ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM (U01 HL 70526)

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PART A:

PRINCIPAL INVESTIGATOR'S SUMMARY

Part A. Principal Investigators Summary

I. Program Accomplishments

A major theme of the UCLA component of the Animal Models of Diabetes Complications consortium (AMDCC) is the development of insulin resistant mouse models in which insulin resistance, the metabolic syndrome or type 2 diabetes accelerate atherosclerosis. Within this framework we have further attempted to develop models that not only have an increase in extent of atherosclerotic lesions, but also a progression of lesion histology from fatty streaks to necrotic lipid cores or cholesterol clefts with fibrous caps surrounded by a proteoglycan matrix. Several models now appear promising and are described in more detail in Part B:

- ***Elderly LDLR*** $^{-/-}$ Three months of western diet administered to 10-12 mo old LDLR$^{-/-}$ mice induces obesity and type 2 diabetes with a marked acceleration in both extent and progression of atherosclerotic lesions compared to younger (2-3 mo old) LDLR$^{-/-}$ given the identical diet for the same amount of time. Younger LDLR$^{-/-}$ also develop diabetes on the Western diet, but these animals only get 3-4% of their aortic surface area covered with lesions which are generally fatty streaks. In sharp contrast, the older animals get 20% of aortic surface covered with lesions, which are clearly advanced (see attached tables). While, diabetes does not appear to accelerate lesions in the younger animals; we are determining whether there is an effect of diabetes in older animals. Three months of low fat diet (normal chow) following the Western diet did not induce lesion regression in the older LDLR$^{-/-}$; however, preliminary data suggests three months of low fat diet plus enalapril (angiotensin converting enzyme inhibitor) could regress lesions.

- ***Muscle-specific PPARγ deletion*** Hevener and colleagues (*Nat Med*, 9: 1491, 2003) reported that skeletal muscle specific knockout of PPARγ (MKO$^{fl/fl}$) results in progressive insulin resistance that is associated with hyperinsulinemia, hypertriglyceridemia, hyperleptinemia, weight gain and decreased adiponectin similar to the metabolic syndrome in man. We have obtained these animals and are currently breeding them onto a LDLR$^{-/-}$ background. Because these animals closely mimic human metabolic syndrome, we believe that if they develop accelerated atherosclerosis, this model will be important for defining specific mechanisms.

- ***Apolipoprotein AII transgenic (ApoAIItg)*** This model develops severe hypertriglyceridemia with obesity, insulin resistance, hyperinsulinemia, hypertension, increased free fatty acids and other lipid and apolipoprotein changes (except decreased HDL) that mimic the metabolic syndrome. The HDL in that model is also proatherogenic (Catelliani et al, *Diabetes*, 50: 643, 2001, Castellani; Castelliani et al, *Jour. Lipid Res.*, (submitted). After

breeding, we just obtained animals with ApoAIItg on the $APOE^{-/-}$ background, and we are also putting the transgene onto an $LDLR^{-/-}$ background. If this metabolic syndrome model also accelerates atherosclerosis, it should serve as a key model to identify mechanisms of accelerated vascular injury.

- ***Angiotensin II (AngII) infusion*** Osmotic mimic prep delivery of Ang II in pressor or subpressor doses enhances the extent of lesions (10-40 fold) and progression of lesions in genetically prone mice. We recently demonstrated that the large acid phosphoprotein adhesion molecule, osteopontin, was substantially responsible for AngII acceleration of atherosclerosis (Bruemmer, et al, *JCI*, 112:1318, 2003). Knockout of osteopontin in an $APOE^{-/-}$ background attenuated AngII induced lesions, but did not affect atherosclerosis extent in eight month old chow fed $APOE^{-/-}$ animals. We found that macrophage osteopontin (vs. that in endothelial or vascular smooth muscle cells) was responsible for the atherosclerosis acceleration in response to AngII. These studies are particularly interesting since lesions from humans with diabetes have increased expression of osteopontin compared to lesions from humans without diabetes. Thus, expression of osteopontin may be a marker of diabetes-accelerated atherosclerosis. AngII has also been shown to stimulate 5 lipoxygenase (5LO). We reported that knockout of 5LO attenuates atherosclerosis in non AngII infused animals (Mehrabian, et al, *Circ Res*, 91:120, 2002) and that a polymorphism of 5LO is associated with human atherosclerosis (Dwyer et al, *NEJM*, 350:29, 2004). We are currently in the process of creating a 5LO transgenic animal on an $LDLR^{-/-}$ background. We will also determine whether 5 LO is increased in models of accelerated atherosclerosis. We plan to employ AngII infusion in some of our models to accelerated progression of lesions.

2. Interrelationship of Projects

Project leaders include:
Willa Hsueh, MD PI
Aldons Lusis, PhD Co PI
Richard Davis PhD, Co PI
Alan Collins, PhD, Co PI
Larry Castelliani, PhD
Margarete Mehrabian, PhD
Susanne Nicholas MD, PhD

There is extensive interaction of all the investigators within the UCLA component of the Consortium. Of note: Drs. Hsueh and Collins' laboratory provides AngII infusion, en face lesion analysis and blood pressure measurements; Dr. Castelliani's laboratory measures all of the lipids, apolipoproteins and HDL atherogenicity; Dr. Lusis and Davis' laboratory provides congenic mouse strains

(discussed below); Dr. Nicholas' laboratory provides measurements of albuminuria and glomerular filtration rates. All investigators meet regularly and have shared in accompanying Dr. Hsueh to the AMDCC meetings.

3. Collaborations with Other Consortium Groups Includes:

- Use of AIN diets developed by the Rockefeller group
- Phenotyping of a cardiac specific PPARγ knockout developed by us and phenotyped for cardiac metabolic endpoints by the Utah group

4. Pertinent non AMDCC collaborators

- Drs. Jerrold Olefsky and Andrea Hevener's group at UCSD on the muscle specific PPARγ KO.

Part B. Project Reports by Responsible Investigators

Project 1: *Elderly LDLR$^{-/-}$ mice (Alan Collins)*

We developed a novel model of accelerated atherosclerosis associated with diabetes. Male LDLR$^{-/-}$ mice were placed on a western diet at the age of 10-12 months for 3 months. These mice became grossly obese (50g mean body weight) and exhibited hyperglycemia (388 mg/dl) after an overnight fast. The atherosclerosis was markedly accelerated compared to 2-3 mo old mice placed on 3 mos of Western diet (17.4% vs. 4.3%, $p<0.001$, of the aortic surface covered by lesion). Histological analysis of these lesions indicated that a large proportion of them were in advanced stages of disease, containing cholesterol clefts, necrotic lipid cores and fibrous caps (Figure 1.1 and 1.2). The mice were then placed on a normal chow diet for a further 3 months to determine if these lesions would regress (Figure 1.3). The control mice did not exhibit any regression of lesions despite significant weight loss (35 g). The atherosclerosis increased to 19.0 % and the mice remained hyperglycemic, although to a lesser degree (225 mg/dl). The addition of the ACEi, enalapril, resulted in significant regression of the pre-existing atherosclerosis (11.8%, $p<0.05$) in the absence of improved hyperglycemia (266 mg/dl), Figures 1.4 and 1.5. The lesions from the enalapril treated mice also exhibited impressive decreased amounts of lipid within the lesions by histological analysis. This study suggests that: 1) the elderly LDLR$^{-/-}$ mouse with diabetes is a model of accelerated atherosclerosis, 2) advanced atherosclerotic lesions in mice can regress, 3) the renin angiotension system may be important in regression.

Figure 1.1

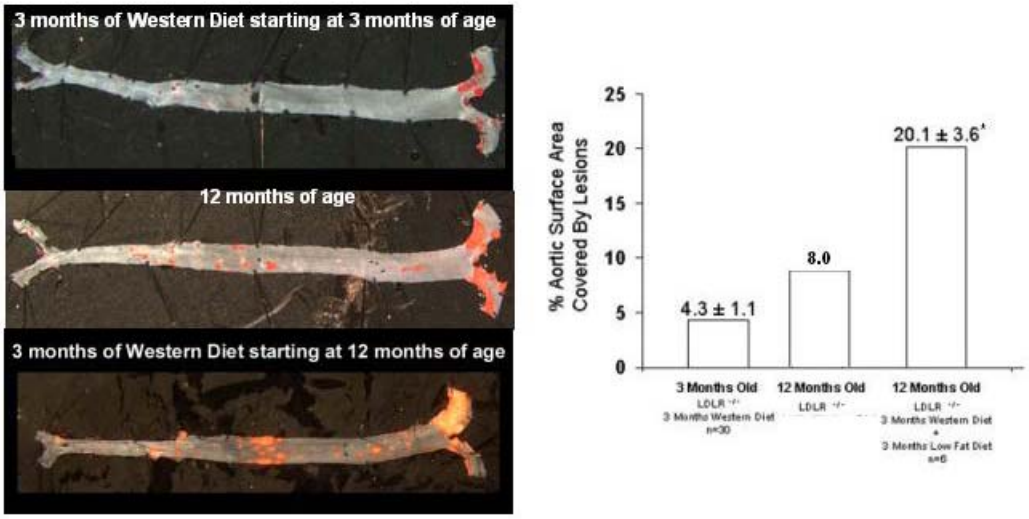


Figure 1.2

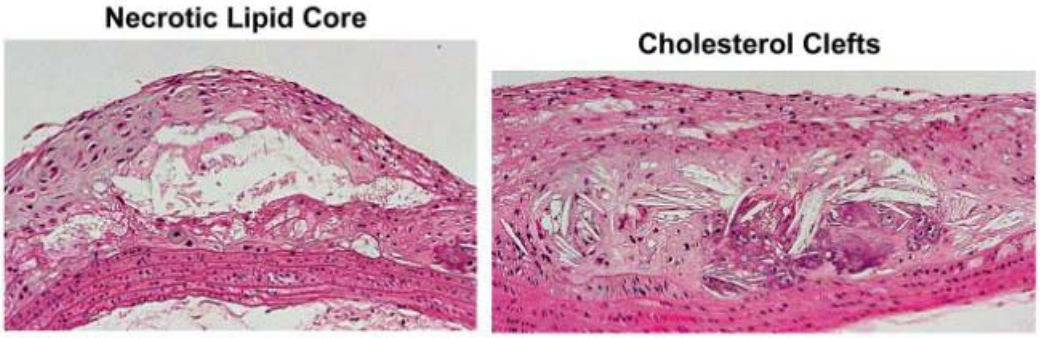


Figure 1.3

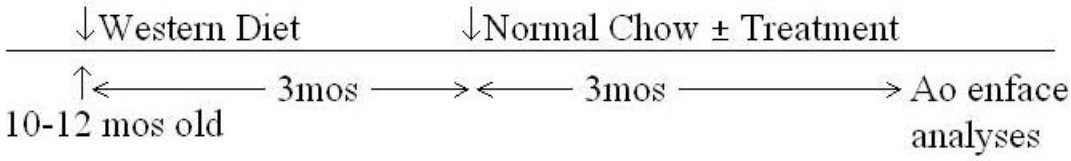


Figure 1.4

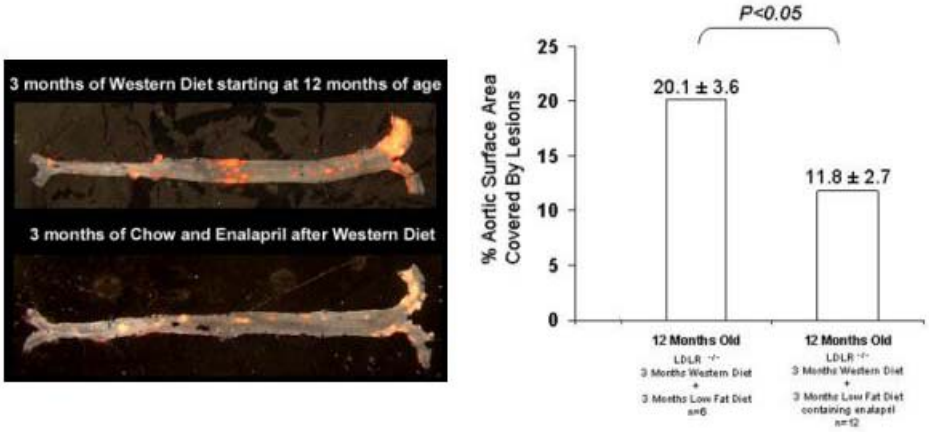
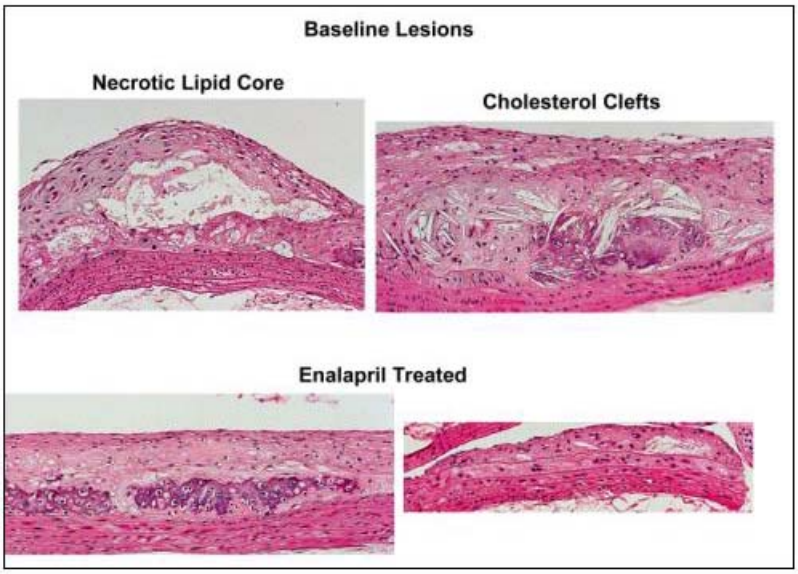


Figure 1.5



Project 2: *Muscle specific PPARγ KO (Willa Hsueh)* We are presently breeding mice with skeletal muscle specific PPARγ knockout ($MKO^{fl/fl}$) into $LDLR^{-/-}$. Characterization of the mice is shown in Table 1 from (Hevener et al, *Nat Med,* 9:1941, 2003).

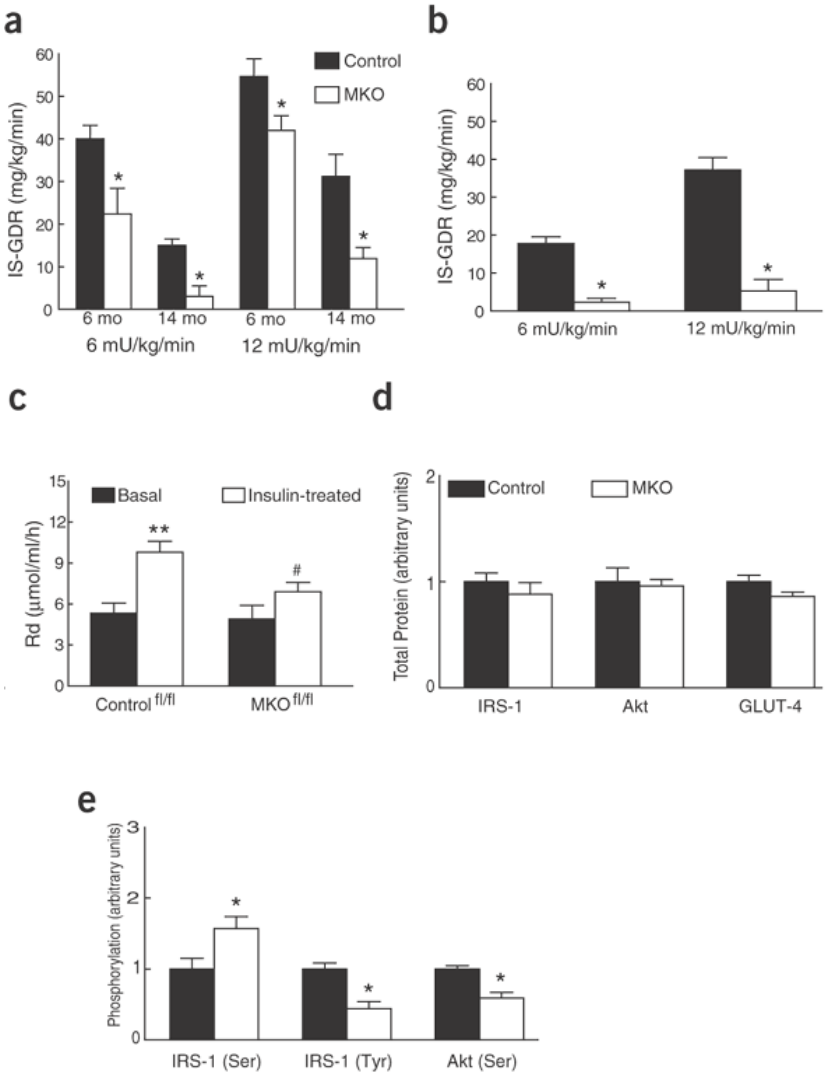
These animals develop skeletal muscle insulin resistance, but not liver or adipose insulin resistance as occurs in type 2 diabetes, Figure 2.1 A-E. These animals develop the metabolic syndrome, which should enhance atherosclerosis in mice, similar to its effect in humans. Like humans with metabolic syndrome, the mice have a decrease in circulating adiponectin (Acrp-30, 50% decrease in mice), which could contribute to accelerated atherosclerosis. Adiponectin inhibits inflammation and protects from atherosclerosis, as well as increases insulin action in skeletal muscle and liver. If the $MKO^{fl/fl}$ X $LDLR^{-/-}$ mice develop accelerated atherosclerosis, we plan to virally deliver adiponectin (adenoviral-adiponectin) to the liver to increase circulating adiponectin levels in order to determine whether a rise in adiponectin attenuates the atherosclerosis.

Table 1 Characteristics at baseline and during glucose clamp studies, at 6 and 14 months of age, with or without TZD treatment

Genotype	6 months untreated Control$^{fl/fl}$	6 months untreated MKO$^{fl/fl}$	14 months untreated Control$^{fl/fl}$	14 months untreated MKO$^{fl/fl}$	14 months untreated Control$^{fl/-}$	14 months untreated MKO$^{fl/-}$	14 months TZD-treated MKO
Body weight (g)	28 ± 1.1	33 ± 1.1	41 ± 2.8	44 ± 2.0	38 ± 4.0	43.7 ± 1.2	46.9 ± 1.5
Blood glucose (mg/dl)							
Basal	108 ± 6.47	123 ± 4.6	131 ± 5.6	139 ± 8.0	137 ± 7.0	134 ± 9.0	115 ± 4.6**
Clamp	140 ± 3.5	140 ± 2.1	148 ± 1.9	147 ± 2.06	148 ± 2.7	145 ± 6.3	146 ± 1.5
Insulin (ng/ml)							
Basal	0.6 ± 0.07	1.21 ± 0.12#	0.87 ± 0.2	1.5 ± 0.15#	0.81 ± 0.09	1.67 ± 0.3#	0.73 ± 0.1**
Clamp	9.9 ± 1.37	10.7 ± 0.58	9.4 ± 0.6	9.45 ± 1.2	8.2 ± 0.78	9.7 ± 1.4	8.6 ± 1.1
Plasma triglycerides (mg/dl)	85 ± 5.4	78 ± 2.5	135 ± 11	209 ± 16#	118 ± 12	200 ± 13#	117 ± 19**
Liver triglycerides (nmol/g)	—	—	6.3 ± 2	12.9 ± 3.5#	5.9 ± 2.4	11.1 ± 3.3#	34.8 ± 3.02**
Muscle triglycerides (nmol/g)	—	—	5.7 ± 2	8.4 ± 3.5	5.1 ± 2.4	7.8 ± 3	9.07 ± 1.77
Leptin (ng/ml)	4.8 ± 1.48	12.4 ± 2.1#	3.76 ± 1.0	4.0 ± 1.3	4.3 ± 0.32	11.6 ± 3.7#	
Acrp-30 (μg/ml)	25.4 ± 1.4	19.2 ± 1.28	21 ± 2.2	9.5 ± 1.6#	14 ± 1.4	8.8 ± 0.6#	25.5 ± 4.5**
Liver weight (g)	1.16 ± 0.06	1.12 ± 0.05	1.27 ± 0.12	1.5 ± 0.16	1.22 ± 0.09	1.84 ± 0.12#	1.9 ± 0.09*
Epididymal fat pad weight (g)	0.51± 0.07	1.17 ± 0.2#	1.4 ± 0.12	1.69 ± 0.08	1.11 ± 0.16	2.41 ± 0.3#	2.35 ± 0.18
Heart weight (g)	0.16 ± 0.015	0.14 ± 0.01	0.165 ± 0.02	0.196 ± 0.015	0.15 ± 0.008	0.174 ± 0.02	0.18 ± 0.01

Data for TZD-treated MKO animals was combined for simplicity. Biochemical parameters and tissue weights are represented as mean ± s.e.m. #, $P < 0.05$ for control versus MKO (within groups of like background); *, $P < 0.05$ for untreated MKO$^{fl/fl}$ versus TZD-treated MKO mice; **, $P < 0.05$ for both untreated versus TZD-treated MKO groups.

Figure 2.1



Project 3: *AngII infusion and osteopontin knockout (Drs. Willa Hsueh, Alan Collins)* Ang II infusion into $LDLR^{-|-}$ or $APOE^{-|-}$ mice profoundly accelerates atherosclerosis (Figures 3.1 A, B). We are currently further analyzing the aortic root and innominant artery to determine the extent and progression of lesion formation in these areas. We bred mice with genetic deficiency of osteopontin ($OPN^{-|-}$) into $APOE^{-|-}$ and attenuated AngII-accelerated atherosclerosis (3 mo old mice on normal chow diet plus AngII infusion, 2.5 μg/kg/min for one month), but not atherosclerosis in untreated mice (8 mo old mice on normal chow diet); See reference 1 in Publications. These data suggest OPN is a critical mediator of accelerated atherosclerosis. Using bone marrow irradiation, we further demonstrated that AngII infused $APOE^{-|-}$ that were irradiated and transplanted with $OPN^{+|+}$ stem cells had no attenuation of atherosclerosis, while those transplanted with $OPN^{-|-}$ cells had substantial attenuation of atherosclerosis (Figures 3.2 A, B). Lack of OPN was associated with decreased macrophage migration and increased macrophage apoptosis consistent with the observation that $OPN^{-|-}$ animals had less macrophage accumulation at the sites of lesions. Thus, AngII's actions to increase macrophage OPN expression may contribute to its atherosclerosis acceleration. In models of insulin resistance-accelerated atherosclerosis, we will determine whether there is enhanced lesion and macrophage expression of OPN.

Figure 3.1

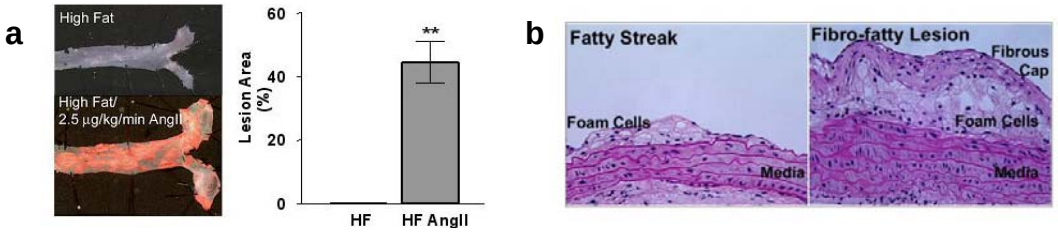
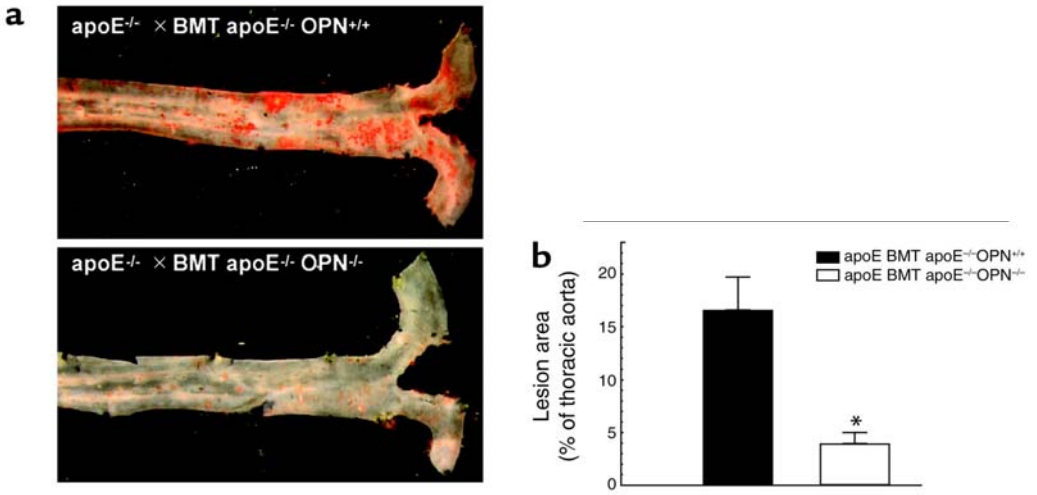


Figure 3.2



We also used the AngII accelerated atherosclerosis model to demonstrate that ligands to peroxisome proliferator activated receptor γ (PPARγ), which are insulin sensitizers that enhance insulin mediated glucose uptake attenuate atherosclerosis by 50-60%; See reference 2 in Publications. Since these agents

did not substantially alter blood pressure or lipids in this model, we suspected that the results were due to direct vascular effects of PPARγ ligands. We further demonstrated that PPARγ ligands attenuate inflammatory events induced by AngII including upregulation of early growth response gene–1 (Egr-1), which regulates expression of vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule (ICAM), and monocyte chemoattractant protein – 1 (MCP-1).

Project 4: AIN Diets (Dr. Alan Collins) The EAC recommended the use of a standardized diet for the induction of atherosclerosis. The western diet commonly used to induce atherosclerosis is not standardized and the components of the diet and results can vary from batch to batch. The use of the Clinton-Cymulsky diets (variants of the AIN76a) would eliminate this batch variability, as the diet is semi-purified having a known dietary composition that does not vary. Since diabetes is associated with obesity in the male $LDLR^{-/-}$ mouse while atherosclerosis appears to be associated with plasma cholesterol, we designed an experiment to equalize cholesterol between a high fat (40% of calories from fat) and low fat (10% calories from fat) in the Clinton-Cymulsky diets.

When the cholesterol in the diet was equalized based on the caloric content of the diet (0.15% in low fat and 0.18% in high fat) the plasma cholesterol and atherosclerosis was significantly higher in the low fat fed mice versus the high fat fed mice. In order to equalize the plasma cholesterol the low fat diet had the cholesterol lowered to 0.1% while the high fat diet's cholesterol was increased to 0.3%. Plasma cholesterol was not significantly different when the low fat with 0.1% cholesterol was used compared to the high fat with 0.18% cholesterol. The high fat fed mice exhibited significantly higher fasted glucose and had a slight increase in atherosclerosis (see FeldmanTables).

An additional experiment was performed to determine if the time at which the diets were begun affected the development of atherosclerosis. Our group normally waits until the mice are fully mature at 3 months of age to start the diets while the Breslow unit begins the diets at 4 weeks of age. The mice are juvenile at 4 weeks of age and this could have been a confounding factor. We found no differences in atherosclerosis between the start times when fed the same diet. (see FeldmanTables).

Infusion of angiotensin II at 2.5 μg/kg/min for 8 weeks increased atherosclerosis in the 2 original diets (low fat 0.15% and high fat 0.18%) with the low fat fed mice having significantly higher cholesterols and atherosclerosis than the high fat fed mice (see FeldmanTables). These results were similar to that found in the non-infused mice. We propose to perform an experiment using the 0.1% low fat and 0.18% high fat in order to equalize cholesterol in the infused mice.

Project 5: GTM Congenics (Drs. Richard Davis and Aldons Lusis)

MOB congenics

The LDL receptor knockout mouse on a western diet is our primary model for diabetes-associated atherosclerosis. As part of our characterization of this model, we are assessing the impact of potential modifying loci. In particular, we are measuring the effects of the multigenic obesity (MOB) loci that we, and others, have linked to diabetes-related phenotypes in mice [1;2]. Notably, the

syntenic locus in humans has repeatedly been linked to a similar set of phenotypes, suggesting a common set of genes with variations that impact obesity and diabetes in both species [1].

To better localize and characterize the MOB locus, we created three congenic mouse strains (Figure MOB.1). Overlapping genomic intervals from the lean CAST/Ei (CAST) strain were introgressed onto an obesity susceptible C57BL/6 (BL6) background to create proximal (15Mb – 73 Mb), middle (63Mb – 165 Mb) and distal (83 Mb – 182 Mb) congenic strains.

The MOB congenics have been assessed for their impact on insulin resistance and atherosclerosis compared to the background strain (C57BL/6). The various congenics have mixed impacts, some enhancing and some ameliorating the insulin resistance phenotypes. This work has recently been published [3].

The congenic strains showed differences in obesity, insulin and lipid traits consistent with the original QTL analysis for the locus. Importantly, characterization of the MOB congenics localized the effects of genes that underlie obesity related traits to an introgressed interval (73-83 Mb) unique to the middle MOB congenic. Conversely, significant differences between the lipid and insulin profiles of the middle and distal MOB congenics implicated the presence of at least 2 genes that underlie these traits. When fed an atherogenic diet, several traits associated with metabolic syndrome were observed in the distal MOB congenic while alterations in plasma lipoproteins were observed in the middle MOB congenic strain.

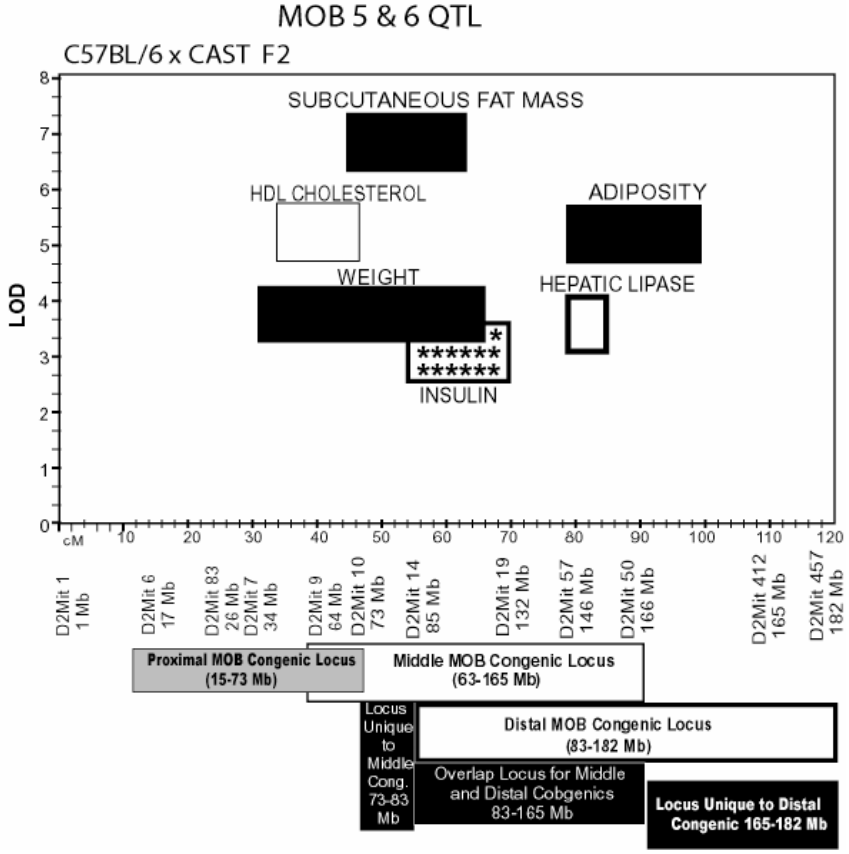


Fig MOB.1

Progress: The MOB congenics have been transferred to the LDLr knockout background and we are now accumulating data on their impact on the major atherosclerosis model of our overall group. We have aortas for lesion analysis that should be complete in the next month. Data for related traits should be available in the same time frame.
Preliminary fat accretion data by NMR indicates that the middle MOB locus decreases fat/lean mass ratio in the LDLR-/- middle MOB congenics. Interestingly, the parental distal MOB congenics show a significant increase in fat/lean mass ratios when compared to LDLR-/- controls, however the LDLR-/- distal MOB congenics show a significant decrease in fat/lean mass when compared to LDLR-/- controls.

Taken together the data suggest that gene/s within the middle MOB locus decrease, fat mass accretion and atherosclerotic lesion formation and improve insulin sensitivity in an environment of caloric and cholesterol enrichment. Conversely, gene/s within the distal MOB locus that is syntenic to the NIDDM3 locus) in humans [1], acts to increase fat mass accretion, insulin resistance and atherosclerotic lesion formation when challenged with a diet enriched for fat and sucrose thereby replicating the phenotype demonstrated by humans.

Work in progress includes assessing LDLr knockout congenics after Angiotensin 2 infusion. Animals for this study should be ready for Azlet pump insertion in 6-8 weeks.

GTM congenics are being independently phenotyped and, based on these phenotypes will be considered for combination with the MOB congenics as additional modifiers of the LDLr ko model.

Reference List

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[2] M.Mehrabian, P.-Z.Wen, J.Fisler, R.C.Davis, A.J.Lusis. Genetic loci controlling body fat, lipoprotein metabolism and insulin levels in a multifactorial mouse model., J.Clin.Invest., 101, (1998) 2485-2496.

[3] D.Estrada-Smith, L.W.Castellani, H.Wong, P.Z.Wen, A.Chui, A.J.Lusis, R.C.Davis. Dissection of multigenic obesity traits in congenic mouse strains, Mamm.Genome, 15, (2004) 14-22.

Project 6: Apolipoprotein AII Transgenics (Dr. Larry Castellani)

Placing the apoAII transgene on an apoE knockout background.

Overview:

Transgenic mice that overexpress mouse apolipoprotein A-II (apoAII) become insulin resistant, obese, and exhibit accelerated atherosclerosis (1,2). These phenotypes occur when the transgene is expressed on a normal C57BL6 background, and while the animals are maintained on a standard low fat chow diet. We recently performed a cross between strains C57BL6 (B) and C3H (H), in which both inbred strains were on an apoE knockout (apoE ko) background. We generated 328 BxH.apoE ko F2 mice (164 males, 164 females), which were placed on a western diet for 16 weeks and sacrificed at 24 weeks of age. In this cross the *apoa2* gene was identified as the major locus affecting plasma concentrations of triglycerides, total cholesterol, HDL cholesterol, unesterified cholesterol, free fatty acids, and glucose (Fig. 1), traits that are all increased in the apoAII transgenic mice. We are presently performing atherosclerotic lesion analysis on these mice, as well as determining plasma concentrations of insulin and apoAII.

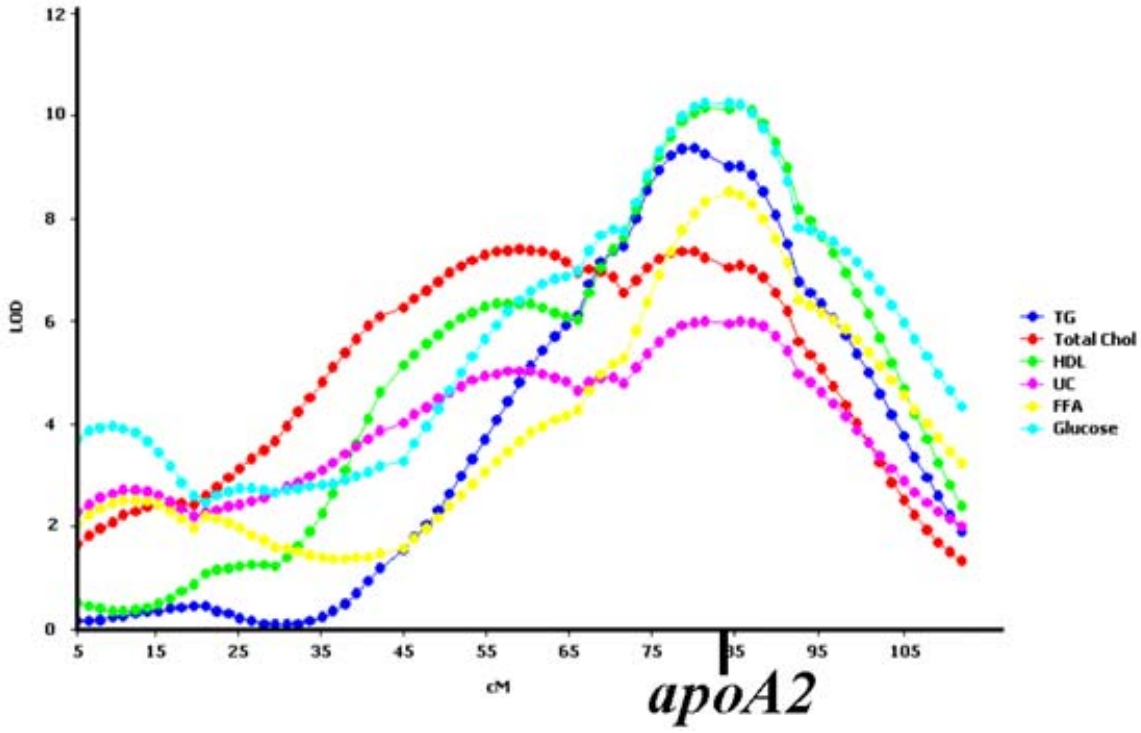

Figure 1. Strains C57BL6 (B) and C3H (H), both on an apoE knockout (apoE ko) background were crossed to generate 328 BxH.apoE ko F2 mice (164 males, 164 females). After 16 weeks on a western diet the animals were sacrificed and plasma concentrations of triglyceride (TG), total cholesterol (total chol), HDL cholesterol (HDL), unesterified choelsterol (UC), free fatty acids (FFA), and glucose were determined. The animals were genotyped using 140 markers

distributed throughout the genome to generate a 10 cm map. The location of the apoA2 gene under the peak lod scores is indicated on the figure.

We previously reported that C3H mice exhibit a 50% increase in plasma apoAII concentrations compared to strain C57BL6 (3). In the present cross, it is the C3H *apoa2* allele that is associated with significant increases in all of the traits described above. Apparently, only a small increase in plasma apoAII concentrations (~50% increase) on the apoE knockout background had very dramatic effects on the traits in question.

Our apoAII transgenic mice exhibit anywhere from a 1 to 6 fold increase in plasma apoAII concentrations (for female heterozygous and male homozygous transgenics, respectively) compared to control mice. In light of the results of our BxH.apoE ko cross described above, we are placing the apoAII transgene onto an apoE knockout background. Since the dramatic effects of apoAII described above were observed with only a 50% increase in plasma apoAII concentrations, we expect to see a dramatic exacerbation of the phenotype when we increase plasma concentrations 1 to 6 fold on the apoE knockout background.

Progress/Status of generating the combined apoAII transgenic.apoE ko mice.

We have already crossed the apoAII transgenic mice to apoE null mice and have generated 20 F1 progeny that are heterozygous for both the apoAII transgene and apoE null mutation. Several of these F1 progeny are currently being bred to the apoE knockout mice, which will generate mice that are heterozygous for the apoAII transgene on the apoE knockout background. Some of these combined apoAII transgenic.apoE ko mice will immediately be placed on the western diet as described above, and the effects on insulin resistance/diabetes and atherosclerosis will be examined. Based on the results of our cross, we anticipate that the apoAII transgenic/apoE ko mice will have a marked increase in all aspects of the apoAII transgenic phenotype. We expect to have combined apoAII transgenic.apoE ko mice that can be started on the western diet approximately 5/15/04.

Additional aspects of the atherosclerosis phenotype in the apoAII transgenic mouse model.

We previously reported that the apoAII transgenic mice develop increased lesions, even when maintained on a low fat chow diet (2). Our lesion findings were later confirmed in another transgenic mouse model over-expressing human apoAII (4). We went on to demonstrate that HDL from the apoAII transgenic mice could not protect LDL from becoming oxidized in a human aortic cell co-culture model of the artery wall, and were in fact, themselves, pro-inflammory (5). This pro-inflammatory nature of apoAII enriched HDL was later confirmed in another study (6).

We recently treated apoAII transgenic mice with rosiglitazone, which significantly ameliorated their insulin resistance (table 1). Preliminary data indicates that the HDL from the apoAII transgenic mice were still pro-inflammatory even though the insulin resistance was significantly improved (data not shown). Patients with type 2 diabetes have been shown to be at increased risk for atherosclerosis, even when their hyperglycemia is controlled. Our preliminary findings suggest that the apoAII transgenic mouse model may also be a case in which atherosclerosis is still increased even though insulin

resistance/hyperglycemia is controlled. We are placing the apoAII transgenic mice on thiazolidinedione treatment for a longer period of time in order to examine the effect of normalizing insulin sensitivity/glucose on lesion development, oxidative stress, etc. These studies will also be repeated with the combined apoAII transgenic.apoE ko mice when they are available.

Table 1. Treatment with rosiglitazone ameliorates insulin resistance in the apoAII transgenic mice. Fasting plasma lipids, glucose, and insulin were determined in a group of 14 apoAII transgenic mice before (Pre-treatment) and after (Post-treatment) administration of rosiglitazone in the diet (4mg/kg) for one week.

	Pre-treatment (n=14)	Post-treatment (n=14)
total cholesterol (mg/dl)	252±20	216±17
HDL cholesterol (mg/dl)	176±12	137±9 *
triglycerides (mg/dl)	212±28	98±14 *
FFA (mg/dl)	41±2	32±2 *
glucose (mg/dl)	142±9	109±8 *
insulin (pg/ml)	1880±318	718±110 *

* indicates values that were significantly different from pre-treatment values, $p<0.05$.

Potential involvement of apoAII in renal disease/hypertension.

ApoAII transgenic mice exhibit several aspects of the metabolic syndrome including hyperlipidemia, insulin resistance, obesity, and increased atherosclerosis. As described above, we have demonstrated that apoAII transgenic mice exhibit increased oxidative stress that may adversely affect vascular wall cell metabolism. As another component of the metabolic syndrome, we had examined blood pressure in the apoAII transgenic mice. In three separate determinations of blood pressure on a group of 12 apoAII transgenic and 12 control mice, we observed on two of the three dates, that blood pressure was significantly increased in the apoAII transgenic mice compared to controls (increased ~12 mm Hg). We have since acquired an automatic tail cuff blood pressure monitor for the mice and are in the process of repeating our study of blood pressure. Oxidative stress, insulin resistance, hypertension, and renal disease are interrelated in a complex manner that is not understood. With respect to the hypertension/renal disease, we have discovered another potential link between apoAII and renal disease/hypertension.

We have been examining the role of apoAII in insulin resistance and lipid metabolism in a study population consisting of 390 Hispanic family members of 77 hypertensive probands. This population shows significantly increased susceptibility to obesity and insulin resistance. Fasting plasma apoA-II levels were positively correlated with insulin resistance and plasma concentrations of triglycerides, HDL cholesterol, LDL cholesterol, total cholesterol, and apolipoprotein B. The correlations between plasma apoAII levels and these traits in this study population parallels the effects of apoAII overexpression in transgenic mice. Interestingly, we also observed a highly significant correlation

between apoAII and plasma creatinine concentrations ($p<0.00001$). A search of the literature revealed another study in which plasma apoAII concentrations were highly correlated with plasma creatinine (7). In that study, plasma apoAII was also the best predictory of the extent of coronary atherosclerosis. We tested plasma creatinine concentrations in the mice but were unable to demonstrate a difference between the apoAII transgenic and control mice. However, we recently learned that mouse serum contains chromagens that interfere with standard picric acid-based assays for serum creatinine, and can increase “apparent” creatinine concentions 5 fold. Therefore, we are going to use a recently described HPLC method to re-analyze plasma creatinine in the apoAII transgenic mice (8).

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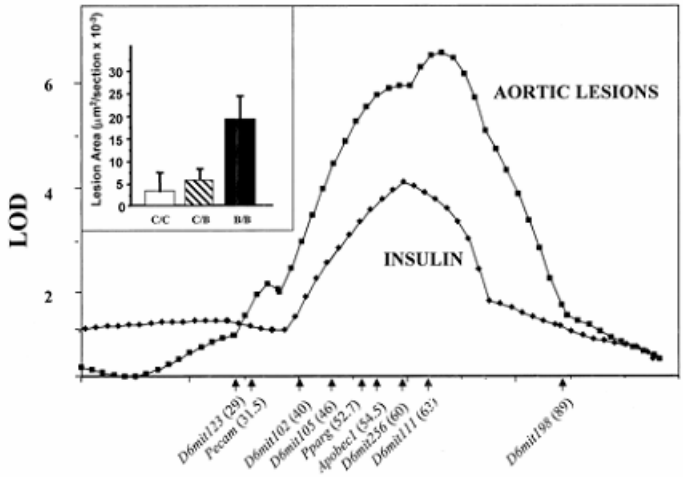
Project 7: 5-lipoxygenase Transgenics (Dr. Margarete Mehrabian)

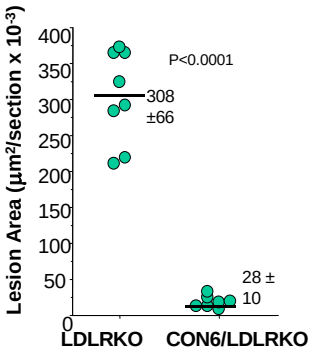
In published work from our lab we identified a quantitative trait locus (QTL) locus on mouse chromosome 6 that confers resistance to the development of both early and advanced atherosclerotic lesions and has a strong affect on plasma insulin levels [[1]] (Fig 5LO.1, below). The QTL was confirmed by construction of a congenic strain (CON6) in which the chromosomal 6 segment from CAST was introgressed onto a LDLR null background.

Fig. 5LO.1 : Lod score curves for QTL on mouse chromosome 6 controlling lesion size and plasma insulin in the B6 X CAST intercross. Inset shows that CAST alleles are protective for lesion formation and exert a dominant effect (C = CAST allele, B = B6 allele).



As shown in Fig 5-LO.2, CON6 mice have a very significant reduction ($p<0.0001$) in aortic lesion formation despite similar hypercholesterolemia in both the congenic and controls. In fact, resistance to aortic lesion formation by the chromosome 6 locus is greater than that of any knockout or transgenic reported. Moreover, the CON6 strain exhibited significantly decreased insulin levels as compared to B6 mice ($P<0.0001$). In addition, we have used bone marrow transplantation to demonstrate that the CON6 locus affects atherosclerosis susceptibility, at least in part, through cells derived from the bone marrow, presumably, monocyte/macrophage or other cells of myeloid lineage. Impact of the chromosome 6 locus on atherosclerosis has been confirmed others in separate mouse cross [2]. Moreover, the same region has been suggested to influence the onset of diabetes in the NOD mouse model [3].

Fig 5LO.2

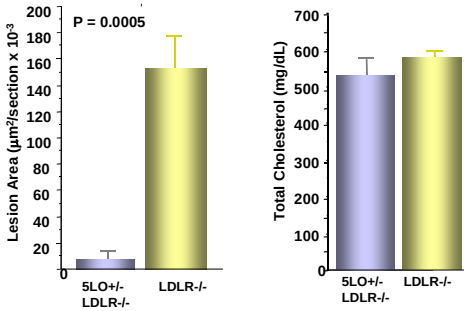


Identification of 5-LO as a candidate gene for the CON6 locus: The bone marrow transplantation experiments with CON6 implied the involvement of monocyte/macrophages or other leukocytes, leading us to examine 5-lipoxygenase (5LO), which is also located in the CON6 interval [4]. Arachidonate 5LO is the key enzyme in the oxidative biosynthesis of a class of eicosanoids known as leukotrienes (LTs), which are potent mediators of vascular constriction and are involved in the recruitment of leukocytes to sites of inflammation. Atherosclerosis has clearly been shown to be an inflammatory disease involving the recruitment of monocytes and lymphocytes to the vessel wall [5-7].

CON6 mice expressed approximately 15% of the 5LO mRNA levels observed in B6 control mice, accompanied by significantly reduced protein and LTB_4 levels. Two amino acid differences between B6 and CON6 were observed in a C-terminus nuclear signaling motif of the protein, I645V and V646I [4]. Interestingly, recent *in vitro* studies creating the same substitutions in the human enzyme have shown dramatic reductions in the expression and activity of 5LO, lending support for the notion that these substitutions are responsible for the altered activity observed in CON6 mice [8].

Furthermore, we found that a heterozygous knockout of 5-LO on LDLR$^{-/-}$ background strikingly reduced atherosclerosis. This reduction in lesion size was very similar to what we previously observed when the CON6 locus was transferred onto the LDLR$^{-/-}$ background and indicates that 5-LO has a dose-dependent effect on lesion size (Fig 5LO.3).

Fig 5LO.3



Again, bone marrow was transplanted from 5-LO$^{+/-}$ into LDL receptor null recipient mice. As previously observed with CON6, there was a 2-fold decrease in atherosclerotic lesion development, suggesting macrophage involvement in lesion formation.

Moreover, like CON6, the heterozygous knockout of 5LO greatly reduced plasma insulin suggesting, that the 5-LO gene also has a role in regulation of insulin levels associated with this locus. Finally, in addition to our mouse studies, we have now obtained preliminary results indicating that a 5LO promoter polymorphism exhibits a very significant association with atherosclerosis and insulin resistance in human populations.

Involvement of 5LO pathway in atherosclerosis and insulin resistance-related phenotypes. 5LO and other genes in the 5LO pathway have been shown to play a complex role in atherosclerosis and perhaps insulin resistance [9-12]. In particular, 5LO activating protein (FLAP), synthesized from the gene ALOX5AP, transfers arachidonic acid to 5LO and is, therefore, critical for leukotriene synthesis. FLAP has been shown to be expressed in several types of inflammatory cells in a highly regulated manner. Excitingly, a recent publication in Nature Genetics strongly implicates FLAP with susceptibility to myocardial infarction and stroke in independent populations in Iceland and Britain [13].

Because of the strong, dose dependent decrease in atherosclerosis in CON6 and the heterozygous 5-LO knockout mice, we anticipate that a 5 LO-transgenic will dramatically enhance the atherosclerotic process. This transgenic is now under construction and will assessed for its impact on atherosclerosis and related phenotypes in the LDLr ko.

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Animal Model/Background Strain	LDLR-/- on C57BL/6J
Protocol (describe age at start and sacrifice, diet, etc)	Angiotensin-II infused on Western Diet (Research Diets D12079a) started at 3 months of age, completed at 5 months of age
Primary Screening	
Insulin Resistance	**No**
Type 2 Diabetes mellitus	**No**
Type 1 Diabetes mellitus	**No**
Fasting Glucose (mg/dl)	221±17.9
Fasting Insulin (ng/ml)	0.481±0.070
Lesion Quantification	
en face (% of aortic surface) compared to controls	44.4±6.3% 40-100 fold increase
Aortic root (μm/section) compared to controls	In progress
Brachial cephalic/innominant (μm/section) compared to controls	In progress
Lesion complexity (Schmidt score)	In progress
Secondary Screening	
Total Plasma Cholesterol (mg/dl)	1123±40
Plasma Triglycerides (mg/dl)	106±8.0
Plasma HDL-C (mg/dl)	88±4.2
Body Weight (g)	22.6±0.88
Body Fat (%)	ND
Blood Pressure (mmHg)	159±3
Evidence of inflammation in the lesions	
Circulatory Inflammatory Markers	Increased EGR-1, TNF-α, MCP-1, ICAM-1
Presence of Calcification	In progress
Gene Expression (include specific genes of interest)	
Other comments	Baseline data for Angiotensin infusion
Collaborators	

Animal Model/Background Strain	Skeletal muscle PPARγ knockout on C57BL/6J
Protocol (describe age at start and sacrifice, diet, etc)	Western Diet at 6 and 14 months of age
Primary Screening	
Insulin Resistance	**Yes**
Type 2 Diabetes mellitus	**Yes**
Type 1 Diabetes mellitus	**No**
Fasting Glucose (mg/dl)	123±4.6 (6 months) 139±8.0 (14 months)
Fasting Insulin (ng/ml)	0.6±0.07 (6 months) 1.5±0.15 (14 months)
Lesion Quantification	
en face (% of aortic surface) compared to controls	In progress
Aortic root (μm/section) compared to controls	In progress
Brachial cephalic/innominant (μm/section) compared to controls	In progress
Lesion complexity (Schmidt score)	In progress
Secondary Screening	
Total Plasma Cholesterol (mg/dl)	In progress
Plasma Triglycerides (mg/dl)	78±2.5 (6 months) 209±16.0 (14 months)
Plasma HDL-C (mg/dl)	In progress
Body Weight (g)	33±1.1 (6 months) 44±2.0 (14 months)
Body Fat (%)	In progress
Blood Pressure (mmHg)	In progress
Evidence of inflammation in the lesions	
Circulatory Inflammatory Markers	
Presence of Calcification	In progress
Gene Expression (include specific genes of interest)	
Other comments	We have obtained these mice form Jerry Olefsky and are breeding them into LDLR-/-
Collaborators	

Animal Model/Background Strain	ApoE/OPN-/- on C57BL/6J
Protocol (describe age at start and sacrifice, diet, etc)	Angiotensin-II infused on normal chow started at 3 months of age, completed at 5 months of age
Primary Screening	
Insulin Resistance	**No**
Type 2 Diabetes mellitus	**No**
Type 1 Diabetes mellitus	**No**
Fasting Glucose (mg/dl)	156±30
Fasting Insulin (ng/ml)	0.614±0.049
Lesion Quantification	
en face (% of aortic surface) compared to controls	5.1% ± 0.7% 70% decrease
Aortic root (μm/section) compared to controls	In progress
Brachial cephalic/innominant (μm/section) compared to controls	In progress
Lesion complexity (Schmidt score)	In progress
Secondary Screening	
Total Plasma Cholesterol (mg/dl)	447±22
Plasma Triglycerides (mg/dl)	63±5
Plasma HDL-C (mg/dl)	30±6
Body Weight (g)	24±2
Body Fat (%)	In progress
Blood Pressure (mmHg)	161 ± 2
Evidence of inflammation in the lesions	
Circulatory Inflammatory Markers	Increased MCP-1
Presence of Calcification	In progress
Gene Expression (include specific genes of interest)	
Other comments	We will be placing the OPN-/- on LDLR-/- to determine its effects in an insulin-resistant / type 2 diabetic model of atherosclerosis
Collaborators	

Animal Model/Background Strain	LDLR-/- on C57BL/6J			
Protocol (describe age at start and sacrifice, diet, etc) started at 1 month of age, completed at 4 months of age	Low fat Clinton-Cymulsky (AIN76a) with 0.1% cholesterol added	Low fat Clinton-Cymulsky (AIN76a) with 0.15% cholesterol added	High fat Clinton-Cymulsky (AIN76a) with 0.18% cholesterol added	High fat Clinton-Cymulsky (AIN76a) with 0.3% cholesterol added
Primary Screening				
Insulin Resistance	**No**	**No**	**Yes**	**Yes**
Type 2 Diabetes mellitus	**No**	**No**	No	**Yes**
Type 1 Diabetes mellitus	**No**	**No**	**No**	**No**
Fasting Glucose (mg/dl)	180±14	210±13	240±41	311±11
Fasting Insulin (ng/ml)	In progress	In progress	In progress	In progress
Lesion Quantification				
en face (% of aortic surface) compared to controls	4.7% ± 0.44%	9.7% ± 1.9% 70% decrease	3.7% ± 0.4%	5.8% ± 1.1%
Aortic root (μm/section) compared to controls	In progress	In progress	In progress	In progress
Brachial cephalic/innominant (μm/section) compared to controls	In progress	In progress	In progress	In progress
Lesion complexity (Schmidt score)	In progress	In progress	In progress	In progress
Secondary Screening				
Total Plasma Cholesterol (mg/dl)	In progress	In progress	In progress	In progress
Plasma Triglycerides (mg/dl)	In progress	In progress	In progress	In progress
Plasma HDL-C (mg/dl)	In progress	In progress	In progress	In progress
Body Weight (g)	28±1	25±1	32±2	31±1
Body Fat (%)	In progress	In progress	In progress	In progress
Blood Pressure (mmHg)	103±3	102 ± 2	104 ± 2	106 ± 3
Evidence of inflammation in the lesions	In progress	In progress	In progress	In progress
Circulatory Inflammatory Markers	In progress	Increased MCP-1	In progress	In progress
Presence of Calcification	In progress	In progress	In progress	In progress
Gene Expression (include specific genes of interest)				
Other comments				
Collaborators				

Animal Model/Background Strain	LDLR-/- on C57BL/6J			
Protocol (describe age at start and sacrifice, diet, etc) started at 3 month of age, completed at 6 months of age	Low fat Clinton-Cymulsky (AIN76a) with 0.1% cholesterol added	Low fat Clinton-Cymulsky (AIN76a) with 0.15% cholesterol added	High fat Clinton-Cymulsky (AIN76a) with 0.18% cholesterol added	High fat Clinton-Cymulsky (AIN76a) with 0.3% cholesterol added
Primary Screening				
Insulin Resistance	**No**	**No**	**Yes**	**Yes**
Type 2 Diabetes mellitus	**No**	**No**	**Yes**	**Yes**
Type 1 Diabetes mellitus	**No**	**No**	**No**	**No**
Fasting Glucose (mg/dl)	171±18	157±20	232±13	287±12
Fasting Insulin (ng/ml)	In progress	0.492±0.071	0.941±0.149	In progress
Lesion Quantification				
en face (% of aortic surface) compared to controls	3.6% ± 1.0%	5.8% ± 0.6%	4.4% ± 0.7%	4.4% ± 0.5%
Aortic root (μm/section) compared to controls	In progress	In progress	In progress	In progress
Brachial cephalic/innominant (μm/section) compared to controls	In progress	In progress	In progress	In progress
Lesion complexity (Schmidt score)	In progress	In progress	In progress	In progress
Secondary Screening				
Total Plasma Cholesterol (mg/dl)	660±95	1243±47	875±108	970±99
Plasma Triglycerides (mg/dl)	127±13	145±23	132±14	131±18
Plasma HDL-C (mg/dl)	87±3	97±4	102±6	99±6
Body Weight (g)	30±1	28±1	33±1	34±2
Body Fat (%)	In progress	In progress	In progress	In progress
Blood Pressure (mmHg)	105 ± 1	101 ± 4	105 ± 4	107 ± 4
Evidence of inflammation in the lesions	In progress		In progress	In progress
Circulatory Inflammatory Markers	In progress	In progress	In progress	In progress
Presence of Calcification	In progress	In progress	In progress	In progress
Gene Expression (include specific genes of interest)				
Other comments				
Collaborators				

Animal Model/Background Strain	LDLR-/- on C57BL/6J			
Protocol (describe age at start and sacrifice, diet, etc)	Western diet started at 12 month of age, completed at 15 months of age	normal chow for an additional 3 months	normal chow and enalapril for 3 months	normal chow and pioglitazone for 3 months
Primary Screening				
Insulin Resistance	**Yes**	**Yes**	**Yes** No	**Yes**
Type 2 Diabetes mellitus	**Yes**	**Yes**	**Yes** No	**Yes**
Type 1 Diabetes mellitus	**No**	**No**	Yes **No**	**No**
Fasting Glucose (mg/dl)	388±30	225±25	266±20	257±13
Fasting Insulin (ng/ml)	In progress	In progress	In progress	In progress
Lesion Quantification				
en face (% of aortic surface) compared to controls	17.4±1.4	19.0±1.8	11.7±0.7	14.3±0.9
Aortic root (μm/section) compared to controls	In progress	In progress	In progress	In progress
Brachial cephalic/innominant (μm/section) compared to controls	In progress	In progress	In progress	In progress
Lesion complexity (Schmidt score)	In progress	In progress	In progress	In progress
Secondary Screening				
Total Plasma Cholesterol (mg/dl)	In progress	In progress	In progress	In progress
Plasma Triglycerides (mg/dl)	In progress	In progress	In progress	In progress
Plasma HDL-C (mg/dl)	In progress	In progress	In progress	In progress
Body Weight (g)	50±1.7	35±01.0	31±1.8	±
Body Fat (%)	In progress	In progress	In progress	In progress
Blood Pressure (mmHg)	105±5	106 ± 4	85 ± 4	102 ± 4
Evidence of inflammation in the lesions	In progress	In progress	In progress	In progress
Circulatory Inflammatory Markers	In progress	In progress	In progress	In progress
Presence of Calcification	In progress	In progress	In progress	In progress
Gene Expression (include specific genes of interest)				
Other comments				
Collaborators				

Animal Model/Background Strain	LDLR-/- on C57BL/6J	
Protocol (describe age at start and sacrifice, diet, etc) started at 3 month of age, completed at 4 months of age	Angiotensin-II infused on Low fat Clinton-Cymulsky (AIN76a) with 0.15% cholesterol added	Angiotensin-II infused on High fat Clinton-Cymulsky (AIN76a) with 0.18% cholesterol added
Primary Screening		
Insulin Resistance	**No**	**No**
Type 2 Diabetes mellitus	**No**	**No**
Type 1 Diabetes mellitus	**No**	**No**
Fasting Glucose (mg/dl)	144±23	192±17
Fasting Insulin (ng/ml)	0.634±0.041	0.616±0.091
Lesion Quantification		
en face (% of aortic surface) compared to controls	15-17%	6-9%
Aortic root (μm/section) compared to controls	In progress	In progress
Brachial cephalic/innominant (μm/section) compared to controls	In progress	In progress
Lesion complexity (Schmidt score)	In progress	In progress
Secondary Screening		
Total Plasma Cholesterol (mg/dl)	1350±71	1079±36
Plasma Triglycerides (mg/dl)	301±49	189±28
Plasma HDL-C (mg/dl)	79±7	130±7
Body Weight (g)	18±0.5	20±0.5
Body Fat (%)	In progress	In progress
Blood Pressure (mmHg)	161 ± 2	163 ± 4
Evidence of inflammation in the lesions	In progress	In progress
Circulatory Inflammatory Markers	In progress	In progress
Presence of Calcification	In progress	In progress
Gene Expression (include specific genes of interest)		
Other comments		
Collaborators		