8 mm Hg, respectively), a calcium channel blocker, amlopidine, did not halt the acceleration of atherosclerosis as did Irbesartan (64). Thus, the attenuation of atherosclerosis was specific to the inhibiton of AT1 and not solely a result of lowering SBP.

Finally, links between hyperglycemia, inflammation, and vascular thrombotic complications in diabetic atherosclerosis have been examined. Zuccollo et al demonstrated the atherosclerosis inhibiting effects of S18886, a thromboxane A2 receptor (TP) antagonist. S18886 significantly protected from diabetic lesion progression in STZ-treated apoE-/- mice. It also prevented the decrease in endothelial nitric oxide synthase (eNOS) expression and the increases in several markers of inflammation that normally occur after the induction of diabetes (65). In addition, the role of platelet-derived growth factor in the development of diabetes-induced atherosclerosis was examined by Lassila et al. They showed that tyrosine kinase inhibition by

imatinib lowered the expression of inflammatory cytokines in the aorta and slowed the progression of atherosclerosis in diabetic ApoE-/- mice.

Jointly, these experiments suggest that modulation of vascular function in diabetic animals has generally beneficial effects, and is protective from the acceleration of diabetes-induced atherosclerosis.

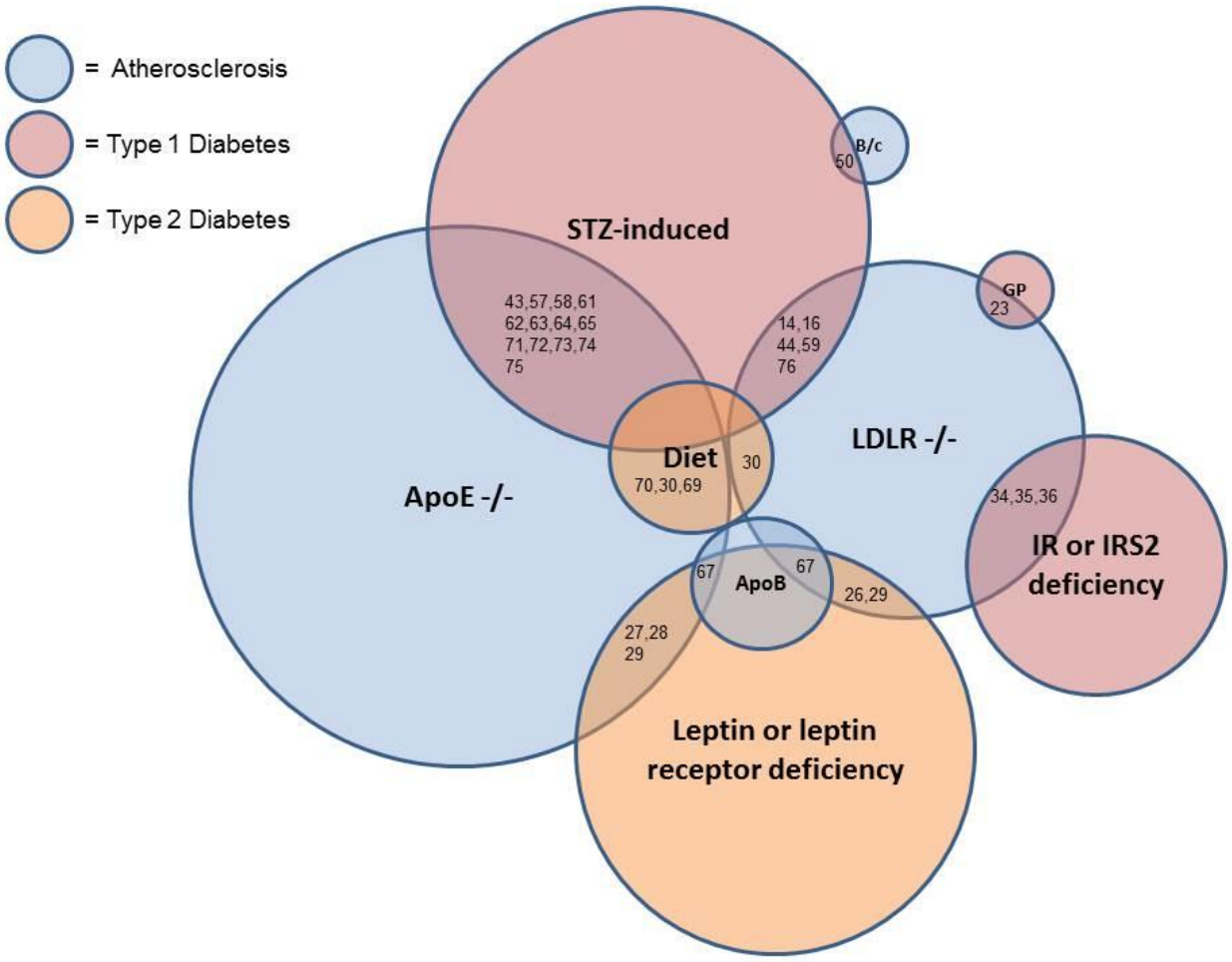

Figure 4.2. **Commonly used mouse models of diabetic atherosclerosis.** Each circle represents a distinct mouse model of Atherosclerosis (Blue), Type 1 Diabetes (Red), or Type 2 diabetes (Orange). Size of each circle is a general reference to the number of studies employing each model. Areas of overlap represent combinations/crosses of these various models (selected studies) and include reference number. Apolipoprotein E (ApoE), Apolipoprotein B (ApoB), Balb/c (B/c), Insulin Receptor (IR), Insulin Receptor Substrate 2 (IRS2), Low density lipoprotein receptor (LDLR), streptozotocin (STZ).

4.9 Expert Commentary

The global epidemic of obesity and metabolic syndrome means millions of additional patients will soon find themselves with a severely increased risk of developing cardiovascular disease (66). This rapidly expanding health burden has created a pressing need for further exploration of the biological mechanisms of diabetic atherosclerosis, and has placed a tremendous value on the discovery of new therapies. Because the pathophysiology of metabolic syndrome and type 2 diabetes is far more complex than that of type 1 diabetes, there are limitations in the use of atherosclerosis prone mouse models to dissect the underlying mechanisms of how the diabetic condition enhances cardiovascular complications.

To study the pathogenesis of diabetic atherosclerosis, and to correctly translate the findings to human disease, further development of models that accurately recapitulate the human diabetic condition are needed. They include models of type 2 diabetes that account for important differences in human versus mouse physiology, as well as models that demonstrate reduced plaque stability, increased thrombosis, and common human vascular events such as myocardial infarction and stroke.

In this regard, the ApoE triple knockout (ApoE-/-;ApoB100/100; ob/ob) and the LDLR triple knockout (LDLR-/-;ApoB100/100;ob/ob) models of metabolic syndrome are worth mentioning since both strains, on normal mouse chow, develop obesity, hyperinsulinemia, hyperlipidemia, hypertension, and accelerated atherosclerosis - the components of the metabolic syndrome (67). The Apob100/100 homozygous mutation in these mice results in a plasma accumulation of atherogenic lipoprotein particles containing apolipoprotein B-100, which is more similar to human lipoprotein profiles. Despite the complexity of these models, both recapitulate

the cluster of disorders central to the metabolic syndrome, and might prove to be useful tools in the search for potential therapies for this uniquely human condition.

One of the inherent limitations of the commonly used models of atherosclerosis is that both the apoE and Ldlr proteins, integral components in the pathophysiologic process of atherosclerosis, are lacking. Models in which these proteins, and other essential factors, are present but functioning sub-optimally may provide more accurate insights and better translate to the human condition. For example, our laboratory has begun to explore the progression of atherosclerosis in mice that carry two copies of the human apolipoprotein E isoforms as well as express human Ldlr (68). These mice develop significant atherosclerotic plaques and demonstrate diabetes-induced increases in aortic lesions when made diabetic by acquiring the Akita mutation. Most importantly, these mice retain all the critical proteins - mainly apoE, LDLR, and insulin receptor - involved in lipoprotein metabolism, in contrast to other athero-prone models that lack one or more of these components.

4.10 Five Year View

Further studies focusing on diabetic atherosclerosis will not only help to determine the underlying factors that are important in the pathogenesis of cardiovascular disease in the setting of diabetes, but may also reveal therapeutic avenues and preventative measures. Creative use of existing mouse models and the generation of new models, particularly those that sufficiently address the human condition, will provide an invaluable platform to examine the specific mechanisms by which diabetes exerts its effect on the vasculature and to test the potential of new therapies and treatments.

Table 4.1 Key Issues. Macrovascular complications of diabetes.

Key Issues
• Diabetes accelerates atherosclerosis and its cardiovascular complications. Atherosclerosis prone mice (ApoE-/- and LDLR-/-) have been used extensively to examine the mechanisms by which hyperglycemia and/or defective insulin actions advance macrovascular disease. • The most commonly used mouse models of diabetic atherosclerosis are STZ-treated ApoE-/- and LDLR-/- mice. • Diabetes generally increases atherosclerotic plaque size in athero-prone mice. The atherosclerosis model employed, age of onset and duration of diabetes, as well as diet, affect lesion progression. • Diabetes induces hyperlipidemia, which is exaggerated by high fat/cholesterol diets, is required for the development of advanced atherosclerotic plaques in mice. Other important lipid-independent atherogenic effects could be masked by this severe dyslipidemia. • Lipid-independent effects of diabetes demonstrated in mouse models of diabetic atherosclerosis include oxidative stress, glucose toxicity, inflammation, endothelial dysfunction, coagulopathy, and dysfunction of the renin-angiotensin system. • Targeted intervention of these processes in mice have produced promising approaches towards the amelioration and prevention of diabetic atherosclerosis. • More “human-like” mouse models are needed that accurately replicate human lipoprotein and glucose metabolism, as well as those that demonstrate the clinical complications of atherosclerosis (decreased plaque stability and rupture).

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Chapter 5

CONCLUSIONS AND SIGNIFICANCE

5.1 ApoE in Diabetic Dyslipidemia: Hyperlipidemia

In Chapter 2, I described the differential roles of apolipoprotein E (apoE) isoforms in modulating diabetic dyslipidemia – a potential cause of the increased cardiovascular disease risk of patients with diabetes. Using LDLR deficient mice crossed with the human apoE mouse models described earlier and employing a chemically induced model of type 1 diabetes, I observed several important phenomena.

First, non-diabetic E3LDLR-/- and E4LDLR-/- mice had similar plasma glucose and lipids, lipoprotein distribution profiles and indistinguishable degrees of atherosclerosis. However, after the induction of diabetes, E4LDLR-/- mice, but not E3LDLR-/-, develop enhanced dyslipidemia, characterized by elevated VLDL TG and LDL cholesterol, delayed clearance of post-prandial triglycerides, and an increased rate of VLDL secretion (Figure 5.1). The increase in plasma VLDL and LDL cholesterol was not a result of a difference between E3 and E4 lipoprotein composition, hepatic receptor expression, or VLDL/LDL clearance rates. Instead, the primary difference noted between the E3 and E4 expressing mice was E4-specific increase in hepatic VLDL secretion. Using *in vivo* techniques as well as cultured primary E3 and E4 hepatocytes as an *in vitro* model system, I determined that the increase in VLDL secretion was a result of larger hepatic fat stores in the E4 mice.

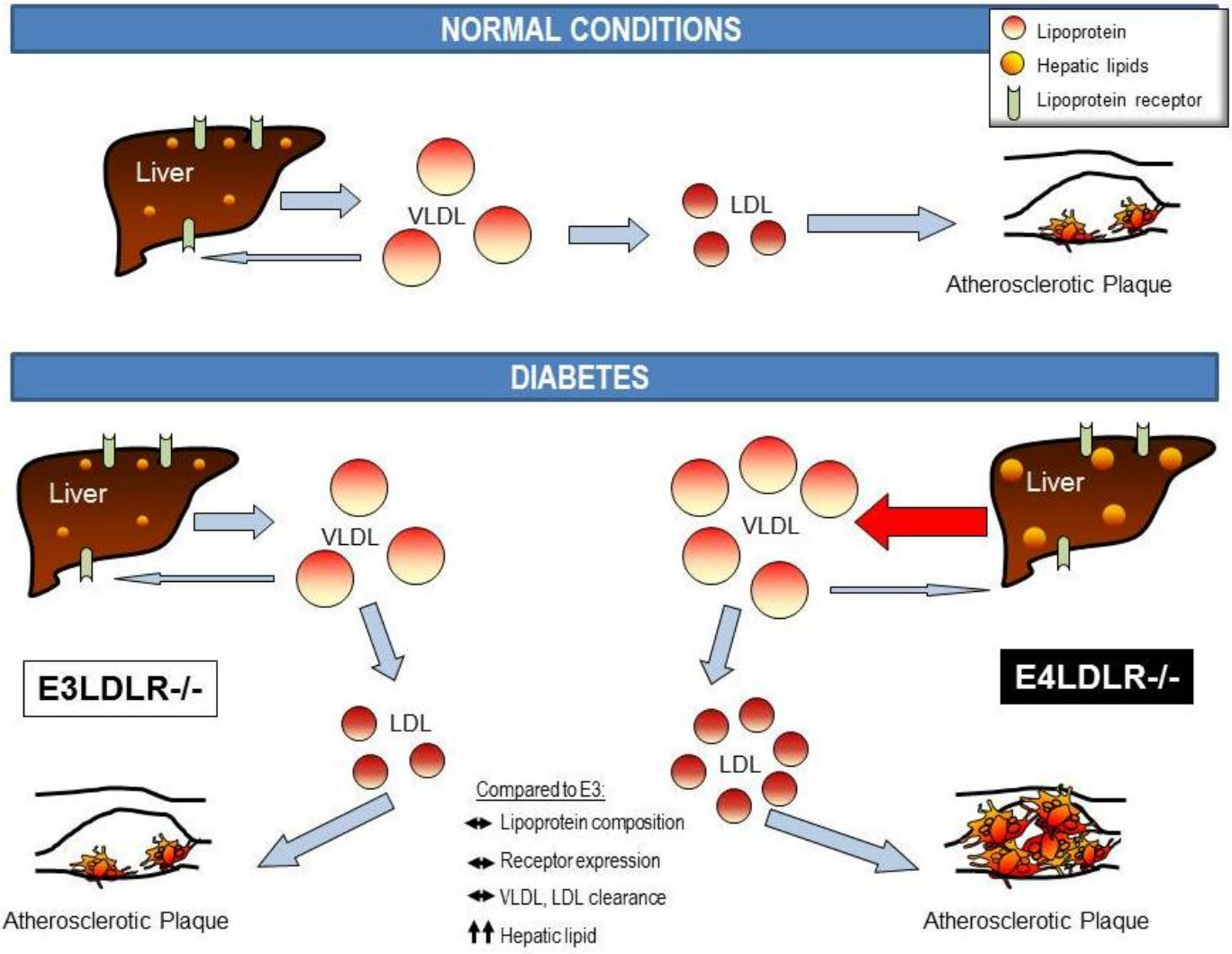

Figure 5.1. **Severe dyslipidemia and atherosclerosis in diabetic E4LDLR-/- mice.** Induction of diabetes leads to an increase in hepatic VLDL secretion (red arrow) in E4LDLR-/- mice. The increase in VLDL secretion leads to an accumulation of plasma VLDL (large gray circles). As circulating VLDL is lipolyzed it becomes the denser, cholesterol-rich LDL particles (small gray circles). LDL contributes to the development of the atherosclerosis. Thus, the diabetes-induced increase in LDL in the diabetic E4LDLR-/- mice leads to larger atherosclerotic plaques.

The severe dyslipidemia in the diabetic E4LDLR-/- mice is associated with a calorimetric profile indicative of lower lipid utilization, suggesting that the accumulation of hepatic fat stores in these mice could be a result of a decrease in lipid burning. When primary hepatocytes isolated from E4LDLR-/- mice were cultured in high glucose, they accumulated more intracellular lipid. This accumulation was not a result of a difference in fatty acid uptake, glucose uptake, de novo lipogenesis, or glucose oxidation between the E3 and E4 expressing hepatocytes. However, there was a striking reduction in fatty acid oxidation in the E4 hepatocytes only when exposed to the hyperglycemic conditions (Figure 5.2). The reduction in fatty acid oxidation in the E4 expressing livers was associated with a significant shift in oxidative signaling pathways. Diabetic E4LDLR-/- mice had a reduced phospho-ACC / ACC ratio, which is indicative of increased lipogenesis and decreased fatty acid oxidation. E3LDLR-/- mice showed a diabetes-induced decrease in FASN gene expression as expected. However, diabetic E4LDLR-/- mice maintained high levels of FASN expression before and after the induction of diabetes. Thus, lipid accumulates in the E4LDLR-/- livers during diabetes via two metabolic pathways: high, unresponsive levels of fatty acid synthesis and a significant reduction in fatty acid oxidation.

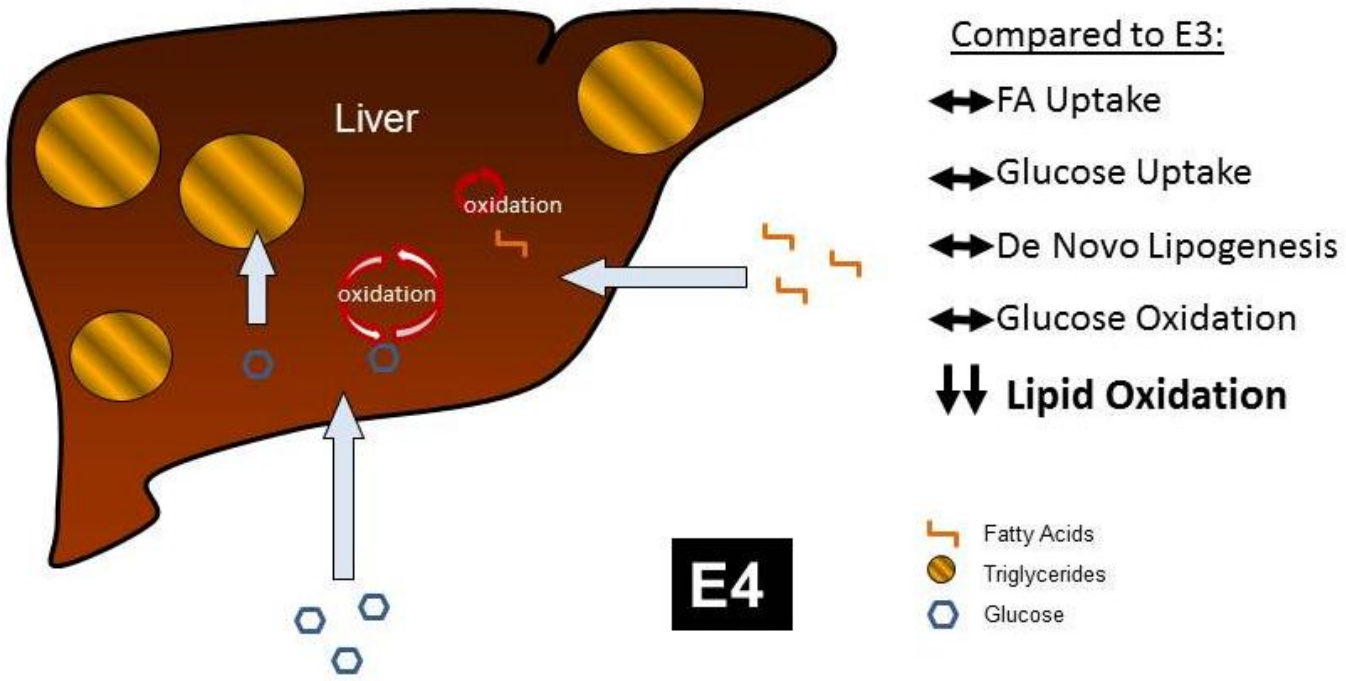

Figure 5.2. **Mechanism of hepatic lipid accumulation in diabetic E4LDLR-/- mice.** Reduced fatty acid oxidation in E4LDLR-/- hepatocytes contributes to the accumulation of hepatic lipid. Ldlr-/- hepatocytes expressing apoE4 accumulated twice as much lipid as those expressing E3 when cultured in a high glucose environment. Metabolic studies involving C^{14} labeled glucose and fatty acids demonstrated that this accumulation was not due to an increase in fatty acid uptake, glucose uptake and de novo lipogenesis, nor a decrease in glucose oxidation. Instead, the E4 hepatocytes accumulated lipid due to a 40% reduction in rates of fatty acid oxidation.

In summary, I showed that dyslipidemia and atherosclerosis were greatly exaggerated in diabetic LDLR-/- mice expressing human apoE4 (E4LDLR-/-) compared to those with human apoE3 (E3LDLR-/-), despite a similar degree of hyperglycemia. Diabetes increased VLDL triglycerides and LDL cholesterol in E4LDLR-/- mice twice as much as in E3LDLR-/- mice, and diabetic E4LDLR-/- mice had delayed clearance of post-prandial triglycerides, larger hepatic fat stores, and increased VLDL secretion. Diabetic E4LDLR-/- mice demonstrated a decreased reliance on lipid as an energy source based on indirect calorimetry and had a hepatic metabolic profile indicative of reduced fatty acid oxidation and increased fatty acid synthesis.

Strikingly, the presence of the E4 allele resulted in severe dyslipidemia and atherosclerosis independent of its interaction with LDLR. This E4-specific aggravation of diabetic dyslipidemia is central to the liver and appears to be a result of a novel role of apoE in the regulation of hepatic lipid metabolism. That is, ApoE4 expressing livers had reduced fatty acid oxidation, which contributed to the accumulation of tissue and plasma lipids and subsequent atherosclerosis.

5.2 ApoE in Diabetic Dyslipidemia: Normolipidemia

In Chapter 3, I investigated a similar issue as in Chapter 2, this time taking a very different approach. Instead of using a hyperlipidemic mouse model like the LDLR-/-, I employed mice that overexpressed the human LDLR gene, resulting in a model with very low plasma cholesterol. Previous work in our laboratory has shown the importance of the apoE-LDLR interaction in terms of regulating lipoprotein metabolism and the development of atherosclerosis. By crossing human apoE mice that overexpress the LDLR to strains carrying the type 1 diabetes 'Akita' mutation, I was able to examine the role of apoE in diabetic dyslipidemia in a more physiologically relevant (normolipidemic) setting.

Additional factors contribute to the increased CVD risk for patients with diabetes beyond the diabetic dyslipidemia described in earlier chapters. These include factors such as inflammation, endothelial dysfunction, hypertension, and a pro-thrombotic state (1). However, due to the limitations of currently used disease models, past research has often failed to adequately separate the effects of these important factors from the overwhelming dyslipidemic response that occurs in response to diabetes in these models (2). The main hurdles for researchers studying vascular pathologies during diabetes have been an inability to consistently demonstrate a diabetes-induced increase in atherosclerosis, and in the cases where they are able, difficulties distinguishing between lipid-dependent and lipid-independent effects of diabetes on atherosclerosis (2-3). Moreover, the mouse models of atherosclerosis currently available all have plasma cholesterol levels that far exceed the physiological range in human patients. Therefore, an important goal has long been the generation of a model of diabetic atherosclerosis in which the CVD inducing effects of diabetes are not obscured by severe hyperlipidemia.

In Chapter 3, I introduced a new mouse model of atherosclerosis that clears these hurdles, allowing researchers for the first time to study the atherogenic etiology of diabetes in a metabolic setting that accurately reflects the human condition. The diabetic E4h mouse develops spontaneous atherosclerotic plaques in spite of low total plasma cholesterol levels (<175 mg/dl). The main advantages of this new model are outlined in Table 5.1 and include: 1) consistent development of atherosclerotic plaques induced by diabetes, 2) no toxic side effects of chemical induction of diabetes, 3) all the components of lipoprotein metabolism are present (and "humanized"), 4) no additional high fat or high cholesterol diet is necessary to induce atherosclerosis, and 5) atherosclerosis occurs in the absence of hyperlipidemia (Table 5.1).

Athero Model	Diabetes induced by:	ApoE preent?	LDLR present?	Does diabetes increase athero?	Cholesterol (mg/dl)	Ref.
ApoE-/-	STZ	no	yes	yes	500 - 1,300	# 17-19
ApoE-/-	leptin def.	no	yes	yes	580	# 20
Ldlr-/-	STZ	yes	no	no	950 - 1000	# 21
Ldlr-/-	leptin def.	yes	no	yes	1,700	# 22
E4h	**Akita**	**yes, human**	**yes, human**	**yes**	**< 120**	unpub.

Table 5.1. **Advantages and disadvantages of common models of diabetic atherosclerosis.** Six of the most commonly used models of diabetic atherosclerosis and the E4h model are compared in regards to several important factors. Abbreviations: apolipoprotein E deficiency (apoE-/-), $Ins2^{+/-}$ Akita mutation (Akita), high fat diet (HFD), low density lipoprotein receptor deficiency (Ldlr-/-), leptin deficiency (db/db and ob/ob models) (leptin def.), streptozotocin (STZ), unpub (unpublished data).

In addition to providing a new model of diabetic atherosclerosis, this study was able to address the importance of the apoE-LDLR interaction during diabetes. While increasing LDLR expression in the presence of apoE3 during diabetes had no detrimental effects, doing so in the presence of apoE4 resulted in a significant increase in VLDL and decrease in HDL cholesterol – two harmful changes to lipoprotein metabolism that are commonly associated with diabetes in human patients. In essence, the effects of diabetes on dyslipidemia were amplified in mice expressing apoE4 (Figure 5.4).

Diabetes had no effect on total plasma cholesterol in E3 and E3h mice, but induced a 20 and 50% increase in the E4 and E4h mice, respectively. Still, average total plasma cholesterol in all the diabetic groups remained <120 mg/dl, compared to average total cholesterols ranging from 300-1700 mg/dl in other common models of diabetic atherosclerosis. The cause of atherogenesis in the diabetic E4h mice instead arose from the specific types of lipoproteins that accumulated in these mice and in particular the cholesterol distribution among them. The accumulation of cholesterol-rich VLDL and VLDL remnant particles in the diabetic E4h appears to be a result of an increase in hepatic VLDL secretion. Previous work has implicated both apoE and LDLR in the process of apoB lipidation and VLDL secretion (4). However, the impact of overexpressing the LDLR on VLDL secretion during diabetes had not been examined. Interestingly, overexpressing the LDLR in E4 mice resulted in the secretion of primarily apoB48-containing, triglyceride-poor, cholesterol-rich VLDL (Figure 5.5).

In summary, this study highlights the importance of adequately regulating VLDL secretion during diabetes and the significance of the quality (VLDL/LDL versus HDL, protein and lipid composition of various lipoprotein fractions and the distribution of cholesterol among the various fractions) versus solely quantity of lipoproteins.

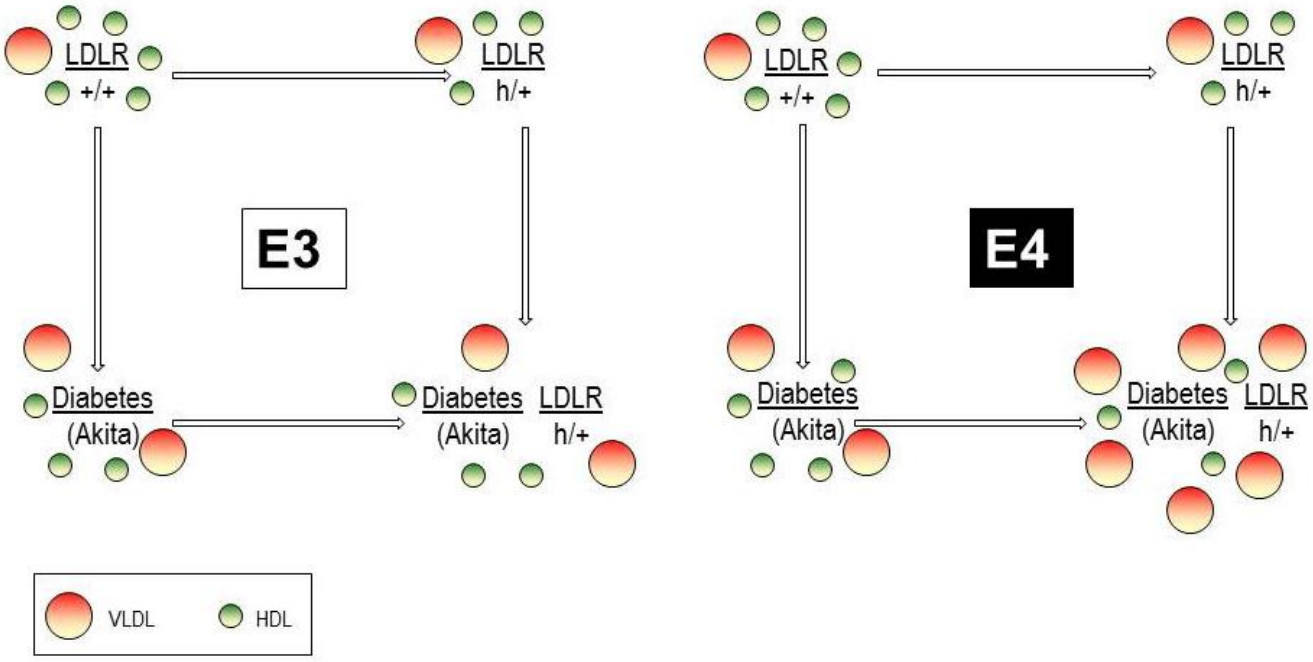

Figure 5.3. **Summary of plasma lipids in non-diabetic and diabetic E3 and E4 models.** Arrows moving from left to right indicate an increase in LDLR expression as a result of the h/+ genetic manipulation. Arrows moving from top to bottom indicate the induction of diabetes as a result of the $Ins2^{+/-}$ "Akita" mutation. Diabetes induces an increase in VLDL cholesterol in the presence of E3 and E4. Likewise, an increase in LDLR reduces HDL cholesterol in both E3 and E4 expressing mice. However, atherosclerosis occurs only in the presence of both severely elevated VLDL cholesterol and reduced HDL cholesterol, as is the case in the diabetic E4 h/+ mice.

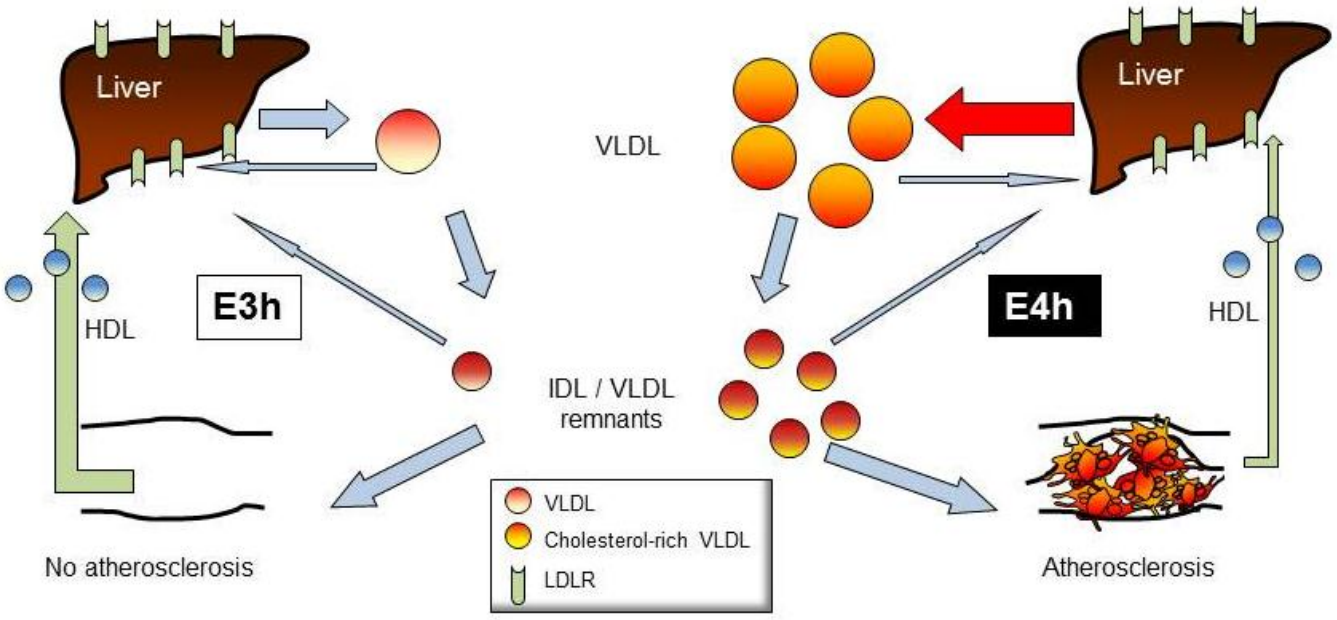

Figure 5.4. **Proposed mechanism of cholesterol accumulation in diabetic E4h mice.** Diabetic E4h secrete more cholesterol-rich VLDL compared to diabetic E3h mice. Combined with their low HDL cholesterol levels, and thus likely impaired reverse cholesterol transport, the diabetic E4h mice develop atherosclerosis.

5.3 Tying it all together: Diabetic Atherosclerosis and the Role of ApoE

In order to effectively study the process of diabetic atherosclerosis and more importantly in order to appropriately translate the implications to the human condition, models that consistently and accurately reflect diabetes and atherosclerosis in human patients are needed. In order to address this issue while examining the role of apoE in diabetic dyslipidemia and atherosclerosis, I employed the 'humanized' E3LDLR-/- and E4LDLR-/- mice described in Chapter 2. Using these models, I demonstrated a dramatic exacerbation of diabetes-induced hyperlipidemia in the presence of the E4 allele.

However, as described in Chapter 4, one of the major hurdles for researchers has been the confounding issue of diabetes-induced hyperlipidemia and the consequent difficulty in separating its effects on the progression of atherosclerosis from other lipid-independent factors. Thus, while the LDLR deficient mice employed in the study described in Chapter 2 provided an important new look at the role of apoE in diabetic dyslipidemia, it is difficult to completely reconcile the data with the human condition due to the hyperlipidemia associated with the LDLR-/- model. In Chapter 3, I introduced a new mouse model of diabetic atherosclerosis that consistently develops atherosclerosis despite low plasma cholesterol, thereby circumventing this difficult issue. To our knowledge, this is the first mouse model of diabetic atherosclerosis with normal plasma lipids, providing researchers with an important new tool to begin to discriminate between diabetes-induced hyperlipidemia and lipid-independent effects of diabetes. In addition, this new model may allow for simpler study of other complications of diabetes such as diabetic nephropathy or diabetic retinopathy. Interestingly, I observed a diabetes-induced development of atherosclerosis in the presence of apoE4, but not apoE3. As noted above, this was due to a distinct, apoE4-specific shift

in the type and composition of lipoproteins circulating during diabetes. Importantly, I demonstrated that the presence of apoE4 led to more severe diabetic dyslipidemia at both ends of the LDLR spectrum; LDLR deficiency in Chapter 2 and increased LDLR expression in Chapter 3. Together, this work not only highlights the dramatic effects of even subtle changes in lipoprotein distributions during diabetes, but also underscores the essential role of apoE, and the importance of APOE genotype, on the pathogenesis of diabetic dyslipidemia and atherosclerosis.

The studies described here raise important questions about the role of inuslin in regulating these apoE4-specific effects. Are the effects described in these studies a result of insulin deficiency, insulin resistance and defects in insulin signaling, or hyperglycemia alone? Epidemiologic studies have demonstrated a clear association between APOE genotype and glucose metabolism (5-12). Previous studies in our lab also strongly suggest that apoE4 is associated with obesity and insulin resistance (13-14). Therefore, future studies aimed at further dissecting the specific metabolic pathways by which apoE4 exerts its detrimental effects will be of great importance. Some such investigations are already underway in our laboratory. For instance, I have co-authored two papers in which our laboratory demonstrated that apoE4 mice fed a Western type diet are more prone to become insulin resistant because they have a reduced ability to store fat in adipose tissue depots compared to apoE3 mice (13-14). Reduced functionality of adipose tissue during a metabolic disturbance such as a high fat diet is rooted in an impaired activation of the adipogenic genetic program. Although the mechanism underlying this phenomenon has yet to be fully determined, reduced insulin sensitivity in the presence of apoE4 could have a significant impact on lipid metabolism. This is particularly of interest when considering hypoinsulinemic conditions such as the genetic (Akita) and chemical (STZ) models of type 1 diabetes employed in Chapters 2 and 3.

As diabetes reaches epidemic proportions across the world, millions of people will face dramatically increased risk of developing cardiovascular disease (15-16). Diabetic dyslipidemia stands out as a potential cause of this increased risk of diabetic patients. I have shown here that ApoE, an important mediator of normal lipoprotein metabolism and atherosclerosis, plays a role in modulating diabetic dyslipidemia and diabetic atherosclerosis as well. Already at an increased risk of developing CVD, approximately 60 million Americans carrying the E4 allele may experience more severe diabetic dyslipidemia due to an E4 specific shift in metabolic homeostasis. Taken together, the studies described here suggest a mechanism by which ApoE modulates both glucose and lipid metabolism during diabetes, thereby effecting the progression of atherosclerosis. In the metabolic processes of glucose and lipoprotein regulation, and specifically in diabetic dyslipidemia where the two substantially overlap, the role of ApoE has broad and significant implications for a wide spectrum of disease states.

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C h a p t e r 6

(S u p p l e m e n t a l)

ABSENCE OF HYPERLIPIDEMIA IN LDL RECEPTOR-DEFICIENT MICE HAVING APOLIPOPROTEIN B100 WITHOUT THE PUTATIVE RECEPTOR-BINDING SEQUENCES

(This chapter consists of material from a manuscript reprinted with permission from *Artierosclerosis Thrombosis and Vascular Biology*; 2008 Oct;28(10):1745-52; titled "*Absence of hyperlipidemia in LDL receptor-deficient mice having apolipoprotein B100 without the putative receptor-binding sequences*" by **Lance A Johnson**, Michael K Altenburg, Rosemary L Walzem, Lori T Scanga and Nobuyo Maeda)

6.1 Summary

The objective of this study is to examine the effects of apoB100 structure, specifically a mutation in the LDLr binding region, on the production of LDL and development of atherosclerosis in vivo. *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice lacking the LDLR and apoB editing enzyme accumulated LDL in plasma and developed severe atherosclerosis when they had wild-type apoB100. In marked contrast, in *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice carrying the Apob100-β mutation, in the 2 putative LDLR-binding domains of apoB prevented both LDL accumulation and atherosclerosis. Intestinal absorption of lipids and triglyceride secretion from the liver were not affected. However, the VLDL particles with apoB100-β were larger in volume by about 70%, and carried approximately four times as much apoE per particle. ApoB100-β synthesis rate in the primary hepatocytes was normal, but its intracellular degradation was enhanced. Additionally, mutant apoB100 VLDL cleared from the circulation more quickly in vivo through apoE-LRP-mediated mechanism than VLDL with wild-type apoB100. In contrast, uptake of the 2 VLDL by macrophages were not different.

While conformational change to apoB100 during conversion of VLDL to LDL exposes LDLR binding domains and facilitates LDLR-mediated lipoprotein clearance, it may also inhibit LRP-mediated VLDL uptake and contribute to LDL accumulation in familial hypercholesterolemia. Mice that lack the LDLR and ApoB editing enzyme (Ldlr$^{-/-}$ *Apobec1*$^{-/-}$) accumulate LDL and develop severe atherosclerosis. Conversely, *Ldlr*$^{-/-}$ *Apobec1*$^{-/-}$ mice carrying Apob100-β with altered sequences in the putative LDLR-binding domains of apoB neither accumulate LDL nor develop atherosclerosis. This finding highlights a potential therapeutic target for patients with familial hypercholesterolemia.

6.2 Introduction

Apolipoprotein (apo) B is an essential component of VLDL, LDL, and chylomicrons. ApoB normally exists in 2 forms, apoB100 and apoB48; both are the products of the same gene. ApoB100 comprises 4536 amino acids, synthesized in the liver, and secreted into the circulation as a structural component of VLDL. ApoB48 is 48% of full-length apoB, and is formed as a result of posttranslational editing of *ApoB* mRNA by the apoB editing complex (apoBEC), which changes Gln at codon 2153 to a stop codon (1). ApoB48 is synthesized in the small intestine and is required for the packaging of lipids into chylomicrons. Whereas human liver makes exclusively apoB100, a large proportion of message in the mouse liver is edited and consequently mice produce both apoB48 and apoB100 from the liver (2).

In addition to maintaining the structural integrity of lipoprotein particles, apoB100 also functions as a ligand for the LDLR and is therefore a primary determinant of circulating LDL cholesterol levels. The LDLR-binding domain of apoB100 has not been fully defined; however, biochemical, immunochemical, and genetic evidence suggests that it is a region of net positive change located in the carboxyl-terminal portion of apoB100. Two sequences, residues 3147 to 3157 and 3359 to 3367, are enriched in basic amino acid residues and have been proposed as putative LDLR-binding domains in both species (3). The sequence at 3359 to 3367 is highly conserved among mammalian species and is also similar to the LDLR-binding site of apoE. Also, Boren et al showed that the removal of positive charges from residues 3359 to 3367 by site-directed mutagenesis renders the LDL containing the modified apoB defective in LDLR binding (3).

To define the regions of apoB that bind the LDLR, we previously introduced mutations into the mouse *Apob* gene (4). The apoB100-β protein is the same length as apoB100 but contains 2 peptide sequences for human β-globin in place of the residues 3147 to 3157 and 3359 to 3367. The modification also drastically reduced the net positive charges and amphipathic helicies of the 2 domains. We expected that the mice producing apoB100-β would model defective apolipoprotein B100 in humans by accumulating binding-defective LDL in plasma (5). However, we found that the $apo^{B100\text{-}}\beta/^{B100\text{-}}\beta$ mice have slightly, but not significantly lower than normal, total plasma cholesterol and HDL cholesterol, and the amount of plasma LDL was not different from that in wild-type mice (4). One explanation is that these 2 regions are not essential for apoB100 binding to the LDLR in vivo. The interpretation, however, is complicated because mice normally have very little apoB100-containing LDL particles in circulation. In addition, the production of apoB48 from the liver and the efficient clearance of apoB48-containing remnants mediated by apoE make the metabolism of apoB100 difficult to study in vivo in mice.

The present study examined the effect of apoB100-β-containing LDL by introducing the mutation onto a background of $Ldlr^{-/-}Apobec1^{-/-}$ double mutants; a model of human familial hypercholesterolemia with severe atherosclerosis. $Apobec1^{-/-}$ mice that lack the mRNA editing enzyme produce only apoB100 (7), whereas $Ldlr^{-/-}$ mice that lack LDLR accumulate LDL cholesterol in plasma (6, 7). Surprisingly, when these mice also carry the apoB100-β mutation, they are completely protected from hypercholesterolemia and atherosclerosis that normally occurs in $Ldlr^{-/-}Apobec1^{-/-}$mice.

6.3 Methods

Animals and Diets

The *apoB100-β* allele codes for "VHLTPVEKSAVT" and "KEFTPPVQAAYQ" instead of "LSVKAQYKKNSD" and "GTSRLMRKRGLK" of the wild-type *apoB100* allele at residues 3143 to 3154 and 3356 to 3366, respectively.[4] *Ldlr*$^{-/-}$ mice (B6;129S7-Ldlr$^{tm1Her/J}$) were obtained from the Jackson Laboratory. *Apobec1*$^{-/-}$ mice were obtained from Dr Eddy Rubin at the Lawrence Berkeley National Laboratory (7). Three strains of mutants were crossed to generate mice that are heterozygous for the *Apob* locus and doubly homozygous for the *Apobec1* and the *Ldlr* loci. These mice were then crossbred, and *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with *Apob* genotypes of 100/100 (wild type), 100/100-β (heterozygous), and100-β/100-β (homozygous) were generated for experiments. Their genetic backgrounds were complex mixes between C57BL/6J, 129/SvEv, and129/Ola. Animals were maintained on normal chow (NC; 4.5% fat, 0.022% cholesterol; Prolab Isopro 3000; Agway Inc), or were fed a high-fat Western-type diet (HFW; 21% fat, 0.2% cholesterol; TD 88137; Harlan Teklad). Mice in all experiments were age-matched within 3 weeks. All procedures for the handling of mice were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Biochemical Analyses and Atherosclerosis Evaluation

Mice were fasted 4 hours before analysis. Liver and fecal lipids were extracted with chloroform/methanol.[8] Plasma lipids, lipoprotein distribution, and triglyceride secretion rate, were determined as described (9). Lipoprotein particle diameters were determined by dynamic light scattering analysis using a Microtrac 250 (10). Peritoneal macrophages and hepatocytes

were isolated as described (11, 12). The VLDL (d <1.006 g/mL) and LDL (d=1.06 to 1.10 g/mL) fraction was isolated from pooled plasma by ultracentrifugation and labeled with 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate (DiI C18; Molecular Probes Inc) (13), or with ^{125}I (Iodine-125 Radionuclide, Perkin Elmer) (14), for clearance assays. Fibroblasts were kindly provided by Dr J. Herz at the University of Texas Southwestern Medical Center. Cellular lipids were extracted with isopropanol and measured with a microscope fluoromete r (13). Gene expression in the liver was analyzed by real-time polymerase chain reaction (PCR), and quantification of atherosclerosis was carried out as described (11).

Data Analysis

Values are reported as mean±SEM unless otherwise stated. Data were analyzed by ANOVA using JMP software (SAS Inc).

6.4 Results

ApoB100-β causes marked reduction of LDL in Ldlr$^{-/-}$Apobec1$^{-/-}$ mice.

Ldlr$^{-/-}$Apobec1$^{-/-}$ mice with wild type apoB100 had high levels of plasma cholesterol and triglycerides on NC, and further increased plasma lipids on a HFW diet (Table 6.1). In contrast, both plasma cholesterol levels in the *Ldlr$^{-/-}$Apobec1$^{-/-}$* mice that are heterozygous and homozygous for the apoB100-β mutation were reduced in an allele dose-dependent manner. The protective effect of the apoB100-β mutation compared to controls was retained when mice were fed a HFW diet, although plasma cholesterol levels increased about 3 fold in all mice. Plasma levels of

triglyceride, free cholesterol and phospholipids in mice with apoB100-β were also significantly lower than those with apoB100 mice.

When plasma lipoproteins from male mice on normal chow diet were analyzed by fast protein liquid chromatography (FPLC), over 60% of the plasma cholesterol was in the LDL fraction in *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with wild type apoB100. In contrast, a striking absence of LDL-cholesterol was noted in the plasma of *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice homozygous for apoB100-β (Figure 6.1A). Mice with one copy of *apoB100-β* had approximately half the amount of LDL as those with wild type *apoB100*. All mice had very low levels of VLDL cholesterol, and there was no difference in the amount of HDL. The possession of apoB100-β resulted in a similar reduction of triglycerides in the LDL fraction (Figure 6.1B). SDS gel electrophoresis of lipoprotein fractions from plasma of mice fed a HFW diet showed that the distribution of apoB100-β among various classes of lipoproteins was similar to that of normal apoB100 with the highest concentration in the LDL range (1.02 g/ml>d>1.04 g/ml, Figure 6.1C). However, total apoB100-β in these mice was much less than wild type apoB100, since samples of three times of apoB100-β plasma volume was loaded compared to apoB100 plasma. Total plasma apoE was also less in mice with apoB100-β than in mice with apoB100, but the ratio of apoE/apoB on the lipoprotein particles in the apoB100-β mice was about four times higher than in apoB100 mice.

While there was no difference in adipose tissue weight, the liver weight per body weight was slightly but significantly smaller in mice with apoB100-β ($P<0.005$, Figure 6.1D). Hepatic intracellular cholesterol pools in the two groups of mice were not significantly different after 2 months on HFW diet. In contrast, the liver triglyceride content of *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ males with apoB100-β was significantly lower than in mice with wild type apoB100 ($P<0.001$, Figure 6.1E).

Fecal cholesterol and triglyceride levels of mice after 2 months on HFW diet were not significantly different (Figure 6.1F). These data suggest that the apoB100-β mice are also protected from liver steatosis. Under light microscopy, however, liver sections from mice on HFW contained similar degrees of fatty droplets and no remarkable difference was observed between the *Apob* genotypes (data not shown).

	Apob genotype	Normal Chow Diet		High Fat Western Diet			
		TC (mg/dl)	TG (mg/dl)	TC (mg/dl)	TG (mg/dl)	FC (mg/dl)	PL (mg/dl)
♀	**100/100**	380±31(15)	78±6 (15)	1,005±35 (24)	134±14 (20)	253±37 (15)	627±23 (10)
	100/100-β	196±7 (10)	36±3 (10)	476±82 (6)	124±56 (6)	143±36 (10)	342±21 (10)
	100-β/100-β	93±6 (15)	23±3 (15)	292±24 (19)	60±5 (15)	84±14 (8)	236±8 (8)
♂	**100/100**	478±23 (7)	99±7 (7)	1,161±26 (11)	599±22 (11)	n.d.	n.d.
	100/100-β	256±10 (8)	60±9 (8)	n.d.	n.d.	n.d.	n.d.
	100-β/100-β	126±7 (8)	39±4 (8)	329±20 (7)	149±29 (7)	n.d.	n.d.

Table 6.1. **Plasma lipids in Ldlr-/-Apobec1-/- mice with wild type apoB100 or apoB100-β mutation**. Data are mean±S.E. in mg/dl. Plasma samples were collected from mice after a 4 hr fast. The numbers in parentheses are the number of animals. F, females; M, males; TC, total cholesterol; TG, triglycerides; FC, free cholesterol; PL, phospholipids. n.d., not determined. Effects of *Apob* genotypes are highly significant in all categories ($P<0.0001$)

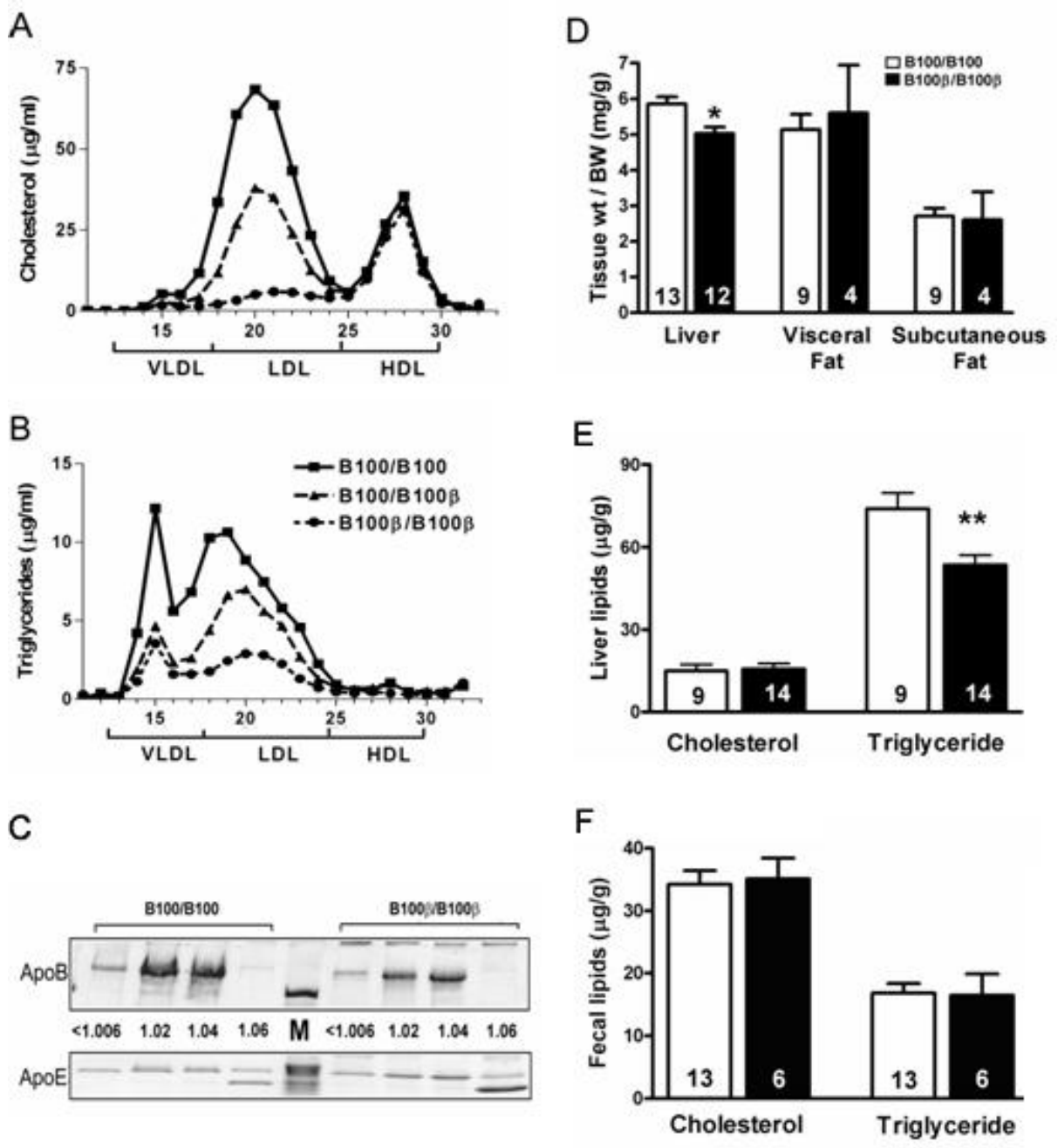

Figure 6.1. **Plasma and tissue lipids**. Plasma lipoprotein distribution by FPLC (A) cholesterol, (B) triglycerides. (C) SDS-PAGE of apoB100, apoB-100β, and apoE in VLDL to LDL (1.006 – 1.06 g/ml) density fractions. Samples equivalent to 5 µl of apoB100 plasma and 15 µl of apoB100-β plasma were loaded. M, weight markers. (D) Tissue weight normalized to body weight, (E) liver lipid contents and (F) fecal lipids. Number of animals is in each bar. Error represents SEM. *, $P<0.005$, **, $P<0.001$.

Ldlr$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100-β secrete larger VLDL

A possible source of the disparity in plasma LDL levels is a difference in hepatic VLDL production rates. To estimate the secretion rate of triglyceride-rich lipoprotein (TRL) particles from the liver, we injected Triton WR1339 (Tyloxapol) intravenously into mice to inhibit lipolysis and uptake of TRLs, and measured plasma triglycerides (Figure 6.2A). Although the basal triglyceride levels differ in the two groups of mice, triglyceride secretion rates were nearly identical at 280-300 μg/ml/hr regardless of whether they have apoB100 or apoB100-β.

We next analyzed VLDL particle size at 2hr post-Tyloxapol injection. The size of particles in the <1.006 g/ml density fraction was significantly different between the two groups of *Ldlr*$^{-/-}$ *Apobec1*$^{-/-}$ mice; the mean±SD diameter of apoB100-β VLDL particles were larger (55.0±15 nm) than those with wild type apoB100 (45.6±14 nm). Based on the difference in the diameter, we estimate that the average apoB100-β VLDL has approximately 46% more surface area and 76% greater volume than that of the normal apoB100 VLDL. Assuming that the triglyceride content of a particle is relative to its volume, this implies that the number of VLDL particles secreted from the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ liver with apoB100-β is approximately 60% that from the liver with wild type apoB100.

To examine the production and degradation of apoB100 proteins, we conducted a pulse-chase experiment with radiolabelled methionine in the primary hepatocytes isolated from the *Ldlr*$^{-/-}$ *Apobec1*$^{-/-}$ mice with apoB100 and with apoB100-β (Figure 6.2B). Immunoprecipitable apoB protein in the apoB100-β cells after the 30-minute-pulse was not significantly different from that in apoB100 cells, suggesting that the initial synthesis rates are not different. After 4hr chase in the medium with excess of cold methionine, however, the immunoprecipitable apoB protein both in the medium and associated with cells was significantly less in the apoB100-β hepatocytes. Thus, the

mutated apoB protein is degraded more quickly, leading to the reduced number of VLDL particles secreted from the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ liver with apoB100-β.

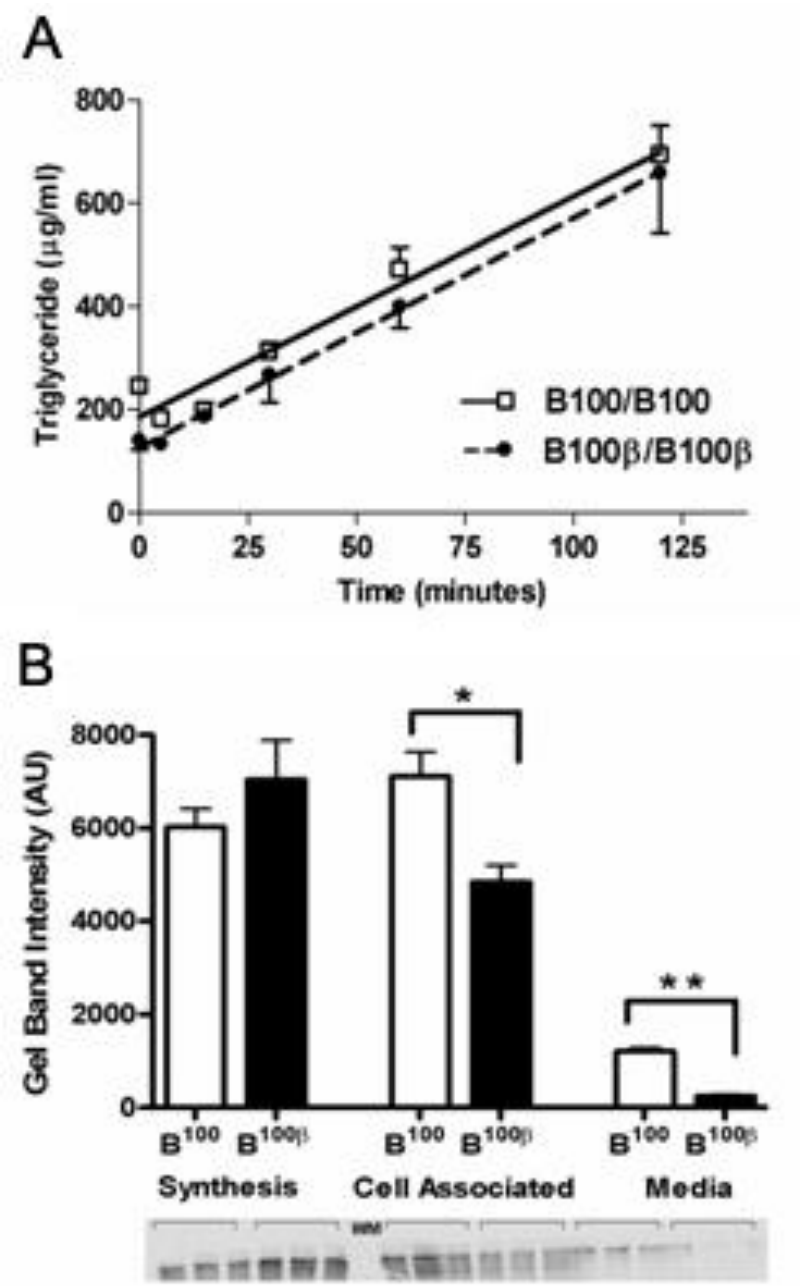

Figure 6.2**. Hepatic TG secretion and hepatocyte apoB metabolism**. (A) Hepatic triglyceride secretion rates. Female mice (5-8 month old fed a HFW, n=11) were injected via tail-vein with Triton WR-1339 and plasma triglyceride levels were measured post-injection. (B) ^{35}S Pulse-chase analysis in primary hepatocytes. ApoB100 bands in the Gel were quantified using Image J software. Error represents SEM. *, $P<0.005$, **, $P<0.001$.

B100-β VLDL is efficiently cleared from the circulation

The lack of LDL accumulation in the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100-β is not proportional to the amount of VLDL particles secreted in these mice compared to that in mice with wild type apoB. To test a hypothesis that apoB100-β VLDLs are cleared more efficiently thn apoB100 VLDL, we isolated VLDL from *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with either apoB100 or apoB100β, labeled them with ^{125}I, and injected them into *Ldlr*$^{-/-}$ mice via the tail vein. Monitoring plasma clearance of ^{125}I labeled VLDL over a 2 hour period showed that ^{125}I VLDL with apoB100-β are cleared faster than VLDL with wild type apoB100 (Figure 6.3A). To determine the specific tissue loci of the cleared VLDL, we repeated the ^{125}I-VLDL turnover, this time measuring radioactivity in various tissues after 20 minutes. Of ^{125}I-VLDL cleared, the majority was found in the liver. The distribution of ^{125}I-VLDL with apoB100β did not differ from that of ^{125}I-VLDL with apoB100 in the five organs measured (Figure 6.3B).

In order to assess the conversion of VLDL to smaller particles *in vivo*, plasma samples were isolated from mice two hours after ^{125}I-VLDL injection, pooled, and separated into VLDL, IDL, and LDL fractions using ultracentrifugation. Proportions of counts in the density fractions containing each class of lipoproteins were similar between mice received ^{125}I-VLDL with apoB100 and those with apoB100-β (Figure 6.3C). Furthermore, the apoB100-β VLDL particles incubated with post-heparin plasma released FFA at rates of 12±1 nmol FFA/min compared to apoB100 VLDL at 11±1 nmol FFA/min (Figure 6.3D), suggesting that the lack of putative LDLR binding domain sequences of apoB does not affect the lipolysis of VLDL in *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ apoB100-β mice.

Taken together, these experiments suggests that the absence of hyperlipidemia in LDLR-deficient mice having apoB100 without the putative receptor-binding sequences is likely because

their VLDL particles are quickly cleared from the circulation before they become small, cholesterol-enriched LDL particles.

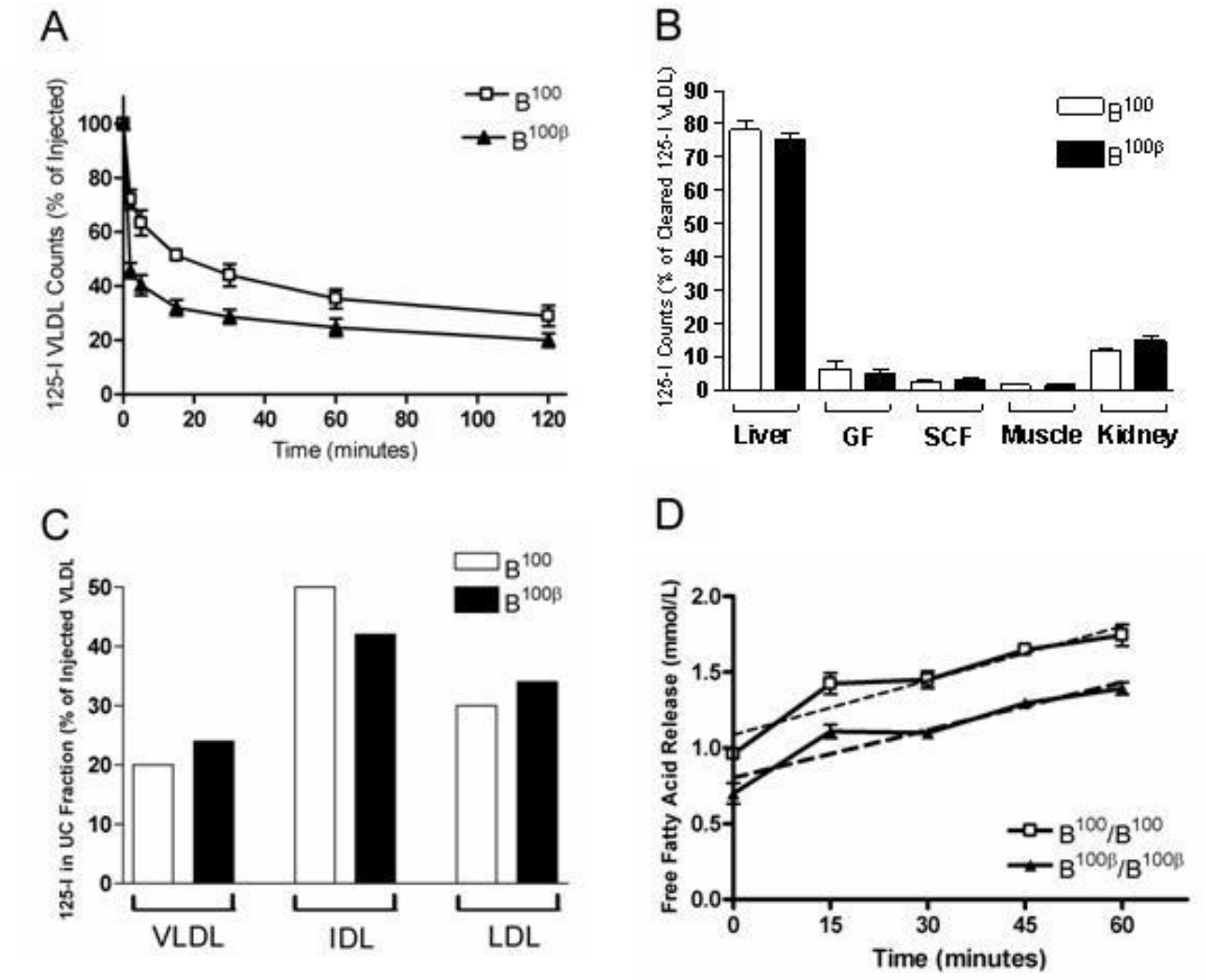

Figure 6.3. **Lipoprotein clearance and lipolysis**. (A) Clearance of ^{125}I labeled apoB100 or B100-β VLDL in *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice. n > 3 for each group. (B) Organ uptake expressed as the percentage of total ^{125}I-VLDL in all organs measured. n = 3 for each group. (C) Conversion of injected ^{125}I-VLDL after 2hrs, expressed as a percentage of injected VLDL. (D) FFA release in heparin treated plasma from *Ldlr*$^{-/-}$ *Apobec1*$^{-/-}$ mice with apoB100 or apoB100-β measured over 1 hour.

Role of apoE-LRP mediated clearance

To examine the specific roles of the LRP on apoB100-β particle uptake, we measured uptake of DiI labeled VLDL and LDL in mouse fibroblasts deficient in either LDLR (LDLR-/-LRP+/+), in LRP (LDLR+/+LRP-/-), or in both LDLR and LRP (LDLR-/-LRP-/-, negative control cells). Two hours after DiI-labeled apoB100-LDL was added to the medium, the uptake into LDLR+/+LRP-/- cells was significantly higher than in LDLR-/-LRP-/- negative control cells (Figure 6.4A). The uptake of apoB100-LDL by the LDLR-/-LRP+/+ cells was also higher than in negative control cells. Uptake of apoB100-β LDL was not increased by the expression of either LDLR or LRP. The opposite pattern of uptake was observed in studies with VLDL. In 30 min, DiI-labeled apoB100-β-VLDL was efficiently taken up by cells expressing LDLR, and particularly LRP, while no such increase over the uptake by negative control cells was found in cells given DiI-labeled-apoB100 VLDL (Figure 6.4B).

We next blocked hepatic LRP function using Ad-RAP to determine the role of the LRP in the clearance of apoB100-β VLDL *in vivo*. While basal cholesterol and triglyceride levels in plasma are significantly lower in *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100-β than those with normal apoB100, the levels five days after Ad-RAP injection were not different between the two groups of mice. FPLC analyses showed a similar accumulation of cholesterol in the VLDL fraction after 5 days in both groups (Figure 6.5). These data imply that apoB100-β VLDL is removed by receptors inhibited by RAP, such as LRP. We also examined the contribution of HSPG binding by incubating LDLR-/-LRP-/- cells at 4º C with Dii-VLDL. The amount of VLDL released from the surface by heparinase after 2h was not significantly different (Figure 6.6), suggesting that apoB100-β does not affect the binding ability of VLDL to proteoglycans.

To gain further insight into the apparent enhancement of lipoprotein clearance in *Ldlr*$^{-/-}$ *Apobec*$^{-/-}$ mice with apoB100-β, we analyzed the expression of the *Apob*, *Apoe* and *Lrp1* genes in the liver by real-time PCR. While there was no *Apob* genotype effect on the mRNA levels for *Apob* and *Lrp1*, liver expression of *Apoe* was approximately twice as high in mice with apoB100-β as in mice with wild type apoB100 (Figure 6.7). These data, combined with the higher apoE:apoB protein ratio, suggest that the increased production of apoE protein may be contributing to the accelerated clearance of apoB100-β containing VLDL-remnants and the resistance to hyperlipidemia in the apoB100-β mice even in the absence of LDLR.

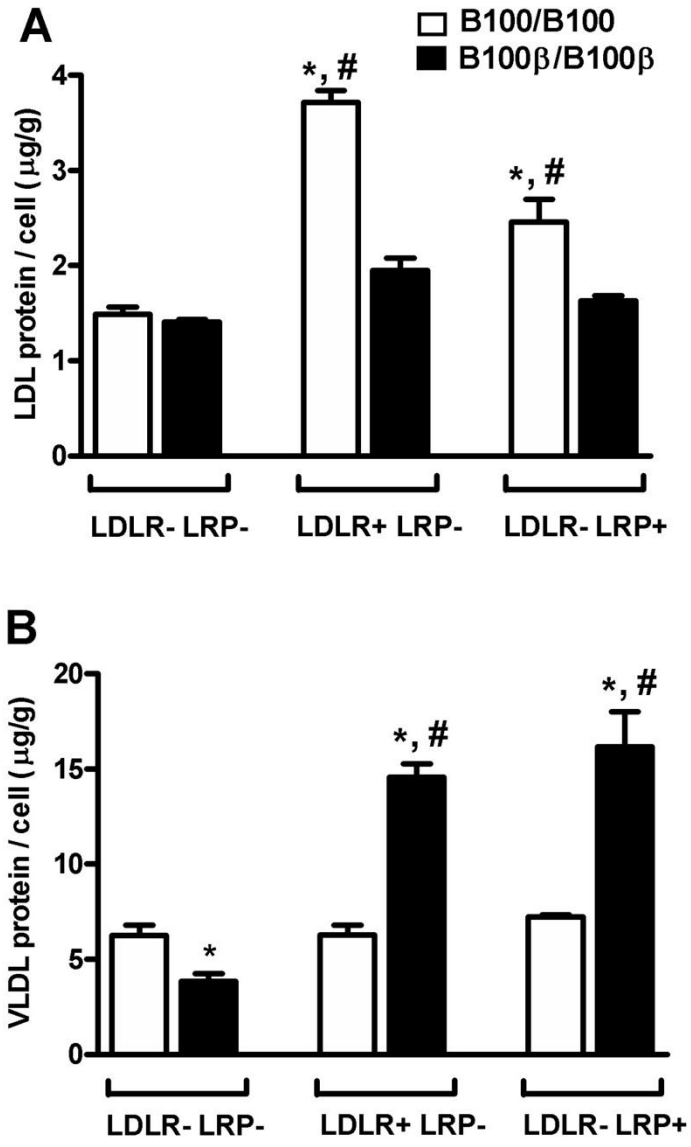

Figure 6.4. **Uptake by fibroblasts of DiI-labeled lipoproteins with apoB100 or apoB100-β.** (A) Cells were incubated with DiI-labeled LDL 37˚ C for 2 hr or (B) VLDL for 30 min. Error represents SEM. *P<0.05, **P<0.005 against uptake in LDLR- LRP cells. #, P< 0.05 between genotypes within the same cell system.

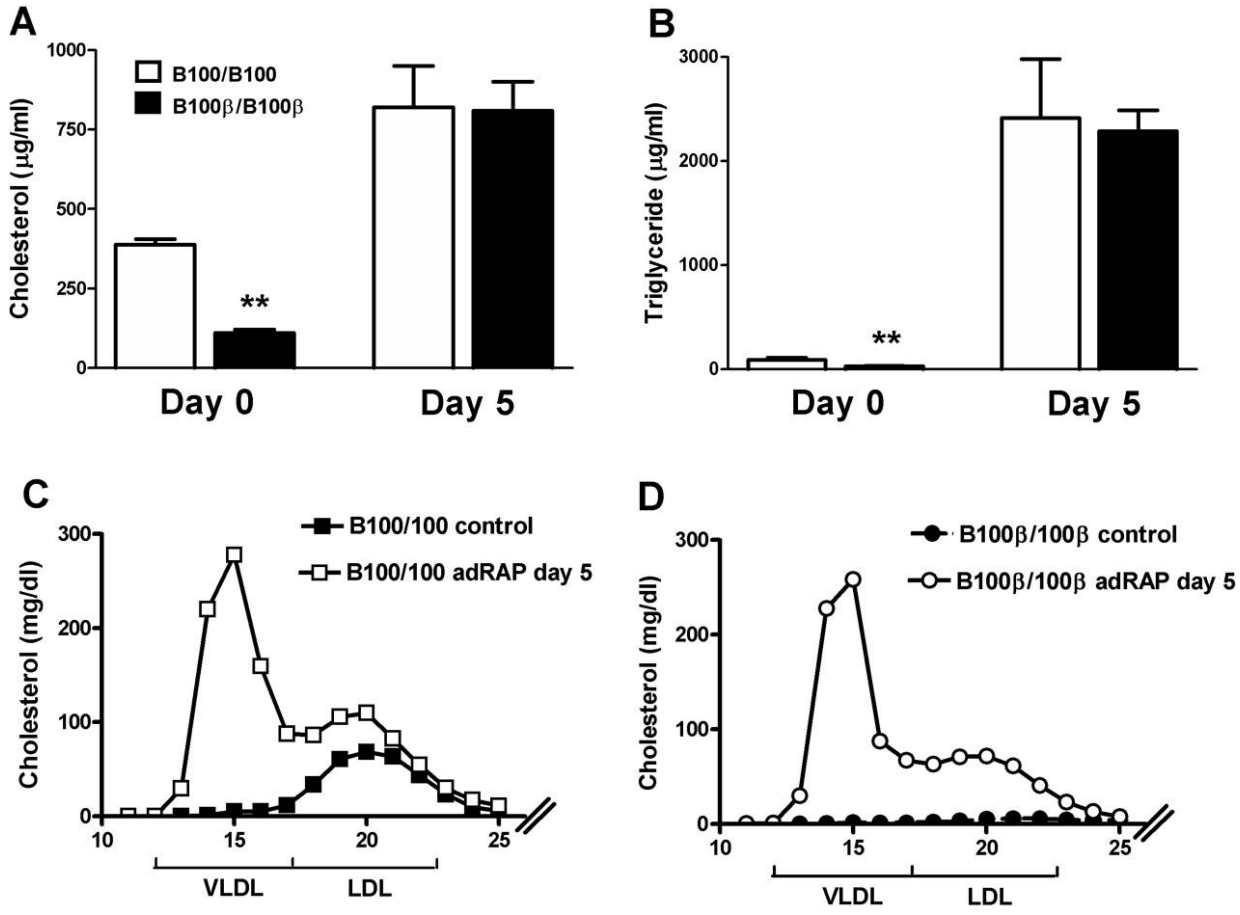

Figure 6.5. **VLDL and LDL cholesterol distribution after RAP mediated blocking of LRP.** Total plasma cholesterol (A) and triglyceride (B) at 0 and 5 days after injection of adRAP. White bars and black bars indicate *Ldlr*$^{-/-}$*Apobec*$^{-/-}$ mice with wild type apoB100 and those with apoB100-β, respectively. The distribution of plasma VLDL and LDL cholesterol in *Ldlr*$^{-/-}$*Apobec*$^{-/-}$ mice with wild type apoB100 (C) and in those with apoB100-β (D). Blood samples were collected from mice after a 4 hr fast prior (filled symbols) and at 5 days after injection of adRAP (open symbols). A total of 50 μl plasma, pooled from groups of 3 male mice, was size-fractionated by FPLC and cholesterol amount in each fraction was measured. ** denotes $P<0.005$ between mice with apoB100 and mice with apoB100-β.

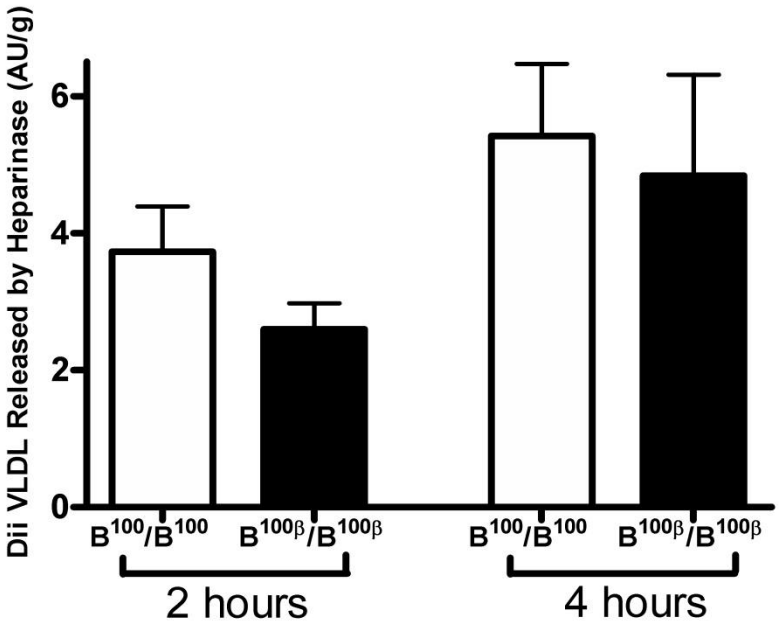

Figure 6.6**. Heparinase releasable VLDL.** Dii-VLDL released by LDLR-/-LRP-/- fibroblasts after treatment with heparinase. Cells were incubated at 4˚ C for 2 hr with Dii-VLDL. VLDL was isolated from Ldlr-/-Apobec1-/- mice homozygous for apoB100 or apoB100-β.

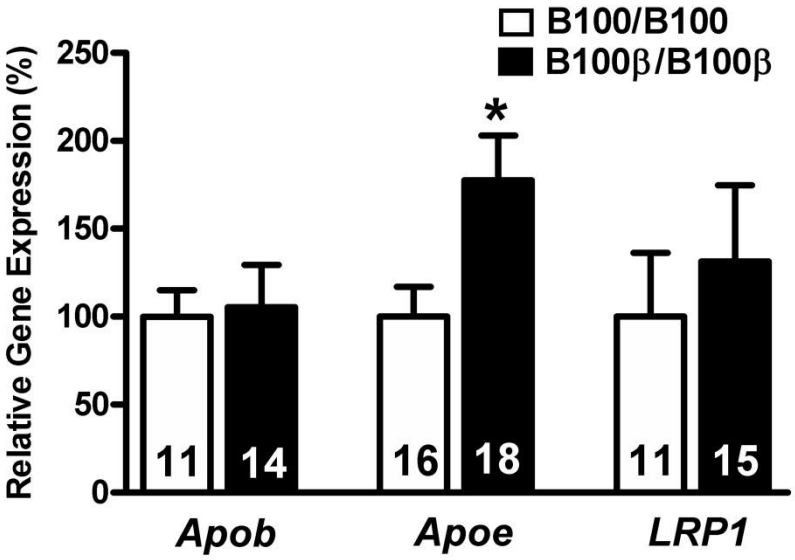

Figure 6.7. **Hepatic *Apoe*, *Apob* and *Lrp1* gene expression**. Liver tissues were isolated from 6-8 month old mice fed a HFW diet for 1-2 months. mRNA amounts are normalized to β-actin and expressed relative to the mean of B100/B100 mice as 100%. Error represents SEM. The number of animals is shown inside each bar. * $P<0.002$.

$Ldlr^{-/-}Apobec1^{-/-}$ mice with apoB100-β are protected from development of atherosclerosis

High levels of LDL are a well-documented risk factor for atherosclerosis. $Ldlr^{-/-}Apobec^{-/-}$ female mice with apoB100 had a significant size of lesions (34±6 $x10^3\mu m^2$), even when they were on NC diet and were as young as 4-5 months old (Figure 2.8A). In marked contrast, the apoB100-β mutation demonstrated a significant athero-protective effect. While three out of five $Ldlr^{-/-}Apobec^{-/-}$ mice heterozygous for apoB100-β had visible plaques (16±7 $x10^3\mu m^2$), there were absolutely no plaques seen in mice homozygous for the apoB100-β mutation. The overall effect of the *Apob* genotype on plaque development was $P<0.002$ by ANOVA. Feeding a HFW diet for 2 months accelerated the plaque development in the $Ldlr^{-/-}Apobec^{-/-}$ mice with apoB100 (mean lesions size 54±8 $x10^3\mu m^2$; n=5). In contrast, there were virtually no lesions present in apoB100-β mice but only very small foam cell aggregations (1.7±0.8 $x10^3\mu m^2$, $P<0.002$).

To examine whether direct VLDL scavenging by macrophages rather than LDL accumulation is responsible for the dramatic differences in atherosclerosis, we isolated peritoneal macrophages from $Ldlr^{-/-}$ mice and incubated with equal amounts of DiI-labeled VLDL in the medium. Uptake of the DiI-VLDL with apoB100 by the macrophages was a little more enhanced compared to that with apoB100-β, but the differences were not statistically significant (Figure 6.8B). A similar result was obtained in the macrophages isolated from wild type mice (data not shown), indicating that the scavenging by macrophages of the VLDL is not affected by the apoB100-β mutation.

Taken together our data demonstrate that, even in the absence of LDLR, a mutation in the putative receptor binding domains of apoB prevents LDL accumulation, and dramatically reduces atherosclerosis.

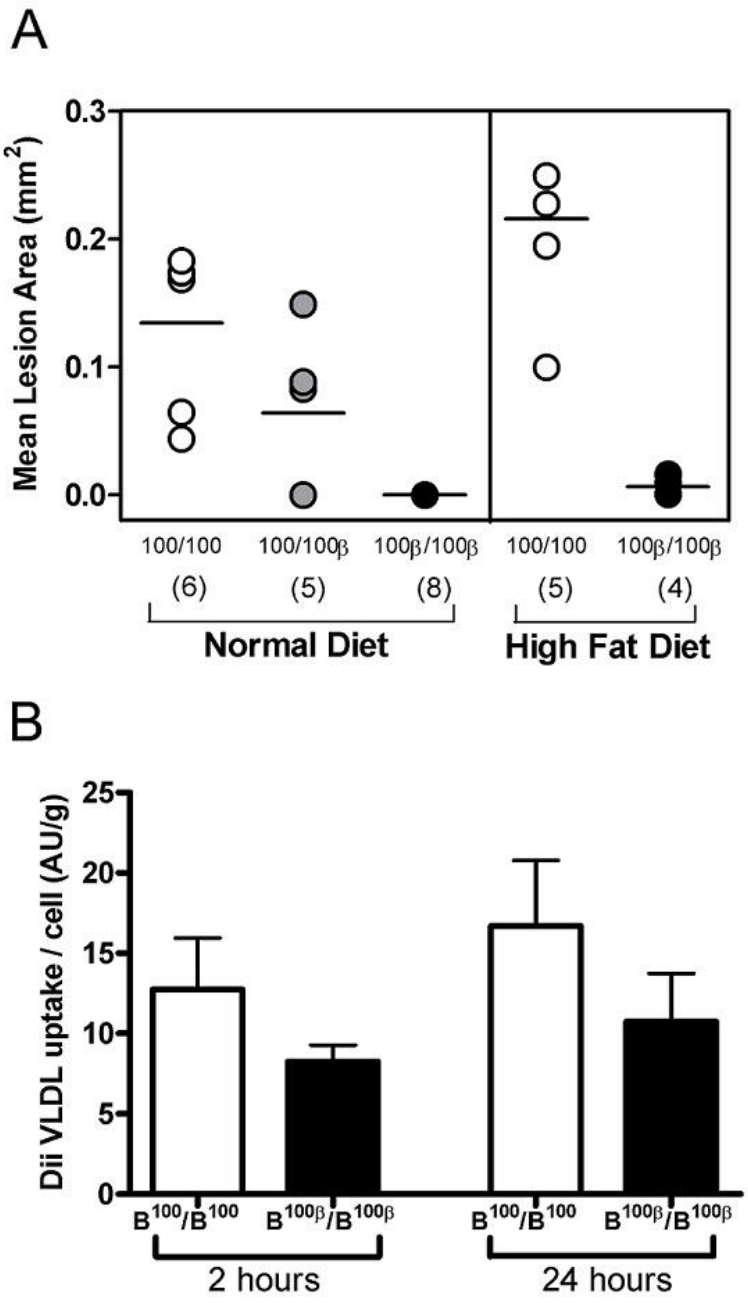

Figure 6.8. **Atheroslcerosis and macrophage VLDL uptake**. (A) Atherosclerotic plaque sizes at the aortic roots of 4 month old female *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice. Number of animals is in parentheses. (B) Macrophage VLDL uptake. *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ macrophages were incubated with DiI-labeled VLDL with apoB100 or with apoB100-β. Cellular florescence is expressed as Arbitrary Units (AU) per cell gram of cell protein.

6.5 Discussion

LDL is generated in the circulation from VLDL produced by the liver following lipolysis and exchange of surface apolipoproteins. During this conversion, conformational changes occur in its structural protein, apoB100, allowing for the exposure of domain(s) that interact with LDLR (15-17). Exposure of the receptor binding domain and subsequent binding of apoB100 to the LDLR is the major pathway for the clearance of LDL cholesterol by the liver, as illustrated by the marked accumulation of LDL in plasma of patients and in animals lacking LDLR (18-21). Particles that lack full length apoB, such as apoB48-containing chylomicron remnants, can acquire apoE which mediates efficient clearance of these particles by the LDLR, LRP and other receptors which may act in concert with proteoglycans (22).

To investigate the mechanisms for the uptake of apoB48- and apoB100-containing lipoproteins by the LDLR and by the LRP, Veniant et al previously characterized plasma lipoproteins in the *Ldlr*$^{-/-}$ mice homozygous for an "apoB48-only" allele or homozygous for an "apoB100-only" allele (24). The authors concluded that the LDLR plays a significant role in the clearance of both apoB100 and apoB48 containing lipoproteins, and that the LRP is important for apoB48-containing lipoproteins but has little if any capacity to remove apoB100-containing lipoproteins from the plasma. The "apoB100-only" *Ldlr*$^{-/-}$ mice are phenotypically identical to the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with wild type apoB100 we used in the current study. Interestingly, the plasma lipids and lipoprotein distribution in *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with mutant apoB100-β are very similar to "apoB48-only" *Ldlr*$^{-/-}$ mice, despite that apoB100-β retains the full length of the apoB protein. Both strains of mice have no substantial accumulation of LDL particles, suggesting that apoB100-β remnants, like apoB48-only remnants, are cleared via LRP in the absence of LDLR and

that the length of apoB protein does not influence this process. However, while Veniant et al showed that LRP inhibition with RAP of "apoB48-only" *Ldlr*$^{-/-}$ mice leads to higher VLDL levels than in "apo-B100-only" *Ldlr*$^{-/-}$ mice (23), VLDL accumulation in the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100 and those with apoB100-β were similar after LRP inhibition, suggesting that additional mechanisms may present in the protective effects noted in the apoB100-β producing mice.

The *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100-β do not accumulate substantial LDL particles in plasma even when fed a HFW diet. This lack of LDL accumulation occurs despite the inability of apoB100-β-containing LDL to be cleared *in vitro*. Importantly, the livers of *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100-β appear to produce a smaller number of larger VLDL particles than the livers of the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100, despite equal expression of the *Apob* gene and protein synthesis. However, the reduction of LDL cholesterol in the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100-β is more than the reduction of apoB secreted. This is in contrast to the report by Crooke et al. that *Ldlr*$^{-/-}$ mice treated with an apoB antisense oligonucleotides had a reduction of apoB mRNA by 74% but still had 48% levels of LDL-cholesterol compared to the pretreatment levels (24). It has long been recognized that the larger VLDL particles are removed faster and less likely converted to LDL than smaller VLDL, and a larger surface area of apoB100-β VLDL may allow more apoE to associate with the particle and facilitate LRP mediated uptake (25, 26). *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB100-β have plasma lipoproteins containing four-fold higher apoE protein per particle, and two fold higher *Apoe* gene expression in the liver than mice with apoB100. All together, these changes favor the enhanced clearance of apoB100-β containing particles via the LRP.

We also observed an enhanced degradation of apoB100-β in primary hepatocytes from the *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice with apoB-100β in culture. Whether the accelerated degradation of

apoB100-β results from its abnormal protein folding or is the consequence of enhanced turnover has yet to be determined. While a limited apoB protein available for lipoprotein assembly could account for the larger size of VLDL, a question remains as to whether apoB100-β fails to form subsets of VLDL particles that are pre-destined to form LDL particles. Studies have demonstrated that a substantial amount of newly synthesized apoB protein is degraded rather than secreted, and that its interaction with LDLR channels apoB toward pre-secretory degradation (27-30). Reuptake of newly synthesized lipoproteins by LDLR can also attenuate VLDL secretion, and both apoE and apoB are important for this process (31, 32). Loss of these regulations results in an increased secretion of apoB proteins and smaller, underlipidated VLDL particles in humans and mice that lack functional LDLR (33).

The metabolism of lipoproteins with apoB100-β mutation is consistent with other observations. For example, truncations of apoB on the C-terminal side of amino acid 3500 result in more efficient clearance of VLDL (34). Individuals heterozygous for a R3480P mutation in apoB exhibit hypobetalipoproteinemia because of a reduced conversion of VLDL to LDL, despite that this mutation caused reduced binding of LDL to the LDLR (35). Similarly, milder than expected hyperlipidemia in individuals with familial defective apolipoprotein B-100 due to mutations at R3500 has been attributed to an enhanced removal of apoE-containing VLDL and decreased production of LDL (36, 37). The apoB100-β mutation may also affect a process of structural/conformational change of apoB100 that is important for the *in vivo* generation of LDL particles as well as for LDLR binding, although interpretation is complex because amino acid changes disrupting amphipathic helices represented by sites A and B likely cause additional conformational changes of apoB on LDL and VLDL. Systematic replacements of the basic LDLR binding sequences with acidic or neutral residues would provide a potentially less disruptive and

comprehensive approach. Chatterton et al. hypothesized that the apoB100 "bow", where a segment of apoB100 crosses over itself between amino acid residues 3000 and 3500, inhibits interaction of apoB100 protein with LDLR, hence inhibiting clearance (38). Since the apoB100-β mutation at amino acids 3147-3157 and 3359-3367 are within the proposed bow crossing structure, a mutation in these sequences may physically block or otherwise disrupt "bow" structure formation.

There is little doubt that the exposure of the positively charged domains of apoB100 to the lipoprotein surface following conformational changes is required for the effective clearance of LDL through LDLR. Considering the overall consequences of the mutations in the second half of the apoB100, however, it is tempting to speculate that the exposure of the positively charged domains of apoB100 may also inhibit the accumulation of apoE on the particles required for their apoE-mediated uptake via LDLR and/or LRP. This is consistent with the hypothesis raised by Veniant et al that the presence of the carboxyl half of apoB100 (amino acids 2153-4536) on the surface of the lipoprotein prevents the lipoprotein particle from binding a "sufficient dose of supplemental apoE" that is necessary for the lipoprotein particle to escape circulation via uptake by the LRP (23). Lack of the putative LDLR binding domains in apoB100-β may also prevent the secretion of newly packaged but underlipidated particles by enhancing the degradation of apoB through enhanced interactions between apoE and LDLR/LRP.

In conclusion, we have demonstrated that the mutation in the LDLR binding domains of apoB100 dramatically protects mice from both hypercholesterolemia and atherosclerosis that develop in the absence of LDLR. Our observations raise an intriguing possibility that an interference of the exposure of the putative LDLR-binding domains to the lipoprotein surface may indeed enhance remnant clearance through apoE-mediated mechanisms. This may be applicable as

a potential therapeutic approach for preventing LDL accumulation in patients with familial hypercholesterolemia.

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