A cell-type specific approach to assess the contribution of dysregulated nutrient handling to atherosclerosis

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2015

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University of Washington

Abstract

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The severity of atherosclerosis, which underlies the majority of cardiovascular disease, is determined by a wide variety of factors. Though many of these factors are well studied, a complete understanding of how to best slow or reverse development of atherosclerosis is lacking. Blood glucose is positively associated with an increased incidence of cardiovascular disease in several studies. However, elevations in blood glucose in humans are often accompanied by additional cardiovascular disease risk factors such as elevated lipids in people with concurrent metabolic syndrome and/or increased circulating cytokines in people with diabetes. In the following body of work, the contribution of cell type-specific dysregulation of glucose uptake and fatty acid handling to atherosclerosis was investigated in models of metabolic syndrome and diabetes.

Features of metabolic syndrome (including weight gain, increased plasma lipids and elevated blood glucose) can be induced in mice by combining low-density

lipoprotein receptor (LDLR) deficiency with a high fat, high carbohydrate diet with added cholesterol. By using a mouse model of metabolic syndrome, factors of metabolic syndrome could be held constant and additional glucose uptake was introduced in a cell-type specific manner. *Here we hypothesized that smooth muscle cells were preferentially influenced by increased glycolytic flux leading to accelerated atherosclerosis in a model of metabolic syndrome.* Increased glucose uptake in smooth muscle cells (SMCs) or macrophages *in vivo* was made possible by specific overexpression of the insulin-independent glucose transporter, GLUT1, in either of these cell types. Increased glucose uptake alone in either of these cell types did not facilitate atherosclerosis initiation or progression. However, increased glucose uptake in SMCs in combination with traits of metabolic syndrome allowed accelerated development of lesions, which were larger, contained more free cholesterol and had increased lesion SMC and glycosaminoglycan content. This effect was specific to SMCs over myeloid cells. In an additional study using the same metabolic syndrome model, mice having GLUT1 overexpressed in myeloid cells did not differ from controls.

The phenotype of type 1 diabetes mellitus includes both increased markers of systemic inflammation and high blood glucose, both of which have been the focus of several recent publications. For studies of diabetes mellitus in mice, glucose is increased and cell-type specific modifications are made to reduce the inflammatory potential of immune cells or their target receptors. It has previously been shown that enzymes involved in intracellular fatty acid handling are increased in myeloid cells by diabetes, and that modifications to fatty acid metabolism in macrophages can block inflammation and atherosclerosis without lowering blood glucose. These studies suggest that fatty acid

handling is a strong factor in acceleration of the disease, at least in mice. Here we investigated the role of acyl-CoA thioesterases 7 (ACOT7) in diabetes. The role of this enzyme is poorly understood. *We hypothesized that ACOT7 is induced in macrophages by diabetes and that it contributes to diabetes-associated macrophage inflammation and atherosclerosis*. ACOT7 was increased in macrophages in a mouse model of diabetes. Overexpression of ACOT7 increased inflammatory mediators in activated macrophages *in vitro*. However its deletion from bone marrow derived cells *in vivo* had only a minor reducing effect on inflammatory mediators and did not reduce diabetes-accelerated atherosclerosis.

Together, these studies provide novel information on the role of glucose and fatty acid handling in cell types involved in atherosclerosis associated with metabolic syndrome and diabetes. Cell-type specific modifications in combination with systemic drivers of atherosclerosis can fine-tune knowledge not only of which pathways show promise as therapeutic targets, but which cell types should be targeted for maximal effectiveness of future treatments.

Acknowledgements

The following body of work was not completed singlehandedly, but rather with help from many people whom have contributed both directly and indirectly.

My mentor, Karin Bornfeldt, has been indispensible by offering guidance for my projects. I have had my fair share of successful experiments showing me that science works! I've also had an equal portion of experiments that have left me utterly confused. Karin's positive attitude has continued to propel these projects forward because the enzymes and transporters that we have chosen to study, "must be doing something!" Members of the Bornfeldt lab, including: Jenny Kanter, Shelley Barnhart and Farah Kramer have provided their expertise and assistance for some of the more ambitious days that we have organized. Jenny and Shelley have also made time for me whenever a coffee break is needed. My committee members: Karin, Bornfeldt, William Mahoney, Kelly Smith, Jay Heinecke and Joseph Beavo have provided additional suggestions during our meetings that have helped me to consider new angles to investigate in these projects.

My husband has been supportive of my decision to go to graduate school and has never wavered his support. Having completed his PhD as I was just about to embark on my own intellectual adventure, he understands the mental stresses and benefits of putting so much time and effort into deeply understanding such a small piece of the whole puzzle.

My family has shaped the person that I am today. Though my parents never had the opportunity to pursue advanced degrees themselves, they understand the benefits of doing so and have always been proud of my younger sister, Hayley, and myself for making the decision to continue higher education. My dad, through his work on restoring

and modifying old cars and inventing useful contraptions for his cabinet shop has instilled the idea that with some effort one can accomplish just about anything. Having recently earned a PhD in Molecular Medicine herself, my little sister is always up for discussing the fascinating advances in molecular biology as well as the challenges that we both have come across in this field.

Finally I would like to acknowledge my friends and those I've met in the mountains. I am fortunate to be surrounded by so many ambitious people. Most of my friends were pursuing advanced degrees prior to my entry into graduate school. Going to graduate school was highly encouraged in my group of close friends. I have also met countless people while climbing, some I have shared a single day with and others I have had a multitude of adventures with. Each of them has inspired me to continue to find the next great challenge. The desire to seek out these challenges, both physical and intellectual is now a part of my personality that will continue to push me forward for years to come.

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Abbreviations

ABCA1: ATP-binding cassette, sub-family A, member 1

ACCORD: Action to Control Cardiovascular Risk in Diabetes

ACOT7: acyl-CoA thioesterase 7

ACSL1: acyl-CoA Synthetase 1

ADVANCE: Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation

CRP: C-reactive protein

CREDIT: Cardiovascular Risk Evaluation in people with type 2 Diabetes on Insulin Therapy study

CVD: cardiovascular disease

DCCT: Diabetes Control and Complications Trial

DOX: doxycycline

ECM: extracellular matrix

EDIC: Epidemiology of Diabetes and Complications study

FABP4: fatty acid binding protein 4

FFA: free fatty acid

GLUT1: glucose transporter 1

HA: hyaluronan

HbA1c: glycated haemoglobin

HDL: high-density lipoprotein

IL-1β: interleukin 1 beta

IL-6: interleukin 6

KO: knockout

LDL: low density lipoprotein

oxLDL: oxidized low density lipoprotein

PDGF: platelet-derived growth factor

PLA2: phospholipase A2

PGE_2: prostaglandin E2

ROS: reactive oxygen species

rtTA: reverse tet-controlled transcriptional activator

SAA: serum amyloid A

SMC: smooth muscle cell

SM-GLUT1: mouse model with GLUT1 overexpressed under the SM22α promoter

SRA: scavenger receptor A

SGLT2: sodium-glucose cotransporter 2 (SGLT2)

STZ: streptozotocin

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

TLR4: toll-like receptor

TNFα: tumor necrosis factor alfa

TRE: tetracycline response element

UCP2: uncoupling protein 2

VCAM-1: vascular cell adhesion molecule 1

Chapter 1

Atherosclerosis- a multifactorial disease exacerbated by diabetes

Atherogenesis

Cardiovascular disease (CVD) is the leading cause of death worldwide and in 2011 cost $320.1 billion (hospital associated costs and loss of future productivity) in the United States alone, more than any other diagnostic group (1). Atherosclerosis underlies the majority of cardiovascular complications, therefore a detailed understanding of factors that control development and progression of atherosclerosis is of the utmost importance.

Atherosclerosis is a complex disease involving multiple cell types in the vascular wall (Fig. 1.1). In humans, endothelial dysfunction and diffuse intimal thickening precede early lesion development (2,3). Endothelial dysfunction as the possible initiating event, was first considered in the Response-to-Injury hypothesis. First proposed as desquamation of the endothelium, the Response-to-Injury hypothesis stated that loss of endothelial cells and exposure of the collagen beneath would allow circulating platelets to adhere, aggregate and release platelet-derived growth factor (PDGF)(4). In turn PDGF would promote underlying smooth muscle cell (SMC) proliferation and remodeling (4). As it became understood that the endothelial cell layer is intact in early atherosclerotic lesions (5), the Response-to-Injury hypothesis was amended to include endothelial dysfunction (6). Endothelial dysfunction in the form of decreased nitric oxide availability (a potent vasodilator) and increased permeability and expression of adhesion proteins allows monocytes to better adhere to the endothelium and transmigrate into the intima. Here, monocytes are retained and differentiate into macrophages. It has also been noted that in regions prone to atherosclerosis, diffuse intimal thickening results in an intima rich

in SMCs with increased deposition of extracellular matrix (ECM) proteoglycans that also precedes macrophage accumulation in humans. The Response-to-Retention hypothesis proposes that these proteoglycans retain low-density lipoprotein (LDL) in the intima which contributes to the progressing lesion (7). The bound LDL is modified by reactive oxygen species (ROS) to form oxidized LDL (oxLDL). OxLDL is both chemoattractive to monocytes (8) and can be taken up by intimal macrophages in this modified form by CD36 and scavenger receptor A (SRA). Following uptake of modified LDL, these lipid-laden macrophages (known as foam cells by their foamy appearance) secrete cytokines attracting more monocytes to the lesion. Other immune cells, such as T cells and neutrophils, can also be found in the intima, but macrophages make up the majority of immune cells in the lesion. These macrophage-rich early lesions begin early in life and are present in one third of children by age 9 (9). Lesion regression is possible through cholesterol (and lipid) efflux from lesion macrophages to high-density lipoprotein (HDL) for excretion by the liver, a process known as reverse cholesterol transport. Cholesterol efflux is mediated largely by ATP-binding cassette, sub-family A, member 1 (ABCA1) interacting with Apo-A1 proteins to transfer lipids from peripheral cells to HDL and ABCA1 expression greatly influences atherosclerosis (10). However, in some circumstances, early lesions may continue to progress into larger, clinically relevant lesions. As they progress, features of advanced disease develop. Macrophages continue to enter the lesion and accumulate cholesterol, leading to cell death in some of these macrophages, which leaves an acellular core rich in free cholesterol (11-13). Arterial SMCs begin to migrate, proliferate and secrete ECM glycosaminoglycans forming a fibrous cap over the lesion. This is thought to be an attempt to heal the wounded artery.

However, once these advanced features occur, the lesion can no longer completely regress (14). Advanced lesions can grow large enough to slow blood flow through the artery, leading to angina in the affected patient. Most of the complications from atherosclerosis occur when the lesion becomes unstable and ruptures, forming a thrombus that can block blood flow in downstream arteries. If blood flow is blocked to the brain or heart muscle, myocardial infarction or stroke will occur in the affected patient. Though the process by which a lesion begins and evolves into advanced disease is well documented, why some lesions advance, while other remain stable or regress is less understood. This is why atherosclerosis is an active area of research with recent advancements in knowledge and treatment being made.

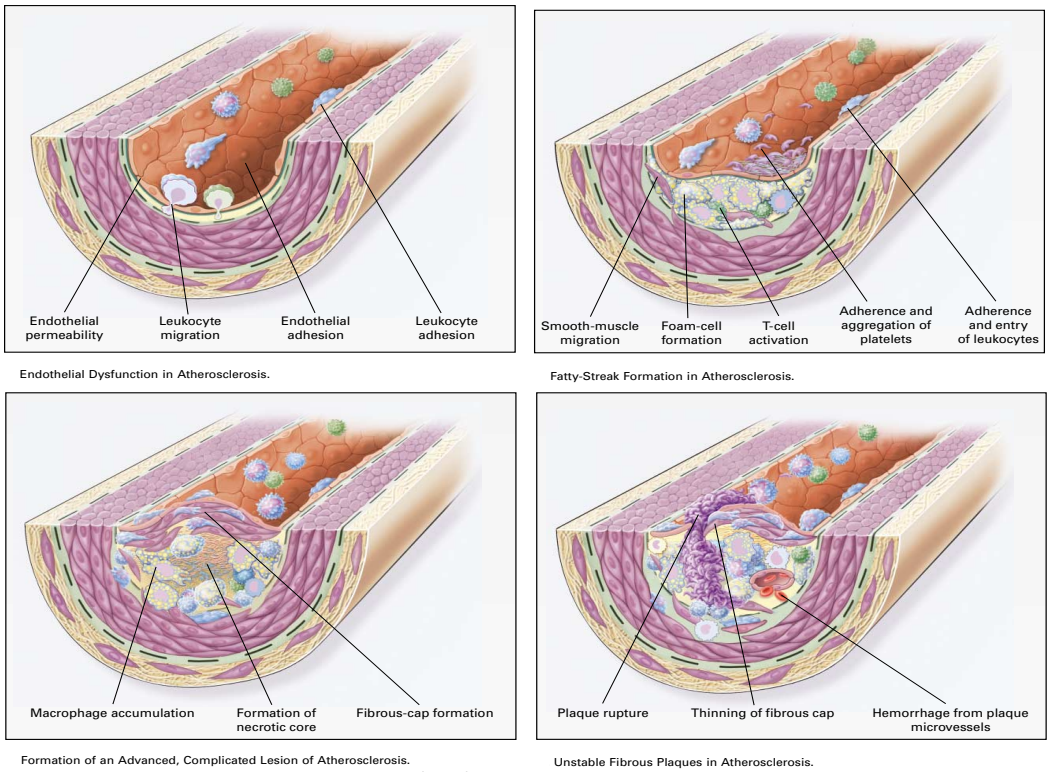

Figure 1.1. Etiology of atherosclerosis. Reproduced with permission from (Ross *NEJM* 1999), Copyright Massachusetts Medical Society.

Recent advances in SMC research

The means by which SMCs contribute to atherosclerosis and vascular remodeling has received some attention in the last couple of years. Arterial SMCs contribute to atherogenesis by increased proliferation, migration, cross-talk with immune cells and by becoming lesion foam cells (15). The Response-to-Injury hypothesis noted that damage to the endothelium could lead to remodeling of the vessel wall by increased proliferation and migration of SMCs. Mechanical disruption of the endothelium, particularly in surgeries such as sent placement or balloon angioplasty, is of particular relevance today. In 20% and 30% of patients receiving these respective surgeries, denudation of the endothelium leads to growth of SMCs and recurrence of the blocked artery (16). Drug-eluting stents are now being used to help control SMC proliferation following some of these surgeries. The drugs currently in use are anti-proliferative agents as sirolimus or paxitaxel (taxol, a drug originally isolated from the Pacific Yew, that inhibits SMC proliferation by microtubule stabilization). These drugs not only inhibit SMC proliferation, but also endothelial cell proliferation, slowing the repair of the initial endothelial cell damage and leaving the patients at greater risk for thrombosis. Recent work has involved finding ways in which endothelial cell and SMC proliferation are differentially regulated (17-19). This work could lead to better drug targets to promote growth and healing of the endothelium while limiting SMC proliferation.

It is now understood that the endothelium is often intact prior to atherosclerotic disease and SMC proliferation occurs as a result of cell signaling. SMCs can both increase proliferation after receiving signals from other cell types and can actively signal

through paracrine and autocrine mechanisms to neighboring SMCs. SMC cross-talk can create a feed-forward loop by amplifying signals from their environment and, in disease models, dysregulated migration, proliferation and neointimal thickening can occur. A few recent articles highlight conditions in which SMCs produce chemokines that promote migration and recruitment of additional SMCs, and remodeling of the vessel wall (20-22). Beyond amplification of signals by neighboring SMCs, cellular cross-talk among multiple cell types can occur, activating these other cell types and enhancing proliferative behavior in SMCs. Recently, SMCs have been shown to activate monocytes (23), macrophages (24), and dendritic cells (25) in this manner through release of soluble growth factors and cytokines. In turn, SMCs receive migration and proliferation signals from these neighboring cell types.

Other recent work has demonstrated the SMC to have a more dynamic phenotype that goes beyond its traditional roles of proliferation, migration and even paracrine cell signaling. Co-staining experiments have found populations of SMCs in lesions that can adopt a more macrophage-like phenotype, with ~40% of the $CD68^{+}$ cells being of SMC origin (26). In the same study the authors propose that reduction in ABCA1, specifically in intimal SMCs, as the lesions advance leads to accumulation of lipids and that they contribute to the number of lesion foam cells. Conversely, lesion SMCs can arise from progenitor cells. Progenitor cells which can adopt a SMC-like phenotype also exist in the adventitia. These are capable of migration to the intima and can contribute to growing lesions (27). The ability to alter phenotype, even transforming to a new cell type underscores the complex nature of these lesion SMCs.

Diabetes and metabolic syndrome accelerate atherosclerosis

Atherosclerosis is a multifactorial disease and is associated with lifestyle habits such as smoking (28) and lack of exercise. In addition, hyperlipidemia, hypercholesterolemia, hypertension and diabetes, which have a genetic component in addition to environmental factors are also highly correlated with disease occurrence (1). Oftentimes, multiple risk factors occur together, and have cumulative influence on disease risk (28,29). With the rising number of people diagnosed with diabetes, the relationship between this disease and atherosclerosis is of particular interest. Currently, 1 in 10 US adults has diabetes (1). Of these, the CDC reports that approximately 5% have type 1 diabetes mellitus (T1DM), 90-95% have type 2 diabetes (T2DM) and the remaining 1-5% have other rare forms of diabetes. In all of these forms of diabetes, insufficient insulin signaling to target tissues (primarily adipose tissue and skeletal muscle), leads to failure of the insulin-dependent glucose transporter, GLUT4, to translocate to the plasma membrane, decreasing glucose uptake by these tissues and resulting in elevated blood glucose. The onset of T2DM is most often later in life and though there are genetic factors involved, many cases are attributable to life-style habits such as poor diet and lack of exercise. Development of T2DM occurs over time and is a combination of both insulin resistance by target tissues and loss of beta cell mass and function (30). People at risk for T2DM will first present with prediabetes. Prediabetes is categorized as impaired glucose tolerance, modestly increased fasting glucose (100-125 mg/dL) or presence of metabolic syndrome (a clustering of 3 out of 5 CVD risk factors, which include: elevated fasting glucose (>100mg/dl) and triglycerides, high blood pressure, increased central adiposity and low HDL cholesterol (31)). During this time, interventions such as lifestyle changes can

reverse prediabetes in some subjects. However, if these measures are not taken, eventually the insulin-producing beta cells in the pancreas will fail and advanced T2DM with the need for exogenous insulin develops. T1DM occurs most often in children and is caused by failure to produce insulin as a result of an irreversible autoimmune attack on beta cells. Lack of insulin in these patients requires intervention early in life with exogenous insulin injections to control blood glucose. Unlike those with T2DM, patients with T1DM often have normal blood lipid profiles. Increased systemic inflammation often accompanies both T1DM (32) and T2MD (33), however the sources of inflammation may differ between the two disease states. Adipose tissue macrophages are thought to significantly contribute to inflammation in obese subjects with T2DM (34).

Though type 1 and type 2 diabetes are very different diseases in terms of cause and blood lipid levels, both of these forms of diabetes are associated with a 2-4 fold increase in cardiovascular disease (1,35) with the underlying cause mainly attributable to atherosclerosis. Several mechanisms have been proposed to account for this interesting observation, two of which will be covered in more detail here:

1) *uncovering how elevated glucose affects specific cell types in the vasculature, and (2) by what mechanism T1DM promotes systemic inflammation*. Though elevated glucose and inflammation can be observed in both forms of diabetes (32,33,36), the process by which T1DM and T2DM contribute to atherosclerosis is dependent on other factors as well that differ between the two disease phenotypes. Those with type 1 diabetes lack insulin and those with type 2 diabetes have additional traits that influence atherosclerotic disease such as high blood pressure, hyperlipidemia, hypercholesterolemia, and can be overweight/obese. To avoid some of these confounding factors, much of the work

presented here will be focused on glucose effects of diabetes. This will be accomplished by using a mouse model of type 1 diabetes without elevated cholesterol (37). Additionally, to assess glucose effects in a model of metabolic syndrome, which is permissive to atherosclerosis (38), glucose uptake will be altered in specific cell types while systemic traits of metabolic disease are unchanged. This method will highlight the interplay between increased glucose usage in cells and other factors associated with metabolic disease.

Glucose effects, elevated lipids and inflammation likely combine to accelerate atherosclerosis (6). Interestingly, despite being exposed to the same systemic factors seen with diabetes, all vascular cell types respond differentially to these stimuli. Monocytes and macrophages are greatly influenced by enzymes, such as acyl-CoA synthetases (ACSLs) and fatty acid binding proteins (FABPs) that interact or modify fatty acids to promote inflammation (37,39,40), whereas some of these same enzymes in endothelial cells, such as ACSL1, are not pro-atherosclerotic (41). Conversely, endothelial cells are thought to be more susceptible to changes in intracellular glucose (42) whereas increased glucose flux has little effect on myeloid-derived cells (43). Finally, vascular SMCs, which have long been studied for their proliferative capabilities in advancing lesions, also have important signaling functions affecting other cell types in lesions through crosstalk with these cell types (15) or by secreting components of the ECM (44). We only recently gained useful models to ask whether high glucose directly influences the chemokines and secreted factors in SMCs *in vivo*.

Lasting effects of increased blood glucose on the artery wall

Elevated glucose (hyperglycemia – often measured as glycated hemoglobin, HbA1c) is the hallmark feature of diabetes and there is some evidence that hyperglycemia might promote atherosclerosis in people with diabetes. A large human study known as the Diabetes Control and Complications Trial (DCCT) followed patients with type 1 diabetes who received either intense blood glucose control for an average 6.5 years resulting in a glycated haemoglobin (HbA1c) of 7.4 or standard treatment and an average HbA1c of 9.1 at the end of the study. Blood glucose control was continued in both groups of patients for an additional 12 years, known as the Epidemiology of Diabetes and Complications study (EDIC), with the intensely treated group and standard group reaching HbA1c values of 7.9 and 7.8 respectively. Despite blood glucose levels normalizing between these two groups, those that received the initial intense treatment continued to have better cardiovascular outcomes, with the better controlled group having a 42 percent reduction in all cardiovascular disease outcomes and a 57 percent reduction in the first occurrence of a disease outcome. The increased incidence of disease in those with initially higher glucose despite normalization of blood glucose between the two groups later in the study was coined "metabolic memory". The contribution of high glucose to cardiovascular disease in those with type 2 diabetes is less clear. Multiple large-scale studies in people with type 2 diabetes have failed to show a positive effect of glucose lowering, and even indicating a negative effect (45-47). However, glucose lowering has been successful in reducing cardiovascular disease risk in type 2 diabetics if started early in the disease process (48), again arguing for metabolic memory. To directly test the "metabolic memory" hypothesis, changes in gene expression were measured by microarray in the

aortic arch of mice were followed during hyperglycemia and following normalization of blood glucose (49). One in 4 genes that were differentially expressed during initial hyperglycemia did not return to basal expression following glucose normalization, showing a persistent effect of transient hyperglycemia. A pathway analysis of the nonreversed genes suggest ongoing tissue remodeling despite return to normoglycemia. In another study, epigenetic changes resulted in increased NF-κB mediated expression of *Ccl2* and *Vcam-1* mRNA levels in endothelial cells following 6 hours of elevated blood glucose in non-diabetic mice that persisted for at least 6 days after normalization of blood glucose (50). It is unknown if metabolic memory exists in humans.

Another study investigated the effects of increased blood glucose on cytokines in people with impaired glucose tolerance as well as control subjects (51). Artificially raising plasma glucose to 15 mmol/L for 5 hours or giving multiple pulses of intravenous glucose led to increased circulating cytokines with greater and longer lasting effects in those with impaired glucose tolerance. These effects could be ameliorated with concurrent antioxidant therapy using glutathione suggestive of a role for reactive oxygen species in glucose-induced cytokine production.

Lowering blood glucose in animal models of atherosclerosis has been used to test the role of glucose in diabetes-accelerated atherosclerosis. It was recently shown that high glucose inhibits regression of atherosclerotic lesions. In a mouse model of diabetes using the beta cell toxin streptozotocin (STZ) to induce hyperglycemia, a subset of mice was treated with a sodium-glucose cotransporter 2 (SGLT2) inhibitor to lower blood glucose by blocking its reuptake in the kidneys. This achieved blood glucose levels similar to non-diabetic mice. The group determined that monocytosis, present in STZ

treated mice, could be reversed with concurrent SGLT2 inhibition (52). One limitation of this study is that one cannot rule out whether glucose effects in other cell types also contributed to monocytosis. Nonetheless this argues that glucose could have a direct effect on at least one cell type or could lead to extracellular modifications that have secondary effects.

GLUT1 overexpression- a cell-type specific method to test glucose effects

Blood glucose enters cells through glucose transporters (GLUTs) located on the plasma membrane. There are 14 members of the GLUT family, each with different expression on particular cell types, substrate specificity and kinetic properties (53). In the setting of diabetes, it has been proposed that the cell types making up the artery as well as circulating immune cells are most sensitive to high glucose (42). These cells primarily express an insulin-independent glucose transporter (GLUT1), and may have increased glucose uptake when extracellular glucose concentration is high. To directly test the role of high intracellular glucose in specific cell types *in vivo*, we overexpressed GLUT1 under both the CD68 promoter and the SM22α promoter (for expression in myeloid cells and SMCs, respectively). This transporter is primarily localized to the plasma membrane in the basal state. GLUT1 has a Km for glucose of 5 mM and is therefore active at physiological glucose levels in that range (54). Because intracellular glucose is rapidly converted into early glycolytic intermediates, intracellular glucose concentrations are much lower than that of the extracellular environment (55), and an increase in the number of GLUT1 molecules on the cell surface necessitates increased glucose uptake. We have previously overexpressed GLUT1 in myeloid cells (43), are currently phenotyping

GLUT1 overexpression in SMCs and have plans for GLUT1 overexpression in endothelial cells.

In myeloid-derived cells, GLUT1 overexpression led to stably increased glucose uptake *in vivo* and increased flux through glycolysis and the pentose phosphate pathway (43). The mice were fed a semi-purified low fat diet known to allow development of atherosclerotic lesions. In this model, increased glucose uptake by these cells did not recapitulate the inflammatory phenotype of diabetic macrophages, nor did it accelerate atherosclerosis (43). This argues that if high blood glucose is responsible for the proinflammatory phenotype in macrophages, it is through secondary effects. On the contrary, the Makowski group reported a proinflammatory effect of GLUT1 overexpression in RAW264.7 macrophages (56). Further, in their study GLUT1 was increased in adipose tissue macrophages after high-fat feeding and correlated with greater cytokine production by these macrophages. These studies are difficult to compare given that each used a separate macrophage cell line and diet. The potential for increased GLUT1 expression to drive cytokine production in fat-fed mice is an interesting possibility that will be further investigated in Chapter 2.

Involvement of SMCs in the lesion is a key feature of more advanced atherosclerotic disease. The effect of increased glucose in SMC cultures has been extensively investigated, but is still debated in the literature. One such example involves regulation of GLUT1, its effect on intracellular glucose and the resulting phenotype from increased intracellular glucose. Culturing SMCs in high glucose has been shown to down-regulate plasma membrane GLUT1 and was thought to be a compensatory mechanism to protect the cells from high extracellular glucose (57,58). Later, down-

regulation of GLUT1 was found to not be sufficient to normalize intracellular glucose (58) in hyperglycemic conditions and can likely not fully compensate for increases in extracellular glucose. Phenotypically, forced glucose uptake by overexpression of GLUT1 *in vitro* was found to increase proliferation and resistance to apoptosis (59); however, in another study, a direct effect of glucose on isolated aortic porcine SMCs could not be observed (60). These studies highlight how difficult glucose effects SMCs is to study *in vitro* because the phenotype of SMCs is significantly altered in culture from a contractile to synthetic state (61). For example, though glucose lowers GLUT1 *in vitro*, *Slc2a1* mRNA (the gene for GLUT1) expression in SMCs does not appear to be altered *in vivo* by high glucose. In my work, I have investigated GLUT1 regulation *in vivo* by isolating the SMC-rich medial area of the aorta from a mouse model of diabetes with high blood glucose and their normoglycemic controls. I have not observed regulation of this receptor at the mRNA level in response to high blood glucose (Figure 1.2). Recently, mouse models have been developed to study increased glucose flux in smooth muscle cells *in vivo* and may provide better insight to how glucose affects this cell type (62). These models again use overexpression of GLUT1 under the SM22α promoter to drive glucose uptake in this cell type. Increased glucose uptake in SMCs promotes neutrophil accumulation in lesions following femoral artery injury along with increased levels of CCL2 (62). Given this phenotype, altered glucose uptake may have a role in some of the SMC changes that are involved in furthering atherosclerosis. Experimental data presented here (Chapter 2) will investigate this possibility in detail.

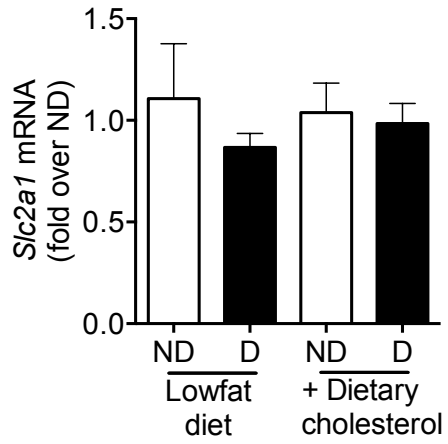

Figure 1.2. GLUT1 expression in the medial layer of diabetic aortas. *Slc2a1* mRNA (gene for GLUT1) expression in the medial layer of the aorta from STZ treated diabetic mice (D) or their nondiabetic (ND) controls.

Though a mouse model for GLUT1 overexpression in endothelial cells has not been developed, glucose is believed to contribute to endothelial dysfunction. Culturing human aortic endothelial cells in the presence of high glucose leads to significantly increased release of the cytokine, interleukin-1 beta (IL-1β) (63). Further, in a large body of work, Michael Brownlee proposes a unifying hypothesis in which high glucose leads to accumulation of reactive oxygen species (ROS) in endothelial cells. In this hypothesis, overproduction of ROS has multiple detrimental effects in endothelial cells which can accelerate atherosclerosis, including: increased flux through the polyol pathway, increased advanced glycation end products and their receptors, increased activity of protein kinase C and overactivity of the hexosamine pathway. The role of high glucose on endothelial dysfunction is discussed in a review by Giacco and Brownlee (64). We have overexpressed GLUT1 in endothelial cells *in vitro* and have found increased expression of vascular cell adhesion molecule 1 (VCAM-1), suggesting glucose-specific effects in endothelial cells, which may contribute to diabetes-accelerated atherosclerosis (Figure 1.3). This finding is corroborated by increased VCAM-1 expression in human

aortic ECs in response to high glucose (50), resulting from ROS-mediated epigenetic alterations of NF-κB subunit transcription. Though the direct effects of increased glucose uptake in endothelial cells *in vivo* by GLUT1 overexpression has not yet been tested, this mouse model is on the horizon.

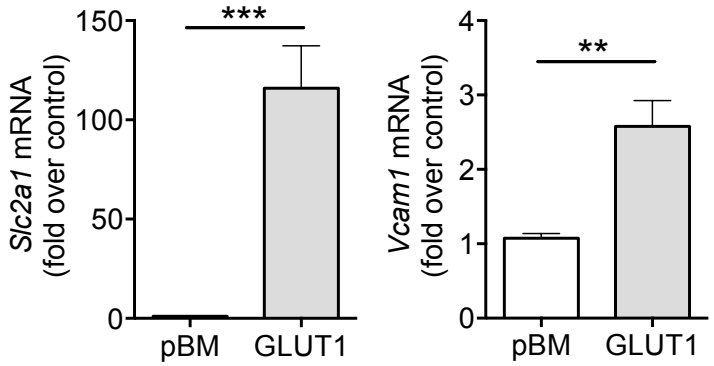

Figure 1.3. GLUT1 overexpression in primary mouse heart endothelial cells increases *Vcam1* mRNA expression. A retrovirus was used to deliver a pBM vector containing a mouse *Scl2a1* gene (GLUT1) or an empty pBM vector (pBM) to serve as a control.

Though overexpression of GLUT1 is a useful tool to increase glucose uptake in specific cell types, the endogenous regulation of GLUT1 should not be ignored. GLUT1 expression at the plasma membrane in SMCs can be reduced in times of high glucose (57,58). Likewise, the demand for increased glucose uptake in times of hypoxia has been shown to increase mRNA expression of the GLUT1 gene, *Slc2a1* (65). Proinflammatory stimuli also cause significant upregulation of *Slc2a1* mRNA in macrophages, reflective of an increased demand for glucose in order to adopt a classically activated macrophage phenotype (66). Since exogenous glucose concentration, oxygen availability and systemic inflammation could be altered by our models, we instead used CD68 and SM22α promoters to continually overexpress GLUT1 regardless of these external stimuli.

Effects of altered immune cells and cytokines in diabetes-accelerated atherosclerosis

Increased blood glucose is not the only systemic change observed in people with diabetes that may lead to accelerated atherosclerosis. Another important feature associated with diabetes is elevated markers of systemic inflammation, such as C-reactive protein (CRP) (32) and serum amyloid A (SAA) (33). Increased circulating cytokines, including interleukin-6 (IL6) and tumor necrosis factor alpha (TNFα) have also been detected in human subjects and mouse models of both type 1 (37) and type 2 (33,38) diabetes. Though there is evidence for increased circulating cytokines being derived from non-circulating cells in T2DM (67), several studies maintain a link between cytokines and increased immune cell activity in the diabetic state. Leukocytosis observed in humans (68,69) and in models of diabetes, may account for the increase in cytokines. Lowering blood glucose in a mouse model of T1DM was sufficient to reverse the monocytosis phenotype (52) supporting this potential link between greater circulating cytokines and glucose. Alternatively, it is widely accepted that increased circulating cytokines may be a reflection of the activity of monocytes/macrophages. Toll-like receptors (TLRs) sense pathogen-associated molecular patterns, which are present on a wide variety of invading viral and bacterial pathogens, leading to activation the innate immune response. Greater expression of TLR2 and TLR4 on monocytes in humans with T1DM and T2DM as well as in mouse models of the disease, suggest the presence of more inflammatory macrophages (70-72). In support, isolated macrophages from mouse models of T1DM release more cytokines than those from non-diabetic controls (37). Importantly, reducing

the proinflammatory effects of diabetes can slow lesion progression in mouse models of the disease (37,73).

Increased TLR4 signaling in macrophages from people with T1DM could in itself be a direct mechanism for greater cytokine production. Devaraj et al. (70) determined that monocytes from type 1 diabetics had enhanced expression of TLR4 and that this correlated with increased secretion of TNFα and IL-1β from isolated monocytes. This same group went on to show in a mouse model of T1DM that knockout of TLR4 attenuated the proinflammatory state of diabetes (72). Further, another group demonstrated that atherosclerosis progression in a mouse model of T1DM was slowed with a TLR4 antagonist and this also reduced detection of cytokines within aortic lesions (73). In addition to detection of pathogen-associated molecular patterns, TLR4 detects other ligands endogenous ligands such as: ECM components and heat shock proteins (74) leading to activation of kinases and, ultimately, transcription factors. Signaling through TLR4 upregulates inflammatory cytokines such as TNFα and IL-6 and other inflammatory mediators such as prostaglandins. Therefore, it is understandable that perturbation of TLR4 signaling could have such a profound effect on cytokine production. However, there are other avenues in which diabetes can alter cytokine signaling that should be addressed.

Fatty acids can mediate immune cell activation, contributing to atherosclerosis

Fatty acids can direct immune cell functions through many mechanisms. Arachidonic acid (AA) is an unsaturated long-chain fatty acid that serves as the precursor to the lipid mediators known as eicosanoids. These can have both pro and anti-

inflammatory properties. Other fatty acids, such as palmitate and oleate when applied directly to cultured cells have opposing effects on macrophage activity. General fatty acid metabolism is linked to macrophage polarization. Lastly, FABPs control trafficking of fatty acids within the cell and promote macrophage inflammation and atherosclerosis. Described below is a model indicating several possible fates of intracellular fatty acids and how they may alter the inflammatory status in macrophages (Fig. 1.4)

Fatty acid mobilization is a key regulatory step in the synthesis of prostaglandins and other eicosanoids. Upon activation by inflammatory stimuli, phospholipase A2 (PLA_2) translocates to the Golgi, ER and nuclear membranes where it can act on its substrate phospholipids, leading to the liberation of the fatty acid in the sn_2 position (primarily AA) and generation of lyso-phospholipids (75). The free AA is then available for eicosanoid synthesis by either 5-lipoxygenase or cyclooxygenase. However, regulation of the pool of free AA within the cell can limit the amount of eicosanoid produced and free AA within the cell is tightly controlled by re-esterification and ultimately reincorporation into phospholipids by lysophospholipid acyltransferases. As one might expect, perturbations which inhibit fatty acid reincorporation have a profound effect on increasing eicosanoid synthesis (76), demonstrating the importance of regulating this free AA pool. ACSL1 is another enzyme upregulated downstream of TLR4 signaling and is increased in diabetic humans and in the STZ model of T1DM. ACSL1 appears to be involved in esterification of newly obtained AA as it enters the cell and not re-esterification of AA that has been released by PLA_2. In support of this, ACSL1 deficiency leads to reduced turnover of AA-containing phospholipid species (77) while also attenuating the production of PGE_2 (37). Importantly, knockout of ASCL1 in the

STZ model of T1DM partially reverses the proinflammatory phenotype of the diabetic macrophages (37).

In addition to modulation of inflammation by eicosanoid species, other fatty acids also potentiate production of cytokines by immune cells or dampen the responses to proinflammatory stimuli. *In vitro* experiments have noted increased gene expression and secretion of cytokines by palmitate and anti-inflammatory roles for oleate in macrophages (78,79).

Fatty acid metabolism may also contribute to whether a macrophage adopts a classically activated, proinflammatory phenotype or an alternatively activated phenotype that contributes to the resolution phase of the inflammatory response. Proinflammatory macrophages utilize more glucose whereas alternatively activated macrophages rely more on oxidative metabolism of fatty acids (66).

Recent work has identified macrophage fatty acid binding protein 4 (FABP4) in inflammation and atherosclerosis (39,80,81). One way in which FABP4 may alter inflammation and atherosclerosis is through control the pool of free fatty acids (FFAs) that are available as ligands for PPARs. In work by Xu and colleges, FABP4 loss or inhibition increased FFAs and transcription of uncoupling protein 2 (UCP2) (81). UCP2 can reduce ER stress and inflammation. Its increased expression in FABP4 deficient macrophages appeared to depend on PPARγ activation by FFA ligands (81).

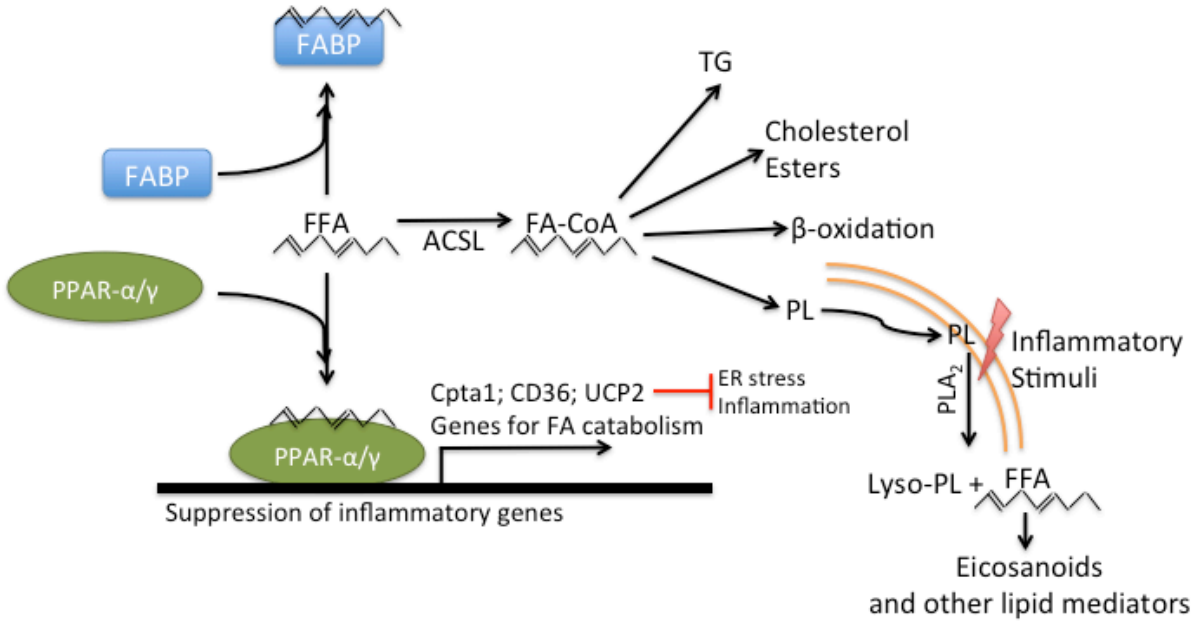

Figure 1.4. Fates of intracellular free fatty acids (FFA). Intracellular FFA can either be esterified be acyl-CoA synthetases (ACSLs) or become ligands for PPARs. If esterified, FA-CoAs can be used for energy by β-oxidation or used to synthesize triglycerides (TGs), cholesterol esters, or membrane phospholipids (PLs). Upon activation by an inflammatory stimulus, phospholipase A2 (PLA_2) can release FFA from the membrane, which can be acted upon by additional enzymes to produce eicosanoids and other lipid mediators. If FFA becomes a PPAR ligand, the activated PPAR can suppress inflammatory genes, or activate target genes for FA catabolism.

Accelerated atherosclerosis- a complex interplay of multiple factors

Through the following studies and work of others, I will demonstrate the complexity of factors that determine atherosclerosis disease severity in both SMCs and macrophages and I will highlight the distinct roles that each of these cells types have in accelerating atherosclerosis. New research will be presented that will lead to a more complete picture of how SMCs and macrophages may become dysregulated in a manner to promote atherosclerosis. In Chapter 2, GLUT1 overexpression in SMCs will be used to further investigate glucose effects in SMCs in a model of atherosclerosis and metabolic syndrome. I hypothesize that *GLUT1 overexpression in SMCs will accelerate atherosclerosis, demonstrating that the glucose-mediated effects of diabetes are driven, at least in part, by this cell type*. In Chapter 3, the role of fatty acid modifications with regard to macrophage inflammation will be further tested by manipulating expression of acyl-CoA thioesterase 7 (ACOT7) *in vitro* and *in vivo*. This enzyme catalyzes the cleavage of fatty acids from their CoA moiety. I propose that there are other fatty-acid modifying enzymes, in addition to ACSL1, which are altered in macrophages by the diabetic state. *I hypothesize that ACOT7 is one of these enzymes and that changes in the expression of ACOT7 can alter the inflammatory activity of macrophages.*

Chapter 2-
Smooth muscle expression of the glucose transporter GLUT1 selectively promotes atherosclerosis in a mouse model of metabolic syndrome

Summary

Systemic metabolic factors, such as dyslipidemia, are largely responsible for the increased cardiovascular disease (CVD) risk and atherosclerosis associated with metabolic syndrome, but the role of arterial metabolism in atherosclerosis is poorly understood. Smooth muscle cell (SMC) metabolism is governed in part by the glucose transporter GLUT1, which is induced by inflammatory mediators and hypoxia, and is increased in human lesions of atherosclerosis.

We therefore investigated if increased expression of GLUT1 in SMCs leads to increased atherosclerosis in a mouse model of metabolic syndrome. Mice deficient in the low-density lipoprotein receptor (*Ldlr*$^{-/-}$ mice) carrying a GLUT1 transgene under control of the smooth muscle SM22α-promoter (SM-GLUT1 mice) and littermate *Ldlr*$^{-/-}$ controls were fed a high-fat high-sucrose diet with added cholesterol to induce a metabolic syndrome-like phenotype. SM-GLUT1 did not affect the obesity, dyslipidemia or glucose intolerance in this model of metabolic syndrome. GLUT1 overexpression caused increased glucose uptake, increased glucose flux through glycolysis, and increased levels aortic sorbitol and hyaluronic acid, indicative of increased glucose flux through the sorbitol and hexosamine pathways. The mice also had aortic accumulation of amino acids. Strikingly, metabolic syndrome SM-GLUT1 mice developed larger and more advanced lesions in the aorta and the brachiocephalic artery more rapidly than their *Ldlr*$^{-/-}$ littermate controls. However, SM-GLUT1 mice fed low-fat diets did not exhibit differences in atherosclerosis size or severity.

Together, these results demonstrate that increased GLUT1 expression in SMCs results in increased glucose utilization and metabolic changes that promote atherosclerosis in a metabolic syndrome-selective manner, and suggest a novel mechanism whereby arterial metabolism promotes atherosclerosis.

Introduction

Atherosclerosis, which is the underlying cause of most CVD, is a common complication that occurs with metabolic syndrome. Metabolic syndrome is a clustering of 3 out of 5 CVD risk factors, which include: elevated fasting glucose (>100 mg/dl) and triglycerides (>150 mg/dl), high blood pressure (>130 mmHg systolic; >85 mmHg diastolic), increased central adiposity (waist circumference >102 cm in men, > 85 cm in women) and low HDL cholesterol (<40 mg/dl in men, <50 mg/dl in women) (31). Lipids are important factors promoting atherosclerotic lesion initiation and progression. The response-to-retention hypothesis states that LDL retention in the subendothelial layer precedes lesion initiation. Uptake of modified LDL by early lesion macrophages promotes cytokine release by these macrophages and contributes to recruitment of additional immune cells to the lesion. It follows that LDL lowering is an effective treatment for reducing CVD risk in high risk groups, such as patients with metabolic syndrome but also including subjects without clinically high LDL cholesterol (82). However, atherosclerosis is a multifactorial disease with several other factors influencing risk. In addition to the important contributions of elevated lipids to CVD risk, other systemic metabolic changes are likely to contribute to CVD risk associated with metabolic syndrome.

Whereas it is clear that systemic changes in metabolism leading to altered lipid levels contributes to CVD risk, the role of arterial metabolism in atherosclerosis is poorly understood. SMC metabolism is governed in part by the glucose transporter GLUT1, which is induced by inflammatory mediators (66) and hypoxia (65), and expression is increased in human lesions of atherosclerosis. We therefore addressed this question by overexpressing GLUT1 (gene name *Slc2a1*) under control of the smooth muscle-selective promoter SM22α in a mouse model of the metabolic syndrome. We recently demonstrated that overexpression of GLUT1, which results in increased glucose uptake and flux through glycolysis, by myeloid derived cells is not sufficient to increase cytokine production, nor is it sufficient to facilitate atherosclerosis in *Ldlr*$^{-/-}$ mice (43) fed a low-fat diet.

The role of arterial metabolism and increased glucose uptake through GLUT1 on atherosclerosis associated with metabolic syndrome is unknown. In the current study, *Ldlr*$^{-/-}$ mice were fed a diabetogenic diet with added cholesterol (DDC), which is high in fat and sucrose and contains added cholesterol. These mice develop many features similar to metabolic syndrome in humans including: weight gain, increased triglycerides, elevated blood glucose in male mice and a decreased HDL/LDL ratio (compared to chow-fed mice) (38). A mouse model in which overexpression of GLUT1 in SMCs is achieved by expressing *Slc2a1* under control of the SM22α promoter was recently generated (62). These mice are prone to greater levels of circulating CCL2 and neutrophil accumulation in a femoral artery injury model (62). We crossed these mice with *Ldlr*$^{-/-}$

mice to study whether increased glucose flux in SMCs is a factor in the severity of atherosclerosis in a model of metabolic syndrome. In addition, we compared the findings in these experiments to findings in mice with metabolic syndrome and GLUT1 overexpression in myeloid-derived cells to verify smooth muscle cell-specificity for these glucose effects. We demonstrate that increased GLUT1 expression in SMCs results in increased glucose utilization and metabolic changes that promote atherosclerosis in a metabolic syndrome-selective manner, and suggest a novel mechanism whereby arterial metabolism promotes atherosclerosis.

Experimental Procedures

Animals- To test GLUT1 overexpression in SMCs, experimental animals were generated by crossing transgenic mice heterozygous for the *Slc2a1*gene under control of the SM22a promoter (62) with *Ldlr*$^{-/-}$ mice (both on the C57BL/6 background). The resulting transgenic pups were backcrossed into the *Ldlr*$^{-/-}$ colony, producing transgenic mice that were also deficient in the *Ldlr* gene allowing for a lipoprotein profile in which atherosclerosis can develop (83,84). Mice were maintained by crossing male *Ldlr*$^{-/-}$ mice containing the *Slc2a1* transgene to *Ldlr*$^{-/-}$ females. For all studies *Ldlr*$^{-/-}$ mice that overexpress *Slc2a1* in one allele were used as experimental mice (SM-GLUT1) and their *Ldlr*$^{-/-}$ littermates served as controls (WT). To test GLUT1 overexpression in macrophages, GLUT1 was overexpressed in BMDCs under the CD68 promoter as previously described (43). Briefly, bone marrow stem cells were transduced with CD68-GLUT1 or CD68-EGFP as a control and transplanted into lethally irradiated (10 Gy) C57BL/6 recipients ($5x10^6$ cells injected retro-orbitally). After being allowed 7 weeks to recover, bone marrow was harvested again and transplanted to irradiated 8–10 week-old

male *Ldlr*−/− mice. Male and female mice, 8-12 weeks of age, were used for experiments. In a separate study, C57BL/6 mice were made diabetic by streptozotocin (STZ) injections. STZ (mixed anomers no. S0130, 50 mg/kg; Sigma) was dissolved in freshly made citrate buffer (0.1 M, pH 4.5) and injected intraperitoneally for 5 consecutive days. The mice were monitored for development of diabetes (defined as blood glucose >250 mg/dl). Diabetic mice received insulin (Lantus, Sanofi-Aventis, Bridgewater, NJ) as needed to prevent excessive weight loss and ketonuria. After induction of diabetes, the mice were fed a semi-purified low-fat diet for the duration of the 6-week study. This diet has been described previously (83).

For atherosclerosis studies, mice were fed a high-fat high-sucrose diet with added cholesterol known to produce a phenotype characteristic of metabolic syndrome (38). Animals that did not respond to the diet by either failing to gain weight or failing to elevate plasma cholesterol were removed from analysis for all subsequent measurements; this included 5 WT and 5 SM-GLUT1 mice. Body weight and blood glucose were monitored every 4 weeks. A glucose tolerance test (GTT) was done after 13 weeks of DDC feeding by i.p. injection of dextrose (1mg/kg, Baxter, Deerfield, IL). Blood glucose was monitored throughout the study and for the GTT, using One Touch Ultra test strips (LifeScan). Plasma lipids were determined by colorimetric assays according to manufacturers' instructions: triglycerides (Sigma), and cholesterol (Wako). All experiments were performed in accordance with an approved University of Washington Institutional Animal Care and Use Committee protocol.

Isolation and maintenance of aortic SMCs- Thoracic aortas were harvested from 3-4 week old mice, cleaned of residual fatty tissue under a dissecting scope and incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% fungizone, 1% penicillin/streptomycin, 2 mg/ml BSA (Sigma #A-4378), 1 mg/ml Collagenase type II (Worthington, CLS-2), 0.375 mg/ml Soybean Trypsin Inhibitor (Worthington) and 0.2305 mg/ml Elastase (Worthington, LS00290) for 3 min. After incubation, the aortas were put back into ice cold DMEM supplemented with 1% fungizone and 1% penicillin/streptomycin. Aortas were then cut longitudinally, the adventitia peeled away and the endothelial cells removed with a sterilized cotton swab. The aortic media was cut into 5 mm pieces placed in DMEM supplemented with 1% fungizone, 1% penicillin/streptomycin, 20% fetal bovine serum (FBS), 0.8 mg/ml Collagenase type II and incubated in a 37°C water bath for ~20 minutes. The supernatant was removed after centrifugation, the pellet washed once with DMEM, and then resuspended in DMEM supplemented with 1% fungizone, 1% penicillin/streptomycin, 20% FBS, 0.8 mg/ml Collagenase type II, and 0.44 mg/ml Elastase and incubated in a 37°C water bath for 45-50 minutes with periodic mixing. The digested cells were resuspended in DMEM supplemented with 1% fungizone, 1% penicillin/streptomycin, 1% non-essential amino acids (NEAA), 20% FBS and plated onto tissue culture plates. Cells were maintained in DMEM supplemented with 1% fungizone, 1% penicillin/streptomycin, 1% NEAA, 20% FBS (10% FBS after passage 3).

Overexpression of GLUT1 in mouse SMCs- GLUT1 was overexpressed using a pBM retroviral vector. Isolated mouse aortic SMCs were serum starved then incubated for 24

hrs in the presence of viral media (diluted 1:30 in standard growth media), 1M HEPES and 5.5μg/ml polybrene.

Isolation and maintenance of BMDMs- Bone marrow-derived macrophages (BMDMs) were harvested from freshly obtained bones by syringe flushing with DMEM media supplemented with 1% fungizone and 1% penicillin/streptomycin. Cells were purified of erythrocytes and maintained in DMEM media (450 mg/dl glucose) containing 1% fungizone, 1% penicillin/streptomycin, 7% FBS and 30% L-cell conditioned media. Cells were allowed to differentiate for 7-10 days prior to being used in experiments.

Lesion analysis, histochemistry and aortic lipid measurements- At the end of the study, tissues were collected for histology in 4% PFA, following perfusion with PBS. Aortas were immediately flushed with RNAlater (Life Technologies) using a 1 ml syringe, then dissected out down to the femoral bifurcation and stored in RNAlater at 4° C. The aortic sinus and brachiocephalic artery (BCA) from each animal were paraffin-embedded and serial sectioned for histological analysis by Movat's pentachrome stain as described previously (85). Cross-sections were also analyzed by TUNEL staining to assess apoptosis, or expression of specific tissue markers including: Ki67 for proliferating cells, GLUT1, smooth muscle a-actin, and Mac2 as a macrophage marker. Hyaluronan was measured in BCA sections by using a biotinylated HA binding protein (86). Adjacent BCA cross-sections were pretreated with HA lyase for 1 h at 37° as a control. Aortas was opened longitudinally and measured for *en face* lesion area while in RNAlater. Only thickened deposits on the endothelial side were counted positive as lesion area.

Following lesion quantification, lipids were extracted from the aortas using a 3:2 mixture of hexane:isopropanol for 2x13 min at 4° C. Extracted lipids were dried at 37° C under nitrogen and resuspended in PBS with sodium cholate and triton-X. Immediately after lipid extraction, aortas were homogenized (tissue tearor), digested with protease K (Qiagen, Valencia, CA) and mRNA was isolated from each aorta (Qiagen RNAeasy kit). Extracted lipids were later analyzed for cholesterol content using Amplex Red Cholesterol Assay Kit (Life Technologies) and triglyceride content (Sigma).

Glucose metabolism and metabolomics- Mouse aortic SMCs were isolated and plated in high glucose DMEM supplemented with 1% penicillin/streptomycin, 1% NEAA and 20% FBS. Glucose uptake was measured as uptake of H^3-2-deoxyglucose (deoxy-D-glucose, 2-[1,2-H^3(N)]-;25Ci/mmol; PerkinElmer, Santa Clara, CA)
over a 24 h period. Lactate release into the media was measured by a colorimetric assay (Sigma) over a 48 h period. Metabolomics was performed on isolated whole aortas from DDC fed male mice, quickly stripped of the endothelium and adventitia. In a separate study, aortas were isolated from C57BL/6 mice made diabetic by streptozotocin and non-diabetic controls. To make sure that the effects of hyperglycemia were maintained, the aortas from diabetic mice were dissected in the presence of 25 mM glucose DMEM while aortas from non-diabetic mice were dissected in 5.6 mM glucose DMEM. Freshly isolated, cleaned aortas were snap frozen one at a time to minimize any changes to metabolic species that may occur post-harvest. Aortas were homogenized in PBS by one rapid freeze/thaw cycle in combination with a tissue tearor. Targeted metabolomics was performed through liquid chromatography tandem-mass spectrometry (LC-MS/MS).

Targeted aqueous metabolite profiling analysis was performed using an Agilent 1260/AB-Sciex 5500 Qtrap LC-MS/MS instrument and protocols based on hydrophilic interaction chromatography. This system provides detailed information on metabolites involved in glycolysis, the TCA cycle, pentose phosphate shunt, as well as amino acid, fatty acid, and nucleic acid metabolism. The targeted multiplex-LC-MS/MS method provides quantitative measurements with excellent reproducibility (typical median CV value = 7-8%) of these metabolites in a single 20-min run.

Real-time PCR- Gene expression was quantified by real-time PCR. RNA isolation and the real-time PCR protocol was performed as described previously (37). Briefly, RNA was isolated using Qiagen (Valencia, CA) RNeasy or Macherey-Nagel (Bethlehem, PA) Nucleospin RNA kits according to manufacturer's protocol and treated with DNase1 (1 μg/sample, Thermoscientific, Waltham, MA) to remove trace DNA. Real-time PCR was performed using SYBR Green 1 detection method (Thermoscientific). Cycle threshold (Ct) values were normalized to *Rn18s* and presented as fold over control. See table 1 for list of primers used.

Gene name	Forward primer	Reverse primer
Rn18s	CATTAAATCAGTTATGGTTCCTTTGG	CCCGTCGGCATGTATTAGCT
Slc2a1	CAGTTCGGCTATAACACTGGTG	GCCCCCGACAGAGAAGATG
Cd11b	TGTGAGCAGCACTGAGATC	ATGGCTCCACTTTGGTCTC
CyclinD1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
Has1	CTTTCAAGGCACTGGGCGACTCC	TCCAGCAGTGCCATGGGGTCTAG
Has2	GCCTTCAGAGCACTGGGGCGAA	TCCACAGATGAGGCAGGGTCAAGC
Cxcl1	TGGCTGGGATTCACCTCAAG	CCGTTACTTGGGGACACCTT
Abca1	CTCAGAGGTGGCTCTGATGAC	CCCATACAGCAAGAGCAGAAG
Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT

Table 2.1 Primer sets for real-time PCR.

Statistical analysis- Statistics for all studies were performed using Prism GraphPad software (La Jolla, CA). Unpaired two-tailed Student's t-test was used to compare two conditions, while multiple groups were compared by one-way ANOVA with a Tukey post hoc test or two-way ANOVA. Error bars indicate SEM, except for the results in Table 2, where SDs are reported Statistics for all studies were performed using Prism GraphPad software (La Jolla, CA). Unpaired two-tailed Student's t-test was used to compare two conditions, while multiple groups were compared by one-way ANOVA with a Tukey post hoc test or two-way ANOVA. * = $p<0.05$, ** = $p<0.01$, *** = $p< 0.001$, **** = $p<0.0001$.

Results

GLUT1 overexpression in SMCs results in increased glucose flux and amino acid accumulation in aortas of metabolic syndrome mice- Aortic SMCs were isolated from transgenic *Ldlr*$^{-/-}$ mice overexpressing full-length human *Slc2a1* cDNA under control of the SM22α promoter (SM-GLUT1) and *Ldlr*$^{-/-}$ littermate controls. SMCs isolated from SM-GLUT1 mice exhibited increased glucose uptake as measured by H^3-2-deoxy-glucose uptake and increased lactate release, as compared with SMCs from littermate controls (Fig 2.1. a,b).

We next investigated the effects of GLUT1 overexpression on aortic metabolism by a metabolomics approach. Freshly isolated aortic media specimens from metabolic syndrome mice with and without SM-GLUT1 overexpression were analyzed. As shown in Table 2, glucose levels were increased in SM-GLUT1 mice as compared to the controls, demonstrating that GLUT1 is active *in vivo*. No significant changes were observed in glycolytic intermediates, but a significant increase in sorbitol was present in

SM-GLUT1 mice. Furthermore, several amino acids were present at elevated levels in SM-GLUT1 aortas, with a preferential effect on branched-chain amino acids (Table 2.2).

A separate study was carried out to investigate whether the changes observed in SM-GLUT1 mice are similar changes that may occur with increased blood glucose levels. For this study, we took advantage of streptozotocin-diabetic mice and controls on the C57BL/6 background. As also shown in Table 2.2, diabetic mice did not show the same metabolic signature as the SM-GLUT1 mice, suggesting that the effects observed due to GLUT1 overexpression differs from that of systemic elevation in blood glucose levels.

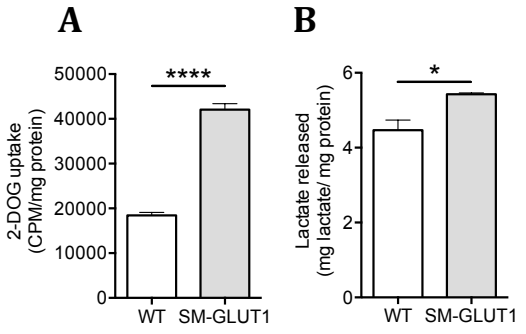

Figure 2.1 GLUT1 overexpression in SMCs led to increased glucose uptake (a) and lactate release (b) from isolated aortic SMCs grown in culture. Results are expressed as mean ± SEM (n=3-6) Statistical analysis was performed by two-tailed unpaired Student's t-test (E-J); *p<0.05; ****p<0.0001.

Metabolite	KEGG ID	HMDB ID	PubChem	CAS	Metabolic pathway	Non-diabetic (n=4)	Diabetic (n=5)	DDC (n=5)	DDC SM-GLUT1 (n=4)
Glucose	C00031	HMDB00122	79025	50-99-7	Glycolysis	**54,453 ± 26,125**	**171,635 ± 30,206*****	**198,321 ± 37,361**	**311,518 ± 76,878***
Lactate	C001861	HMDB00190	612	79-33-4	Glycolysis	5,202 ± 2,497	3,493 ± 581	**8,632 ± 1,405**	**11,701 ± 2,268***
Phosphoenolpyruvic Acid (PEP)	C00074	HMDB00263	1005	138-08-9	Glycolosis	37,386 ± 18,030	27,872 ± 9,742	13,830 ± 4,146	14,568 ± 6,161
D-Glyceraldehyde-3-phosphate (D-GA3P)	C00661	HMDB01112	729	142-10-9	Glycolysis	1,085 ± 586	799 ± 172	712 ± 218	806 ± 198
Glucose 1-phosphate (G1P); Glucose 6-phosphate (G6P); Fructose 6 phosphate (F6P); Fructose 1-phosphate (F1P)	C00103; C00092; C00085; C01094	HMDB01586; HMDB01401; HMDB00124; HMDB01076	439165; 5958; 69507; 10400369	59-56-3; 56-73-5; 643-13-0; 15978-08-2	Glycolysis	5,471 ± 2,479	3,381 ± 714	6,876 ± 921	8,448 ± 1,402
Fructose 1,6 bi-phosphate (F16BP); Fructose 2,6 bi-phosphate (F26BP); Glucose 1,6 biphosphate (G16BP)	C00354; C00665; C01231	HMDB01058; HMDB01047; HMDB03514	172313; 105021; 82400	488-69-7; 79082-92-1; 10139-18-1	Glycolysis	3,707 ± 1,834	3,123 ± 555	2,567 ± 821	2,696 ± 205
Ribose-5-P	C00117	HMDB01548	439167	3615-55-2	Pentose phosphate pathway	2,147 ± 1,513	1,324 ± 435	515 ± 81	675 ± 232
Phosphoribosyl pyrophosphate (PRPP)	C00119	HMDB00280	7339	7540-64-9	Pentose phosphate pathway	797 ± 377	953 ± 506	1,911 ± 1,070	4,299 ± 2,224
Sorbitol	C00794	HMDB00247	107428	6706-59-8	Sorbitol pathway	230 ± 96	150 ± 25	**539 ± 131**	**932 ± 251***
Fumaric Acid; Maleic Acid	C00122; C01384	HMDB00134; HMDB00176	444972; 444266	100-17-8; 110-16-7	TCA Cycle	10,243 ± 4,641	9,779 ± 3,490	12,371 ± 3,540	20,515 ± 8,689
Succinate	C00042	HMDB00254	1110	110-15-6	TCA Cycle	39,965 ± 19,681	29,105 ± 8,934	30,793 ± 5,470	40,916 ± 13,571
Oxaloacetate	C00036	HMDB00223	970	328-42-7	TCA Cycle	1,518 ± 491	1,396 ± 627	3,304 ± 692	4,739 ± 1,769
Glycine (Gly)	C00037	HMDB00123	750	56-40-6	Amino Acid	348 ± 148	248 ± 112	1,511 ± 1,566	4,834 ± 4,920
Alanine (Ala)	C00041	HMDB00161	5950	56-41-7	Amino Acid	1,095 ± 636	571 ± 121	550 ± 59	754 ± 187
Serine (Ser)	C00065	HMDB00187	5951	56-45-1	Amino Acid	873 ± 375	513 ± 83	**931 ± 121**	**1,407 ± 278***
Proline (Pro)	C00148	HMDB00162	145742	147-85-3	Amino Acid	32,892 ± 16,259	21,071 ± 5,287	1,248 ± 261	1,838 ± 580
Valine (Val)	C00183	HMDB00883	6287	72-18-4	Amino Acid	2,924 ± 1,379	1,574 ± 221	**8,852 ± 1,967**	**14,742 ± 4,551***
Threonine (Thr)	C00188	HMDB00167	6288	72-19-5	Amino Acid	662 ± 327	395 ± 95	**1,836 ± 482**	**3,253 ± 1,090***
Leucine (Leu)	C00123	HMDB00687	6106	61-90-5	Amino Acid	31,166 ± 15,291	19,516 ± 3,219	**44,299 ± 12,167**	**71,703 ± 18,854***
iso-Leucine (*iso*-Leu)	C00407	HMDB00172	791	73-32-5	Amino Acid	31,106 ± 15,687	18,952 ± 3,384	**45,026 ± 1,1390**	**74,461 ± 21,981***
Asparagine (Asp)	C00152	HMDB00168	6267	70-47-3	Amino Acid	6,149 ± 2,669	3,316 ± 756	137 ± 15	171 ± 35
Glutamine (Glu)	C00303	HMDB00641	5961	56-85-9	Amino Acid	113,690 ± 52,558	59,202 ± 12,741	**140,142 ± 26,002**	**221,256 ± 62,086***
Lysine (Lys)	C00047	HMDB00182	5962	56-87-1	Amino Acid	**6,817 ± 3,067**	**3,035 ± 796***	14,864 ± 2,847	21,343 ± 6,008
Glutamic acid (Glu A)	C00025	HMDB03339	611	56-86-0	Amino Acid	5,781 ± 2,624	3,020 ± 959	1,087 ± 248	1,219 ± 429
Methionine (Met)	C01733	HMDB00696	6137	63-68-3	Amino Acid	3,872 ± 1,957	1,771 ± 972	4,954 ± 2,988	9,629 ± 3,022
Histidine (His)	C00768	HMDB00177	6274	5934-29-2	Amino Acid	**15,065 ± 6,440**	**6,954 ± 2,680***	**19,956 ± 4,606**	**36,902 ± 11,742***
Phenylalanine	C02057	HMDB00159	6140	63-91-2	Amino Acid	22,637 ± 11,712	13,618 ± 2,135	**63,131 ± 17,581**	**103,277 ± 30,376***
Arginine	C00062	HMDB00517	232	1119-34-2	Amino Acid	143,332 ± 65,890	92,482 ± 20,066	**51,316 ± 7,200**	**82,413 ± 23,755***
Tyrosine (Tyr)	C00082	HMDB00158	6057	60-18-4	Amino Acid	9,594 ± 4,701	5,826 ± 870	**22,149 ± 5,280**	**37,236 ± 11,356***
Aspartic Acid	C00049	HMDB00191	5960	56-84-8	Amino Acids	1,621 ± 682	888 ± 269	126 ± 42	161 ± 36
Tryptophan (Try)	C00078	HMDB00929	6305	73-22-3	Amino Acids	3,282 ± 1,755	1,841 ± 678	6,601 ± 2,939	11,420 ± 3,694
Reduced glutathione	C00051	HMDB00125	124886	70-18-8	Oxidative Damage	435 ± 195	210 ± 98	169 ± 108	128 ± 72
Oxidized glutathione	C00127	HMDB03337	975	27025-41-8	Oxidative Damage	**2,339 ± 926**	**803 ± 497***	257 ± 222	322 ± 182

Table 2.2. Metabolomics analysis of aortic smooth muscle from a mouse model of diabetes and a mouse model of metabolic syndrome. Statistical analysis was performed Student's t-test; *p<0.05; **p<0.01

Mice fed a high-fat high-sucrose diet develop a metabolic syndrome phenotype, which is not affected by SM-GLUT1 overexpression- In male mice, DDC feeding resulted in significantly increased plasma triglycerides (Fig. 2.2a) and plasma cholesterol (Fig. 2.2b). During 16 weeks of DDC feeding, the animals also gained weight (Fig. 2.2c), had elevated blood glucose (Fig. 2.2d) and had developed glucose intolerance by week 13 (Fig. 2.2e). Female mice had a similar phenotype with DDC feeding as male mice with elevated plasma triglycerides (Fig. 2.2f), plasma cholesterol (Fig. 2.2g) and weight gain throughout the study (Fig. 2.2h). Females, however, did not develop high glucose or glucose intolerance to the same degree as male mice (Fig. 2.2i,j). As an additional control for an effect of metabolic syndrome, mice were given a semi-purified, low-fat diet known to result in atherosclerosis over time. Mice on the low-fat diet had a similar phenotype in terms of body weight gain, blood glucose and plasma cholesterol and triglycerides (Fig. 2.2k,n) to those fed standard chow. Importantly, there was no effect of genotype between SM-GLUT1 and WT mice for any of these measurements.

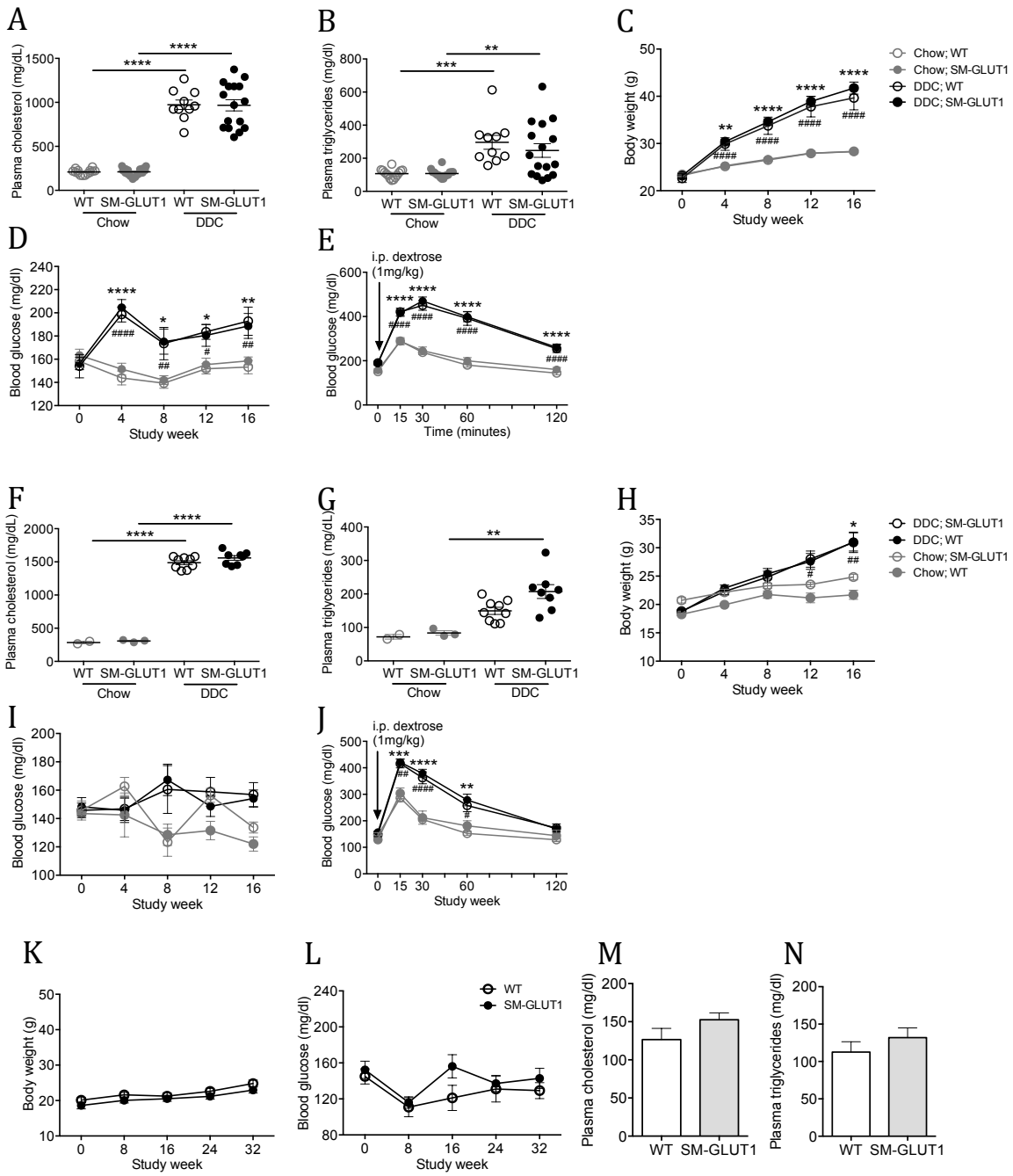

Figure 2.2. DDC feeding in male mice with an LDLR$^{-/-}$ background exhibits features of metabolic syndrome: Elevated plasma cholesterol (a), triglycerides (b), body weight (c), and blood glucose (d). A glucose tolerance test also revealed impaired glucose clearance (e). Results are expressed as mean ± SEM (n=9-16). Female mice fed DDC develop similar elevated plasma cholesterol (f), triglycerides (g), and body weight gain (h), but do not have elevated blood glucose (i) and have a lesser degree of glucose intolerance than male mice (j). Results are expressed as mean ± SEM (n=2 chow 8-10 DDC). Female mice on low-fat diet do not gain weight (k), or have changes in blood glucose (l), and have similar values for plasma cholesterol (m) and triglycerides (n) as chow-fed mice. Results are expressed as mean ± SEM (n=10-11) Statistical analysis was performed by ANOVA with Tukey post test; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. * indicates WT and # indicates SM-GLUT1 more than one comparison is possible.

Metabolic syndrome SM-GLUT1 mice exhibit accelerated atherosclerosis- We next investigated if GLUT1 overexpression in SMCs could exacerbate atherosclerosis in the aortas of male and female mice after either chow or DDC feeding. The entire aorta was quantified for each animal, with representative images of the aortic arch of some animals shown (Figure 2.3a). Neither genotype developed lesions with the chow diet. DDC feeding had little effect on WT mice at the 16 week time point; these were primarily fatty streaks and were not significantly different from either of the chow-fed groups. SM-GLUT1 mice had more advanced lesions in the aorta and were statistically larger that both of the chow-fed groups and WT mice fed DDC (Fig. 2.3b). Similar aortic lesions were observed in both male and female mice, so the data are pooled here. Additional experiments for this study were performed in male mice only. Lipids extracted from aortas of male mice support the increased lesion area in the SM-GLUT1 mice fed DDC. These animals had more cholesterol content in the aorta (Fig. 2.3c), which correlated well with lesion size (Fig. 2.3d). With DDC feeding, both WT and the SM-GLUT1 mice had slightly elevated cholesterol esters reflective of foam cell content in early lesions (Fig. 2.3e). Most of the aortic cholesterol in the DDC fed SM-GLUT1 mice was due to significantly more free cholesterol compared to the WT mice (Fig. 2.3f), which would be found in lesions with more advanced features (13) such as necrotic cores and cholesterol clefts. Aortic triglyceride content was also slightly elevated in DDC-fed SM-GLUT1 mice (Fig. 2.3g).

Following lipid extraction, mRNA was isolated from the entire aorta to analyze gene expression. Gene expression in aortas demonstrate effects of DDC such as elevated *Abca1, Cxcl1* and *Ccl2* (Fig. 2.3h-j), suggesting increased lipid loading and aortic inflammation in mice with metabolic syndrome. *Cd11b* expression, likely reflective of the increased lesion size, was only elevated in SM-GLUT1 mice fed DDC and not their littermate controls (Fig. 2.3k).

The glycosaminoglycan, hyaluronan (HA) can be found in atherosclerotic lesions from humans and mice (87). It is composed of repeating units UDP-N-acetylglucosamine and UDP-glucuronate, both of which can be derived from glucose through the hexosamine pathway. Therefore, we investigated if hyaluronan synthase genes were also increased, which would indicate a possibility for greater HA production in these lesions. *Has2* mRNA was not affected by diet or by GLUT1 overexpression, whereas *Has1* mRNA was significantly elevated with the combination of DDC feeding and GLUT1 overexpression (Fig. 2.3l-m), suggesting both high fat diet and increased glucose flux in SMCs are necessary to induce expression of this gene.

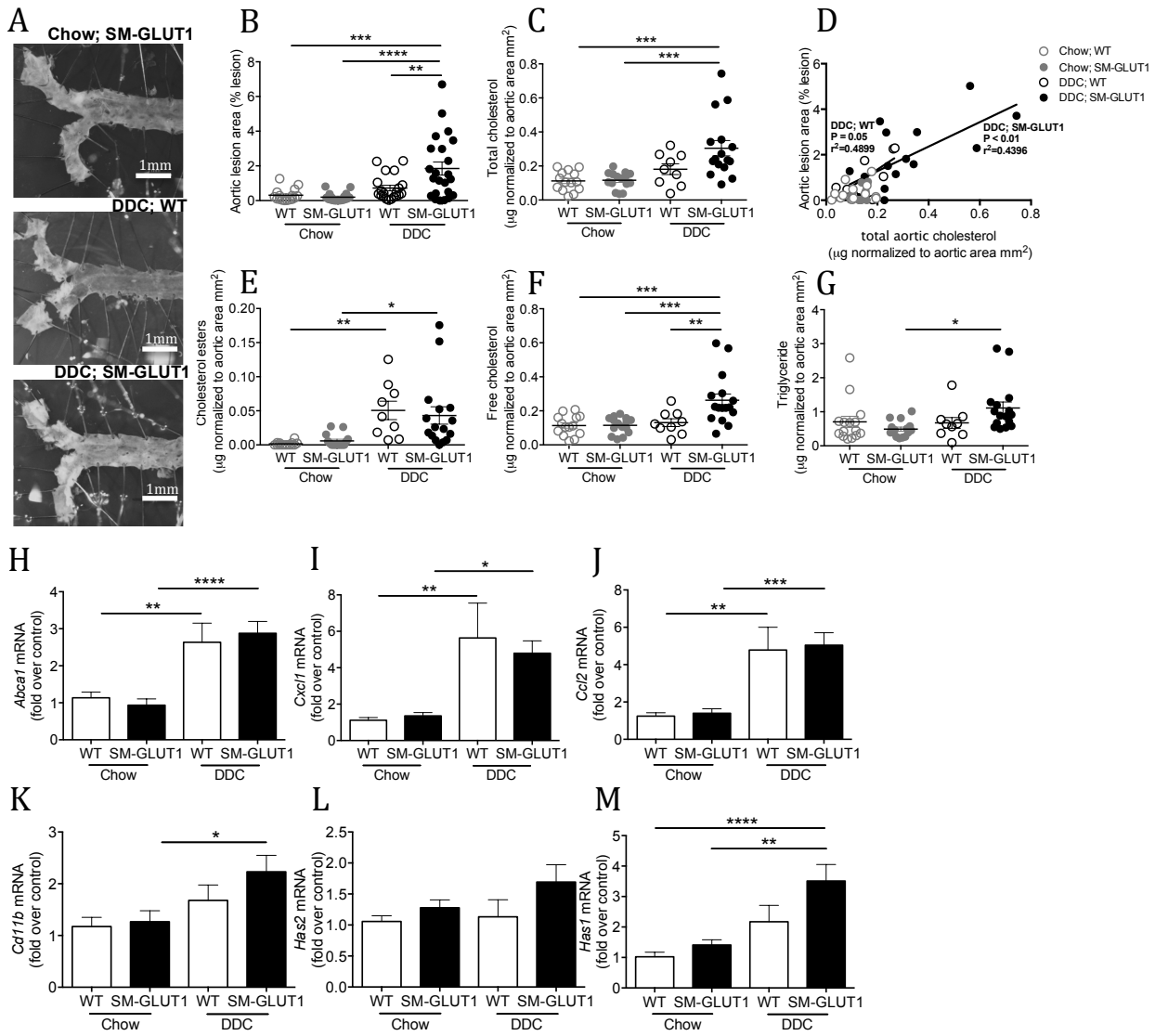

Figure 2.3. SM-GLUT1 mice fed DDC have larger aortic lesions (a,b). Lipids extracted from the whole aorta revealed greater cholesterol content (c), correlating with aortic lesion area (d) in SM-GLUT1 mice fed DDC. DDC feeding led to universal increased cholesterol ester content (e), whereas SM-GLUT1 mice fed DDC had greater free cholesterol (f) and triglyceride (g) content in lipids extracted from aortas. Gene expression as measured by qPCR in homogenized whole aortas from study animals: *Abca1* (h), *Cxcl1* (i), *Ccl2* (j), *Cd11b* (k), *Has2* (l), *Has1* (m). Results are expressed as mean ± SEM (n=16-23 for Aortic lesion and n=9-16 for additional comparisons) Statistical analysis was performed by ANOVA with Tukey post test; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Lesions in the brachiocephalic artery demonstrate features of more advanced lesions and accumulation of hyaluronan in metabolic syndrome SM-GLUT1 mice- Morphological features of the lesions were further characterized by histology of BCA cross-sections. The entire BCA was sectioned and max lesion area was determined for each animal. Representative images for each

group are shown (Fig. 2.4a). SM-GLUT1 mice fed DDC had significantly larger lesions than both of the chow-fed groups, whereas the WT mice fed DDC did not reach significance (Fig. 2.4b). DDC feeding in both groups of mice led to an increased presence of glycosaminoglycans in the medial regions of all sections scored and more positive sections for lesion glycosaminoglycans were observed only in the SM-GLUT1 group fed DDC (Fig. 2.4c-d). Sections adjacent to the max lesion were stained with a Mac2 or SM actin antibody for quantification of lesion macrophage and smooth muscle content, respectively. The lesions were primarily composed of these two cell types (Fig. 2.4e). Several lesions in both the SM-GLUT1 and WT groups fed DDC contained macrophages, but neither group reached statistical significance compared to their chow-fed counterparts (Fig. 2.4f). SM-GLUT1 mice did, however, have more lesion SMCs compared to both of the chow-fed control groups (Fig. 2.4g), suggesting that GLUT1 overexpression in SMCs contributed an earlier occurrence of these more advanced features. With the finding of increased glycosaminoglycans in the BCAs of SM-GLUT1 group fed DDC and increased *Has1* mRNA expression in the aortas of this same group, we decided to further investigate HA levels in the BCAs by immunohistochemistry using an HA binding protein (HABP). Pretreatment with HA lyase prior to addition to HABP was used as a control in several samples to show specificity of the HABP to HA (Fig. 2.4h). Total HA was elevated in the medial, but not lesion area of the BCA near the max lesion (Fig. 2.4i,j). When normalized for medial and lesion area, SM-GLUT1 mice fed DDC had a greater percent of the medial area stain positive for HA suggesting higher HA production by these cells (Fig. 2.4k,l). To determine if GLUT1 overexpression in SMCs directly contributes to HA secretion by these cells, GLUT1 was over expressed in isolated primary aortic SMCs. Retroviral overexpression of GLUT1 led to increase HA secretion by these SMCs *in vitro* (Fig. 2.4m).

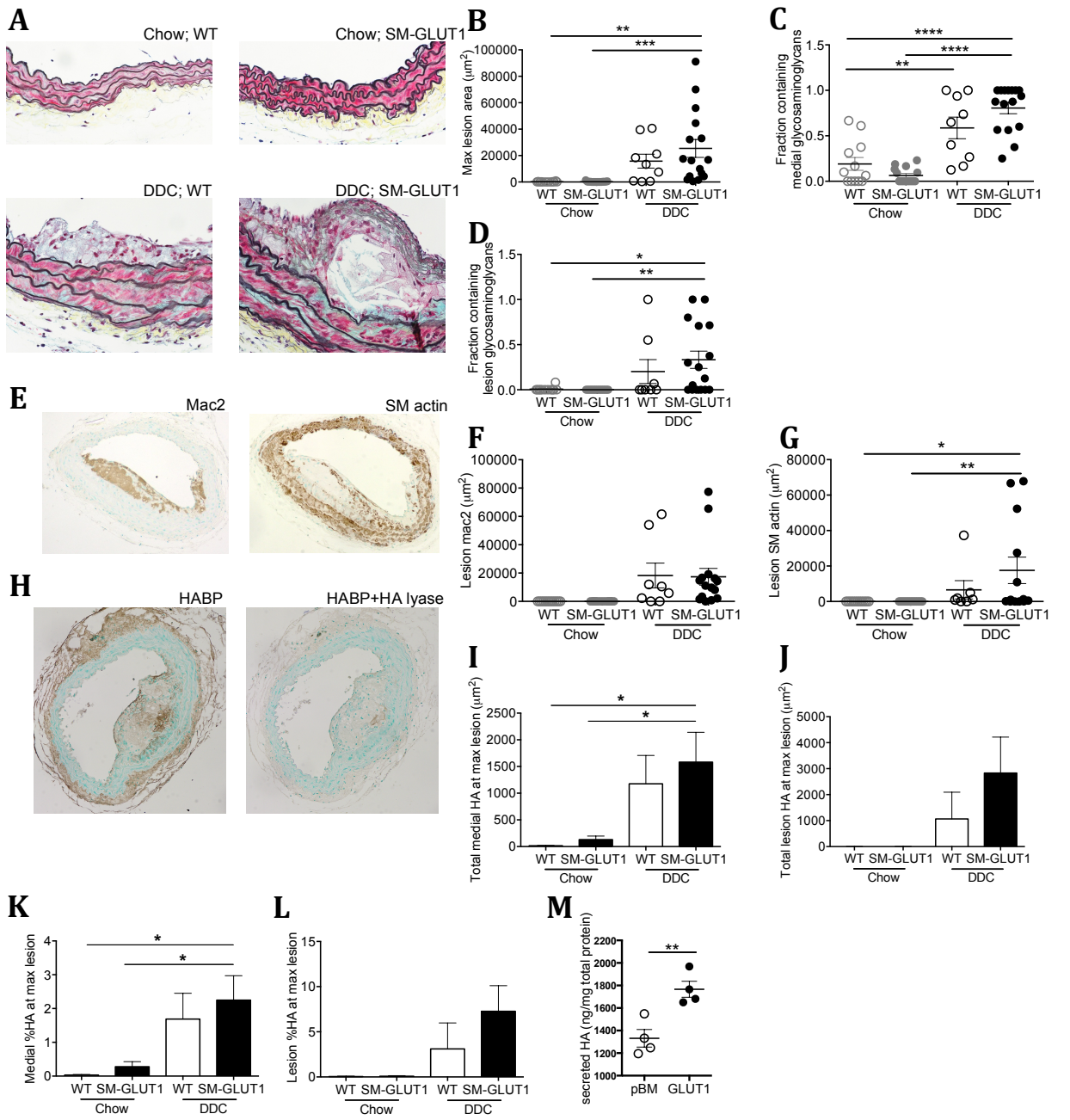

Figure 2.4. Brachiocephalic arteries of SM-GLUT1 mice fed DDC exhibit features of more advanced lesions. Movat pentachrome staining (a) was used to locate and measure max lesion (b), and the frequency of medial (c) and lesion (d) glycosaminoglycans in the BCA sections. Immunohistochemistry for Mac2 and SM actin near max lesion: representative images (e), lesion mac2 (f) and lesion SM actin (g). Quantification of hyaluronan near max lesion: representative images of HA staining and HA lyase control stain (h), total medial (i) and lesion (j) HA, and medial (k) and lesion (l) HA normalized to total area. HA secreted from isolated aortic SMCs with GLUT1 overexpressed by retrovirus or empty vector (pBM) as a control (m). Results are expressed as mean ± SEM (n=8-16) Statistical analysis was performed by ANOVA with Tukey post test; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

GLUT1 expression is not necessary or sufficient to drive SMC proliferation- We next investigated if GLUT1 expression correlated with proliferation of SMCs and whether increased GLUT1 expression is sufficient to promote SMC proliferation. SMCs are known to change from a contractive phenotype *in vivo* to a synthetic proliferative phenotype in culture (61). This phenotypic change is believed to also occur in atherosclerotic lesions, as SMCs migrate from the media to the intima and form a fibrous cap. Indeed, mouse aortic SMCs exhibited a dramatic increase in Cyclin D1 (*Ccnd1*) mRNA (Fig. 2.5a) when grown in culture. However the change in proliferation was not accompanied by a corresponding increase in *Slc2a1* mRNA expression (Fig. 2.5b), suggesting that upregulation of GLUT1 is not required for proliferation. We further investigated if overexpression of GLUT1 could induce proliferation. GLUT1 was overexpressed in isolated aortic SMCs using a retrovirus to deliver a GLUT1 containing plasmid (Fig 2.5c). Transduction of SMCs with this retrovirus resulted in a marked increase in *Slc2a1* mRNA, as compared to SMCs transduced with the empty pBM vector control (Fig. 2.5c), but did not change *Ccnd1* mRNA levels (Fig. 2.5d). Concurrent treatment with oxLDL, which is likely increased in the intima of DDC-fed mice, did not have an effect (Fig. 2.5c,d). Similarly, proliferation was not driven by GLUT1 overexpression *in vivo*. In mRNA extracts from aortas of SM-GLUT1 and WT study animals following 16 weeks of a metabolic-syndrome like phenotype, there was no change in *Ccnd1* (Fig. 2.5e) arguing that increased proliferation is not the mechanism by which SM-GLUT1 mice have accelerated atherosclerosis. This finding has been corroborated with no change in Ki67 staining in BCA cross-sections of the same animals when normalized to either medial or lesion area (data not shown).

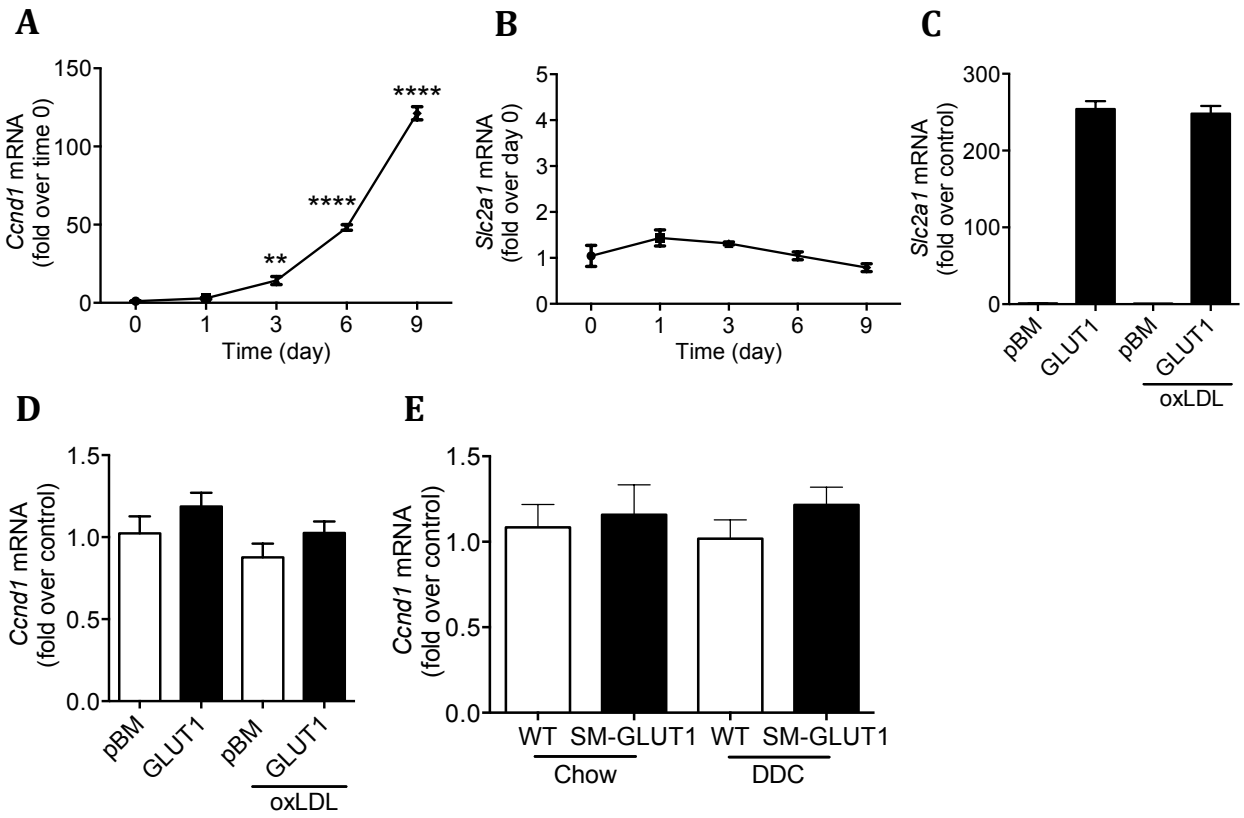

Figure 2.5. Proliferation is independent of GLUT1 expression. Mouse aortic SMCs in culture adopt a synthetic, proliferative phenotype (a), which does not require *Slc2a1* mRNA upregulation (b). Forced overexpression of GLUT1 in mouse aortic SMCs was achieved with a phoenix retroviral vector (c), but did not alter the proliferative status of these cells as measured by C*cnd1* mRNA (d). Results are expressed as mean ± SEM (n=3-6). Statistical analysis was performed by ANOVA with Bonferroni post test (A, B); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. *Ccnd1* mRNA in aortas from experimental mice after 16wks DDC (e). Results are expressed as mean ± SEM (n=9-16). Statistical analysis was performed by ANOVA with Tukey post test.

The effect of GLUT1 is specific to the combination of glucose in SMCs and a metabolic syndrome phenotype **-** Lesions in the aorta and BCA in WT mice primarily consisted of fatty streaks at the early time point of 16 weeks on DDC. Sinus lesions are known to advance more rapidly in WT mice (43,88,89). Lesions in the sinus were quantified for both WT and SM-GLUT1 mice fed chow or DDC for 16 weeks. Advanced lesions were observed in response to diet for both WT and GLUT1 mice and though a similar trend for increased lesion size with GLUT1 overexpression existed in the sinus, there was no significant difference between the two groups (Fig. 2.6a,b). This could suggest that lesions in the sinus are not governed by the same factors as those in the

aorta or BCA. To test whether DDC feeding was required for accelerating aortic atherosclerosis in SM-GLUT1 mice, another group of mice were fed a semi-purified low-fat diet for 12 weeks and 32 weeks. Unlike the chow diet, in which atherosclerosis will not occur, 12 weeks on this low-fat diet can produce very small early lesions and 32 weeks on the same diet will lead to large lesions with advanced morphology. There was no effect of the SM-GLUT1 genotype in aortic lesion area at either time point (Fig. 2.6c), suggesting that high fat feeding is required to observe accelerated atherosclerosis caused by GLUT1 overexpression in SMCs. Further, since SM-GLUT1 did not have an effect on advanced lesions in either of these regions, GLUT1 overexpression may preferentially influence early developing lesions when in combination with a high-fat diet.

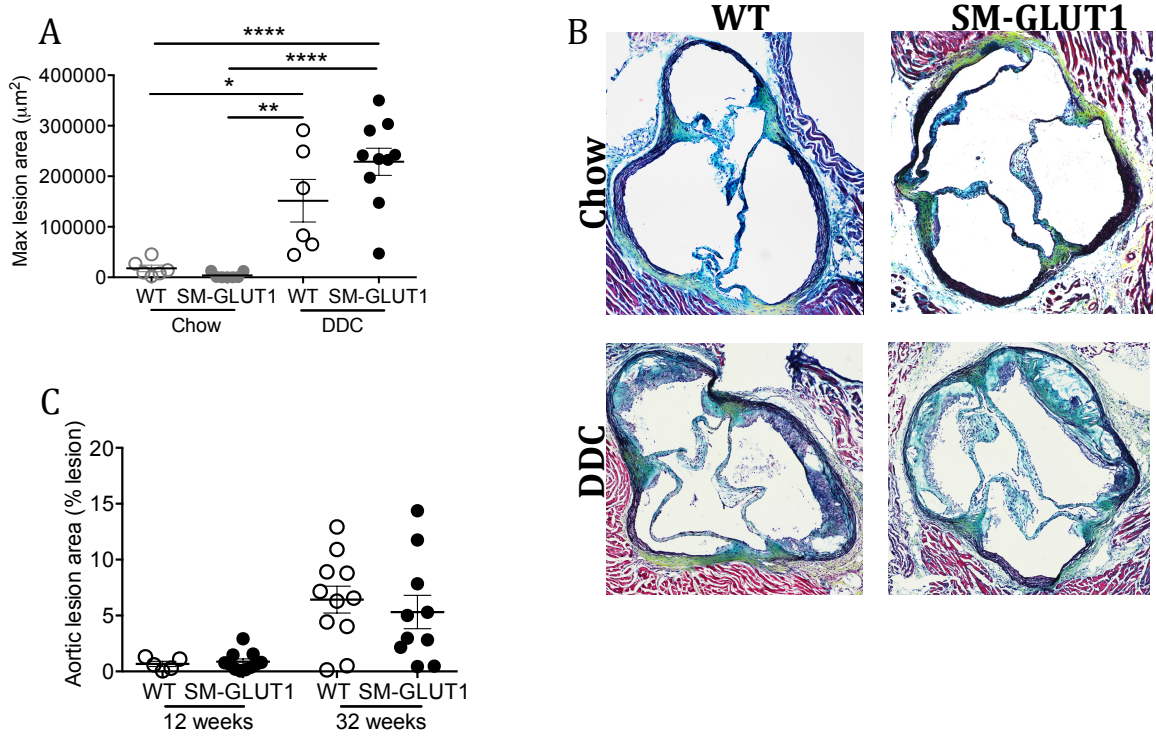

Figure 2.6. Lesions in the sinus are present in both WT and SM-GLUT1 mice after 16 weeks of DDC. Metabolic syndrome is required for GLUT1-specific effects in the aorta. Sinus lesions were not significantly different in SM-GLUT1 mice compared to WT mice after 16 weeks of DDC feeding (a,b) (n=6-10). Low-fat feeding produces advanced lesions over 32 weeks. However, GLUT1 overexpression in SMCs did not have an effect on these lesions, nor their smaller precursors at the 12 week time point (n=5-11) (c). Statistical analysis was performed by ANOVA with Tukey post test; *p<0.05; **p<0.01; ****p<0.0001.

GLUT1 overexpression in myeloid cells does not mimic the effect of SM-GLUT1 mice on atherosclerosis- Finally, to determine the cell-type specificity of GLUT1 overexpression on atherosclerosis in metabolic syndrome mice, Ldlr$^{-/-}$ mice were transplanted with bone marrow stem cells transduced with a retroviral vector encoding mouse GLUT1 under control of the CD68 promoter, as described previously (43). Isolated bone-marrow-derived macrophages from these animals had similar fold changes in glucose uptake and lactate release as the SM-GLUT1 animals (Fig. 2.7a,b). Like the SM-GLUT1 mice, no effects of M-GLUT1 were observed on plasma cholesterol (Fig. 2.7c), triglycerides (Fig. 2.7d), body weight gain (Fig. 2.7e), blood glucose level (Fig. 2.7f), or glucose intolerance (Fig. 2.7g). However, unlike SM-GLUT1, M-GLUT1 had no effect on atherosclerosis in metabolic syndrome mice (Fig. 2.7h).

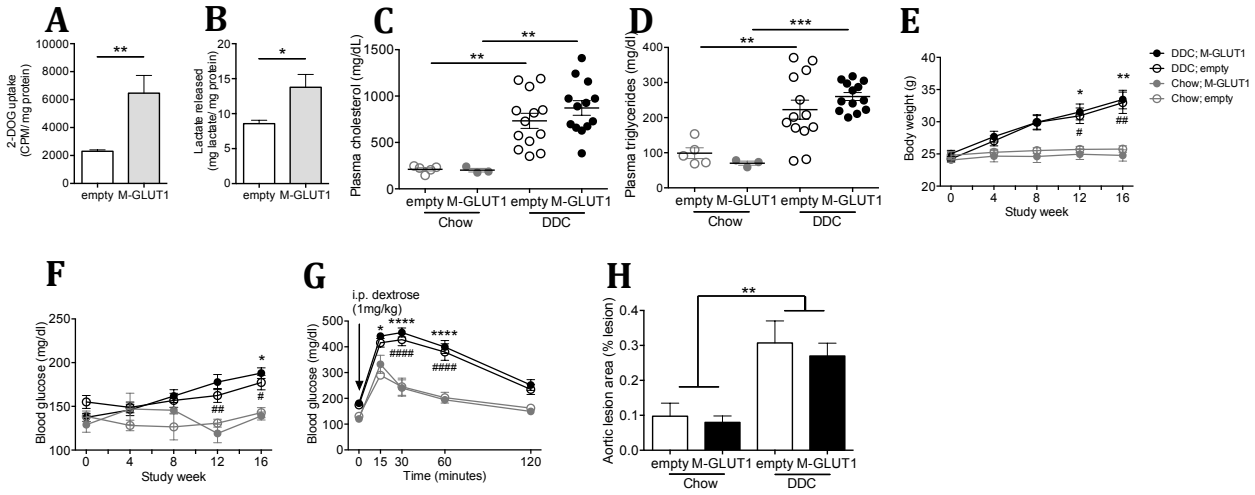

Figure 2.7. GLUT1 overexpression in myeloid cells. GLUT1 overexpression in myeloid-derived cells led to increased glucose uptake (a) and lactate release (b) in BMDMs from these mice. Results are expressed as mean ± SEM (n=3-6) Statistical analysis was performed by two-tailed unpaired Student's t-test (E-J); *p<0.05; **p<0.01. DDC feeding in male mice exhibits features of metabolic syndrome irrespective of genotype: elevated plasma cholesterol (c), triglycerides (d), body weight (e), and blood glucose (f). A glucose tolerance test also revealed impaired glucose clearance (g). En face analysis of aortas demonstrated no effect of M-GLUT1 (h). Results are expressed as mean ± SEM (n=3-13). Statistical analysis was performed by ANOVA with Tukey post test; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. * indicates WT and # indicates SM-GLUT1 more than one comparison is possible.

Discussion

Metabolic syndrome is a combination of risk factors that predict the occurrence of CVD and often precedes development of T2DM. We tested the role of dysregulated arterial SMC glucose metabolism in combination with systemic factors of metabolic syndrome. Our study found a combinatorial effect of GLUT1 overexpression in SMCs and systemic changes in a model of metabolic syndrome that together lead to accelerated atherosclerosis.

Here, we demonstrate that increased expression of GLUT1 in SMCs drives glucose flux, which correlates with increased deposition of the extracellular matrix component, HA, often found in vascular lesions (90,91). The composition of the extracellular matrix in the vessel wall influences phenotypic behavior of vascular cells including SMCs and macrophages. HA is a non-sulfated glycosaminoglycan that may accelerate atherosclerosis through activation the NFκ-B pathway in macrophages (92), and by promoting migration and proliferation of SMCs (93). HA is synthesized by any of three hyaluronan synthases (*Has1-3*). Availability of its two alternately repeating substrates, UDP-*N*-acetylglucosamine and UDP-glucuronic acid is required for HA synthesis and increased availability of glucose can lead to greater HA synthesis by vascular SMCs (94). Expression of *Has1*, in particular, is positively regulated by its substrate, UDP-*N*-acetylglucosamine (95). Thus, it is plausible that increased glucose uptake may increase hyaluronan precursors allowing for both additional substrate for HAS enzymes and upregulation of *Has1* mRNA which in turn could increase HA deposition.

Surprisingly, GLUT1 overexpression did not appear to influence lesion size in animals fed chow or fed a low-fat diet permissive to slower development of atherosclerosis. Though atherosclerotic lesions in low-fat fed mice can become large with advanced morphology, the rate

of progression of these did not change in SM-GLUT1 mice. Likewise, SM-GLUT1 mice fed chow did not have increased HA deposition nor increased *Has1* mRNA, which suggests additional factors present in metabolic syndrome are required for this to occur.

We suspect that a combinatorial effect of metabolic syndrome is necessary for dysregulated glucose metabolism in SMCs to accelerate atherosclerosis. There are several mechanisms in which this could occur. In addition to regulation by glucose, disrupted cholesterol homeostasis is also thought to contribute to HA accumulation (96) by stabilization of HAS enzymes at the cell surface. Additionally, human aortic SMCs treated with oxLDL increase HA production (97). In support of a requirement of dyslipidemia to observe hyaluronan-mediated atherosclerosis, similar results were obtained when HAS2 was overexpressed in SMCs (44). Here atherosclerosis was absent in HAS2 transgenic mice on a C57BL/6J background, but when crossed into a dyslipidemic ApoE null background HAS2 transgenic mice developed accelerated atherosclerosis (44). Alternatively, metabolic syndrome may be required to induce dysfunction of the artery wall, allowing for monocytes to adhere and transmigrate into the intima. Gene expression of chemokines in whole aortas demonstrate diet-induced arterial alterations. Mice with metabolic syndrome had greater expression of *Ccl2* and *Cxcl1* mRNA regardless of genotype, suggesting the possibility of glucose effects being more pronounced in conditions that independently facilitate monocyte activity. CCL2 promotes monocytosis and monocyte accumulation in artery wall of $ApoE^{-/-}$ mice (98) whereas CXCL1 facilitates increased leukocyte adhesion to the endothelium with mild oxLDL treatment (99). GLUT1 overexpression also increased sorbitol content in aortas of DDC fed mice. Transgenic repletion of human aldose reductase, which catalyzes the reduction of glucose to sorbitol, in diabetic mice pre-disposes

them to accelerated atherosclerosis. Thus, increased flux through the sorbitol pathway may be another means by which SM-GLUT1 mice develop larger lesions.

The combination of GLUT1 overexpression and metabolic syndrome increased total cholesterol content in the aorta. It has been demonstrated that high glucose can suppress ABCA1 expression, inhibiting cholesterol efflux (100). Lesion SMCs have less ABCA1 than medial SMCs or macrophages, which is thought to contribute to foam-cells of SMC origin (26). However, expression of aortic *Abca1* mRNA was not affected by GLUT1 overexpression, and accumulated cholesterol was primarily in the form of free cholesterol. This suggests that accumulation of cholesterol was not a result of decreased cholesterol efflux by SMCs, but rather increased necrotic core area, which is a feature of more advanced lesions. Follow-up measurements of necrotic core area will be needed to verify that it is the major contributor to increased cholesterol content in these aortas.

Our earlier work has demonstrated that increased glucose uptake alone in macrophages cannot in itself drive atherosclerosis (43). However in recent work by Freemerman and colleagues, GLUT1 expression correlated with cytokine production in adipose tissue macrophages (56) in mice fed a high fat diet. We investigated if a synergistic effect could exist between fat feeding and GLUT1 overexpression in macrophages that may accelerate atherosclerosis via inflammatory activation. In the same model of metabolic syndrome as in our SM-GLUT1 studies, atherosclerosis in mice that received bone marrow expressing GLUT1 under the CD68 promoter did not differ from mice that received bone marrow containing empty vector. This does not refute that increased GLUT1 expression in adipose tissue macrophages may drive inflammation, but it does suggest that macrophages of the artery wall are less effected by GLUT1 overexpression than are SMCs. Further, the combination of GLUT1 overexpression in both

SMCs and macrophages supports a specific effect of dysregulated glucose metabolism in SMCs that is harmful to the artery wall in our model.

In summary, we have demonstrated that increased GLUT1 expression in SMCs results in increased glucose utilization and metabolic changes that promote atherosclerosis in a metabolic syndrome-selective manner, and suggest a novel mechanism whereby arterial metabolism promotes atherosclerosis.

Acknowledgments

This study was supported by NIH grants HL062887, HL092969 and a grant from the American Heart Association, 14GRNT20410033, to KEB. Part of the study was supported by the Diabetes Research Center at the University of Washington, P30 DK017047. The metabolomics studies were performed by the Analytic Core of the University of Washington NORC supported by P30 DK035816. VZW was supported by the Samuel and Althea Stroum Fellowship in Diabetes Research.

Chapter 3-

Acyl-CoA Thioesterase 7 Induction Promotes Cytokine Production in Macrophages

Summary

Macrophages respond to toll-like receptor 4 (TLR4) stimulation by adopting an inflammatory phenotype, attracting other immune cells and killing pathogens. Acyl-CoA thioesterase 7 (ACOT7), an intracellular enzyme that converts acyl-CoAs to free fatty acids, is induced by the TLR4 ligand lipopolysaccharide (LPS) in macrophages. We demonstrate that ACOT7 is induced by LPS through TLR4 and its adapter protein MyD88, and is also induced in macrophages in a mouse model of diabetes. We therefore investigated whether ACOT7 overexpression or ACOT7-deficiency alters the inflammatory status in LPS-stimulated macrophages *ex vivo* and *in vivo* in a mouse model of diabetes-accelerated atherosclerosis. ACOT7 induction led to a drastic increase in thioesterase activity toward arachidonoyl-CoA with smaller increases in activity toward palmitoyl-CoA and oleoyl-CoA, and a concomitant increase in cytokine secretion in LPS-stimulated macrophages and suppression of peroxisome proliferator-activated receptor-γ (PPAR-γ) target genes. Conversely, ACOT7-deficiency resulted in decreases in arachidonoyl-CoA thioesterase activity and suppression of inflammatory activation following TLR4 stimulation, although the effects were much smaller than those of ACOT7 overexpression. Hematopoietic ACOT7-deficiency reduced *Tlr4* mRNA levels in leukocytes from non-diabetic mice, but did not protect against the effects of diabetes on inflammation and atherosclerosis. These data suggest that ACOT7 is induced by TLR4-MyD88 activation and diabetes in macrophages, that arachidonoyl-CoA is a preferred ACOT7 substrate, and that ACOT7 induction serves to amplify the TLR4 response through inhibition of PPAR-γ activity. This study adds ACOT7 to the group

of fatty acid handling proteins that promotes inflammation through suppressing PPAR-γ activation in innate immunity.

Introduction

Macrophages are an essential component of the innate immune response. When activated by pathogens, macrophages secrete cytokines contributing to immune cell recruitment and activation. Once the pathogen is cleared, this inflammatory response is resolved. Dysregulation of the inflammatory response however, underlies several disease processes. It is becoming increasingly clear that intracellular fatty acid handling plays an important role in modulating the inflammatory response (37,39,40,81,101). Thus, macrophages deficient in the fatty acid binding protein FABP4 (aP2) exhibit reduced activation of NF-κB and a concomitantly increase in peroxisome proliferator-activated receptor-γ (PPAR-γ) activation in macrophages (39), and inhibition of FABP4 prevents atherosclerosis in mice (40). Similar results have been found by deleting FABP5 (101). The anti-inflammatory effects of FABP-deficiency are likely due to increased intracellular levels of free unsaturated fatty acids, or their metabolites, which act as PPAR-γ ligands (81). Furthermore, acyl-CoA synthetase 1 (ACSL1), an enzyme that converts free fatty acids into their acyl-CoA derivatives, is induced by TLR4 activation in macrophages and its deletion in myeloid cells results in a less inflammatory phenotype and protection against atherosclerosis in diabetic mice (37). ACSL1 acts primarily on unsaturated fatty acids in macrophages, and ACSL1-deficiency leads to reduced levels of arachidonoyl-CoA and increased levels of free unsaturated fatty acids (37).

We chose to further investigate the role of unsaturated fatty acid handling in regulation of inflammation and atherosclerosis by studying one of the acyl-CoA thioesterases (ACOTs)

induced by lipopolysaccharide (LPS) in macrophages, ACOT7. ACOTs hydrolyze the thioester bond on acyl-CoAs, but their cellular functions have just recently begun to be elucidated. There are 15 known ACOTs in mice, each with differential tissue and intracellular localization, transcriptional regulation and acyl-CoA substrate preference (102-105). Only a few studies to date have investigated the function of ACOTs *in vivo* (106-111). These studies have revealed that ACOTs are important mediators of fatty acid and acyl-CoA channeling into specific fates, and that the processes mediated by ACOTs appear to require rapid and dynamic interconversion between free fatty acids and acyl-CoAs. Thus, ACOT2 promotes hepatic fatty acid oxidation, which requires acyl-CoA formation (108). ACOT13-deficiency protects against hepatic triglyceride accumulation in fat-fed mice and increases energy expenditure following cold exposure (109,110), and likewise ACOT11-deficiency protects against obesity by increasing energy expenditure (111). ACOT15 is involved in cardiolipin remodeling (106). ACOT7 is highly expressed in neurons (112,113) and a neuron-specific ACOT7-deficient mouse exhibits elevated levels of lysophosholipid and sphingosine in the brain (107). ACOT7 has preferential activity toward arachidonoyl-CoA (20:4-CoA) in macrophages (114), but also acts on other long-chain acyl-CoAs (114,115). Expression of an active form of ACOT7 has been found to suppress prostaglandin D_2 (PGD_2) and PGE_2 production under basal conditions in a macrophage cell line (114). Contrary to what one would predict based on the enzymatic activity of ACOT7, but consistent with the emerging understanding of the cellular functions of ACOTs, ACOT7-deficiency results in markedly increased levels of free unsaturated fatty acids in neurons (107), suggesting a possible role for ACOT7 in suppressing PPAR activity by reducing its endogenous ligands.

We investigated the role of ACOT7 in two models of inflammation in which altered fatty acid handling in macrophages is thought to contribute to the pro-inflammatory phenotype: LPS

stimulation *in vitro* and an *in vivo* model of diabetes. Our results demonstrate that ACOT7 is induced by TLR4 through its adapter protein MyD88 and by diabetes in macrophages, and that ACOT7 promotes an inflammatory phenotype and suppresses PPAR-γ target genes in these cells.

Experimental procedures

Mice- All mice were on the C57BL/6 background. *Acot7*$^{-/-}$ mice were generated by crossing *Acot7*$^{fl/fl}$ mice (107) to mice expressing Cre recombinase from the germline (Jax 006054) (116). Mice carrying a deleted allele of *Acot7* were then bred to homozygosity in the absence of Cre recombinase. Female LDL receptor-deficient (*Ldlr*$^{-/-}$) mice for atherosclerosis studies were obtained from Jackson Labs (Bar Harbor, ME). Bones from *Tlr4*$^{-/-}$ mice were a generous gift from Dr. Linda Curtiss, the Scripps Research Institute, and bones from *Myd88*$^{-/-}$ mice and TRIF-deficient (*Ticam1*$^{-/-}$) mice were generously provided by Dr. Alan Aderem, Seattle Biomedical Research Institute and Dr. Kelly D. Smith, University of Washington, Seattle. Bones from wildtype littermates were used as controls.

Isolation and maintenance of bone marrow-derived macrophages- Bone marrow-derived macrophages (BMDMs) were harvested from freshly obtained bones, or from bones shipped overnight on wet ice, by syringe flushing with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% fungizone and 1% penicillin/streptomycin. Cells were purified of erythrocytes and maintained in DMEM (450 mg/dL glucose) containing 1% fungizone, 1% penicillin/streptomycin, 7% fetal bovine serum (FBS), and 30 % L-cell conditioned medium.

Generation of a retroviral vector for ACOT7 overexpression- Murine *Acot7* cDNA (Origene, Rockville, MD) was cloned into the retroviral pBM-IRES-PURO (pBM) vector. Phoenix ecotropic cells (Allele Biotechnology, San Diego, CA) were transfected with the empty

pBM vector, or pBM-ACOT7 vector by $CaCl_2$ transfection, according to Allele Biotechnology's instructions. Phoenix cells transfected with a pBM-EGFP (enhanced-green fluorescent protein) vector were used to generate additional controls for some experiments. The next day, the cells were passaged into media containing 2 μg/mL puromycin for positive selection, and then maintained in media containing puromycin until virus collection when the cultures were ~90% confluent. J774 macrophages or freshly harvested bone marrow cells were treated with DMEM (450 mg/dl glucose) containing 0.45 μm syringe-filtered retroviral media as well as HEPES (60 mmol/L), polybrene (4 μg/mL), 1% fungizone, 1% penicillin/streptomycin and, in the case of BMDMs, 30% L-cell conditioned media to induce macrophage differentiation. Retroviral media were replaced after 48 h with DMEM (450 mg/dL glucose) containing 1% fungizone, 1% penicillin/streptomycin and 10% FBS for J774 macrophages or with DMEM (450 mg/dl glucose) containing 1% fungizone, 1% penicillin/streptomycin, 7% FBS, and 30% L-cell conditioned media for BMDMs. BMDMs were allowed to differentiate for 7-10 days prior to experiments. For measurements of inflammatory responses, cells were stimulated with LPS (5-10 ng/mL; *E. coli* 0111:B4; List Biological Laboratories, Campbell, CA) and, for BMDMs, IFN-γ (12 ng/mL; eBiosciences, San Diego, CA #14-8311-63) for 18-24 h. In a subset of experiments, cells were treated with inhibitors of eicosanoid production: the cyclooxygenase 2 inhibitor CAY 10404 (500 nM; Cayman, Ann Arbor, MI) or the 5-lipoxygenase inhibitor CJ-13,610 (1-2 μM; Sigma).

Knockdown of ACOT7 in thioglycollate elicited macrophages- For *in vitro* ACOT7 knockdown experiments, macrophages were harvested from the peritoneal cavity 5 days following injection of thioglycollate (5). Prior to plating the macrophages, *Acot7* siRNA (Life Technologies; #4390771; i.d. s88465 and s211904) or negative control #2 siRNA (Life Technologies; #4390846) was introduced by electroporation (Amaxa mouse macrophage

nucleofector kit; Lonza, Cologne, Germany). The samples were electroporated in the Amaza Nucleofector I device using program Y-01, as described previously (77). $3x10^6$ cells/cuvette were electroporated and immediately plated in media supplemented with 10% FBS, and media and floating cells were removed 1 h later for adherence purification of macrophages. All experiments were done using RPMI 1640 medium (11.2 mM glucose) supplemented with 1% penicillin/streptomycin. For measurements of inflammatory responses, cells were stimulated with ultrapure LPS (*E. coli* 0111:B4, 10 ng/mL; List Biological Laboratories) 48 h following electroporation as ~90% knockdown of ACOT7 protein was achieved at this time point.

Real-time PCR, enzyme-linked immunosorbent assays and Western blots- Inflammatory mediators were quantified by real-time PCR and ELISAs. RNA isolation and the real-time PCR protocol were performed as described (37). Briefly, RNA was isolated using Qiagen (Valencia, CA) RNeasy or Macherey-Nagel (Bethlehem, PA) Nucleospin RNA kits according to manufacturers' protocols and treated with DNase1 (1 µg/sample, Thermo Scientific, Waltham, MA) to remove trace DNA. Real-time PCR was performed using the SYBR Green 1 detection method (Thermo Scientific). Cycle threshold (Ct) values were normalized to *Rn18s* and the results were presented as fold over control. Primers for many of the genes investigated have been published (37) and additional primer sets used were as follows: *Acot7* (sense, 5'-TGACCAATAAAGCCACCTTGTG-3'; antisense, 5'-CCTGCTCCTGCCGTAAATACAC-3'); *Acot8* (sense, 5'- CCTCGAGCCGCTAGATGAAG-3'; antisense, 5'-GGCCCATAATTTGACCCCCA-3'); *Acot9* (sense, 5'-ACGGCTTTGGACCTTGAACA -3'; antisense, 5'-CTCCAGACTGTGGATACGCC-3'); *Acsl4* (sense, 5'-CTGGAAAGCAAACTGAAGGC - 3'; antisense, 5'-AGGGATACGTTCACACTGGC-3'); *Cd36* (sense 5'- GCAAAGAAGGAAAGCCTGTG-3'; antisense 5'-CCAATGGTCCCAGTCTCATT-3'); *Cpt1a* (sense, 5'- CCAGGCTACAGTGGGACATT-3';

antisense 5'-GACTTGCCCATGTCCTTGT-3'); *Tlr4* (sense, 5'-TTTGACACCCTCCATAGACTTCA-3'; anti-sense 5'-GAAACTGCAATCAAGAGTGCTG-3'), *Ucp2* (sense, 5'–GTGGTGGTCGGAGATACCAGA-3'; antisense 5'-GGGCAACATTGGGAGAAGTCC-3').

IL-6, TNF-α, IL-1β and CCL2 ELISA kits (eBioscience) and PGE_2 ELISA kits (Cayman Chemical) were used to quantify secreted cytokines or PGE_2 *in vitro*. For detection of ACOT7 protein, total cell lysates (10–20 μg) were loaded onto SDS/PAGE gels, separated, and transferred onto nitrocellulose membranes. Detection was accomplished by using a rabbit polyclonal ACOT7 antibody (1:1,000 dilution, Abcam, Cambridge, UK, ab85151), and a mouse monoclonal β-actin antibody (1:10,000 dilution; Sigma-Aldrich).

Determination of thioesterase activity and acyl-CoA species- Thioesterase enzymatic activity was determined in lysates of BMDMs from mice with hematopoietic ACOT7-deficiency or BMDMs overexpressing ACOT7 (by transduction with an *Acot7* ecotropic retrovirus). Cells were allowed to differentiate in L-conditioned media for 7 days prior to the experiment and then harvested in Western lysis buffer containing protease (Santa Cruz Biotechnology, Dallas, TX, sc29130) and phosphatase inhibitors. Samples were frozen prior to the enzymatic assay at -80°C without degradation. For the assays, 12.5 μg of protein lysate and DNTB [5,5'-Dithiobis-(2-Nitrobenzoic Acid); Cayman Chemical] were incubated with various acyl-CoAs (Sigma) in buffer containing: 50 mM KCl, 10 mM HEPES pH 7.2, 0.025% Titon-X100, and 0.2 mg/mL fatty acid-free BSA. Free CoA reacts with DNTB and was detected by measuring absorbance at 412 nm. Quantification was done by using a standard curve of known CoA concentration (Sigma). In some experiments, ACOT7 was immunoprecipitated prior to the thioesterase activity assay. ACOT7 was immunoprecipitated by using a rabbit polyclonal anti-ACOT7 (ab8515;

1:1000, Abcam). A non-specific rabbit IgG was used for control immunoprecipitations. Briefly, cell lysates at a concentration of 1 mg of protein in 0.5 mL were incubated with 3 μg of ACOT7 antibody or IgG overnight. Target proteins bound by the antibodies were immunoprecipitated by addition of protein G-agarose beads (Protein G-agarose Immunoprecipitation Kit, Roche Diagnostics, Basel, Switzerland) and incubation for 3 h. Antibody-conjugated beads were washed several times according to the manufacturer's protocol prior to the thioesterase assay. Each sample absorbance was normalized to its no substrate control prior to quantification of CoA released. All reactions were performed within the linear range.

Measurements of acyl-CoA molecular species were performed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS), as described previously (43).

Generation of non-diabetic and diabetic mice lacking ACOT7 in bone marrow cells for atherosclerosis study- Bone marrow was harvested from ACOT7-deficient (ACOT7 KO) mice or wildtype controls (107) purified of erythrocytes and transplanted into lethally irradiated (10 Gy) female *Ldlr*$^{-/-}$ recipients ($5x10^6$ cells injected retro-orbitally). The mice were maintained on antibiotic water for two weeks following the transplant and allowed to recover for a total of seven weeks before streptozotocin (STZ) injections. STZ (mixed anomers no. S0130, 50 mg/kg; Sigma) was dissolved in freshly made citrate buffer (0.1 M, pH 4.5) and injected intraperitoneally for 5 consecutive days. The mice were monitored for development of diabetes (defined as blood glucose >250 mg/dl). If mice did not respond to the STZ treatment 14 days after the first injection, they were reinjected for an additional round of 5 days. In the present study, 37/40 mice exhibited blood glucose values >250 mg/dl. Blood glucose was monitored throughout the study using test strips (One Touch Ultra, LifeScan) and diabetic mice received insulin (Lantus, Sanofi-Aventis, Bridgewater, NJ) as needed to prevent excessive weight loss and ketonuria. After

induction of diabetes, the mice were fed a semi-purified low-fat diet for the duration of the 12-week study. This diet has been described previously (83) and is used because diabetic mice do not exhibit hypercholesterolemia as compared to non-diabetic controls when fed this diet. Blood cholesterol was measured using test strips (Cardiochek, Indianapolis, IN). Plasma lipids were determined by colorimetric assays according to manufacturers' instructions: triglycerides (Sigma), NEFAs (Wako, Richmond, VA), and cholesterol (Wako). For mRNA measurements in leukocytes, mRNA was extracted from blood leukocytes according to manufacturer's instructions (Macherey Nagel; Nucleospin RNA kit). Aortas were dissected longitudinally and stained *en face* with Sudan IV for visualization of aortic lesions (83). Lesions were quantified in a blinded manner as percent area of the entire aortic area.

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Washington.

Statistical Analysis- Statistical analyses for all studies were performed using GraphPad Prism 5 software (La Jolla, CA). Unpaired two-tailed Student's t-test was used to compare two conditions, while multiple groups were compared by one-way ANOVA with a Tukey post hoc test or two-way ANOVA. Grubbs's test was used to identify statistical outliers within the *in vivo* leukocyte data. Error bars indicate SEM. For enzymatic activity assays, differences in slope determined significance. * = $p<0.05$, ** = $p<0.01$, *** = $p< 0.001$, **** = $p<0.0001$

Results

LPS induces ACOT7 through TLR4/MyD88- ACOT7 has previously been shown to be induced by LPS in macrophages (114). We first verified that in thioglycollate-elicited macrophages and BMDMs *Acot7* mRNA and ACOT7 protein are increased after treatment with LPS (Fig. 1A-D). *Acot7* mRNA levels peaked at 6 h post stimulation, and then declined (Fig. 3.1a). For comparison, *Acsl1* was more strongly induced by LPS (Fig. 3.1a). Furthermore, a significant increase in ACOT7 protein levels was observed up to 48 h after LPS stimulation (Fig. 3.1b). Similar results were obtained in BMDMs stimulated by LPS/IFNγ (data not shown). Moreover, ACOT7-specific 16:0-CoA thioesterase activity was increased by 3.1 ± 0.4-fold in BMDMs stimulated with LPS/IFNγ as compared to IL-4 for 48 h ($p=0.006$; $n=4$). LPS is a ligand of TLR4, which signals through two adapter proteins: Toll/Il-1 receptor domain containing adaptor inducing IFN-β (TRIF; gene name *Ticam1*) and MyD88. We investigated the adapter protein responsible for LPS-induced *Acot7* by taking advantage of BMDMs from *Tlr4*$^{-/-}$ and *Ticam1*$^{-/-}$ mice (Fig. 3.1c). BMDMs isolated from *Tlr4*$^{-/-}$ mice exhibited no increase in *Acot7* mRNA levels after LPS stimulation, as expected, whereas BMDMs from *Ticam1*$^{-/-}$ mice had normal, or even elevated induction of *Acot7* mRNA following LPS stimulation. Therefore, regulation of ACOT7 after LPS treatment in macrophages is entirely dependent on TLR4 signaling, but does not require the TRIF adapter protein. In an additional experiment using BMDMs from *Myd88*$^{-/-}$ mice, we demonstrated that induction of *Acot7* mRNA requires MyD88 (Fig. 3.1d).

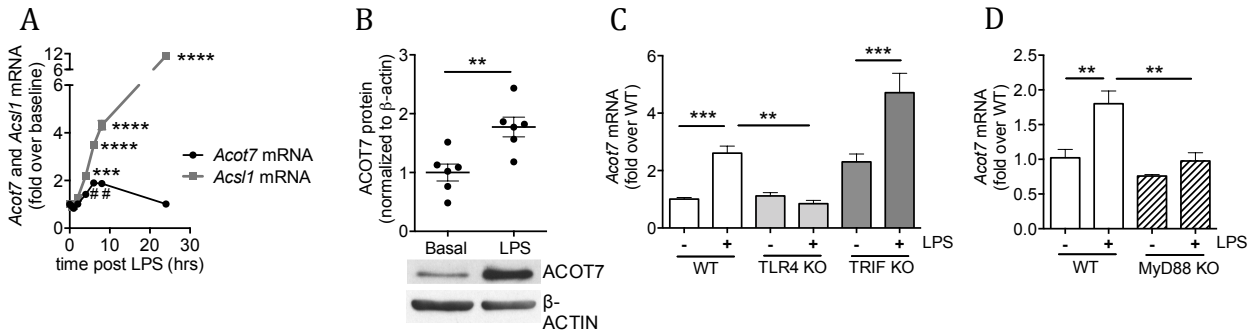

Figure 3.1. ACOT7 is upregulated in response to LPS via the MyD88 arm of the TLR4 pathway. Thioglycollate-elicited macrophages exhibit increased *Acot7* and *Acsl1* mRNA (A) and ACOT7 protein (B) in response to LPS. BMDMs from *Tlr4*$^{-/-}$ (TLR4 KO), *Ticam1*$^{-/-}$ (TRIF KO) (C) and *Myd88*$^{-/-}$ (MyD88 KO) (D) mice were treated with 10 ng/mL ultrapure LPS for 4-6 h. *Acot7* mRNA was measured by real-time PCR and ACOT7 protein was measured by Western blot analysis. Results are expressed as mean ± SEM (n=3-6) Statistical analysis was performed by ANOVA (A, C, D) or two-tailed unpaired Student's t-test (B); **p<0.01; ***p<0.001; ****p<0.0001 versus t=0 for *Acsl1* mRNA (A) or versus indicated group; ##p<0.01 versus t=0 for *Acot7* mRNA (A).

ACOT7 overexpression in macrophages- Overexpression of ACOT7 was achieved in the J774 macrophage cell line and in BMDMs by transduction with a retroviral vector containing mouse *Acot7* cDNA. In J774 macrophages *Acot7* mRNA was ~20-fold overexpressed after transduction with the *Acot7* retroviral vector compared to empty vector (pBM) control (Fig. 3.2a). ACOT7 was overexpressed in BMDMs using the same retroviral vector as compared to a vector delivering EGFP as a control. *Acot7* mRNA was 50-fold overexpressed in these BMDMs compared to EGFP controls (Fig. 3.2b), leading to a large increase in ACOT7 protein expression (Fig 3.2c). The thioesterase activity assay was used to determine if ACOT7 overexpression altered hydrolysis of acyl-CoAs. ACOT7 is known to preferentially cleave long chain acyl-CoAs, so we chose to investigate palmitoyl-CoA (16:0-CoA), oleoyl-CoA (18:1-CoA) and 20:4-CoA. In macrophages overexpressing ACOT7, thioesterase activity toward 16:0-CoA and 18:1-CoA was elevated (1.706±0.059 vs. 9.09±0.39 and 1.35±0.063 vs. 8.49±0.27 nmoles/μg/min, respectively) with the greatest change in activity toward 20:4-CoA (0.948±0.065 vs. 10.85±0.61 nmoles/μg/min) (Fig. 3.2d-f). These data support the notion that, of these, 20:4-CoA is the

preferred substrate of ACOT7 in macrophages (114). Next, the levels of corresponding acyl-CoAs were quantified by LC/ESI-MS/MS. ACOT7 overexpression did not significantly alter the pools of 16:0-CoA, 18:1-CoA or 20:4-CoA in BMDMs (Fig. 3.2g-i). LPS stimulation caused a small significant ($p<0.05$ by unpaired two-tailed Student's t-test) increase in 20:4-CoA levels (Fig. 3.2i), consistent with previous studies (77). Interestingly, even a large increase in ACOT7 activity is not sufficient to reduce overall acyl-CoA levels, suggesting that ACOT7 acts on a specific pool of acyl-CoAs that is rapidly replenished.

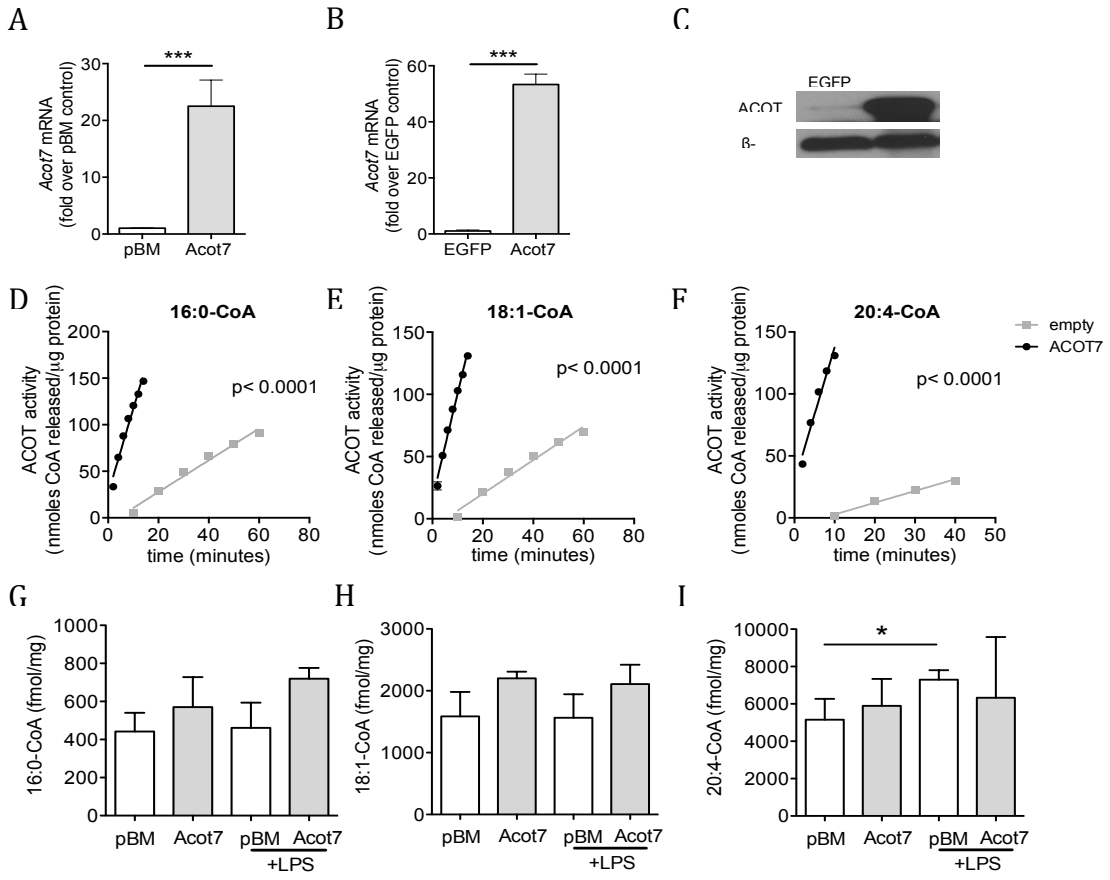

Figure 3.2. ACOT7 overexpression in macrophages results in a marked increase in ACOT activity primarily towards 20:4-CoA. ACOT7 was overexpressed in J774 macrophages transduced with an Acot7 retrovirus, compared to empty pBM vector controls (A). Overexpression of ACOT7 was also accomplished in BMDMs, which resulted in increased *Acot7* mRNA level (B), ACOT7 protein (C), and increased thioesterase activity toward 16:0-CoA (D), 18:1-CoA (E) and 20:4-CoA (F). Acyl-CoA molecular species were measured by mass spectrometry (G-I). Results are expressed as mean ± SEM (n=3) Statistical analysis was performed by ANOVA (D-F) or two-tailed unpaired Student's t-test (A-B); ***$p<0.001$.

Loss of ACOT7 reduces 20:4-CoA thioesterase activity - We next investigated whether loss of ACOT7 in macrophages is sufficient to lead to an overall reduction in 20:4-CoA hydrolysis. Loss of ACOT7 was achieved by harvesting bone marrow from whole-body *Acot7*$^{-/-}$ mice and differentiating these cells into BMDMs, or by transfecting thioglycollate-elicited mouse peritoneal macrophages with *Acot7* siRNA (Fig. 3.3a-d). Both the BMDMs from *Acot7*$^{-/-}$ mice and peritoneal macrophages treated with *Acot7* siRNA exhibited a marked reduction in *Acot7* mRNA (Fig. 3.3a-b). ACOT7 protein was also markedly reduced in both cell populations (Fig. 3.3c-d). There was no compensatory upregulation of other ACOTs expressed in macrophages, including *Acot8* and *Acot9* (Fig. 3.3e-f). However, *Acot9* was significantly induced by LPS, just like *Acot7* (Fig. 3.3f). Furthermore, there was no compensatory regulation of ACSL1 or ACSL4 in ACOT7-deficient macrophages (Fig. 3.3g-h). Consistent with the results on ACOT7-overexpressing macrophages, ACOT7-deficient macrophages exhibited significantly less activity toward 20:4-CoA (1.22±0.075 vs. 0.963±0.046 nmoles/μg/min) as compared to wildtype controls, while activity toward 16:0-CoA and 18:1-CoA was similar to wildtype cells (1.949±0.066 vs. 1.901±0.071 and 1.458±0.074 vs. 1.30±0.076 nmoles/μg/min respectively) (Fig. 3.3i-k). These data demonstrate that ACOT7 preferentially hydrolyzes 20:4-CoA and moreover, that other ACOTs cannot fully compensate for the specific function of ACOT7. However, because of the relatively small decrease in 20:4-CoA hydrolysis in ACOT7-deficient macrophages, other enzymes with ACOT activity must be responsible for a larger part of 20:4-CoA hydrolysis.

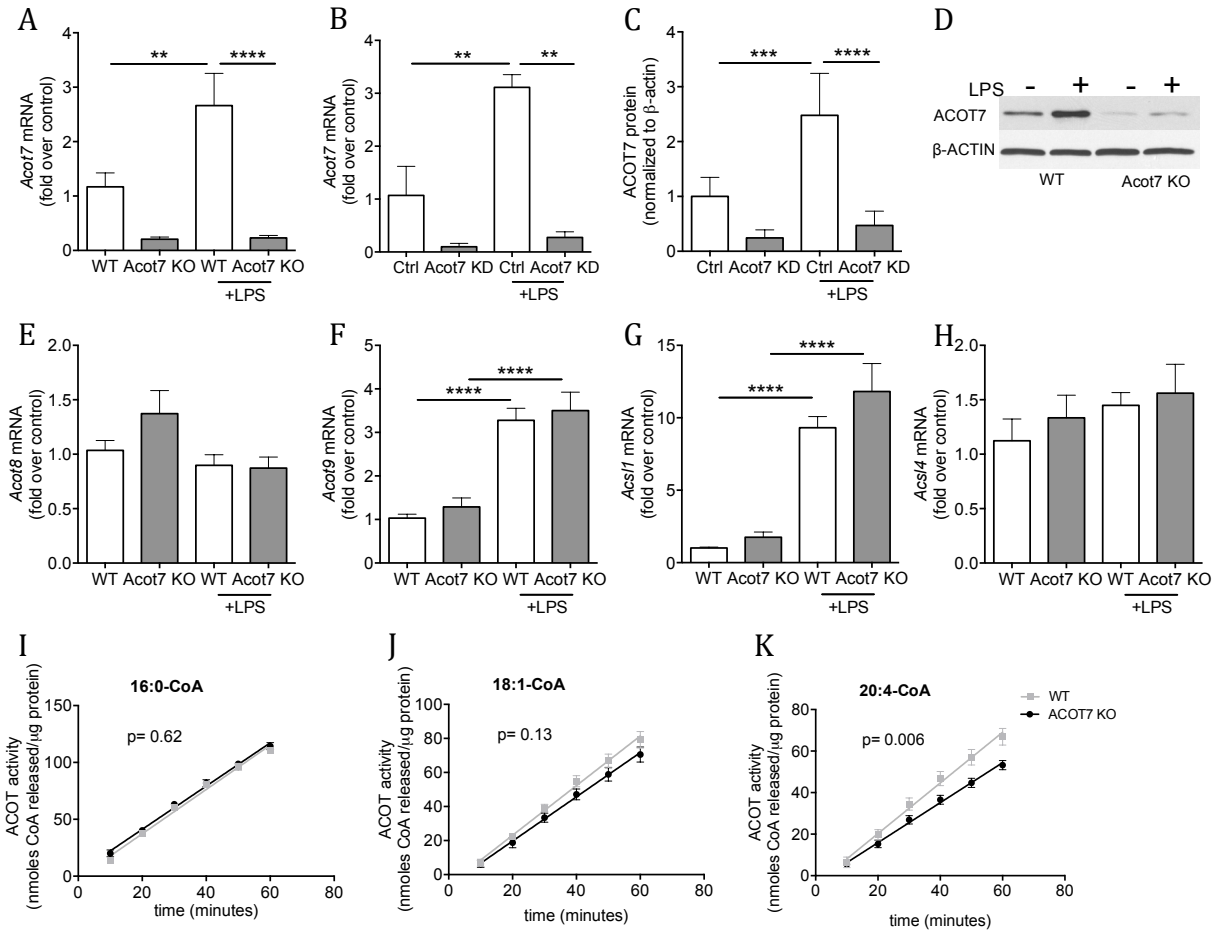

Figure 3.3. ACOT7 knockout and knockdown in macrophages results in reduced ACOT activity toward 20:4-CoA. *Acot7* mRNA levels (A) and ACOT7 protein levels (D) in BMDMs from ACOT7-deficient mice were measured by real-time PCR and Western blot analysis, respectively. *Acot7* mRNA and ACOT7 protein was also markedly knocked down in thioglycollate-elicited macrophages transfected with *Acot7* siRNA as compared to macrophages transfected with a control siRNA (B-C). BMDMs from *Acot7*$^{-/-}$ mice did not exhibit compensatory changes in *Acot8*, *Acot9*, *Acsl1* or *Acsl4* (E-H). Thioesterase activity toward 16:0-CoA, 18:1-CoA and 20:4-CoA was measured in macrophage cell lysates (I-K). Results are expressed as mean ± SEM (n=7-8) Statistical analysis was performed by ANOVA; **p<0.01; ***p<0.001; ****p<0.0001.

ACOT7 overexpression promotes LPS-induced cytokine production and inhibition of PPAR-γ target genes, while loss of ACOT7 has a minor anti-inflammatory effect- We next investigated if overexpression or knockdown of ACOT7 could alter the overall inflammatory status of macrophages stimulated with LPS. First, overexpression of ACOT7 was achieved in J774 macrophages. Increased secretion of IL-1β, TNF-α and IL-6 was observed in LPS-

stimulated J774 macrophages overexpressing ACOT7 whereas CCL2 secretion was not changed (Fig. 3.4a-d). PGE_2 was not affected by ACOT7 overexpression, either under basal conditions or after LPS stimulation (Fig. 3.4e). Overexpression of ACOT7 in BMDMs had similar effects, with increased *Il6* mRNA (Fig. 3.4f).

Other proteins involved in intracellular fatty acid handling, such as FABP4, FABP5 and ACOT11, have been shown to modulate activation of PPAR-γ (39,81,101,111). Consistently, overexpression of ACOT7 reduced mRNA levels of the PPAR-γ target genes *Cd36* and *Cpt1a*, especially in LPS-stimulated cells (Fig. 3.4g-h). ACOT7 overexpression did not significantly reduce mRNA levels of *Ucp2* (Fig. 3.4i), a protein recently implicated in endoplasmatic reticulum stress and the inflammatory effects of FABP4 (81).

Because ACOT7 has been described to inhibit prostaglandin production (114) and to further investigate a potential role of eicosanoids in ACOT7-associated inflammatory activation, the cyclooxygenase 2 and 5-lipoxygenase inhibitors CAY 10404 and CJ-13,610 were applied to LPS stimulated cells. Both inhibitors reduced secretion of IL-6 (Fig. 3.4j), and produced the same percent inhibition in both ACOT7 overexpressing cells and control cells, suggesting that the proinflammatory effects of ACOT7 overexpression were not mediated through either cyclooxygenase 2- or 5-lipoxygenase products.

Knockdown of ACOT7 in thioglycollate-elicited macrophages by transfection with *Acot7* siRNA resulted in a small but significant reduction in LPS-induced *Il6* mRNA, but no significant inhibition of *Tnfa* mRNA (Fig. 3.4k-l). The slight reduction in *Il6* mRNA did not translate into reduced secretion of IL-6 or TNF-α, as measured 18-24 h after LPS stimulation (Fig. 3.4m-n). Similar to what was observed with ACOT7 overexpression, ACOT7 knockdown had no effect on *Ccl2* mRNA expression or PGE_2 release (Fig. 3.4o-p).

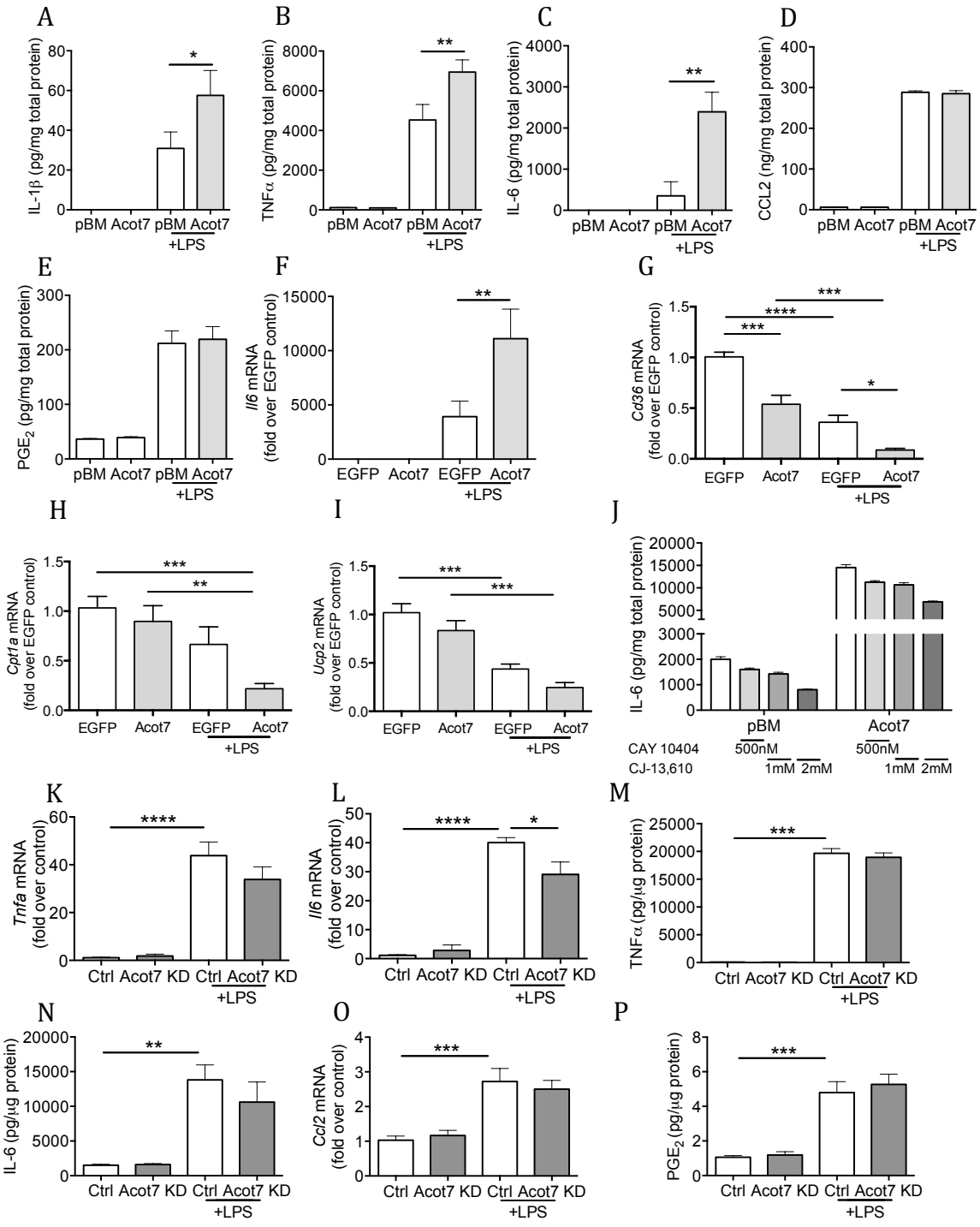

Figure 3.4. ACOT7 promotes cytokine production and suppresses expression of PPAR-γ target genes in macrophages. J774 macrophages overexpressing ACOT7 and control cells were stimulated with LPS or were left unstimulated for 24 h. Secretion of IL-1β (A), TNF-α (B), IL-6 (C), CCL2 (D) and PGE_2 (E) was measured by ELISAs. BMDMs overexpressing ACOT7 or EGFP were stimulated with LPS or were left unstimulated for 24 h, and *Il6, Cd36, Cpt1a* and *Ucp2* mRNA levels were measured by real-time PCR (F-I). Inhibition of COX2 (by 500 nM CAY 10404) or 5-LO (by 1 and 2 μM CJ-13,610) reduced IL-6 secretion in LPS stimulated macrophages (J), however the percent change was no different

in cells infected with the *Acot7* vector over those receiving the empty pBM vector. BMDMs in which ACOT7 had been knocked down by siRNA (and BMDMs treated with an siRNA control) were used to evaluate the effect of ACOT7-deficiency on cytokine production and PGE_2 secretion. The BMDMs were stimulated with 10 ng/mL LPS or left unstimulated. *Tnfa* (K), *Il6* (L) and *Ccl2* (O) mRNA levels were measured by real-time PCR. Secretion of TNF-α (M), IL-6 (N) and PGE_2 (P) was measured by ELISA. Results are expressed as mean ± SEM (n=3-6) Statistical analysis was performed by ANOVA; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

ACOT7 is modestly increased in macrophages from diabetic mice but ACOT7 and diabetes act through different mechanisms to promote inflammation - TLR4 stimulation is thought to contribute to the pro-inflammatory phenotype of macrophages in diabetes (70,72). We therefore investigated whether ACOT7 is upregulated in macrophages in a mouse model of diabetes and if ACOT7 could modulate inflammation and atherosclerosis *in vivo*. Thioglycollate-elicited macrophages from diabetic mice expressed modestly more *Acot7* mRNA and ACOT7 protein than did their non-diabetic littermates (Fig. 3.5a-b). To test the effect of ACOT7-deficiency in bone marrow-derived cells in diabetic mice, bone marrow from *Acot7*$^{-/-}$ mice or age-matched wildtype control mice was transplanted into irradiated *Ldlr*$^{-/-}$ recipient mice. Following recovery, a subset of mice received STZ to induce diabetes and the mice were then fed a semi-purified low fat diet for the duration of the 12-week study (Fig. 3.5c). A complete knockout of macrophage ACOT7 protein was achieved and sustained for the duration of the experiment in mice transplanted with bone marrow from *Acot7*$^{-/-}$ mice (Fig. 3.5c). Diabetic mice exhibited increased plasma triglyceride levels, blood glucose, and decreased body weight, but had no changes in plasma non-esterified fatty acids or cholesterol. Importantly, hematopoietic ACOT7-deficiency did not alter any of these parameters (Fig. 3.5d-h). At the end of the study, leukocytes from mice transplanted with *Acot7*$^{-/-}$ bone marrow maintained significant knockdown of *Acot7* mRNA, as compared to those transplanted with wildtype bone marrow (Fig. 3.5i). Levels of *Il1b* and *S100a9* mRNA in leukocytes were used to evaluate low-grade inflammation

associated with diabetes. *S100a9* and *Il1b* were elevated in mice with diabetes (p=0.05 in both cases), but loss of hematopoietic ACOT7 did not alter these markers of inflammation in diabetic mice (Fig. 3.5j-k). However, hematopoietic ACOT7-deficiency resulted in a significant reduction of *Tlr4* mRNA in non-diabetic mice and a trend towards reduced *S100a9* mRNA levels in non-diabetic mice (Fig. 3.5j,,m). Another ACOT induced by LPS in macrophages, *Acot9*, was not significantly regulated by ACOT7-deficiency or diabetes (Fig. 3.5l). Most mice developed early macrophage-rich atherosclerotic lesions after 12 weeks of diabetes, but hematopoietic ACOT7-deficiency did not alter the effect of diabetes on atherosclerosis (Fig. 3.5n). Non-diabetic mice had no lesions or very small lesions at this time-point (Fig. 3.5n).

Together these results suggest that whereas ACOT7 promotes expression of some inflammatory mediators under non-diabetic conditions, ACOT7 does not mediate diabetes-accelerated atherosclerosis or inflammation associated with diabetes.

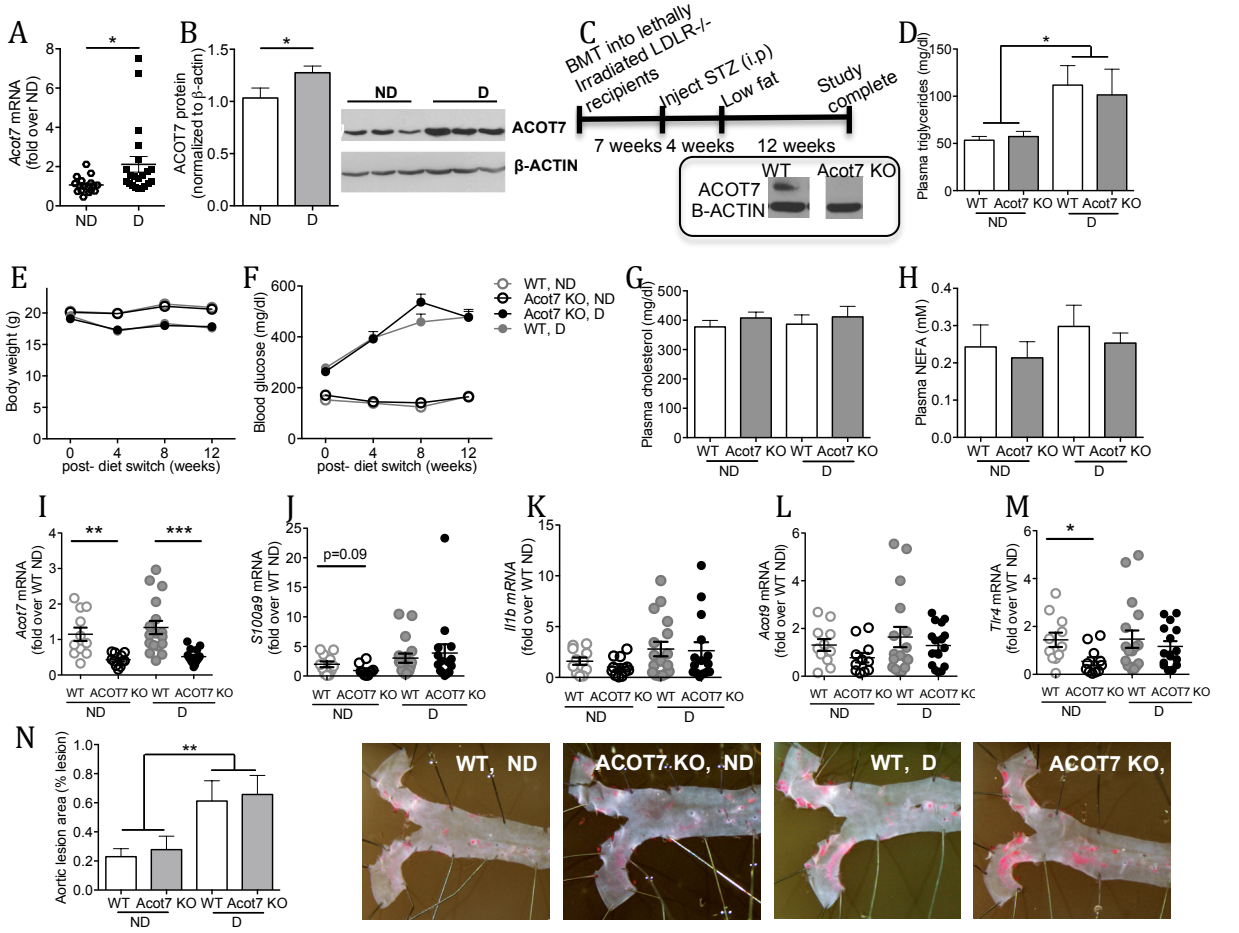

Figure 3.5. Loss of ACOT7 in bone marrow-derived cells results in reduction of some inflammatory mediators, but is not sufficient to prevent inflammation or atherosclerosis stimulated by diabetes. *Acot7* mRNA (A) and ACOT7 protein (B) were measured in thioglycollate-elicited macrophages from diabetic and non-diabetic *Ldlr*$^{-/-}$ mice by real-time PCR and Western blot analysis, respectively. Lethally irradiated female *Ldlr*$^{-/-}$ mice received bone marrow transplants (BMT) from *Acot7*$^{-/-}$ mice or wildtype controls, were allowed to recover for 7 weeks, and were then injected with streptozotocin (STZ) to induce diabetes. Some mice were injected with citrate and were used as non-diabetic controls. All mice were then fed a low-fat semipurified diet for 12 weeks (C). Isolated BMDMs harvested at the end of the study from mice that received *Acot7*$^{-/-}$ bone marrow had no detectable ACOT7 protein, showing a near complete chimerism (C). The bands shown are from the same blot and exposure times. Plasma triglycerides were measured by a kit from Sigma at the end of the study (D). Body weights (E) and blood glucose (F) were measured every 4 weeks. Plasma cholesterol and NEFA were measured at the end of the study by a cholesterol E assay and a NEFA assay from Wako, respectively (G-H). At the end of the study, blood leukocytes were used for real-time PCR measurements of *Acot7* (I), *S100a9* (J), *Il1b* (K), *Tnfa* (L) and *Tlr4* (M). Atherosclerosis was evaluated by Sudan IV staining of the aorta using *en face* preparations, and expressed as percent lesion area of total aortic area (N). Representative en face preparations of the aortic arch are shown. Results are expressed as mean ± SEM (n=10 non-diabetic, n= 15-17 diabetic) Statistical analysis was performed by Student's t-test (A) or ANOVA; *p<0.05; **p<0.01; ***p<0.001. D, diabetic mice; ND, non-diabetic mice

Discussion

Modulation of the immune response by fatty acids and enzymes that control their metabolism and subcellular localization within the cell is an area of research that has attracted significant attention in recent years. There are now several examples of proteins involved in intracellular fatty acid handling that are induced by inflammatory stimuli in macrophages and modulate inflammatory phenotypes of these cells (37,39,77,81,101). Interestingly, these fatty acid-handling proteins might act primarily to restrict levels of free unsaturated fatty acids in the cell, perhaps controlling the availability of PPAR ligands, as has been shown for FABP4, FABP5 and ACOT11 (39,81,101,111,117). The present study adds ACOT7 to this group of proteins. Thus, ACOT7 is induced by LPS in macrophages and inhibits PPAR-γ target gene expression. Our study is the first to investigate the effect of ACOT7-deficiency in macrophages. The results demonstrate that loss of ACOT7 in mouse macrophages is sufficient to reduce overall thioesterase activity toward 20:4-CoA, without reducing thioesterase activity toward 16:0-CoA or 18:1-CoA, and that ACOT7 overexpression greatly increased thioesterase activity toward 20:4-CoA and had a less dramatic effect on 16:0-CoA and 18:1-CoA. This demonstrates that ACOT7 acts on 20:4-CoA in macrophages and that other enzymes with ACOT activity are not able to completely compensate for loss of ACOT7. Given the differential subcellular localization of ACOTs, ACOT7 may have a greater regulatory role of thioesterase activity in certain acyl-CoA pools *in vivo*. ACOT7 has been found to exhibit primarily a cytoplasmic localization in macrophages ((106) and our unpublished data) and in other tissues (112,118).

Increased activity of ACOT7 has been shown previously to reduce basal levels of PGD_2 and PGE_2 in a macrophage cell line (114). Perturbations that affect fatty acid incorporation into phospholipids can have a profound effect on increasing eicosanoid synthesis (76). It has been hypothesized that the balance of 20:4-CoA/free 20:4 can be influenced by long-chain acyl-CoA hydrolases (119), such as ACOT7. However, our results clearly demonstrate that a large increase in overall 20:4-CoA thioesterase activity in macrophages overexpressing ACOT7 is not sufficient to reduce the steady-state pool of 20:4-CoA, suggesting that 20:4-CoA turnover is rapid, consistent with studies on other ACOTs. Accordingly, neither ACOT7 overexpression nor knockdown affected the ability of macrophages to produce PGE_2. These findings mimic other work in neurons lacking ACOT7. In this tissue, a near complete loss of thioesterase activity did not alter levels of acyl-CoAs, PGE_2 or PGD_2 (107). Our study suggests that rather than affecting eicosanoid production, ACOT7 increases inflammatory activation of macrophages by suppressing PPAR-γ. Indeed, ACOT7-deficiency in neurons results in elevated levels of several unsaturated fatty acids, including arachidonic acid (107), which are likely endogenous PPAR-γ ligands (120). PPAR-γ activation is known to suppress inflammatory signaling, including that induced by TLR4 (121-123). Similar findings have been reported in ACOT11-deficient mice, which exhibit reduced cytokine levels and increased PPAR-γ following fat-feeding (111). We therefore propose that ACOT7 induction is an important part of TLR4 signaling, which promotes the inflammatory response by suppressing endogenous PPAR-γ ligands and PPAR-γ activity.

In neurons, where ACOT7 levels are high, expression of ACOT7 is positively regulated by binding of the transcription factor SREBP-2 to the sterol regulatory element

(SRE2) of the *Acot7* promoter region (124). SREBP-2 is increased in response to low cellular cholesterol levels and induces genes involved in triglyceride and cholesterol synthesis. In rat liver cells, ACOT7 is increased after treatment with a PPAR-α agonist (125). Interestingly, in macrophages *Acot7* transcription and translation is increased following exposure to LPS, therefore ACOT7 expression appears to be regulated by alterative pathways in macrophages, compared to these other tissues. We determined that MyD88 and not TRIF was the adapter protein required for TLR4-mediated ACOT7 induction in these cells. MyD88 signaling activates the transcription factors AP-1 and NF-κB (126). Thus, *Acot7* mRNA transcription in response to LPS in macrophages is likely downstream of one or more of these transcription factors. Similarly, ACSL1 is induced by PPARs in insulin target tissues, but not in macrophages, in which ACSL1 is induced by LPS and other inflammatory mediators (77). This suggests that ACOT7 in macrophages is involved in the inflammatory response and might not have a strong role in neutral lipid homeostasis.

TLR4 is upregulated in monocytes from humans with type 1 diabetes (70,127) and in a mouse model of the disease (72). Given that ACOT7 is induced by TLR4 activation, we hypothesized that expression of ACOT7 in macrophages would be altered in an STZ mouse model of diabetes and that it might influence the increased inflammatory response and atherosclerosis observed in diabetic mice. ACOT7 was indeed upregulated in macrophages from diabetic mice, although the extent of increase was modest. The increase in ACOT7 expression in the diabetic state may be specific to macrophages or myeloid cells, as diabetes did not significantly increase ACOT7 expression in the whole leukocyte population. Moreover, hematopoietic ACOT7-

deficiency reduced levels of *Tlr4* in blood leukocytes, but the effect only reached statistical significance in non-diabetic mice. Hematopoietic ACOT7-deficiency did not inhibit diabetes-accelerated atherosclerosis. Thus, although ACOT7 has pro-inflammatory effects after LPS stimulation, it does not explain the increased inflammation and atherosclerosis associated with diabetes. Though our work describes the mechanism of *Acot7* mRNA induction in response to LPS, it is likely that *Acot7* transcription in macrophages is induced by additional stimuli *in vivo* and could utilize alternate signaling molecules downstream of these stimuli. Future studies will reveal whether ACOT7 plays a role in other immune cells and other inflammatory conditions, such as sepsis or various types of bacterial infection.

In summary, ACOT7 is induced by TLR4-MyD88 activation and diabetes in macrophages, acting primarily on arachidonoyl-CoA in these cells. ACOT7 promotes an inflammatory phenotype linked to the TLR4 pathway and PPAR-γ inhibition. This study adds ACOT7 to the group of fatty acid handling proteins that promotes inflammatory activation and suppresses PPAR-γ activation in innate immunity.

ACKNOWLEDGMENTS

This study was supported by NIH grants HL062887, HL092969 (KEB); DK082841 (SP); NS072241 (MJW). Part of the study was supported by the Diabetes Research Center at the University of Washington, DK017047. Acyl-CoAs were analyzed through the Molecular Phenotyping Core, Michigan Nutrition and Obesity Center (P30 DK089503). VZW was supported by the Samuel and Althea Stroum Fellowship in Diabetes Research.

DISCLOSURES

None

Chapter 4-

Study Limitations and Conclusions

Lessons from glucose effects in SMCs

In my research, I investigated the effects of increased glucose flux in SMCs in a model of atherosclerosis *in vivo*. My findings indicate that the causative role of increased glucose flux in SMCs in advancing atherosclerosis may require a first hit of certain factors present in metabolic syndrome. Timing is also a key determinant for when potential interventions are most effective as demonstrated by "metabolic memory". SMC-type specific overexpression of GLUT1 in a model of atherosclerosis had not been tested before. My research has demonstrated that increased glucose flux in SMCs leads to the advancement of lesions in mice with a metabolic syndrome-like phenotype.

The insulin-independent glucose transporter, GLUT1, is the primary glucose transporter for SMCs, macrophages and endothelial cells. Its expression levels in various cell types governs the preferred energy source for the cell's metabolic activity, with glycolysis favored when GLUT1 is highly expressed (43,128,129). Increased glycolysis allows for anaerobic production of ATP and can be necessary to the cell for survival in times of hypoxia or when a boost of glycolytic activity is necessary, such as responding to inflammatory cytokines. This response is beneficial to the organism in some cases. GLUT1 in heart muscle is increased in a model of pressure-overload hypertrophy (130) and GLUT1 overexpression in another model of pressure overload-induced hypertrophy by transverse aortic constriction preserved mitochondrial function in the heart (129). Conversely, GLUT1 can also be elevated in pathological conditions such as cancer cells,

allowing them continue to proliferate in the face of hypoxia and give them a growth advantage even in the absence of hypoxic conditions (131). Hypoxia is also encountered in atherosclerotic lesions (132). Both insufficient diffusion of oxygen through large plaques and increased metabolic demand by lesion macrophages are thought to contribute to plaque hypoxia (132). GLUT1 has been found to upregulated in atherosclerotic lesions and co-localize with hypoxic areas (65). GLUT1 is also upregulated by cytokines, such as IL-1b (133).

Though human studies cannot directly assess the contribution of GLUT1 to vascular pathologies, multiple studies conducted over the past few decades have been designed to determine the contribution of high glucose in patients with diabetes. There are some discrepancies in these studies concerning whether aggressive glucose lowering will reduce the risk of cardiovascular disease, which may be explained by the timing of these interventions. The DCCT/EDIT and UKDPS trials were conducted in people who were recently diagnosed with T1DM and T2DM respectively, whereas the Action to Control Cardiovascular Risk in Diabetes (ACCORD), Cardiovascular Risk Evaluation in people with type 2 Diabetes on Insulin Therapy study (CREDIT) and Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) studies were in patients with T2DM that had been established for several years and many had pre-existing cardiovascular disease (46,47,134). Early glucose control may prevent long-term cellular alterations that would continue to promote atherosclerosis long after glucose has been normalized. In support, a correlation between microvascular/macrovascular outcomes and variability of visit-to-visit HbA1c as well as maximal fasting glucose measured during the trial was found in the ADVANCE trial

(135). This may be indicative of long-term changes associated with spikes in glucose, which may have gone unnoticed when taking an average measure of glucose using HbA1c in the original trial. An alternative interpretation of the data from human trials could also simply be that high glucose (when present) aids the development of early lesions, but does not have a strong role in the progression of more advanced lesions. Therefore poor glycemic control early on, may significantly increase lesion size. Increased lesion size early in the study would go unnoticed, since the end-point criteria were microvascular and macrovascular complications that likely would not yet be apparent. In this situation, after glucose control is equalized between groups atherosclerosis may continue to grow at a similar rate, but the group with poor glycemic control early on may already be further along in the disease process. Though upregulation of GLUT1 in SMCs is not identical to changes associated with increased blood glucose, these studies do suggest that glucose may have more influence in remodeling of the artery wall early in atherosclerosis.

My research suggests that increased glucose flux in SMCs promotes early changes in the artery wall. The preceding study used a comparatively short time point (16 weeks of a metabolic syndrome-like phenotype) to study GLUT1 overexpression in SMCs. When metabolic syndrome is present, SM-GLUT1 mice had more advanced lesions than their WT counterparts. I have also completed an identical study in which DDC fed, *Ldlr*$^{-/-}$ mice have GLUT1 overexpressed in myeloid cells (M-GLUT1). Unlike the SM-GLUT1 mice, M-GLUT1 mice had no change in lesion size at the 16 week time point. If metabolic memory and/or previously established cardiovascular disease is to blame for the lack of glucose-lowering effects in the CREDIT, ADVANCE and

ACCORD human trials, my findings suggest that the glucose lowering benefits observed in the DCCT/EDIT and UKDPS studies may be due to reduced pathological effects in SMCs. An additional study supportive of this finding, reports long-lasting changes in gene expression in the aortic arch (largely composed of SMCs) in response to high glucose, which were retained long after normalization of blood glucose (49). These data suggest that more focus on the role of glucose in CVD should be directed toward SMCs.

The mechanism in which GLUT1 overexpression in SMCs drives atherosclerosis is likely not through proliferation of SMCs, but rather secreted factors. My work demonstrates that GLUT1 overexpression did not alter proliferation (Fig. 2.5), but could increase aortic *Has1* mRNA (Fig. 2.3m) and HA deposition in the medial BCA of mice with metabolic syndrome (Fig. 2.4i-k) and HA secretion in isolated SMCs (Fig 2.4m). These findings further highlight the dynamic nature of SMCs in atherosclerotic lesions. SMCs are known to alter their phenotype to become more proliferative and migratory both which contribute to the mass of the advancing lesion. Additionally, and less studied, they interact with ECs and immune cells by either cell signaling or deposition of ECM components. In the study of GLUT1 overexpression in SMCs, these cells did not appreciably alter gene expression of cyclin D1 mRNA in response to *in vitro* GLUT1 overexpression or in atherosclerotic aortas *in vivo*. However, proliferating SMCs (as measured by Ki67), where found in larger lesions and likely contributed to the overall size and morphology of these lesions (Fig. 4.1). To be sure that proliferation was not related to SMC-type specific GLUT1 overexpression, I also normalized SMC Ki67 positive staining to lesion SMC area. There was no observable trend with this measurement (data not shown). SMC proliferation correlated well with lesion size, but

was not enhanced by GLUT1 overexpression. This suggests the likelihood that GLUT1 overexpression in SMCs is driving the increase in lesion size by means other than proliferation.

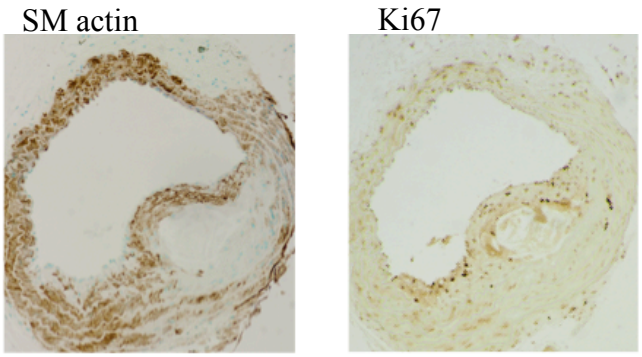

Figure 4.1. Ki67 positive lesion SMCs can be found in atherosclerotic lesions

Increases in Hyaluronan (HA) secretion by GLUT1 overexpression is a strong candidate for the observed phenotype. HA is a non-sulfated glycosaminoglycan which can be found early in atherosclerotic lesions. My study identified increased hyaluronan synthase 1 (*Has1*) mRNA expression could be a possible means of which GLUT1 drives atherosclerosis. Greater availability of its glucose-derived substrate molecules and increased expression of one of its synthases, may cause more HA deposition in SM-GLUT1 mice. Increased HA was observed in the medial region of BCA cross-sections in SM-GLUT1 mice and was still significantly greater than WT controls when normalized to total SMC area. Though HA deposition is known to enhance SMC proliferation and migration (93) neither of these phenotypes were observed in the preceding study. These phenotypes may be limited to a small subset of lesion SMCs and could have been missed with the methods used in this study. Another possibility for the effects of GLUT1 being mediated through HA deposition is activation of inflammatory mediators. Smaller molecular weight HA fragments can activate immune cells through the NFκB pathway

(92). Though macrophage content was not increased in SM-GLUT1 lesions, mRNA levels of CD11b (a marker of myeloid cells) and the cholesterol core, thought to be derived (in part) from dying lesion macrophages (12) contributed greatly to lesion size.

SMCs can directly signal to other cell types through secretion of chemokines that may affect the disease process. Adhikari et al. (62) observed increased selective increase in the macrophage chemo-attractant protein, CCL2, in SM-GLUT1 mice in response to vascular injury. The DDC diet used in our study also increased *Ccl2* mRNA in the aortas of animals. This likely aided the recruitment of monocytes to these lesions, compared to the chow-fed mice. Given the mRNA measured is from total aortic extracts, it is not possible to quantify how much of these gene expression changes are occurring in SMCs and how much they are in lesion macrophages. Diet-induced macrophage recruitment, followed by HA-mediated enhanced activation of recruited macrophages is a plausible mechanism by which GLUT1 overexpression accelerates atherosclerosis, specifically in mice with metabolic syndrome.

Limitations encountered and future directions to assess the role of increased glucose flux in SMCs

I have established that GLUT1 overexpression in SMCs contributes to lesion size and morphology in atherosclerosis associated with a metabolic syndrome phenotype. Clarification on the mechanism by which this occurs will be the focus of future experiments. Testing whether increased glucose uptake in SMCs has a preferential role in lesion initiation vs. progression would help to clarify when interventions in arterial metabolism should be the primary focus of treatment for people with metabolic

syndrome. Further assessment of lesion HA and Has enzymes is needed to decide if HA is a good target for inhibiting atherosclerosis initiation in those with elevated risk for CVD. If HA is not the mechanism by which GLUT1 overexpression exerts its effects, then other potential mechanisms must be considered. Finally, there are also a few notable pitfalls with the models used in these studies; a couple of which could be corrected in future studies by generation of a new mouse model. Laser capture micro-dissection would be a useful tool to investigate differential gene expression in microclimates of atherosclerotic lesions.

I have shown that GLUT1 overexpression in SMCs can increase lesion size in the BCA and aortas of mice with features of metabolic syndrome at the early timepoint of 16 weeks. However, these effects of GLUT1 overexpression are not as apparent in the sinus (a region in which advanced lesions occur by 16 weeks of DDC feeding). I suggest here that this is because GLUT1 overexpression aids in lesion initiation, thus its effects are best observed early on. To isolate the effect of increased glucose uptake in SMCs early in lesion initiation a mouse model of transient GLUT1 overexpression in SMCs would be useful. The reverse tet-controlled transcriptional activator (rtTA) system also known as "tet-on" (described in detail here: (136)) would be an excellent tool to achieve temporal control of GLUT1. This would allow increased GLUT1 transcription in the presence of doxycycline (Dox) (Fig. 4.2). A common disadvantage of the "tet-on" system is leaky expression of the target genes, even in the absence of Dox. This can be of huge consequence if the target gene is toxic. This system is ideal, however, for GLUT1 overexpression in different vascular cell types such as macrophages, endothelial cells and vascular smooth muscle cells since GLUT1 is endogenously expressed in these cell types

and slight leakiness would be of little consequence. Using this expression system, temporal overexpression of GLUT1 could be achieved in any cell-type in which a Cre-line exists. A mouse in which the rtTA is preceded by a flox-stop sequence and expressed under the ubiquitous Rosa26 promoter is available from Jackson labs (ROSA26-rtTA-IRES-EGFP). This mouse also has the added benefit of EGFP expression after successful Cre-mediated recombination. Cell-type specificity could be gained through breeding with a Cre-line for that cell type. For example crossing this mouse with a transgenic mouse line with Cre expression driven by the SM22α promoter (Sm22αCre: available from The Jackson Laboratory) would limit rtTA expression to smooth muscle cells. The final step would be to breed these to a mouse in which GLUT1 is expressed under the tetracycline response element promoter (TRE-GLUT1). These mice have recently been generated (129). The addition of Dox to the drinking water in animals that contained all of these elements would lead to binding of rtTA to TRE reversibly turning on transcription of GLUT1 in SMCs. Mice lacking the TRE-GLUT1 allele given Dox and mice expressing the TRE-GLUT1 transgene with Dox omitted would serve as acceptable controls. Using this system it would be possible to increase GLUT1 expression specifically in SMCs during lesion initiation followed by removal of GLUT1 overexpression during lesion progression. SMC-specific GLUT1 overexpression could also be delayed until after lesion initiation. A potential drawback of this breeding scheme is use of the SM22aCre mouse, which has reported expression of Cre in myeloid cells as well as SMCs (137). If necessary, this could be overcome by bone-marrow transplantation of WT or $LDLR^{-/-}$ cells.

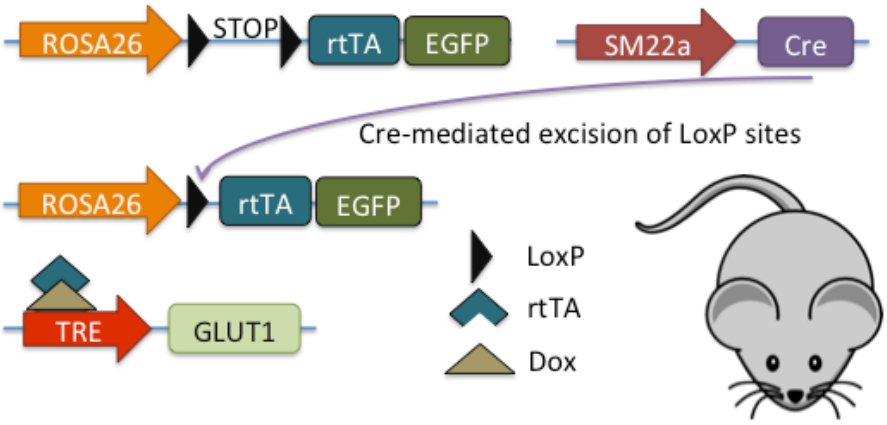

Figure 4.2. Generation of inducible GLUT1 expression in SMCs. A floxed-stop sequence prevents ubiquitous rtTA expression (a). Cre expression is driven by the SM22a promoter, excising the stop sequence and allowing rtTA expression in SMCs (b). In the presence of Dox, rtTA can bind TRE and reversible transcription of GLUT1 will occur (c).

Another possible explanation for why GLUT1 overexpression did not confer a change in phenotype of sinus lesions in DDC fed mice is that our study may be hindered by the choice of the SM22α promoter for GLUT1 overexpression. Though the transgenic mouse with GLUT1 overexpressed under the SM22α promoter was an excellent starting point that showed the promise of glucose effects in SMCs, it may not be the best model in which to study atherosclerosis. The activity of the SM22α promoter varies in atherosclerotic lesions and may compromise GLUT1 overexpression as the lesion progresses. Deletion of SM22α promotes a more proliferative SMC phenotype and increased atherosclerotic lesion area in ApoE deficient mice (138) and in human atherosclerotic lesions SM22α expression is downregulated in intimal SMCs, possibly reflecting a more de-differentiated, less contractile phenotype of these lesion SMCs (139). Though our studies use only a fragment the SM22α promoter for transgenic expression of GLUT1, it may still have similar regulatory elements. This caveat could mean that the same changes that GLUT1 overexpression in SMCs is causing may lead to

the very loss of GLUT1 overexpression as the cells change their phenotype. In this case, GLUT1 overexpression may be lost relatively early in lesion SMCs and these key cells may be relatively similar between SM-GLUT1 and WT mice as soon as lesions progress beyond fatty streaks. The mouse model proposed earlier will also correct for this potential pitfall. In this model, GLUT1 is no longer expressed directly under the SM22α promoter. GLUT1 expression is instead driven by the TRE promoter. This promoter is only active in cells which express rtTA (and concurrent Dox treatment). For the proposed model, all cells that were ever once a contractile SMC and expressed SM22α at any point in time would also induce Cre expression leading to the irreversible recombination of loxP sites and continuous expression of rtTA under the Rosa26 promoter. Since expression of rtTA is irreversible in SMCs, these SMCs would continue to overexpress GLUT1 even after a changing from a contractile to synthetic state. Though we cannot be sure if decreased expression of our GLUT1 transgene throughout lesion development is a concern, repeating these experiments in a second model of SM-GLUT1 overexpression would certainly validate our findings.

Changes in gene expression in whole aortas are useful indicators of potential mechanistic pathways to pursue further. A limitation of this method is lack of cell-type specificity in which these gene changes are occurring. We noticed a significant increase in *Abca1* and *Ccl2* mRNA expression in the aortas in response to DDC feeding with no difference between genotypes. It is possible that the genotype did not have an effect on these genes. Alternatively, the effect of genotype may have been limited to a small subset of cells, such as lesion SMCs and the overall fold change in gene expression could be diluted by averaging the entire aorta. These two genes were likely candidates for a

functional link between GLUT1 overexpression and increased lesion size with greater cholesterol content. ABCA1 facilitates cholesterol efflux from peripheral cells. *In vitro* studies have identified glucose-dependent expression of ABCA1, in which high glucose downregulates ABCA1 in smooth muscle cells (100). In my work, downregulation of ABCA1 would mean enhanced cholesterol loading of lesion SMCs and may explain the finding of increased cholesterol content in aortas from SM-GLUT1 mice fed DDC. Likewise, upregulation of CCL2 in our model was anticipated given that femoral artery injury in SM-GLUT1 mice (in absence of metabolic syndrome) led to increased CCL2 by immunofluorescence and neutrophil accumulation as compared to controls (62), yet in my aortic extracts, there was no effect of genotype. Finally, upregulation of endogenous *Slc2a1* mRNA was not observed, but again we may have failed to observe this given the relatively small quantity of hypoxic cells in the entire aorta. Laser-capture micro-dissection would be a useful tool to separate effects on gene expression in these separate cell types or between populations of lesion SMCs and medial SMCs. We instead chose to extract lipids from these aortas, which was also a useful parameter in determining the composition of these atherosclerotic lesions. In a future study it would be useful to use laser-capture micro-dissection in the aortas of a separate cohort of animals.

HA overproduction is a likely mechanism by which GLUT1 overexpression increased lesion size. My work is suggestive of this, with the presence of increased HA increased in the SMC rich medial area of BCA sections near max lesion. Given that the lesions are already present it is not possible to determine if HA accumulation is the mechanism for the increased lesion size or if HA is increased because the lesion is more advanced. An earlier timepoint of 4 weeks will be used to determine if HA accumulation

precedes lesion progression. HA will be quantified in mouse aortas (the region most affected by genotype) prior to observable lesions. In a concurrent study to verify that increased HA is derived from SMCs, I will overexpressed GLUT1 in isolated aortic SMCs *in vitro* and measured secreted HA as a collaborative project with the laboratory of Dr. Tom Wight, Benaroya Research Institute, Seattle. To mimic the effect of DDC feeding, a subset of cells were treated with oxidized LDL (oxLDL), a possible candidate for the observed synergism with DDC feeding. Treatment with oxLDL had not effect on HA secretion. It is possible that another factor besides oxLDL provides the synergistic effect, in a future experiment cells will instead be treated with DDC or chow-fed serum.

A potential pitfall is the possibility that HA is simply correlative and not causative for increased lesion size in DDC-fed SM-GLUT1 mice. To address this pitfall, other potential mechanisms will continue to be investigated. Cross-talk between SMCs and immune cells is a possibility. SMCs from SM-GLUT1 may secrete chemokines that attract and trap additional macrophages. To investigate immune cell activation, whole leukocyte preparations were taken for mRNA from a subset of mice at the end of the 16 week DDC feeding study. Markers of activation such as *Il1b, Tnfa* and *Il6* mRNA can be quantified. Though this method suffers from a heterogeneous population of leukocytes of which monocytes are only a small portion, it has proven to be useful in detecting changes in monocyte and neutrophil activation (140). An alternative method could be use of laser capture micro-dissection for lesion macrophage mRNA.

Lastly, expression of GLUT1 is sensitive to hypoxia and may be increased in advanced lesions in mice, even in absence of overexpression. Our model may not just be a tool for altering glucose metabolism in SMCs, but may instead be a model which

exacerbates a maladaptive condition already present in atherosclerotic lesions. Additional work would be necessary to verify existence of increased GLUT1 lesion SMCs in our model of atherosclerosis. Laser capture and immunohistochemistry would be useful tools to investigate this possibility. Use of GLUT1-deficient mice would allow for investigation of the endogenous function of GLUT1 in SMCs. A floxed GLUT1 mouse exists (141) which could be crossed with the SM22aCre for irreversible homozygous deletion of GLUT1 or heterozygous knockdown of GLUT1. If GLUT1 expression in SMCs is required for normal development of atherosclerosis, then KO or knockdown should confer some protection from the disease.

Lessons from fatty acid remodeling in macrophages

When investigating the role of fatty acid modifications as regulators of the degree of macrophage inflammation, the complexity of compensation by alternate enzymes also becomes apparent. The enhanced inflammatory potential of macrophages that accompanies atherosclerosis is multifactorial and reversing just one of the changes that can increase macrophage inflammation is not sufficient to completely block the proinflammatory effects of diabetes known to accelerate atherosclerosis.

There are 15 ACOT enzymes in mice (103). Though each has differential tissue and cellular localization, fatty acyl-CoA preference and regulatory influences, there is considerable overlap. My study demonstrated this redundancy in macrophages. When overexpressing ACOT7 *in vitro* it was possible to achieve a large increase in thioesterase activity toward 16:0-CoA, 18:1-CoA and 20:4-CoA. However, the phenotype with respect to KO of the enzyme was minimal. There was no change in thioesterases activity

toward 16:0-CoA and 18:1-CoA and the change in activity toward the preferred substrate 20:4, though significant, was still small. This is likely why KO of ACOT7 in macrophages had a very subtle phenotype with respect to inflammatory potential and did not alter the course of diabetes-accelerated atherosclerosis. Further, though ACOT7 overexpression led to large increases in thioesterases activity in the fatty acyl-CoA species observed, overexpression had no effect on total amounts of these same fatty acyl-CoAs. Failure to change total fatty acyl-CoA species has been observed in two other ACOT studies. Deletion of ACOT7 in neurons led to near complete loss of thioesterases activity, acyl-CoA species were relatively unchanged (107). Similarly, deletion of ACOT11 (highly expressed in brown adipose tissue) has very minimal effects on acyl-CoA species in this tissue type (111).

Instead of altering the pool of acyl-CoA species, ACOT7 expression appears to modulate FFA levels in an unexpected way. In neurons, loss of ACOT7 increased FFAs through compensation by PLA_2 (107). It is possible that similar compensation can exist in macrophages, reducing the pool of FFA with ACOT7 overexpression. My data is suggestive of this possibility, with decreased PPAR-γ target genes when ACOT7 is overexpressed. This may be a novel mechanism by which ACOTs can influence the inflammatory phenotype of macrophages.

Inflammatory mediators are increased in the diabetic state and immune activation contributes to vascular disease under these circumstances. However, the mechanism in which diabetes promotes inflammation is still largely debated. There is likely no single factor that can account for the inflammatory phenotype. The literature on diabetes-induced inflammation is thick with studies that identify connections between particular

pathways and their effects on inflammation: damage from ROS, TLR4-mediated responses and, in our studies, modulation by fatty acid species are just a few of these. The effects of diabetes extend to the entire organism and have multiple targets, likely none of which alone will be able to correct the detrimental effects of diabetes-induced inflammation. ACOT7 is a great example of the wide-reaching effects of diabetes. I observed upregulation of ACOT7 in macrophages from diabetic mice in combination with a role for ACOT7 in expression of inflammatory mediators after LPS treatment *in vitro*. This suggested that ACOT7 may contribute to diabetes-associated inflammation through fatty acid modifications, but deletion of ACOT7 in macrophages did not reduce inflammation in diabetic mice, nor did it protect from atherosclerosis. The contribution of fatty acids and their derivatives as mediators of inflammation in diabetes could be only a small part of the whole picture. Further, with compensation for ACOT7 activity by related enzymes, its overall influence on macrophage biology is likely diminished. Interestingly, another ACOT (ACOT9) was found to be increased in inflammatory macrophages in my study. It is possible that ACOT7 and ACOT9 act together to promote inflammation, and that deleting one of these ACOTs is insufficient for an overall reduction in inflammatory phenotype.

Limitations encountered and future directions for the study of ACOT7

ACOT7 was increased in response to pro-inflammatory stimuli and overexpression led to an exacerbated pro-inflammatory state in macrophages, but only had a minimal role in inflammation when its expression in macrophages was lost or reduced. Future work investigating the endogenous role of ACOT7 would benefit from more tightly controlled overexpression with a greater focus on *in vivo* models. Additional models of

inflammation for study of ACOT7 deletion in macrophages may prove to be more relevant to its endogenous purpose than our model of diabetes. Further, double knockout animals may be necessary to limit compensation by other enzymes.

The level of overexpression of ACOT7 achieved i*n vitro* was far greater than any upregulation observed *in vivo* or that observed when treated with LPS. For this reason, it is difficult to say if endogenous ACOT7 upregulation has a causative role in inflammation. A method to limit the expression level of ACOT7 to that observed with LPS treatment *in vivo* would add relevance to the proinflammatory phenotype. One method to achieve control of expression in each individual cell is by using a vector in which the gene of interest (ACOT7) is linked to an EGFP sequence separated only by an internal ribosome entry site (IRES) (142). EGFP and ACOT7 would be transcribed as one large mRNA but would each give rise to separate proteins. Using this method, the EGFP intensity is linked to the quantity of ACOT7 transcribed and the cells with the desired expression level can be selected for by fluorescence assisted cell sorting. An alternative method to this would be a growing colonies from single cells by limited dilution, which is labor intensive and time consuming.

A second challenge that must be overcome when studying macrophages *in vitro* is the heterogeneity of macrophage populations. It is difficult to know for sure if the population chosen for study is representative of all macrophages or of the population in atherosclerotic lesions. In my work, I observed increased expression of ACOT7 in thioglycollate-elicited macrophages from diabetic mice, but failed to detect upregulation of ACOT7 in resident macrophages (data not shown). ACOT7 activity may promote inflammation in thioglycolate-elicited macrophages or bone-marrow derived

macrophages treated with LPS, but this is not conclusive for a potential role of ACOT7 in our primary end-point of diabetes-accelerated atherosclerosis. This study would have benefitted from a greater emphasis on *in vivo* models. When this project was first begun however, the mouse models for ACOT7 KO were not yet available. Now that these mice exist, future work can be directed at *in vivo* phenotyping of macrophage-specific ACOT7 null mice.

Bone-marrow deficiency of ACOT7 did not alter diabetes-associated inflammation. The greatest endogenous responses achieved for ACOT7 expression were in response to LPS, thus it may have a greater role in TLR4-mediated responses. TLR4 senses cell wall components of Gram-negative bacteria. A more relevant model to study the endogenous role of ACOT7 may be infection with *Salmonella,* which is dependent of the MyD88 arm of the TLR4 pathway for control of bacterial growth (143).

Lastly, when performing future experiments to study the role of ACOT7 in other models such as bacterial infection, compensation by other ACOTs and fatty-acid modifying enzymes must still be taken into account. My data identified *Acot9* mRNA to also be elevated in response to LPS. ACOT9 is among the same hot-dog fold super family as ACOT7 with a similar structure. Though ACOT9 was not found to have specific activity toward 20:4-CoA, it is reported to be a multipurpose ACOT cleaving C2-C20 length acyl-CoA species (144), not much is known about this enzyme leaving a possibility for compensation. Since both ACOT7 and ACOT9 are upregulated in response to LPS a double knockout in a model of bacterial infection may provide more clues to the role of ACOTs in macrophage inflammation.

Final thoughts on atherosclerosis associated with diabetes and metabolic syndrome

The artery wall is a dynamic environment and is constantly remodeled during the life of an organism in response to a wide variety of stimuli received by immune cells, endothelial cells, SMCs and others not discussed here. Certain conditions such as metabolic syndrome and diabetes put one at greater risk for maladaptive remodeling by these cell types. My work has identified roles for dysregulated cell metabolism in the pathogenesis of inflammation and atherosclerosis in the presence of these conditions. The effects of dysregulated cell metabolism where found to be specific to certain cell types. Current research is uncovering new drug targets to limit the detrimental effects that increased glucose and lipids have on the artery wall and my research will allow us to more accurately target these future therapies. However, even with the improved therapies available today, for many people with CVD this is not enough. CVD continues to be the leading cause of death in the United States as well as other developed countries. Though some people will require medical intervention to lower their risk of CVD, for much of the population the increased risk is reversible by lifestyle changes alone. So, if you have made it all of the way to the end of this document, do yourself a favor: have a big helping of vegetables and go for a hike!

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