WILL ABSENCE OF GPAT1 IMPROVE DIET-INDUCED ATHEROSCLEROSIS IN APOE HETEROZYGOUS MICE?

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PEI-CHI WU: Will absence of GPAT1 improve diet-induced atherosclerosis in ApoE heterozygous mice?

(Under the direction of Rosalind Coleman)

This thesis separated into two parts. The first part examined how the lack of GPAT affects atherosclerosis that depends on a western diet. The second part examined whether the lack of GPAT does ameliorates the atherosclerosis developing on an atherogenic diet.

In the first part, we fed $ApoE^{+/-}/GPAT1^{-/-}$ mice and $ApoE^{+/-}$ mice on western diet for 4 months. We found that $ApoE^{+/-}/GPAT1^{-/-}$ mice and $ApoE^{+/-}$ mice did not develop atherosclerotic lesions after 4 months of western diet. $ApoE^{+/-}/GPAT1^{-/-}$ mice had lower serum cholesterol, hepatic triacylglycerol and body weight than $ApoE^{+/-}$ mice. We could not conclude that $ApoE^{+/-}/GPAT1^{-/-}$ mice could improve atherosclerosis.

In the second experiment, we fed $ApoE^{+/-}$ and $ApoE^{+/-}/GPAT1^{-/-}$mice an atherogenic diet (15.8% fat, 1.25% cholesterol, 0.5% cholate) for 90 days. We wanted to see if $ApoE^{+/-}/GPAT1^{-/-}$ mice improve the diet-induced atherosclerosis on these $ApoE^{+/-}$mice. We found out the $ApoE^{+/-}/GPAT1^{-/-}$ mice increased their serum cholesterol and VLDL-cholesterol compared to $ApoE^{+/-}$ mice. The atherosclerotic lesion sizes of $ApoE^{+/-}/GPAT1^{-/-}$ mice and $ApoE^{+/-}$ mice were similar. We concluded that the

absence of GPAT could not improve the diet-induced atherosclerosis.

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ACE	Angiotensin-converting enzyme
APOE	Apolipoprotein E
CETP	Cholesterol ester transfer protein
CM	Chylomicrons
DAG	Diacylglycerol
HDL	High density lipoprotein
HNE	4-hydroxy-2-nonenal
FXR	Farnesoid X receptor
GPAT	Glycerol-3-phosphate acyltransferase
FAS	Fatty acid synthesis
FABP	Fatty acid binding protein
LDL	Low density lipoprotein
LPA	Lysophosphatidic acid
LPL	Lipoprotein lipase
LXR	Liver X receptor
NEM	N-ethylmaleimide
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PDK	Pyruvate dehydrogenase kinase
Pltp	Phospholipid transfer protein
PPAR-γ	Peroxisome proliferator-activated receptor-γ
SRE	Sterol-respone element
SHP	Short heterodimer partner
TGRL	Triacylglycerol rich lipoproteins
VLDL	Very low density lipoprotein

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CHAPTER 1

Introduction

1.1. Atherosclerosis

Atherosclerosis is a major cause of morbidity and mortality and also the third highest risk factor for death in the world. Atherosclerotic cardiovascular disease is a disease with multiple genetic, environmental, and other factors (1-3). For example, many genes which contain encoding apolipoproteins or receptors can cause atherosclerosis. The life-style, cigarette smoking and diet also play an important role contributing to atherosclerosis.

The first animal model for studying atherosclerosis was rabbits in 1908 when Ignatowski (4; 5) described thickening of the intima with formation of large clear cells in the aorta of rabbits fed with a diet rich in animal proteins. In recent years, mouse models of atherosclerosis were generated. The apoE-deficient mice spontaneously develop aortic atherosclerotic plaques similar to those seen in humans (6). The second mouse model of atherosclerosis, lacking the LDL receptor, was produced by Ishibashi *et al* (7). LDL-$R^{-/-}$ mice develop plaques more slowly than do $apoE^{-/-}$ mice fed a chow diet. The beginning of pathology is functional endothelial damage, secondary to mechanical or vascular insult, and is followed by an inflammatory

cascade that involves humoral and cellular mechanisms (8).

The current medical treatment for atherosclerosis is aspirin, Plavix (Clopidogrel bisulfate) , statins (HMG-CoA reductase inhibitor) and other lowing lipid agents (9), capoten(ACE inhibitor), Bystolic (beta blockers), and omega-3-fatty acids. Aspirin inhibits the platelet function by acetylation of the platelet cyclooxygenase (COX) and prevents the binding of the substrate (arachidonic acid) to the catalytic site of the enzyme and results in an irreversible inhibition of platelet-dependent thromboxane formation. The low dose of aspirin has similar effect to high dose of aspirin (10). Because too much aspirin will cause gastrointestinal bleeding, doctors do not recommend using high dose (11). Clopidogrel is more effective than aspirin in preventing atherothrombosis and causes less gastrointestinal bleeding problems than aspirin .

Hyperlipoproteinaemia is also a relevant risk factor for developing atherosclerosis. The Heart Protection Study showed that using lipid agents reduced myocardial infarction, stroke and cardiovascular death and subjects needed fewer revascularizations (12; 13). ACE inhibitors and beta blockers also are used to treat hypertension. ACE inhibitors ameliorate endothelial function and cardiac and vascular remodeling and slow the progression of atherosclerosis by improving the ratio of PAI-1 to tPA in favor of fibrinolysis (14). Omega-3-fatty acids have been demonstrated to have many cardiovascular protective effects including reducing the triacylglycerol content to reduce the cardiovascular disease (15). Moreover, lifestyle

also plays an important role in improving atherosclerosis. For example, regular exercise, diet control and smoking cessation reduces the risk of myocardial infarction and improves survival of patients with atherosclerosis (15; 16).

1.2. Plasma lipid metabolism

In mammals, there are two main metabolic pathways for plasma lipids: one is an exogenous pathway which transports dietary lipid; another is an endogenous pathway which transports lipid derived from the liver. The exogenous pathway includes the absorption and distribution of exogenous dietary lipid. The enterocytes of the small intestine absorb dietary lipids and repackage triacylglycerol, cholesterol, cholesterol esters, phospholipids, and fat soluble vitamins as chylomicrons. Chylomicrons (CM) are transported to the systemic circulation and are processed to chylomicron remnants by lipoprotein lipase (LPL). This processing releases free fatty acid and decreases the amount of triacylglycerol in the particle, while leaving cholesterol ester molecules behind (17) These cholesterol ester-rich chylomicron remnants are removed from the plasma by the LDL receptor. Moreover, chylomicrons do not only contain lipid but also contain several proteins termed apolipoproteins (apo). Nascent chylomicrons contain apoB48, apoA Ⅰ and apoAⅣ. When they go through the thoracic duct and enter into the circulation via the subclavian vein, these nascent CM particles gain apoE and the apoC`s (apoC Ⅰ, apoC Ⅱ, and apoC Ⅲ) from other plasma lipoproteins, particularly high density lipoprotein (HDL) (18).

The endogenous pathway transports endogenously generated lipids from the liver through the plasma to peripheral tissue. In humans, the hepatocytes package lipid into apoB100 containing VLDL particles that are secreted through the space of Disse into the plasma. TAG-rich VLDL is converted by LPL in the capillaries to VLDL remnants (also called IDL). VLDL remnants can then be removed from the circulation by peripheral tissues and the liver via the LDL receptor (19). Alternatively, the VLDL remnants can be further processed by a poorly characterized mechanism to the highly atherogenic lipoprotein LDL (20). These small, dense, cholesterol ester-rich particles do not contain apoE and can be removed by the ligand binding activity of ApoB100 via the LDL receptor.

1.3. ApoE

ApoE is an amphipathic protein which plays a role in lipoprotein trafficking by both stabilizing and solubilizing lipoprotein particles (21). ApoE is a glycoprotein with a molecular size of approximately 34 KD that is synthesized in the liver, brain, and other tissues in both humans and mice (22). It is part of constituent of chylomicrons, VLDL, IDL, and HDL and plays a role as a ligand for the receptor mediated clearance of these particles (6). These particles are important for “reverse-cholesterol transport” which lets cholesterol enter the liver via cholesterol ester transfer protein (CETP) from the peripheral tissues. The cholesterol ester transfer protein plays a role in cholesteryl ester, triacylglycerol, retinyl ester, and phospholipids transfer activities and is also responsible for all of the neutral lipid transfer activity of human plasma,

especially from HDL to VLDL remnants (23). Moreover, apoE is highly expressed in adipocytes and adipose tissue (24). There are two mechanisms by which apoE affect the lipid metabolism in adipocyte. One is that the appearance of apoE could accumulate the triacylglycerol because of the interaction with triacylglycerol rich lipoproteins (TGRLs). Another is that exogenous apoE in VLDL is important for adipocyte triacylglycerol accumulation (23). The inhibition of apoE expression could decrease the lipid accumulation in differentiated adipocyte. The apoE mRNA concentration is also sensitive to the free cholesterol content of the adipocyte. The nuclear receptors LXRα and LXRβ and their oxysterol ligands up-regulate apoE expression in both macrophages and adipocyte (25).

1.4. Human apoE polymorphisms

Human apoE is polymorphic and consists of three major isoforms (apoE-2, apoE-3, and apoE-4) of which apoE-3 is the most common. These isoforms of apoE differ by their amino acid substitutions at two sites, 112 and 158 on the 299 amino acid chain. ApoE-2 has cysteines at both sites, apoE-4 has arginines at both sites, and apoE-3 has a cysteine at site112 and an arginine at site158 (26). This difference in isoform structure plays an important role in apoE function because the polymorphic site at position 158 is the region of apoE that interacts with heparin and the LDL receptor. This amino acid substitution in the LDL receptor binding region decreases the binding affinity of apoE-2 to less than 2% relative to that of apoE-3(27). These differences are also associated with differing levels of risk for atherosclerosis. Many studies

show that apoE-4 increases the plasma cholesterol level 2-5% while the average effect of possession of one apoE-2 allele is to decrease the plasma cholesterol level by 5-8% (28). Because apoE-4 LDL cholesterol level compared to apoE-3 is increased, the increased cholesterol level in apoE-4 LDL increases the risk in atherosclerosis. HDL cholesterol does not vary with the different apoE isoforms. Moreover, the apoE polymorphism has been found to be associated with a higher risk for Type Ⅲ hyperlipidemia, particularly in individuals homozygous for apoE-2. Type Ⅲ hyperlipoproteinemia is a familial human condition characterized by increased plasma cholesterol and triacylglycerol and an increased risk of premature atherosclerosis.

1.5. ApoE knockout mice

In 1992 apoE-deficient mice were generated by deleting the ApoE gene by gene targeting (29). Piedrahita *et al*. inactivated the apoE gene in mouse embryonic stem cells by homologous recombination. The mice lacking apoE develop normally and breed normally. Their body weights compared to wild type mice are the same. However, the apoE-deficient mice have significantly increased total plasma cholesterol levels and triacylglycerol levels in VLDL and reduced plasma high-density lipoprotein (30). In $apoE^{-/-}$ mice, the spontaneous arterial lesions progress with time, as indicated by their increase in size and complexity and by their broader distribution in arterial lesions. Foam cell deposits and admixes smooth muscle and free cholesterol comprise the atherosclerotic lesions by 5 months of age. The arterial

lesions show increased complexity and the formation of fibrous caps as well as calcifications after 8-9 months. The complexity of lesions in the apoE-deficient mice can be used as a model of human disease and make it a desirable system to study environmental and genetic determinants of atherosclerosis. The study by Huang ZH *et al.,* indicated that endogenous apoE modulates adipocyte lipid metabolism and is important for the effect of PPAR-γ agonists on adipocyte lipid metabolism(31). PPAR-γ treatment of adipocyte results in increasing apoE expression and triacylglycerol accumulation. The endogenous adipocyte apoE expression also plays an important role in adipocyte lipid metabolism, increasing adipocyte triacylglycerol mass and synthesis, free fatty acid mass, cholesterol synthesis, and the expression of genes involved in triglyceride droplet metabolism and fatty acid oxidation. The apoE knockout adipocyte reduces the gene expression of genes involved in fatty acid oxidation compared to wild-type adipocytes.

1.6. Influence of diets on the apolipoprotein E knockout mice.

The apoE-deficient mice generated by gene targeting have spontaneously elevated plasma cholesterol levels, even when fed a chow diet (4% fat, 0.022% cholesterol). In contrast, heterozygous apoE $^{+/-}$ mice did not show abnormalities in lipid metabolism when fed a regular chow diet, and no arterial lesions were detected in them (30). Zhang *et al* (32) fed normal mice, heterozygous mutants and homozygous apoE-deficient mice with an atherogenic diet (15.8% fat, 1.25% cholesterol, and 0.5% cholate). The plasma cholesterol levels increased ~2.5 fold in

normal mice, 3.5-fold in heterozygous mutants, and 5-fold in homozygous apoE-deficient mice. The heterozygous apoE-deficient mice fed the atherogenic diet developed 30 times bigger atherosclerotic lesions than normal mice. The atherogenic diet-induced atherosclerotic lesions in apoE$^{-/-}$ mice were more serious than apoE$^{-/-}$ mice fed a chow diet. Plump *et al (33)* fed apoE$^{-/-}$ mice with a Western-style diet (21 % fat, 0.15 % cholesterol) and found that both their plasma cholesterol and their lesion area was three-fold higher than with a chow diet. Other high fat diet had similar effects. In apoE$^{-/-}$ mice, for example, the diet of 13% milk fat and 0.15% cholesterol (34) and the diet of 9% fat and 0.15% cholesterol (35) both increased the plasma cholesterol levels by three-fold. A slightly modified cholesterol content in diet (20% fat from palm oil and 0.1% cholesterol) also can increase plasma cholesterol and mean lesion areas (36). An atherogenic diet (15% cocoa butter as fat, 1% cholesterol and 0.5% cholic acid) induced six-fold to eight-fold increases in plasma cholesterol levels and lesion areas. This atherogenic diet study (31) suggested that the presence of cholic acid may increase the plasma cholesterol levels and accelerate the development of atherosclerosis.

The heterozygous apoE-deficient (apoE$^{+/-}$) mice don't develop the elevated plasma cholesterol levels or atherosclerotic lesion on western diet. If apoE$^{+/-}$ mice are fed with an atherogenic diet (15.8% fat, 1.25% cholesterol. 0.5% sodium cholate), their plasma cholesterol levels increase ~3.5-fold compared to normal mice (32). These data suggest that the apoE$^{+/-}$ mice respond to a particular diet to raise their plasma

cholesterol levels and lesions areas.

However, some diets will improve the atherosclerosis in apoE-deficient mice. Calleja L *e t al* (37) showed that male apoE$^{-/-}$ mice respond to sunflower oils to reduce 1/3 of the size of atherosclerotic lesions, and females respond to palm oil and olive oil both reduce 2-fold lesion size. The lesion reduction in males was accompanied by a 0.06% reduction of triacylglycerol in triacylglycerol-rich lipoproteins. However, the lesion reduction in females also showed an increase of plasma apoA-I. These data suggested that the development of lesions not only depends on the sex differences but also on the diet difference. Black *et al.* (38) showed that apoE$^{+/-}$ mice fed an atherogenic diet (18.5 % triacylglycerol, 1.25 % cholesterol and 0.5 % sodium cholate) supplemented with 1.5g/100g palm tocopherols (palm-E) had 60% lower plasma cholesterol levels than mice fed the atherogenic diet alone. In addition, when apoE$^{+/-}$ mice were fed the atherogenic diet with palm-E supplements they had smaller atherosclerotic lesions than mice fed the atherogenic diet alone or supplemented with palm carotenoids. Ni *et al*. (39) also discovered that mice fed a 10% fat, 1% cholesterol and 0.25% cholate diet with soy protein as the protein source had smaller atherosclerotic lesion development than mice fed a diet with casein as the protein source. Although their plasma cholesterol levels are the same, the lesion sizes in the thoracic aorta in the soy protein isolate group were smaller than those of the casein group. This study showed that the anti-atherosclerotic effect of soy protein doesn't affect serum lipids.

1.7. GPAT

Acyl-CoA : glycerol-3-phosphate acyltransferase (GPAT) plays an important role in the de novo synthesis of TAGs. GPAT catalyzes the acylation of sn-glycerol-3-phosphate to form lysophosphatidic acid (LPA) (40). Lysophosphatidic acid is then esterified to become phosphatidic acid which will form diacylglycerol and triacylglycerol (41). In mammals, triacylglycerol can store the energy, supply the daily calories for humans, and serve as a precursor for the synthesis of phospholipids. Triacylglycerol serves as a precursor for the synthesis of phospholipids in tetrahymena or for acetate-derived antibiotics in *Streptomyces.* The step of GPAT in the synthesis of triacylglycerol may be a rate limiting because GPAT expresses the lowest specific activity of all enzymes in this pathway (42). Abnormal regulation of the synthesis of triacylglycerol is associated with several metabolic disorders (43). Triacylglycerol accumulation in both adipose tissues and nonadipose tissues causes obesity; Triacylglycerol accumulation in muscle and pancreas occurs with insulin resistance (44; 45) and islet cell dysfunction (46). In type 2 diabetes and hepatic steatosis, the hepatic TAG and liver VLDL secretion increase (47; 48).

1.7.1 GPAT isoforms

There are multiple different isoforms of GPAT in mammalian tissues, two located in the endoplasmic reticulum (GPAT3,4), and two located in the outer mitochondrial membrane: GPAT1(which was previously called mtGPAT) and GPAT2 (48; 49). The

GPAT2, 3, 4 and GPAT1 also differ in their acyl-CoA substrate preference. The microsomal GPAT doesn't have a preference between saturated and unsaturated long-chain acyl-CoAs, but GPAT1 prefers C16:0-CoA. GPAT1 was cloned from both mice and rats (50-52). The mouse GPAT1 has 30% identity and additional 42% similarity to a 300-amino acid region of *E.coli* GPAT (51; 53). The cDNA for mouse GPAT1 has an open reading frame of 827 amino acids (50; 54) The rat GPAT1 has 89% homology and 96% identity to mouse GPAT1 and has an open reading frame of 828 amino acids. The mouse GPAT3 and human GPAT3 gene encode 438 and 434 amino acid protein. They are sharing 95% identity. The hGPAT3 and mGPAT3 have <15% sequence identify with mtGPAT1 (55). GPAT4 (initially designated AGPAT6) was first identified in a gene-trap screen in mouse embryonic stem cells (56). GPAT4 contains a cysteine (Cys-325) within a highly conserved domain thought to be important for G3P binding (56).

1.7.2. Difference of GPAT isoforms activities

The microsomal GPAT is sensitive to *N*-ethylmaleimide (NEM) which is a thiol reagent (57) and accounts for 80-90% of total GPAT activity in most tissues and 50-80% of total activity in liver (58) Unlike the microsomal GPATs, GPAT1 is not sensitive to NEM and comprises 10% of the total GPAT activity in most tissues, but 30%~50% of the total activity in the liver (43). GPAT2 is competitively inhibited by dihydroxyacetone phosphate and polymixin B and is not stimulated by acetone. GPAT2 may play an important role for synthesizing mitochondrial phospholipids

(phosphatidylglycerol and cardiolipin) which is similar to the sole bacterial GPAT(*PlsB*) (49). Like GPAT2, GPAT3 is sensitive to NEM, iodoacetamide, heating and acetone. The expression of recombinant GPAT3 increases acylation of G3P specifically, but not LPA, other lysophospholipids, MAG, or DAG. Moreover, GPAT3 mRNA and GPAT activity in differentiated adipocytes is significantly decreased by siRNA directed against GPAT3 (58). GPAT4 contributed 65% of liver NEM-sensitive GPAT specific activity. GPAT4 is also the primary NEM-sensitive GPAT in BAT because the GPAT4$^{-/-}$ male mice lost the 65% NEM-sensitive GPAT activity in BAT (59).

1.7.3. Fatty acid preference of different GPAT isoforms

The other way to distinguish GPAT isoforms is by their preference for specific substrates. In rat liver, kidneys and heart, GPAT1 activity is 3- to 10- fold higher with 16:0-CoA than with 18:1-, 18:2-,18:3-, and 20:4-CoA (60; 61). In contrast, in rat liver, microsomal GPAT activity is similar with 18:1-, 18:2- and 16:0-CoA (60; 61). Recombinant GPAT1 purified from insect cells expressed 2-fold higher activity with 16:0-CoA than with 18:1-, 18:2n6-, 18:3n3-, or C20:4n6-CoA (62). In contrast, mtGPAT2 uses 16:0-CoA as well as 18:1-CoA. Lewin TM *et al.* (49) concluded that the microsomal GPAT does not have a preference between saturated and unsaturated long-chain acyl-CoAs, but GPAT1 prefers C16:0-CoA.

1.8. GPAT 1 knockout mice

GPAT1 is not necessary for embryogenesis, reproduction, or synthesis of milk TAG

because GPAT1$^{-/-}$ mice are healthy and breed normally (63). Hammond *et al.* constructed GPAT1$^{-/-}$ mice and showed that female mice weighed less than controls and had reduced gonadal fat pad weights. Both male and female GPAT $^{-/-}$ mice had lower hepatic triacylglycerol content, plasma triacylglycerol, and very low density lipoprotein triacylglycerol secretion (64).

Lack of GPAT activity would prevent the directing of fatty acyl-CoAs toward triacylglycerol synthesis decrease competition with CPT-1 for acyl-CoAs, and therefore increase the oxidation of fatty acid. When the GPAT1$^{-/-}$ mice were fed a diet high in fat and sucrose, they had two-fold higher plasma β-hydroxybutyrate and acyl-carnitine concentration than did control groups, and hepatic mitochondrial HMG-CoA synthase mRNA was upregulated (64). Moreover, when GPAT1$^{-/-}$ mice were fed a low fat diet, their hepatic acyl-CoA content increased three-fold. This demonstrated that GPAT1 plays an important role in metabolizing acyl-CoA. GPAT1 prefers to esterify saturated fatty acyl-CoAs, especially palmitoyl-CoA, at the *sn*-1 position of glycerol-3-phosphate. In GPAT1$^{-/-}$ mice, the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) contain ~21% less palmitate and 30 and 64% more stearate and oleate (65). PE and PC contain ~40% more arachidonate at the sn-2 position. Arachidonate is also 21 and 67% higher in mitochondrial PE and PC from GPAT1$^{-/-}$ liver than wild-type controls. These increases in arachidonate content and in fatty acid oxidation in GPAT1$^{-/-}$ are associated with a 20% increase in the rate of reactive oxygen species production, a significant increase in sensitivity to the

inducation of the mitochondrial permeability transition, and an increase in both oxidative stress-related hepatocyte apoptosis and in bromodeoxyuridine labeling (65). Deletion of GPAT1 protected mice from developing high-fat-diet-induced hepatic insulin resistance despite a large increase in hepatic acyl-CoA content (66). A study described that a 90% adenovirus-mediated shRNA knockdown of hepatic NEM-resistant GPAT1 activity in *ob/ob* mice decreased liver TAG and DAG content 40-50% and decreased plasma glucose level and plasma cholesterol level content 30-40% (67). The increasing of FA oxidation in the mtGPAT1 knockout mice may shift the balance of energy metabolism towards lipid utilization, making carbohydrate utilization effective to an extra load. Therefore, mtGPAT1 knockout mice fed on high fat diet may ameliorate hepatic insulin sensitivity and whole body glucose tolerance. These studies suggested that inhibiting GPAT1 may improve atherosclerosis. Thus, the goal for this project was to generate apoE$^{+/-}$-GPAT1$^{-/-}$ mice to answer the following questions: Do the apoE$^{+/-}$-GPAT1$^{-/-}$ mice have lower plasma triacylglycerol level and plasma cholesterol level than the apoE$^{+/-}$ mice? Do the apoE$^{+/-}$- GPAT1$^{-/-}$ mice decrease adipose tissue compared to the apoE$^{+/-}$mice? Do the apoE$^{+/-}$- GPAT1$^{-/-}$ mice decrease VLDL triacylglycerol secretion compared to the apoE$^{+/-}$ mice? Do the apoE$^{+/-}$-GPAT1$^{-/-}$ mice develop smaller atherosclerotic lesions than the apoE$^{+/-}$ mice?

This study uses a western diet to induce atherosclerosis on apoE$^{+/-}$ mice. As mentioned before, GPAT$^{-/-}$ mice have lower plasma triacylglycerol, hepatic triacylglycerol content and VLDL triacylglycerol secretion than wild type. If we

generated apoE$^{+/-}$- GPAT1$^{-/-}$ mice, we predicted that lack of GPAT in apoE$^{+/-}$ mice would ameliorate the development of atherosclerosis. We predicted that the plasma triacylglycerol level and hepatic triacylglycerol level on apoE$^{+/-}$- GPAT1$^{-/-}$ mice will be lower than apoE$^{+/-}$ mice because the GPAT is the key regulator in the synthesis of triacylglycerol. We also anticipated that the size of atherosclerotic lesion on apoE$^{+/-}$-GPAT1$^{-/-}$ mice would be smaller than those in apoE$^{+/-}$ mice because the VLDL-TAG secretion would be lower in the GPAT deficient mice. This might improve the lesion formation.

CHAPTER 2

Does absence of GPAT ameliorate atherosclerosis induced on apoE heterozygous mice fed a western diet?

We fed ApoE$^{+/-}$ and ApoE$^{+/-}$/GPAT1$^{-/-}$ mice a western diet for 4 months. The ApoE$^{-/-}$ mice develop diet-induced atherosclerosis on this diet. We wanted to see if ApoE$^{+/-}$/GPAT1$^{-/-}$ mice improve the diet-induced atherosclerosis. We tested the effects on both ApoE$^{+/-}$/GPAT1$^{-/-}$ mice and ApoE$^{+/-}$ mice fed a western diet (consisting of 42% fat, 0.2% cholesterol) for 4 months. We expected to find out that the ApoE$^{+/-}$/GPAT1$^{-/-}$ mice will decrease their plasma triacylglycerol, hepatic triacylglycerol content, plasma cholesterol content and VLDL secretion. Moreover, we hoped that we will see less atherosclerotic lesion area on the ApoE$^{+/-}$/GPAT1$^{-/-}$ mice.

Methods

Animals. Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. In experiment one, ApoE$^{+/-}$and GPAT1$^{+/+}$ heterozygous were generated by crossing the B6-backcrossed ApoE$^{-/-}$ and GPAT $^{+/+}$ mice with wild type B6 animals. ApoE $^{+/-}$ and GPAT1$^{-/-}$ heterozygous were generated by crossing the B6-backcrossed ApoE$^{-/-}$ and GPAT1$^{-/-}$ mice with GPAT1$^{-/-}$ mice. In experiment 1 (Chapter2), after 8 weeks of age, animals were fed on a western diet containing 0.2 % cholesterol and providing 42 % calories as fat (milkfat) (TD88137; Harlan Teklad, Madison Wisconsin, USA) for four months. Mice were weighed at the beginning of diet (d 0) and the end (d 120) of the diets. In experiment 2 (Chapter 3), after 8 weeks of age, animals were fed on a atherogenic diet containing 18.5 % fat, 1.25 % cholesterol, and 0.5 % cholate (TD88051; Harlan Teklad, Madison Wisconsin, USA) for three months. Mice were weighed at the beginning of diet (d 0) and the end (d 90) of the diets.

Plasma lipid assay Blood samples were collected from animals fasted 4 hours by retroorbital bleeding into a tube containing EDTA at d 0, d 60 and d 120. The blood was centrifuged for 10 min at 12,000 x g at 4℃. Plasma was measured for triacylglycerol (GPO-Trinder kit, #339-20, Sigma Chemical) , cholesterol (Cholesterol CⅡ kit, #276-64909, Wako Pure Chemical, Osaka, Japan) and glucose (Glucose Trinder; sigma) using colorimetric enzyme methods.

Lipoprotein separation Plasma lipoproteins were separated by density using FPLC system (Amersham Pharmacia Biotech, Piscataway, NJ). The system fractionated serum lipoprotein by superpose-6 gel column (Pharmacia) which was equilibrated at 0.5 ml/min with running buffer (PBS containing 20 g sodium azide/L). Then 100 µL of mouse serum collected from four mice in one experimental group was injected onto the column. The sample was run at 0.4 mL/min, and 0.5 mL fractions were collected. After discarding the first 10 fractions, the rest of the fractions were assayed for total cholesterol and
triacylglycerol as above.

Morphometric analysis Mice fasted for 4h were sacrificed with an overdose of Avertin. The hearts were perfused with phosphate-buffered paraformaldehyde (4%) under physiological pressure. Segments of the proximal aorta and the portion of the heart containing the aortic sinus were embedded, sectioned, stained with Sudan IVB (Fisher Scientific Co., Milwaukee, WI), and counterstained with hematoxylin. The sections stained with hematoxylin and eosins were used for histological evaluations. Average lesion sizes of four sections were used for the morphometric evaluations to represent the lesion size of each animal. Morphometry evaluation of the lesion size was conducted using Image measure/IP IM 2500 Morphometry system (Phoenix Technology, Federal Way, MA)(32)

mRNA analyses RNA was extracted from liver and fat pads that had been flash-frozen in liquid nitrogen. Total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After 1.5 µg of total RNA were reverse transcribed with an oligo-prime, PCR was performed with a DNA Engine Opticon 2 System using SYBR green QPCR dye kit (Stratagene).

Liver lipids Unperfused liver was removed and flash-frozen in liquid nitrogen. Approximately 0.1 g each of liver samples was homogenized and extracted with chloroform: methanol (2:1v/v) (68). To measure cholesterol and triacylglyceride, lipid aliquots were resuspended in 0.2 mL 10% Triton X-100 in isopropanol before measurement

Total particulate preparations and enzyme assays Livers were sliced into Medium 1 (250 mM sucrose, 10 mM Tris, PH 7.4, 1 mM EDTA, 1 mM dithiothreitol) , homogenized with 15 up-and-down strokes in a motorized Teflon-glass homogenizing vessel, and centrifuged at 100,000 x g for 1 h to obtain total particulate fractions. GPAT activity was assayed in a 200-µl mixture containing 75 mM Tris-HCL, pH 7.5, 4 mM $MgCl_2$, 1 mg/ml bovine serum albumin (essentially fatty acid-free), 1 mM dithiothreitol, 8 mM NaF, 800 µM [H_3] glycerol 3-phosphate, and 80 µM palmitoyl-CoA. The reaction was started by adding 15-30 µg of protein to the assay mix. All assays were performed for 10 min at room temperature.

Result and Discussion

ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had slightly reduced body weight. Because female GPAT1$^{-/-}$ mice have reduced 16% of body weight compared to wild type mice when they were 6 month-old (64), we predicted that apoE$^{+/-}$- GPAT1$^{-/-}$ mice and GPAT1$^{-/-}$ mice might have reduced body weight. ApoE$^{+/-}$- GPAT1$^{-/-}$ mice weighed 14% less than apoE$^{+/-}$mice after 4 months of western diet but the difference was not significant (Figure 2.1). To investigate whether adipose tissue from apoE$^{+/-}$- GPAT1$^{-/-}$ mice and GPAT1$^{-/-}$ mice was decreased, we isolated fat pads from these animals. Fat pads from ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had 51% smaller gonadal fat pads than apoE$^{+/-}$mice (Figure 2.2). Thus, the reason for slightly reduced body weight could be the smaller fat pads.

ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had reduced plasma lipids compared apoE$^{+/-}$mice. In female mice fed the western diet for 120 days, the plasma cholesterol concentration of apoE$^{+/-}$ mice was 27% higher than apoE$^{+/-}$- GPAT1$^{-/-}$ mice (Figure 2.3). But, the concentrations of plasma cholesterol of the two groups were lower than lipid levels that would cause atherosclerosis. In a study by Zhang SH, which fed apoE heterozygous mice on an atherogenic diet for 12 weeks, plasma cholesterol level was 326 ± 153 (32). Because GPAT1$^{-/-}$ mice had lower plasma triacylglycerol and VLDL-triacylglycerol secretion than wild type mice, we expected to see a reduction of plasma triacylglycerol in the apoE$^{+/-}$- GPAT1$^{-/-}$ mice. The concentration of plasma

triacylglycerol of ApoE$^{+/-}$- GPAT1$^{-/-}$ mice was 53% lower than apoE$^{+/-}$mice.

FPLC analysis of lipoproteins. When mice were fed the western diet, the major cholesterol containing fraction was HDL (fractions 28-34). ApoE$^{+/-}$ mice had a higher peak of HDL than the apoE$^{+/-}$- GPAT1$^{-/-}$ mice. ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had higher peak of VLDL than the apoE$^{+/-}$ mice (Figure 2.4). So, apoE$^{+/-}$- GPAT1$^{-/-}$ mice might get atherosclerosis because most of the cholesterol in the apoE-deficient mice was carried by particles with sizes in the VLDL to IDL range (32). However, when high plasma lipids induce atherosclerosis in mice, the major cholesterol containing fraction is VLDL instead of HDL (38). These authors showed that when apoE $^{+/-}$ mice were fed the nonpurified control diet, the major lipoprotein peak was HDL.

ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had lower hepatic triacylglycerol. We examined liver histology and analyzed liver lipid. In apoE$^{+/-}$- GPAT1$^{-/-}$ mice, hepatic triacylglycerol concentration was 30% lower than apoE$^{+/-}$ mice (Figure 2.5). This is consistent with the reduction of GPAT1 activity in the liver which contributes to triacylglycerol synthesis. Our study showed that hepatic triacylglycerol concentration of female GPAT $^{-/-}$ mice was 36% lower than female wild-type mice at the 2 month of age. These data were similar to the data we found from the apoE$^{+/-}$ mice and apoE$^{+/-}$- GPAT1$^{-/-}$ mice.

Histological and morphometric analysis of arterial lesions. To investigate whether lack of GPAT1 can protect against the development of atherosclerosis, the lesion area was assessed at the aortic sinus, the arch, the third intercostals branch and the celiac branch. The atherosclerotic lesions were measured at all four sites. There were no atherosclerotic lesions in apoE$^{+/-}$ mice or apoE$^{+/-}$- GPAT1$^{-/-}$ mice. This is consistent with the lower content of plasma cholesterol and the main peak of HDL-cholesterol. Although Plump *et al.* fed apoE-deficient mice for 4-5 weeks with a western-style diet (21% fat, and 0.15% cholesterol by weight), they found out that the lesion sizes were three times larger than those on normal diet (69).

Thus, after being fed 120 days with a western diet, there were no atherosclerotic lesions in apoE$^{+/-}$mice or apoE$^{+/-}$- GPAT1$^{-/-}$ mice. The plasma cholesterol was low in both groups of mice. The highest peak of cholesterol fraction was HDL. These data showed the western diet cannot induce atherosclerosis in apoE$^{+/-}$mice or apoE$^{+/-}$- GPAT1$^{-/-}$ mice.

Although apoE$^{+/-}$- GPAT1$^{-/-}$ mice decrease 14% body weight, 27% of plasma cholesterol, 53% of plasma triacylglycerol, and 30% of hepatic triacylglycerol compared to apoE$^{+/-}$ mice. There were no atherosclerotic lesions. Thus, we could not conclude that absence of GPAT could improve the atherosclerosis.

Tab 2.1 Weight and plasma triacylglycerol and plasma cholesterol in ApoE +/- female mice and ApoE+/- /GPAT1-/- female mice fed on western diets for 120 days.

Data are presented as mean±SE. For all initial groups, n=8 for all group. For final groups, n=8 for Ee/GG mice, n=6 for Ee/gg mcie. Superscripts "a" indicate significant differences between changes in Ee/GG mice and Ee/gg mice ($p<0.05$). Ee/GG indicates $apoE^{+/-}$ female mice, Ee/gg indicates $apoE^{+/-}/GPAT1^{-/-}$ mice.

	Ee/GG	Ee/gg
Final weight(g)	33.3±2.2	28.8±1.8
Adipose tissue/body weight (%) (inguinal)	2.1±0.3	1.8±0.3
(Gonadal)	7.7±0.4	3.1±1.0
Initial TAG (mg/dL)	32.1±3.7	16.8±6.6 [a]
Final TAG (mg/dL)	48.8±10.9	23.1±3.3
Initial cholesterol (mg/dL)	40.2±2.8	44.1±2.1
Final Cholesterol (mg/dL)	112.5±9.0	81.6±6.9 [a]

Fig 2.1 There was no significant difference between ApoE +/- mice and ApoE+/-/GPAT1-/- mice.
The western diet fed from at 8 weeks to 16 weeks on ApoE $^{+/-}$ mice and ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice. There was no significant different between ApoE $^{+/-}$ mice and ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice.

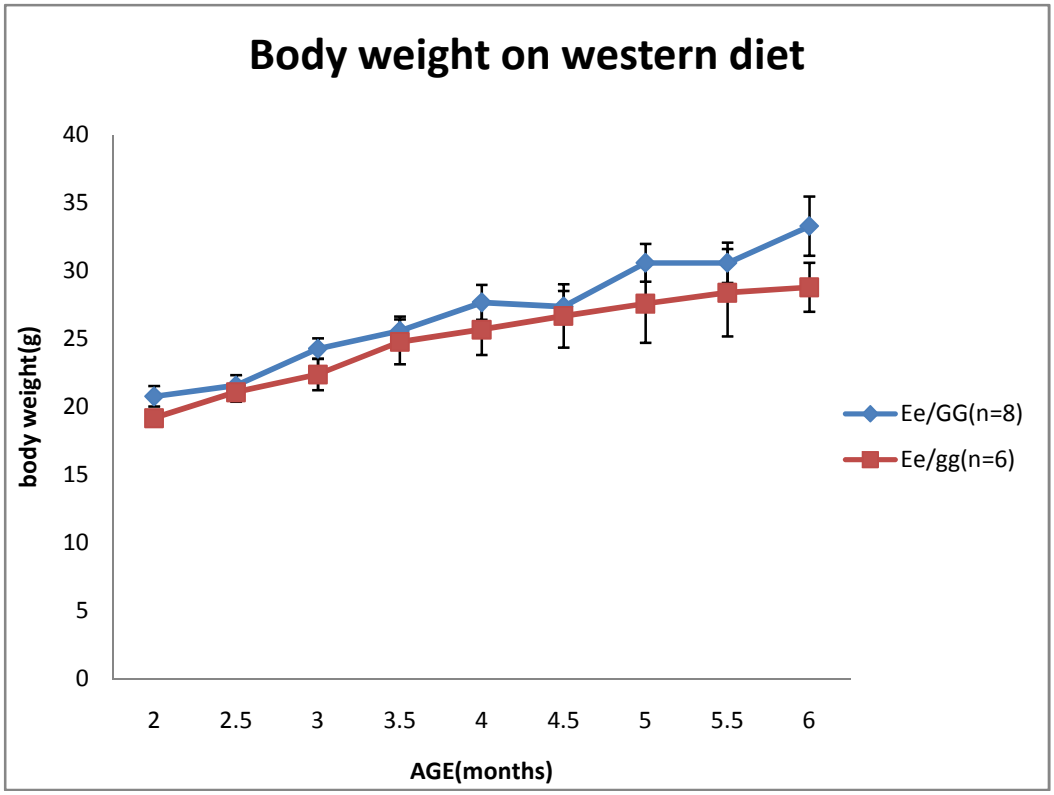


Fig 2.2 GPAT1 knockout in apoE heterozygous mice reduced the fat pad weight in gonadal adipose tissue.
The western diet was provided at 8 weeks to 16 weeks on ApoE $^{+/-}$ mice and ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice. *P<0.05

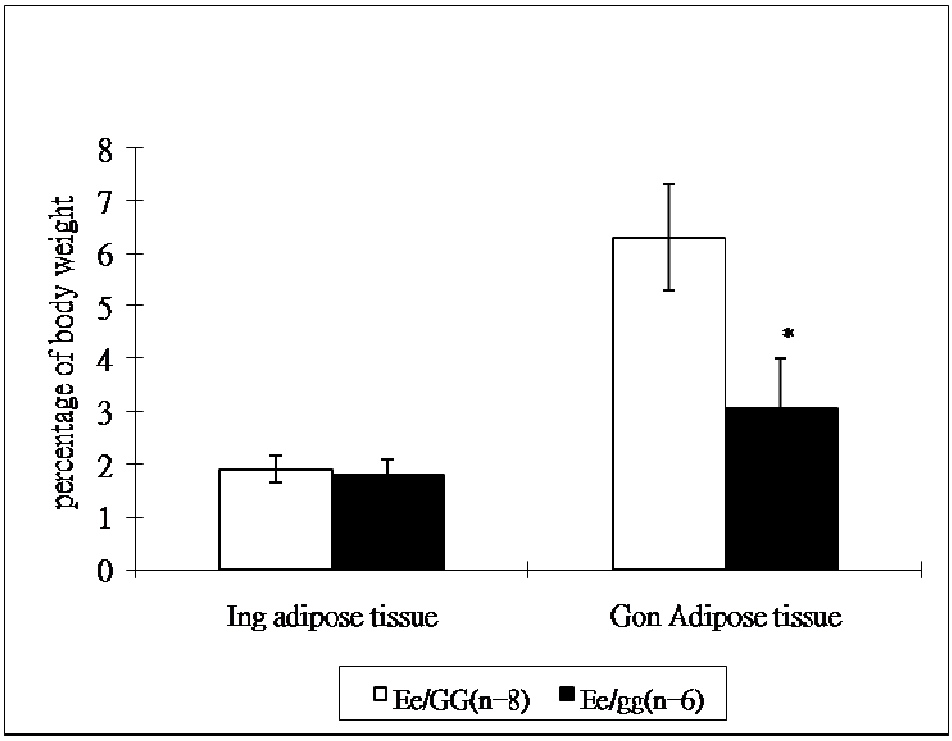
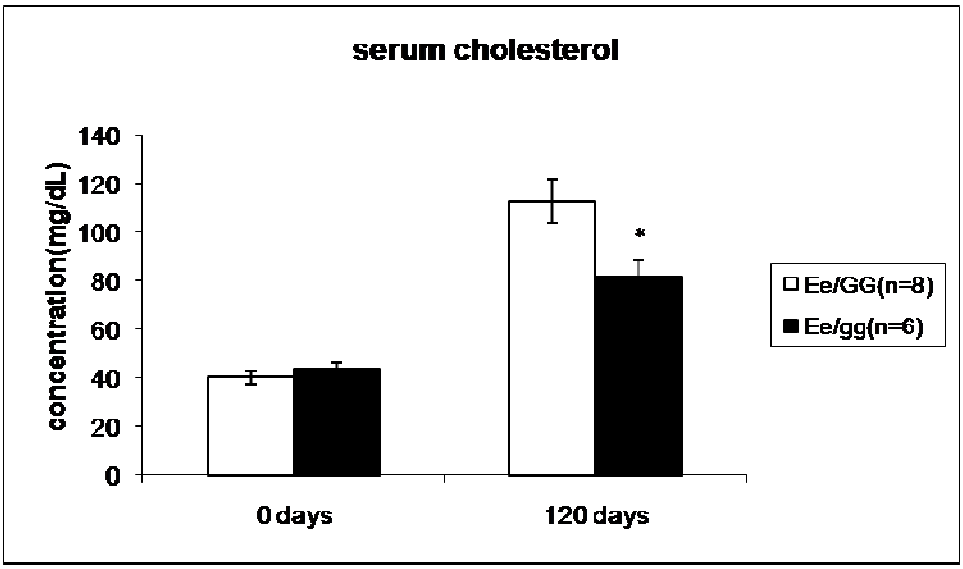


Fig 2.3 ApoE +/- mice and ApoE+/- /GPAT1-/- mice increase serum lipid level. The western diet was provided at 8 weeks to 16 weeks on ApoE +/- mice and ApoE+/- /GPAT1-/- mice.
Serum was obtained in animals at the age of 8 weeks on a chow diet (weeks=0). Mice were started on a western diet and obtained serum at 4 months. Samples were assayed for Cholesterol (A), and Triglycerides (B). * P<0.05

(A) Cholesterol



(B) Triacylglycerol

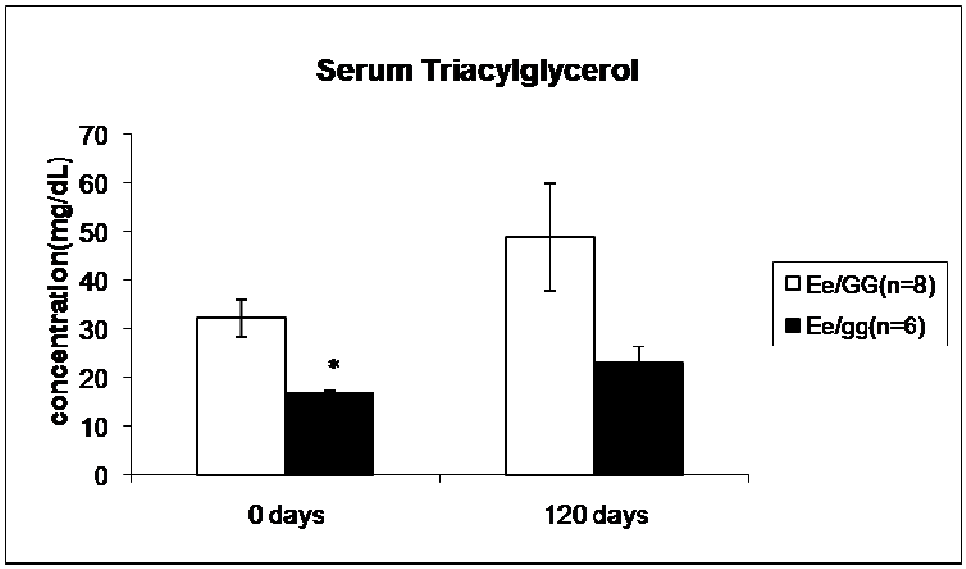


Fig 2.4 ApoE+/- /GPAT1-/- mice had more HDL-cholesterol. Plasma lipoprotein fractions were separated by FPLC.

Cholesterol in each fast-protein liquid chromatography fraction (0.5mL) from plasma (100 L) of ApoE $^{+/-}$ mice (filled diamond) and ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice (filled squares). Each "N" indicates three pooled sample.

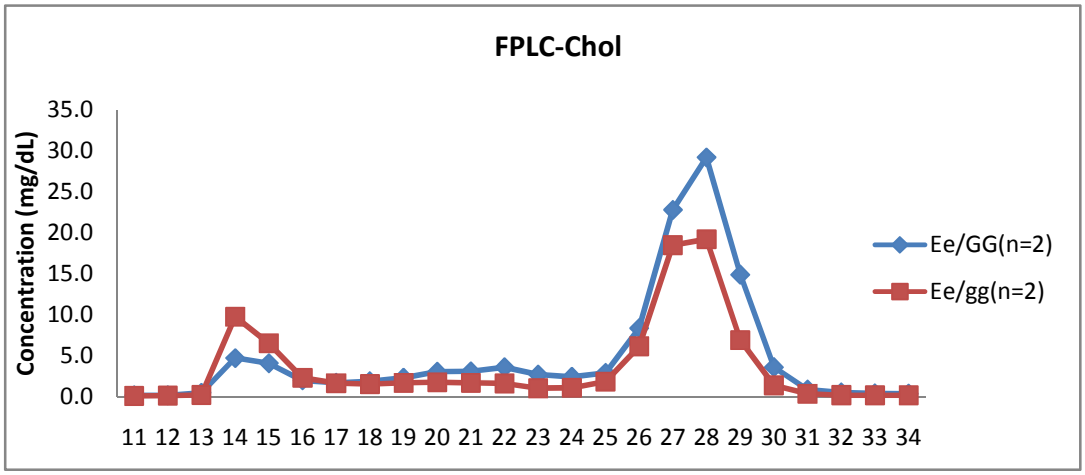


Fig 2.5 Liver triacylglycerol increased in ApoE +/- mice.
The western diet was provided at 8 weeks to 16 weeks on ApoE $^{+/-}$ mice and ApoE$^{+/-}$ /GPAT$^{-/-}$ mice. Results are expressed as mean ±SE. ** P<0.01

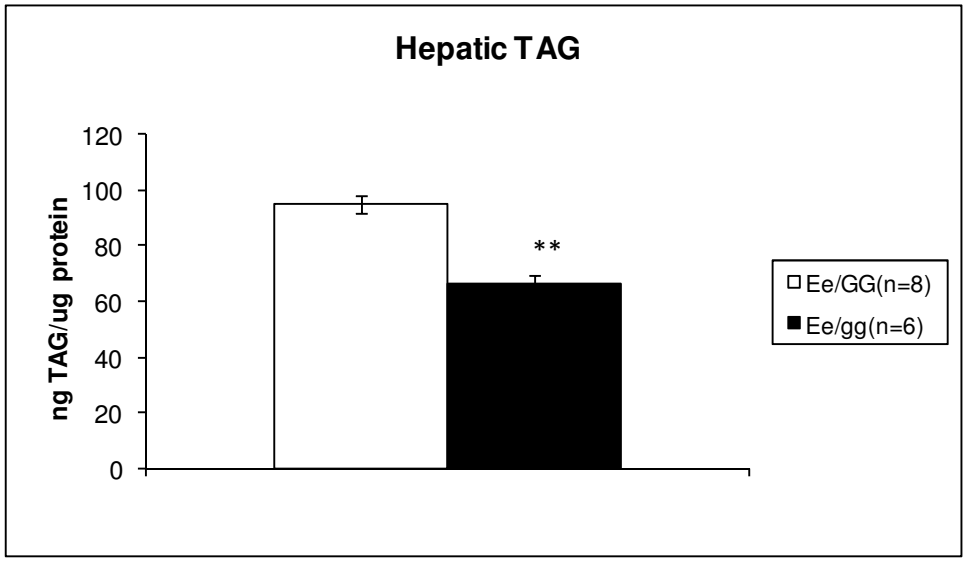


Fig 2.6 Mitochondrial GPAT activity was reduced in ApoE+/- /GPAT1-/- mice. GPAT- specific activity was determined in animal tissue from ApoE $^{+/-}$ mice and ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice as described under "Experimental Procedures."

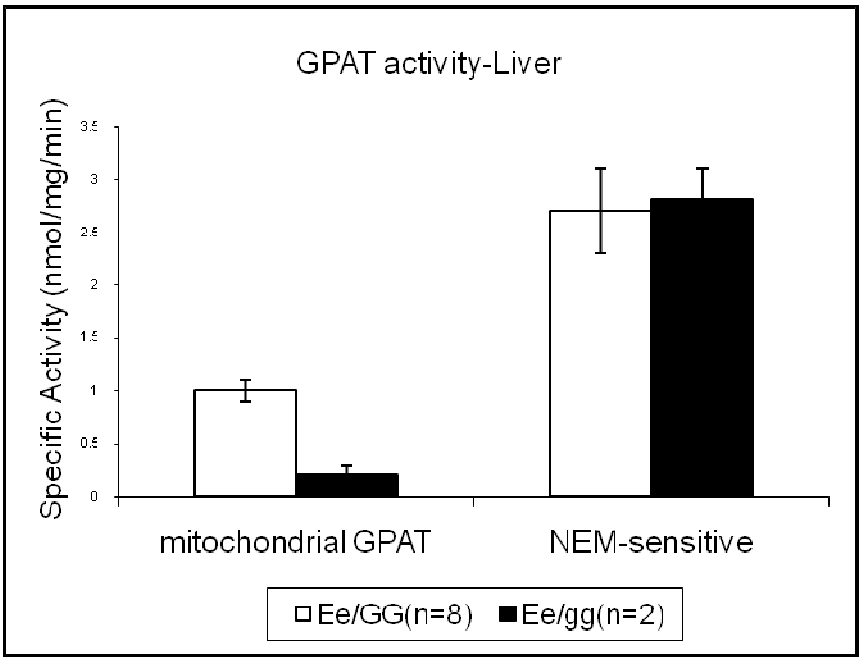
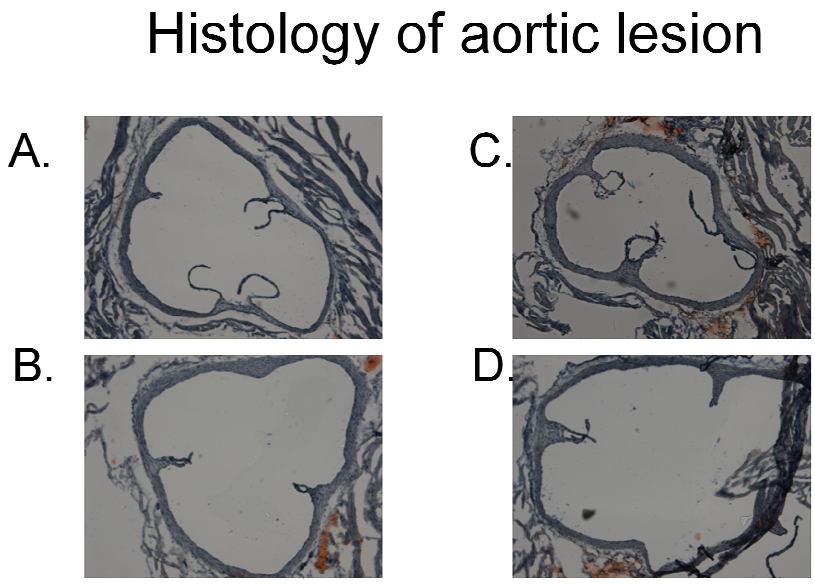


Fig 2.7 Pahtologic evaluations of arterial lesions and lipid depositions of the aortic sinus of ApoE+/- mice and ApoE+/-/GPAT1-/- mice fed a western diet for 4 months.
(A, B) ApoE$^{+/-}$ mice (C, D) ApoE$^{+/-}$/GPAT1$^{-/-}$mice. Sections were stained with Sudan ⅣB and counterstained with hematoxylin.



CHAPTER 3

Does absence of GPAT1 ameliorate atherosclerosis induced on apoE heterozygous mice fed an atherogenic diet?

We fed ApoE$^{+/-}$and ApoE$^{+/-}$/GPAT1$^{-/-}$ mice on a western diet for 4 months because we hypothesized that lack of GPAT1 would improve the atherosclerosis which was induced by western diet. However, the apoE heterozygous mice didn't develop diet-induced atherosclerosis when fed a western diet. Therefore, we changed to another kind of diet to develop diet-induced atherosclerosis. According to the study by Black *et al.*, the atherogenic diet will cause ApoE heterozygous mice to develop atherosclerosis in 90 days. Therefore, we fed ApoE$^{+/-}$ and ApoE$^{+/-}$/GPAT1$^{-/-}$mice on the atherogenic diet (15.8% fat, 1.25% cholesterol, 0.5% cholate) for 90 days. We wanted to see if ApoE$^{+/-}$/GPAT1$^{-/-}$ mice have less diet-induced atherosclerosis than the ApoE$^{+/-}$mice. We expected to find that the ApoE$^{+/-}$/GPAT1$^{-/-}$ mice had lowered their plasma triacylglycerol, hepatic triacylglycerol content, plasma cholesterol content.

Result and Discussion

ApoE$^{+/-}$- GPAT1$^{-/-}$ mice have reduced body weight. Because female GPAT1$^{-/-}$ mice have reduced body weight compared to wild type mice, we predicted that apoE$^{+/-}$- GPAT1$^{-/-}$ mice and GPAT1$^{-/-}$ mice would have reduced body weight. After 3 months of the atherogenic diet, ApoE$^{+/-}$- GPAT1$^{-/-}$ mice weighed 16% less than apoE$^{+/-}$mice and 14% less than GPAT1$^{-/-}$ mice (Table 3.1). Although GPAT1$^{-/-}$ mice had lower body weight than wild type mice (64), the weight of GPAT1$^{-/-}$ mice was similar to that of the apoE$^{+/-}$mice. To determine whether the difference in weight of the ApoE$^{+/-}$- GPAT1$^{-/-}$ mice was due to a difference in adipose tissue mass, we isolated fat pads from these animals. ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had 61% smaller inguinal fat pads compared to apoE$^{+/-}$mice and 42% smaller inguinal fat pads compared to GPAT1$^{-/-}$ mice. ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had 66% smaller gonadal fat pads compared to apoE$^{+/-}$mice and 36% smaller gonadal fat pads compared to GPAT1$^{-/-}$ mice. Thus, the reason of reduced body weight would be smaller fat pads.

ApoE$^{+/-}$- GPAT1$^{-/-}$ mice didn't change plasma lipids. In mice fed the atherogenic diet for 90 days, the plasma cholesterol concentration of apoE$^{+/-}$- GPAT1$^{-/-}$ mice was 39% higher than apoE$^{+/-}$ mice (Table 3.1), but because the values were variable, the difference was not significant. The plasma cholesterol concentration of apoE$^{+/-}$-

GPAT1$^{-/-}$ mice was 40% greater than in the GPAT1$^{-/-}$ mice. Because GPAT1$^{-/-}$ mice have reduced plasma triacylglycerol and VLDL-triacylglycerol secretion compared to wild type mice, we expected to observe a lower plasma triacylglycerol concentration in both apoE$^{+/-}$- GPAT1$^{-/-}$ mice and GPAT1$^{-/-}$ mice. However, the plasma triacylglycerol concentrations were not different between apoE$^{+/-}$ mice and apoE$^{+/-}$- GPAT1$^{-/-}$ mice. The plasma triacylglycerol concentrations were also not different between apoE$^{+/-}$- GPAT1$^{-/-}$ mice and GPAT1$^{-/-}$ mice.

FPLC analysis of lipoproteins. When mice were fed the atherogenic diet, the major cholesterol containing fractions were VLDL (fractions 14-17), intermediate density lipoprotein (IDL) (fractions 17-19), and LDL (fractions 19-27), whereas the HDL peak decreased. ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had a greater area under the curve for VLDL, IDL and LDL than the apoE$^{+/-}$mice (Figure 3.1). This was consistent with the increased serum cholesterol in apoE$^{+/-}$- GPAT1$^{-/-}$ mice compared to apoE$^{+/-}$ mice. These data suggest that lack of GPAT increases serum VLDL-cholesterol.

Histological and morphometric analysis of arterial lesions. To investigate whether absence of GPAT has the potential to protect against the development of atherosclerosis, the lesion area was assessed at the arch, third intercostals branch and celiac branch. There was no significant difference in lesion size between apoE$^{+/--}$ mice and apoE$^{+/-}$- GPAT1$^{-/-}$ mice. The average atherosclerotic lesion size

of apoE$^{+/-}$ mice was 154,161±19,976 μM^2 (Figure 3.2). The average atherosclerotic lesion of apoE$^{+/-}$- GPAT1$^{-/-}$ mice was 213,743 ± 86,896 μM^2. Although the average atherosclerotic lesion of apoE$^{+/-}$- GPAT1$^{-/-}$ mice was 30% greater than apoE$^{+/-}$mice, there was no significant difference between two groups. These data showed that lack of GPAT1 did not lessen the extent of atherosclerosis.

Hepatic lipid. The livers of the mice fed the atherogenic diet were pale and fatty. We examined liver histology and analyzed liver lipid. In apoE$^{+/-}$- GPAT1$^{-/-}$ mice, the hepatic triacylglycerol concentration was 14% lower than apoE$^{+/-}$ mice, but there was no significant difference between groups (Figure 3.3). In GPAT1$^{-/-}$ mice, the hepatic triacylglycerol concentration was 49% lower than apoE$^{+/-}$- GPAT1$^{-/-}$ mice and 53% lower than apoE$^{+/-}$ mice, consistent with the reduction of mitochondrial GPAT activity in the liver which is the rate-limiting enzyme of triacylglycerol synthesis. However, we did not find this consistent by in the apoE$^{+/-}$- GPAT1$^{-/-}$ mice which had similar hepatic triacylglycerol with apoE$^{+/-}$ mice in the liver. Although apoE$^{+/-}$- GPAT1$^{-/-}$ mice had 75% less mitochondrial GPAT activity compared to apoE$^{+/-}$ mice, the hepatic triacylglycerol of apoE$^{+/-}$- GPAT1$^{-/-}$ mice was similar with apoE$^{+/-}$ mice.

We concluded that apoE$^{+/-}$- GPAT1$^{-/-}$ mice reduced body weight compared to apoE$^{+/-}$ mice and GPAT1$^{-/-}$ mice. Because female GPAT1$^{-/-}$ mice have reduced 16% of body weight compared to wild type mice when they were 6 month-old (64), we expected

to see lower body weight in GPAT1$^{-/-}$ mice than in apoE$^{+/-}$ mice. However, the body weight of GPAT1$^{-/-}$ mice was similar to apoE$^{+/-}$ mice. The fat pads of apoE$^{+/-}$-GPAT1$^{-/-}$ mice were very small. As in the previous chapter, the apoE$^{+/-}$- GPAT1$^{-/-}$ mice had smaller the fat pads compared to apoE$^{+/-}$ mice on the western diet. The reason for small fat pads in apoE$^{+/-}$- GPAT1$^{-/-}$ mice could be lack of both apoE and GPAT expression (64; 70). Although there was no difference of plasma cholesterol between apoE$^{+/-}$ mice and apoE$^{+/-}$- GPAT1$^{-/-}$ mice, the cholesterol of apoE$^{+/-}$- GPAT1$^{-/-}$ mice had the highest area under the curve for VLDL, IDL, and LDL. We speculated that apoE$^{+/-}$- GPAT1$^{-/-}$ mice maybe increase the atherosclerotic lesion size.

Tab 3.1 Weight and plasma triacylglycerol and plasma cholesterol in ApoE +/- mice, ApoE+/- /GPAT1-/- mice and GPAT1-/- female mice fed on atherogenic diets for 90 days.

Data are presented as mean ± SE. For all initial groups, n=11 for all group. For final groups, n=9 for Ee/GG mice, n=10 for Ee/gg mcie, and n=11 for gg mice. Superscripts "a" indicate significant differences between changes in Ee/GG mice and Ee/gg mice ($P<0.05$). Superscripts "b" indicate significant differences between changes in Ee/gg mice and gg mice ($P<0.05$). Ee/GG indicates $apoE^{+/-}$ female mice, Ee/gg indicates $apoE^{+/-}/GPAT1^{-/-}$ mice and gg indicates $GPAT1^{-/-}$ female mice.

	Ee/GG	Ee/gg	gg
Final weight(g)	23.6±0.6	19.9±0.7 [a, b]	23.2±0.5
Adipose tissue/body weight (%) (inguinal)	1.1±0.1	0.4±0.1 [a, b]	0.7±0.1 [a, b]
(Gonadal)	1.1±0.1	0.38±0.1 [a, b]	0.6±0.1
Initial TAG (mg/dL)	46.2±3.3	34.7±2.8	27.7±1.5
Final TAG (mg/dL)	12.66±2.0	13.2±4.2	10.5±0.6
Initial cholesterol (mg/dL)	47.8±6.2	82±12.3	54.5±1.4
Final Cholesterol (mg/dL)	244.2±15.3	401.5±82.6	242.7±11.9 [b]

Fig 3.1 ApoE+/-/GPAT1-/- mice had more VLDL-cholesterol.
Cholesterol in each fast-protein liquid chromatography fraction (0.5mL) from plasma (100µL) of ApoE $^{+/-}$ mice (unfilled circle), ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice (filled squares) and GPAT1$^{-/-}$ mice (filled diamond). The ApoE$^{+/-}$/GPAT1$^{-/-}$ mice have similar VLDL-Cholesterol as the ApoE$^{+/-}$ mice. The GPAT1$^{-/-}$ mice have higher peak of HDL-Cholesterol than the ApoE$^{+/-}$/GPAT1$^{-/-}$ mice and ApoE$^{+/-}$ mice.

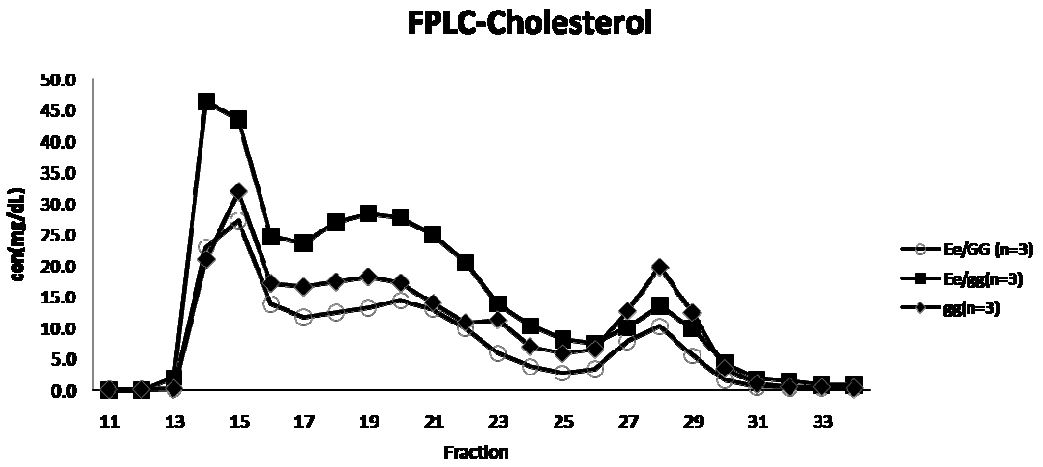


Fig 3.2 Pathologic evaluations of arterial lesions and lipid depositions of the aortic sinus of ApoE+/- mice and ApoE+/- /GPAT1-/- mice fed an atherogenic diet for 90 days.

Cryosectioned tissue was stained with Sudan IV and counterstained with hematoxylin. "A" presented the aortic sinus of $ApoE^{+/-}$ mice. "B" presented the aortic sinus of $ApoE^{+/-}$ /$GPAT1^{-/-}$ mice.

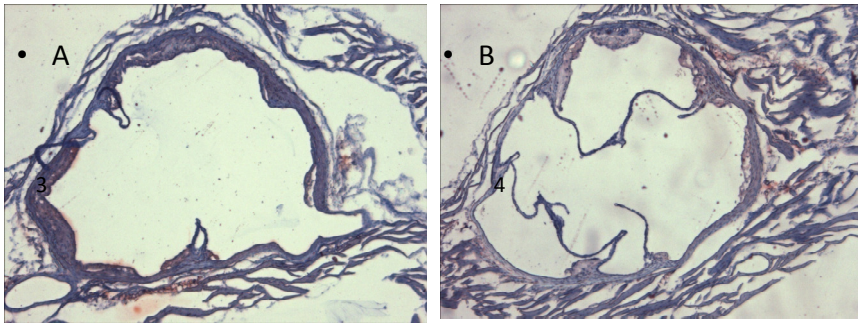


Fig 3.3 Morphometric evaluation of atherosclerotic lesion size at the level of the aortic sinus of ApoE+/- mice and ApoE+/- /GPAT1-/- mice fed an atherogenic diet for 90 days.
Each point represents the mean lesion size of three sections measured in each mouse.

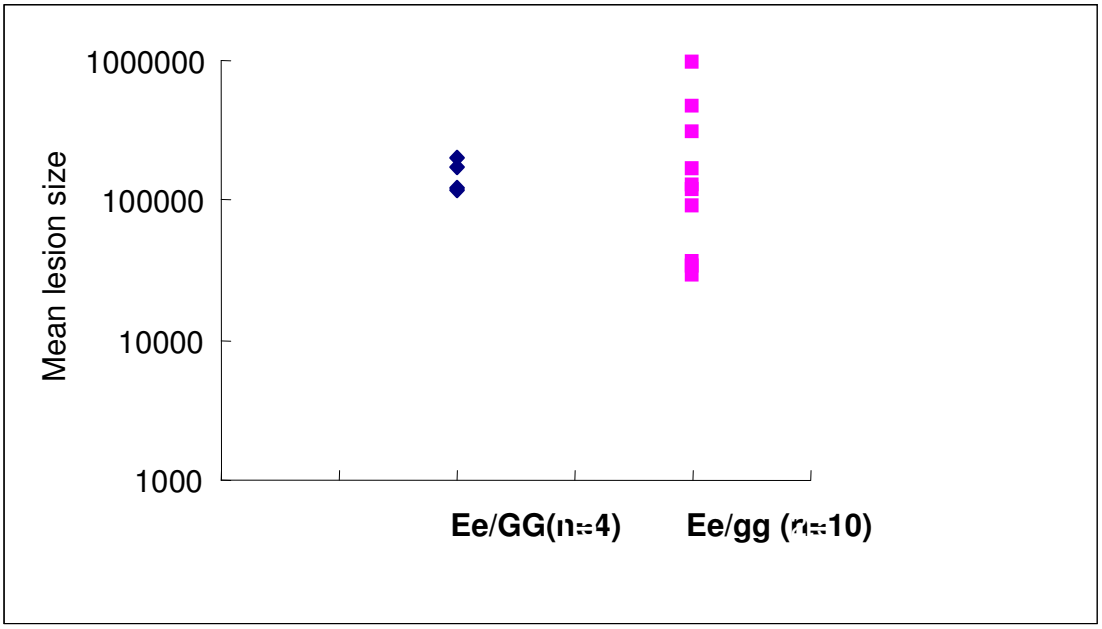


Fig 3.4 The mitochondrial GPAT activity reduced on GPAT1 knockout mice.
GPAT- specific activity was determined in animal tissue from ApoE $^{+/-}$ mice and ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice as described under "Experimental Procedures."
(** P<0.01)

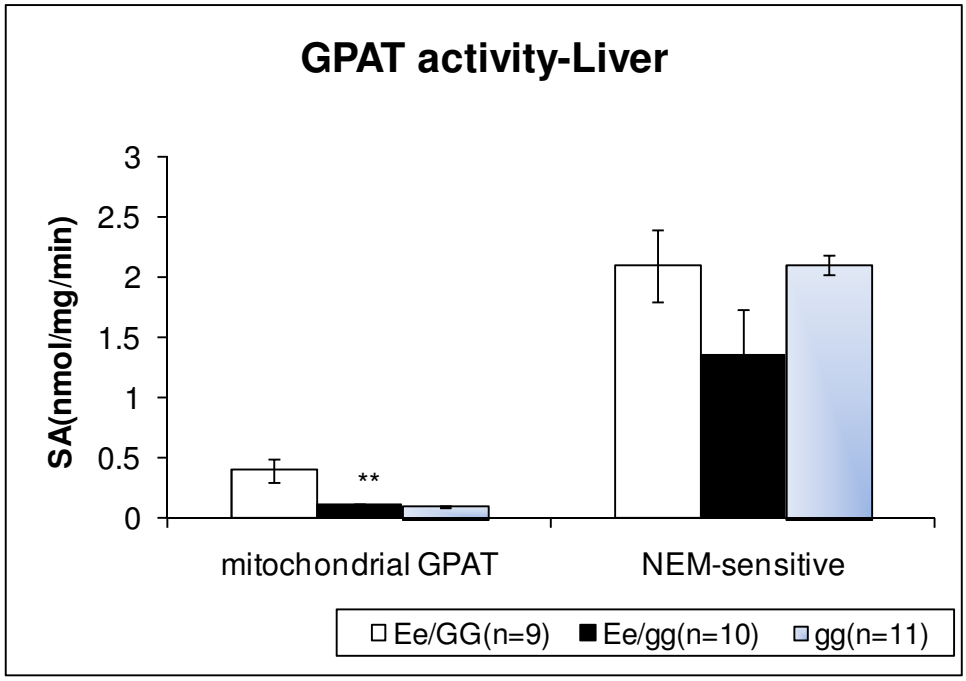
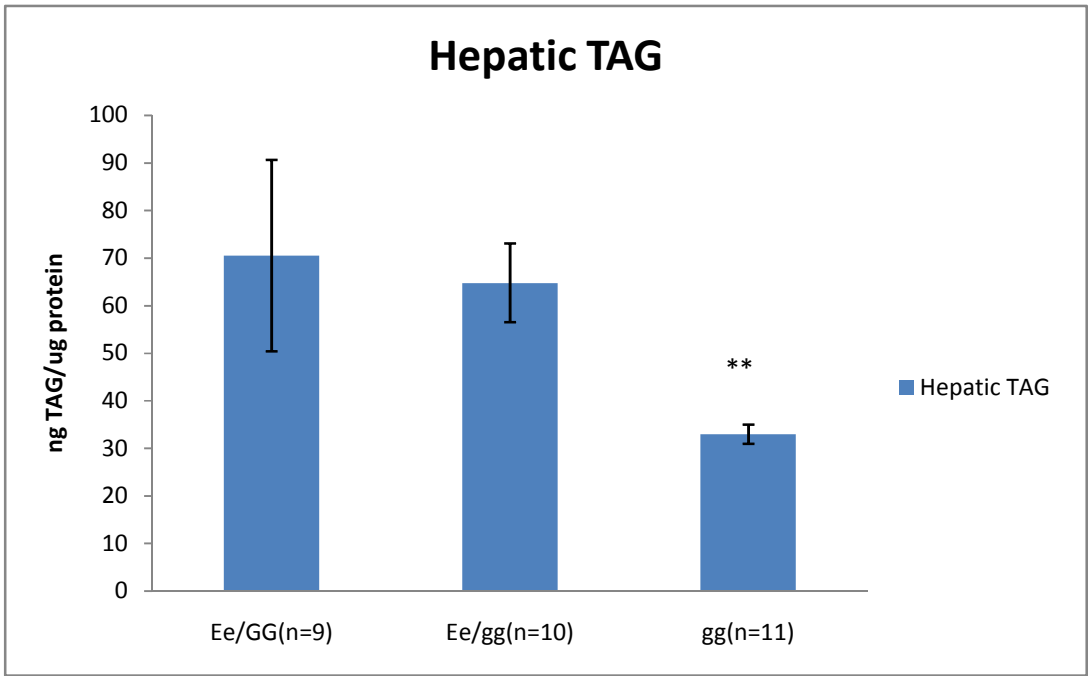


Fig 3.5 Liver triacylglycerol concentration in ApoE +/- mice and ApoE+/- /GPAT1-/- mice fed the atherogenic diet for 90 days.
Liver from the ApoE$^{+/-}$ mice has similar content of hepatic TAG with the ApoE$^{+/-}$/GPAT1$^{-/-}$ mice. But, the GPAT1$^{-/-}$ mice have 49% less content of hepatic TAG than ApoE$^{+/-}$ /GPAT1$^{-/-}$ mice. (** P<0.01)



CHAPTER 4

Discussion

The major finding of this study was that lack of GPAT1 did not diminish atherosclerosis that was induced by diet in apoE$^{+/-}$ mice. GPAT1 is the key enzyme that controls triacylglycerol synthesis and is also up- regulated by SREBP-1c when lipogenesis is enhanced. Although apoE$^{+/-}$-GPAT1$^{-/-}$ mice had an increase in the plasma cholesterol, it was not significant. The lesion size of apoE$^{+/-}$-GPAT1$^{-/-}$ mice was also similar to apoE$^{+/-}$ mice. These results indicated that a knockout of GPAT1 did not improve the atherosclerosis.

Atherosclerosis

The ideal model to investigate whether the GPAT knockout does improve the atherosclerosis would be to use apoE$^{-/-}$ mice. ApoE$^{-/-}$ mice develop atherosclerosis spontaneously, so use of apoE$^{-/-}$ could exclude the diet effect on the study. Because of difficulties in the production of double knockout mice, we decided to use apoE$^{+/-}$-GPAT1$^{-/-}$ mice. When we fed a western diet, ApoE$^{+/-}$ mice and ApoE$^{+/-}$/GPAT1$^{-/-}$ mice did not develop atherosclerosis. Reasons why the apoE$^{+/-}$

mice and apoE$^{+/-}$-GPAT1$^{-/-}$ mice did not develop atherosclerosis may be that the western diet lacks cholate in the diet and contains a low content of cholesterol. When we fed the mice on the atherogenic diet, there was no difference on lesion size in ApoE$^{+/-}$ mice and ApoE$^{+/-}$/GPAT1$^{-/-}$ mice. The composition of atherogenic diet is cholesterol plus cholate. One study showed that cholesterol and cholate components of the atherogenic diet have distinct proatherogenic effects and contribute to the acute inflammatory response and fibrogenesis independently through the different (71). In this study, the authors fed male C57BL/6J mice on five different kinds of diets: chow, atherogenic diet, no cholate, no cholesterol, and no fat. Then, they did microarrays on the liver samples and found that cholate induced phospholipids transfer protein (pltp) which is involved in lipoprotein remodeling. The cholate also induced liver X receptor-β, an oxysterol-binding nuclear hormone receptor that activates several genes involved in cellular cholesterol efflux. Another study on female Zucker rats showed that dietary cholesterol plus cholate down-regulated hepatic lipase (H-TGL). H-TGL facilitates the transfer of cholesterol from IDL to LDL back to the liver (72). The reduction of H-TGL expression was related to a increase in cholesteryl ester in the liver. So, the diet contains cholate would increase liver cholesteryl ester content. The cholic acid also activates FXR to down-regulate the cholesterol 7α-hydroxylase mRNA in C57BL/6 mice (73). When these investigators fed cholic acid to mice, serum LDL and VLDL cholesterol and hepatic cholesterol increased, and the serum triacylglycerol concentration decreased.

These results from (73) were similar to our results. Further, the serum triacylglycerol content of mice fed on atherogenic diet was lower than on western diet. Our results are consistent with a study showing that the addition of sodium cholate prevents a high-fat diet from inducing triacylglycerol accumulation because the cholate down regulates acyl-CoA synthetase1 mRNA through its sterol-response element-1(SRE-1) (74). *Watanabe M et al.* showed that increasing the bile acid pool size increases the activation of farnesoid X receptor (FXR), and increases short heterodimer partner (SHP) levels which, in turn, reduce SREBP-1c expression (75). The reduction of SREBP-1c would decrease triacylglycerol synthesis (Figure 4.1) (75). The possible mechanism for reduced triacylglycerol synthesis could be this FXR-mediated reduction of plasma triacylglycerol content which may occur via FXR-mediated up-regulation of pyruvate dehydrogenase kinase (PDK4) which enhances the utilization of fat rather than glucose as an energy source (76). Another possible mechanism for the reduction of serum triacylglycerol could be that PGC-1α decreases SREBP-1c by increasing the FXR pathway (Figure 2) (77). In this in vitro study with fasting mice, increased hepatic expression of PGC-1α and FXR resulted in a decrease in plasma triacylglycerol.

Oxidative stress and atherosclerosis

What causes the apoE$^{+/-}$-GPAT1$^{-/-}$ mice have to a similar aortic lesion sizes despite a

higher plasma cholesterol concentration? The atherogenic diet will increase lipid-induced oxidative stress, which could cause steatohepatitis and atherosclerosis. From the Haematoxylin & Eosin stain of the liver, we could see the cellular ballooning and couldn't see the original structure of liver lobule. *Matsuzawa N et al.* have reported that the atherogenic diet up-regulates the hepatic expression levels of genes for fatty acid synthesis (SREBP-1c which is a transcriptional regulator of fatty acid synthesis, and fatty acid synthase), oxidative stress, inflammation (electron-transport chain, p38 MAPK signaling pathway, and the FAS pathway and stress induction), and fibrogenesis (transforming growth factor β signaling pathway and matrix metalloproteinase) (78). These authors showed that male C57B1/6J mice fed the atherogenic diet for 24 weeks had more 4-hydroxy-2-nonenal and hepatic protein carbonyls in their liver. 4-HNE is a major aldehyde end-product of membrane lipid peroxidation due to oxidative stress and protein carbonyls are another marker of oxidative stress. Moreover, the reason that apoE$^{+/-}$-GPAT1$^{-/-}$ mice have similar lesion sizes is that maybe the knockdown of GPAT1 cause more oxidative stress. Hammond *et al.* showed that GPAT1 deficient livers have increased oxidative stress and apoptosis (65). The GPAT1$^{-/-}$ livers had more 4-hydroxy-2-nonenal density than wild-type mice in both young and old mice. Further, oxidative stress increases oxidized low-density lipoprotein which is an important factor in the development of atherosclerosis (79). Thus, the apoE$^{+/-}$-GPAT1$^{-/-}$ mice may have similar lesion sizes because GPAT1 knockout livers have more oxidative stress.

Adipose tissue effect

The other major finding of this study was that ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had 61% smaller inguinal fat pads than apoE$^{+/-}$mice and 42% smaller inguinal fat pads than GPAT1$^{-/-}$ mice on the atherogenic diet. ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had 66% smaller gonadal fat pads than apoE$^{+/-}$mice and 36% smaller gonadal fat pads than GPAT1$^{-/-}$ mice on atherogenic diet. We also found a similar result for mice fed the western diet. The possible reasons of ApoE$^{+/-}$- GPAT1$^{-/-}$ mice had smaller fat pads than ApoE$^{+/-}$ mice or GPAT1 $^{-/-}$ mice are unclear. ApoE plays an important role in adipose tissue. ApoE is highly expressed in adipocytes and its expression is up-regulated by PPAR-γ and tumor necrosis factor-α. *Huang HZ et al.,* showed that apoE knockout mice have smaller fat pads and smaller adipocytes compared to wild-type mice (31). They also isolated adipocytes from ApoE $^{-/-}$ mice and wild-type mice. The adipocytes of ApoE $^{-/-}$ mice contained less triacylglycerol than the adipocytes from wild-type mice. They concluded from these results that ApoE modulates adipocyte lipid metabolism and is important for the effect of PPAR-γ agonists on adipocyte lipid metabolism. Another study showed that obese leptin deficient (ob/ob) mice suppress adipose tissue ApoE expression. When they transplanted bone marrow with or without ApoE into ob/ob mice, the ob/ob; ApoE$^{+/+}$ mice had over-10 fold lower atherosclerotic lesion area than ob/ob; ApoE$^{-/-}$ mice (80; 81). Recipients of ApoE$^{+/+}$ marrow demonstrated 3.7-fold

plasma cholesterol and 1.7-fold lower plasma triacylglycerol concentration than ob/ob; ApoE$^{-/-}$ mice.

On the GPAT side, GPAT1 controls the pathway of triacylglycerol synthesis. When GPAT1 is absent in liver, lysophosphatidic acid (LPA) decreases (Coleman unpublished) so a reduction of LPA might down-regulate PPAR-γ. LPA was shown to down-regulate in the PPAR-γ in 3T3F442A cell line, which can differentiate into adipocytes when cultured in an appropriate adipogenic medium (82). LXR expression is regulated by PPAR-γ in adipocytes (83). These authors treated C57BL/6 mice with T0901317, an activator of LXR, and increased the expression of lipogenic genes LXRα, FAS, SREBP-1c, PPAR-γ and aP2. In the LXRα knockdown adipocytes, adipocyte-specific gene expression, including PPAR-γ and aP2, is inhibited. However, LXRα activation of PPAR-γ deficient MEF cells showed that the LXRα activation cannot induce adipogenesis without PPAR-γ. Another study showed that LXRs play an important role in mediating fat metabolism and the control of lipid storage capacity in mature adipocytes (84). When LXR-α is expressed in obese Zucker rats treated with thiazolidinediones, the PPAR-γ target gene Fatty acid binding protein (FABP) increases. In our study, ApoE$^{+/-}$- GPAT1$^{-/-}$ mice lack GPAT1 in liver and may decrease LPA synthesis. The reduction of LPA synthesis in adipocyte might decrease the PPAR-γ activation which would reduce the induction of LXR expression for adipocyte differentiation. Moreover, ApoE$^{+/-}$- GPAT1$^{-/-}$ mice lack

ApoE expression and ApoE is also required for adipocyte differentiation (Figure4.3). Thus, lack of ApoE and GPAT1 might result in smaller fats pad.

On the diet side, there are many studies feeding an atherogenic diet and decreasing the fat pads. But, the mechanism of reduction fat pads is unclear. For example, when wild-type mice are fed an atherogenic diet for 12 weeks, epididymal fat pad weights decreased 64% compared to mice fed a control diet (78). Another study showed that C57BL/6J female mice fed high-oleic safflower oil plus cholate and cholesterol decrease 85% of the WAT weight compared to mice fed the same diet without added cholate and cholesterol (74). These authors did not investigate the reasons why fat pad weights decrease on the atherogenic diet.

In conclusion, we believe that the $ApoE^{+/-}$- $GPAT1^{-/-}$ mice had the smallest fat pad because knockout GPAT1 would down regulate the PPAR-γ and LXR and thus decrease adipocyte differentiation. Because ApoE also controls the adipocyte differentiation, $GPAT1^{-/-}$ mice had larger fat pads than $ApoE^{+/-}$- $GPAT1^{-/-}$ mice.

Future studies. We will want to see if absence of GPAT1 ameliorates atherosclerosis in $ApoE^{-/-}$ mice. $ApoE^{-/-}$ mice develop atherosclerosis spontaneously. Without feeding an atherogenic diet, we could exclude the effect of atherogenic diet on atherosclerosis (such as activation of FXR and LXR). Moreover, we could

exclude the atherogenic diet as a cause of the reduction of fat pad weight. This would be the best model to investigate whether the absence of GPAT1 improves atherosclerosis or not.

Tab 4.1 Composition of the two diets in project 1 and project 2.

	Western diet	Atherogenic diet
Protein	17.3%	19.7%
Carbohydrate	48.5%	40.7%
Fat (source)	21.2% (Anhydrous milkfat)	15.8% (Coca butter)
Cholesterol	0.2%	1.25%
Cholate	0	0.5%

Fig 4.1 FXR-SHP-SREBP-1c regulatory cascade (75).
The cholate in the atherogenic diet would increase the FXR. FXR would increase SHP which decreases the expression of SREBP-1c. The reduction of SREBP-1c would decrease triacylglycerol synthesis in the liver.

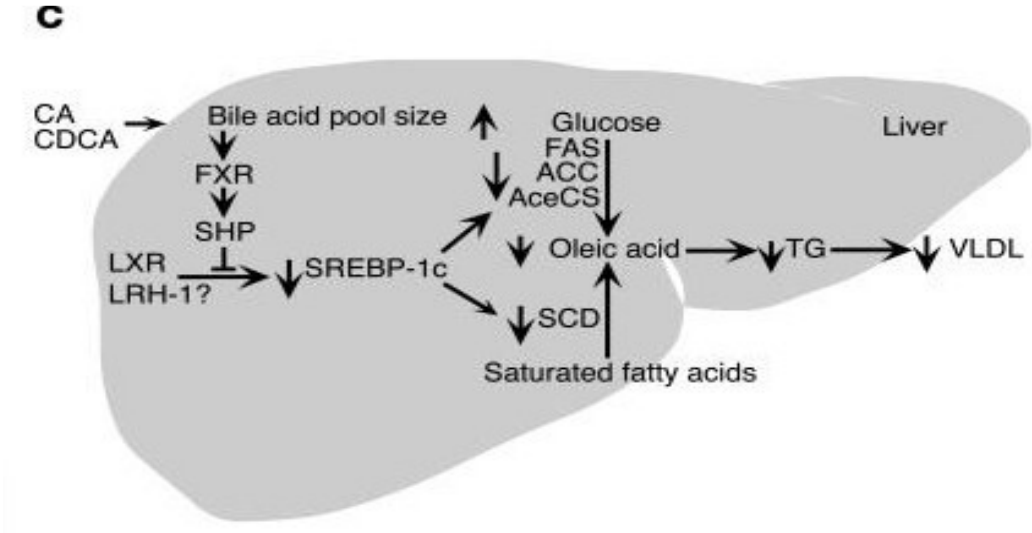


Fig 4.2 A Model for PGC-1α to activate FXR and regulate the triacylglycerol metabolism (77).

The fasting would increase PGC-1α which bound to DR-1 in FXR promoter to induce FXR expression. The activation of PGC-1α will reduce the expression of SREBP-1c and decrease the triacylglycerol synthesis. These could be the reason why atherogenic diet decreases the serum triacylglycerol content.

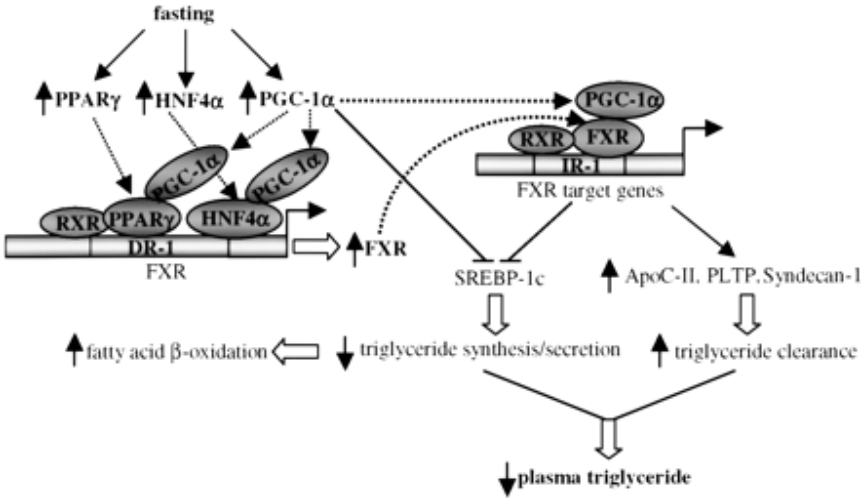
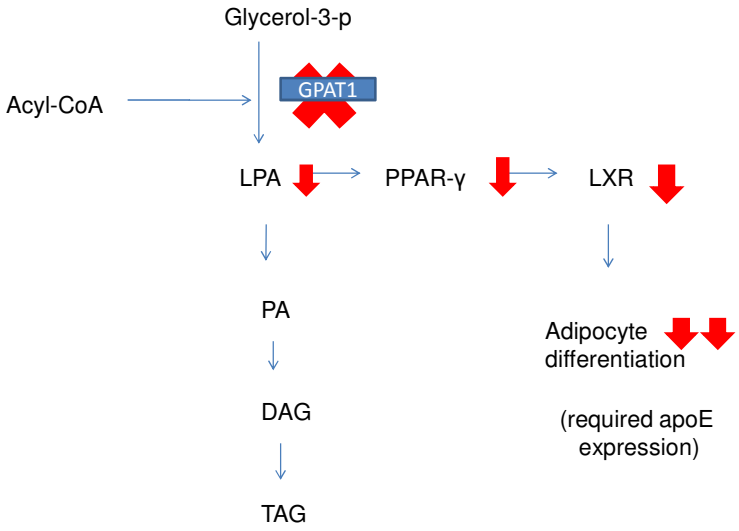


Fig 4.3 The possible mechanism how GPAT1 effect on adipocyte differentiation. When absence of GPAT 1 would decrease the LPA, LPA would down-regulate the PPAR-γ. LXR stimulate adipocyte differentiation through induction of PPAR-γ expression. LXR also up-regulates ApoE expression in adipocytes.



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