Animal Models of Diabetic Complications Consortium
(U01 HL 70526)

Annual Report
(2004)

"Novel Models of Cardiovascular Complications of Diabetes"
UCLA

Principal Investigator
Willa A. Hsueh, M.D.

Willa A. Hsueh, M.D.
UCLA
Department of Medicine
Division of Endocrinology, Diabetes and Hypertension
Los Angeles, CA 90095
Phone: (310) 794-7555 Fax: (301) 794-7654
Email: whsueh@mednet.ucla.edu

Table of Contents

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

The overall goal is to develop mouse models of accelerated atherosclerosis in which components of the metabolic syndrome: insulin resistance or hyperinsulinemia, dyslipidemia or hyperglycemia, accelerate vascular injury.

Our main strategy is to employ an approach 1) in which a gene or gene loci is inserted into the mouse to induce an obesity/insulin resistant/type 2 diabetes phenotype which is then bred into the LDLR$^{-/-}$ strain. or 2) which uses the elderly (12month old) LDLR $^{-/-}$ C57B/6 background. The 4 main animal models on which we are focusing include:

1. Hyplip *(chromosome 15 region from MRL on BALB/C) /*LDLR$^{-/-}$, (Richard Davis, Aldons Lusis)
2. Apolipoprotein AII transgenic apolipoprotein E$^{-/-}$, (Larry Castellani, Aldons Lusis)
3. *Elderly LDLR$^{-/-}$*, (Alan Collins, Rajendra Tangirala, Willa Hsueh)
4. *PPARγ skeletal muscle KO*/LDLR$^{-/-}$, (Rajendra Tangirala, Jerrold Olefsky, Willa Hsueh)

This order is based on the original specific aims of the grant application.

Major achievements have been:

Project 1: *Hyplip 2 (chromosome 15 region from MRL on BALB/C) /LDLR$^{-/-}$*. The *Hyplip2* locus was identified by quantitative trait locus analysis of the F2 intercross between two inbred strains, MRL/*lpr* and BALB/cJ. This locus (maps between markers D15Mit176 and D15Mit16 on chromosome 15) exhibited striking linkage to plasma cholesterol levels and triglyceride levels. In recently published work we described construction and characterization of the congenic strain, where the *Hyplip2* locus from the MRL strain was placed onto the background of BALB strain and was associated with atherosclerosis.

Project 2: *Apolipoprotein AII-transgenic*. We have observed that the apoAII transgenic affect is much more pronounced on the ApoE$^{-/-}$ background. This model has more atherosclerosis than our original ApoAII transgenic strain, and definitely has the potential to produce more severe, complex atherosclerotic lesions.

Project 3: Elderly LDLR $^{-/-}$ discovered that elderly (10-12 months old) LDLR -/- mice have a marked acceleration of the extent and histological severity of their atherosclerosis compared to young (3 month old) LDLR –/- mice following 3 months of a high fat (Western) diet. Despite similar weight gain, in the elderly mice appear to have higher glucose and insulin levels as well as worse oral glucose tolerance test responses, suggesting they have worse type 2 diabetes. The severity and complexity of the lesions

in the elderly mice resembles the same in humans with metabolic syndrome and type 2 diabetes. We are currently characterizing the development of lesions with time and trying to determine whether obesity and diabetes are the cause of the accelerated atherosclerosis in this exciting model.

Project 4: *PPARy skeletal muscle KO.* We are breeding these mice on a LDLR-/- C57B/6 background. We are now bereeding the F2 generation with the F3 generation to obtain mice with the desired genortype.

2. Collaboration within your group:

All of the Investigators at the UCLA site wortk closely together and share technology and phenotyping . Each group develops their own model. However, the Hsueh group (Collins, Tangirala, Lyon) performs en face analysis, uses LINCO-plex technology to measure insulin and circulating inflammatory biomarkers and other factors, while the Lusis group uses aortic root analysis for atherosclerosis quantitation performs the lipid and apolipoprotein analysis, and uses NMR for assessment of obesity. Dr. Hsueh's group also has metabolic cages and performs albumin, creatinine measurements, as well as other assessments of renal function.

3. Collaboration with other AMDCC groups:

1. We have sent MLC2VCRE crossed with PPARγ flox mice to Dale Abel, University of Utah.

2. We are planning on sending animals to Drs. Daneshgari and Feldman for urology and neuropathy assessment. However the UCLA vivarium had an extensive outbreak of parvo- virus, so we can only send animals when they are pathogen free.

4. Pertinent non-AMDCC Collaboration:

We are collaborating closely with Dr. Jerrold Olefsky, UCSD, on project 4 the skeletal muscle PPARγ knockout mouse.

Dr. Ronald Evans is also involved on that collaboration.

5. Address previous EAC comments:

- The previous review asked this group to focus down to 3-4 models. The October 2004 presentation showed that this group is now focusing further phenotyping on four models. One of the models, older LDLR-/- mice (C57BL/6 background) was particularly exciting as advanced lesions with necrotic cores, cholesterol clefts, and smooth muscle caps were clearly forming outside of the aortic root region. Although the plasma cholesterol levels must be quite high (>1,000 mg/dl) to generate lesions in the descending aorta, this model does hold promise of allowing study of advanced lesions in a milieu of diabetes.

We appreciate the reviewer's comments and have markedly expanded our investigation of the elderly LDLR$^{-/-}$ model. The 3 –4 models on which we are focusing include: 1) Elderly LDLR$^{-/-}$ on high fat diet. 2) APO A-II transgenic which causes dyslipidemia and insulin resistance bred into the LDLR$^{-/-}$. 3) Hyplip 2 (chromosome 15 region from MRL on BALB/C) /LDLR$^{-/-}$ resulting in dyslipidemia and hyperrlycemia and possible nephropathy. 4) PPARγ skeletal muscle KO which is an insulin resistant model bred LDLR$^{-/-}$.

- Phenotyping of lesions should include analyses following Movat's trichrome staining, and this procedure has been passed on to Dr. Hsueh and other investigators. This staining procedure, done on fixed tissue, allows simultaneous evaluation of lesion size, proteoglycan, collagen, and elastin contribution to artery wall structure. In addition, lesions moving into the matrix region are easily recognized, and this is opposed to simple lipid staining for which matrix involvement would be invisible.

We are staining aortic sections from young and elderly LDLR$^{-/-}$ with Movats. Results will be reported at the March, 2005 meeting.

- This group needs to move into careful assessment of diabetogenic phenotypes, using oral glucose tolerance tests and insulin sensitivity tests. IPGTT may become more difficult when working with fat animals, and the oral glucose administration is consistent, easy and provides reproducible values.

Oral glucose tolerance tests have been performed on the young vs. old LDLR$^{-/-}$ animals on 3months of a high fat diet and on the Hyplip/LDLR$^{-/-}$ mice. Results will be reported at the March meeting. In addition, we will obtain metabolic data (lipids, OGTT) after 1 and 2 months of the high fat diet in the young vs. elderly LDLR$^{-/-}$ and correlate these with lesion extent and complexity.

- Some results from drug tests show dissociation between lesion size and complexity, and diabetogenic parameters. For instance, mice fed chow versus mice fed chow and treated with GW3965 had similar lesions values, but the drug treated mice were much more insulin resistant, as evidenced by a 2.5-fold greater insulin to glucose ratio. The D4F mice had the lowest glucose, but similar insulin to glucose ratio to Rosiglitazone treated animals. What has this group learned about the relationship between diabetogenic phenotypes and atherosclerosis? Is it too soon to be applying drugs to the models?

We agree that we should extensively investigate the relationship between the diabetogenic phenotypes and atherosclerosis. We will not look at drug effects on the models.

- Young vs. Elderly mice show impressive differences in lesion development. More effort should be directed to how they differ metabolically. The regression studies with different pharmacotherapies, while interesting mechanistically, appear to be outside the scope of the consortium.

We agree that regression studies are beyond the scope of the consortium.

- More extensive phenotyping of the investigated models would be more appropriate. For example, different time points would be important to establish when the extent and complexity of the models begin to diverge from controls. Also, islet cell phenotyping (e.g., counting, morphology) will help determine the effects on insulin production.

We are currently phenotyping at more time points during the 3 months of the Western diet (1 and 2 months). Animals will also be submitted for evaluation of neurologic and urologic phenotypes. We will assess albuminuria in our laboratory. We have also entered discussion with investigators at our UCLA Hillblom islet Center to phenotype the β-cell; the center is led by Dr. Peter Butler.

- The complexity data is a little confusing. Does it summarize lesions that have necrotic cores, cholesterol clefts and fibrous caps or any of those things?

The complexity data includes lesions with either of the following: necrotic cores or cholesterol clefts both with fibrous caps, as suggested by Anna Marie Schmidt.

- The insulin levels in the elderly LDLR-/- mice seem surprisingly low for the high glucose levels. If the glucose levels are confirmed this may be an interesting observation because it could indicate an insulin secretory defect in these mice.

At the end of the 3 months of Western diet, the elderly animals had a fasting glucose nearly twice (285±25 vs. 423±27 mg/dl, $p<0.05$) *that of the young animals with a corresponding insulin that was also double that of the young animals (1199±150 vs. 2609±654 pg/ml, $p<0.05$).*

- AIN diets are of lower priority.

The AIN diet comparison is designed to provide equal cholesterol in a low vs. high fat background. If the animals on low fat diet have less atherosclerotic lesions compared to high fat, the obesity and metabolic syndrome accompanying the high fat diet could likely be considered as contributors to the atherosclerosis. Thus, the elderly model would fit the criteria of the AMDCC.

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Part B:

Update by Project Leaders

Project 1: *Hyplip2*: A mouse model of combined hyperlipidemia.

The *Hyplip2* locus was identified by quantitative trait locus analysis of the F2 intercross between two inbred strains, MRL/*lpr* and BALB/cJ. This locus (maps between markers D15Mit176 and D15Mit16 on chromosome 15) exhibited striking linkage to plasma cholesterol levels and triglyceride levels. In recently published work we described construction and characterization of the congenic strain, where the *Hyplip2* locus from the MRL strain was placed onto the background of BALB strain (1). Analysis of the constructed congenic strain, confirmed the previous QTL findings of *Hyplip2* locus contributing to plasma lipid levels in a cross between inbred strains BALB and MRL. The lipid profile controlled by the *Hyplip2* locus suggests that it may be relevant to FCHL. Our studies also revealed a major impact of this locus on atherosclerosis susceptibility. In addition, we have recently shown that the H*yplip2* congenic mice on a chow diet have elevated glucose levels as compared to BALB/c mice (see Figure).

Future studies of the CON15/LDLR$^{-/-}$ mice on a BALB/c background as a potential model for diabetes-related atherosclerosis and nephropathy.

It will be important to examine whether the glucose levels are determined by the same gene region as the altered lipid levels and increased atherosclerosis. Since male LDLR null mice on a Western diet become insulin resistant, it will be of interest to determine the effect of the CON15 locus (for example, there may be a synergistic interaction) on insulin resistance, atherosclerosis and diabetes associated phenotypes such as nepropathy. We obtained BALB.LDLR-/- mice from Dr. Renee LeBoeuf (Univ. of Washington) and used them to place *Hyplip2* congenic mice onto LDLR-/- background. We are in the final stages of breeding these mice to homozygosity and initiating these studies.

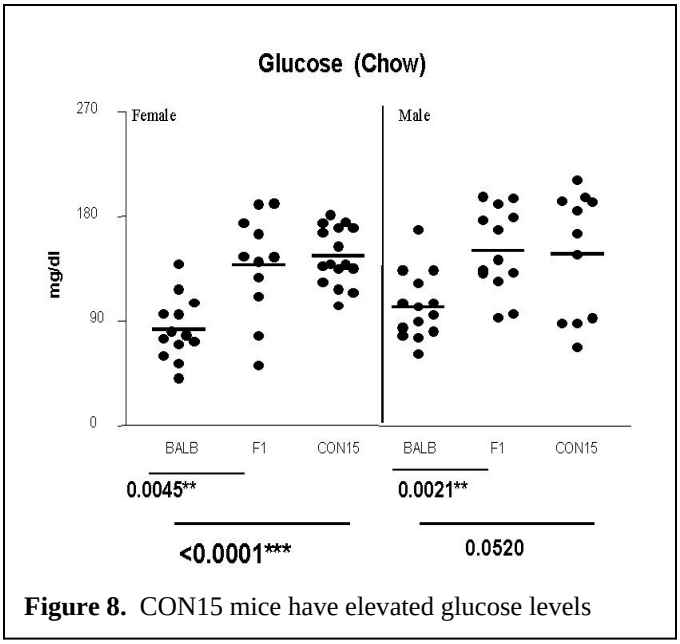


Figure 8. CON15 mice have elevated glucose levels

1. Wang, X., Gargalovic, P., Wong, J., Gu, J. L., Wu, X., Qi, H., Wen, P., Xi, L., Tan, B., Gogliotti, R., Castellani, L. W., Chatterjee, A., and Lusis, A. J. (2004) Hyplip2, a new gene for combined hyperlipidemia and increased atherosclerosis. *Arterioscler Thromb Vasc Biol* 24, 1928-1934

Project 2: Apolipoprotein A-II transgenic mouse model
Responsible Investigators: Lawrence W. Castellani and Aldons J. Lusis.

1. Rationale and Relevance:

Transgenic mice that overexpress mouse apolipoprotein A-II exhibit increased oxidative stress and increased atherosclerotic lesion formation. This mouse model also exhibits hyperlipidemia as well as insulin resistance and increased adiposity. Several human studies have also demonstrated that the apoAII gene has similar effects on lipids, adiposity, and atherosclerosis in humans. Thus, the apoAII transgenic mouse appears to be a relevant model to study atherosclerosis in the context of insulin resistance/diabetes.

2. Summary of Accomplishments:

1. In a cross between strains C57BL6 (B) and C3H (H), in which both inbred strains were on an apoE knockout (apoE ko) background, we demonstrated that the effect of apoAII on plasma lipids and glucose was markedly more severe on the apoE knockout background. This could potentially lead to a model with more severe, complex atherosclerotic lesions than those observed in the current apoAII transgenic strain.

2. We have made combined apoAII transgenic/apoE knockout mice and have confirmed that increased apoAII and the lack of apoE appear to act synergistically to produce a lipoprotein profile that is dramatically more atherogenic than in either the apoAII transgenic or apoE knockout mice alone.

3. We have demonstrated that the insulin resistance in the apoAII transgenic mice responds rapidly to treatment with Rosiglitazone. This is an important finding, not only because it suggests that the mechanism underlying the insulin resistance in the apoAII transgenic mice may share common features with certain types of insulin resistance in humans, but because it presents the possibility of assessing the contribution of the insulin resistance to the increased atherosclerosis observed in this model.

4. We have demonstrated defects in skeletal muscle fatty acid metabolism in the apoAII transgenic mice, which have also been associated with the development of insulin resistance in human skeletal muscle. These defects, at least in part, were reversed by Rosiglitazone treatment. While this finding does not directly relate to the atherosclerosis, it is important in that it further suggests that the mechanism of insulin resistance in this animal model may have relevance to insulin resistance in humans.

3. Plans for the coming year:

1. Assessing lesion development and insulin resistance in the apoAIIT/apoEko mice.

Apolipoprotein A-II (apoAII) transgenic mice that overexpress mouse apoAII become insulin resistant, obese, and exhibit accelerated atherosclerosis. 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. In a cross between strains C57BL6 (B) and C3H

(H), in which both inbred strains were on an apoE knockout (apoE ko) background, 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, in 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 (Figure 1).

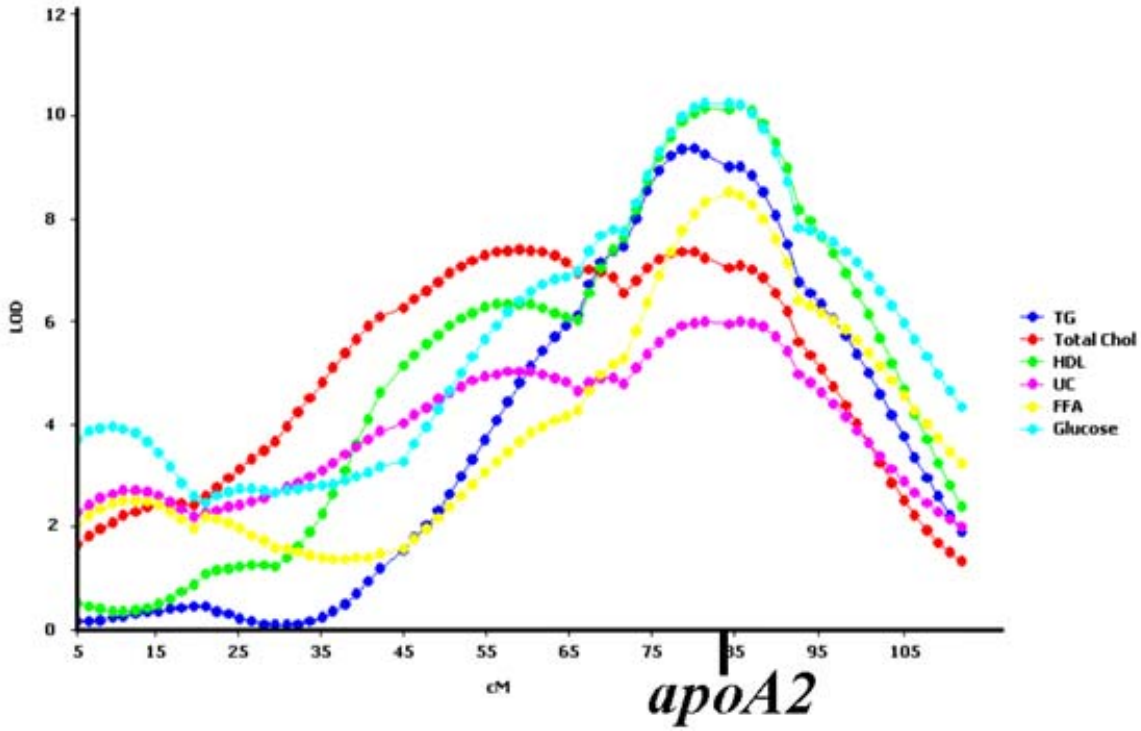


Figure 1. LOD scores for plasma lipids and glucose concentrations over the *apoa2* gene locus on mouse Chromosome 1. Genotyping by Marshfield (140 markers, 10cM map) and ParAllele (2,000 markers, 2 cM map) on 382 F2 mice (164 males, 164 females) from a cross between strains C57BL6 (B) and C3H (H), in which both inbred strains were on an apoE knockout (apoE ko) background. The mice were fed Western diet for 16 weeks and sacrificed at 24 weeks of age.

We previously reported that C3H mice exhibit a 50% increase in plasma apoAII concentrations compared to strain C57BL6. In the present cross, the C3H apoa2 allele was 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.

We now have the apoAII transgene on the apoE knockout background. Consistent with the data in the F2 cross described above, the apoAII transgene on the apoE knockout background has produced a markedly more atherogenic lipoprotein phenotype than that observed in either the apoAII transgenic or apoE knockout mice (Figure 2). As shown below, triglycerides were markedly increased in the combined apoAIIT/apoEko mice compared to the apoEko mice, and there was a trend towards an increase in triglycerides compared to the apoAIIT mice. Total cholesterol was markedly increased in the combined apoAIIT/apoEko mice compared to either

the apoAIIT or apoEko mice. This increase was primarlily in apoB containing lipoproteins since, the apoE null mutation significantly reduced the HDL cholesterol in the combined apoAIIT/apoEko mice compared to the apoAIIT mice. The lipid values presented are from very young mice (~6 weeks old) that had been maintained on a standard low fat mouse chow diet. The lipoprotein profile in the combined apoAIIT/apoEko mice appears to be dramtically more atherogenic than in either the apoAIIT or apoEko mice.

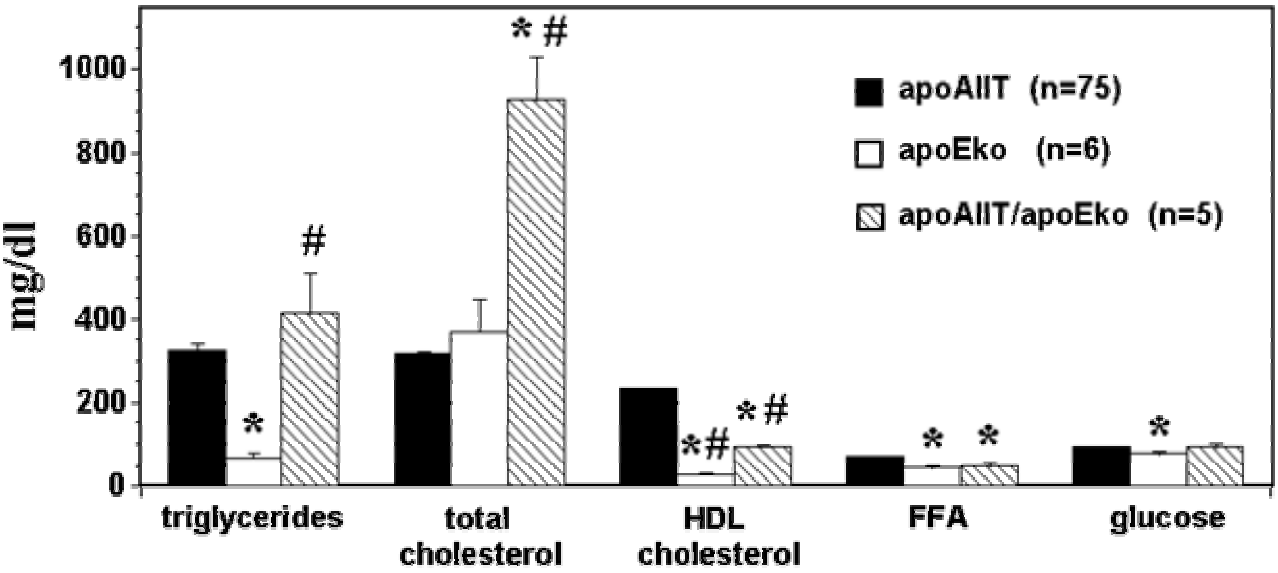


Figure 2. Fasting plasma lipid and glucose concentrations in apoAII transgenic (apoAIIT), apoE knockout (apoEko), and combined apoAII transgenic/apoE knockout (apoAIIT/apoEko) mice. All values are means ± SEM expressed in mg/dl. All animals were 6 weeks (± 1 week) of age and had been maintained on a low fat (4.5%) standard mouse chow diet. * denotes values that are significantly different ($p < 0.05$) than the apoAIIT mice. # denotes values that are significantly different ($p<0.05$) than apoEko mice.

We are currently assessing the extent and severity of lesions in the combined apoAIIT/apoEko mice, as well as the severity of the insulin resistance/diabetes that develops. In the upcoming year we will also determine the effect of thiazolidinedione treatment on lesion formation, to confirm that the insulin resistance is contributing to the lesion development in this model.

2. Assess renal function/disease in the apoAII transgenic mice.

In a Hispanic study population, we observed that plasma concentrations of apoAII, but not apoAI, are positively correlated with all of the lipid/lipoprotein traits as well as the insulin resistance that we observe in our apoAII transgenic mice (Figure 3). Interestingly, we also observed a highly significant correlation between apoAII (but not apoAI) with plasma creatinine concentrations.

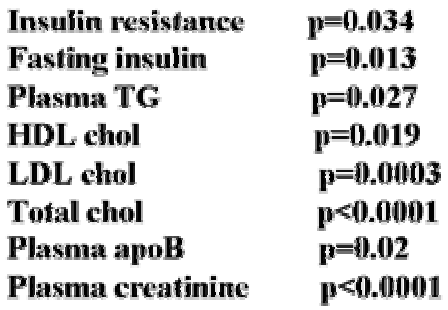


Figure 3. Plasma apoAII concentration correlations in a Hispanic study population of 390 members from 77 families of Hispanic descent. Plasma apoAII concentrations are positively correlated with several of the same traits that are affected by increasing apoAII in the transgenic mice.

A search of the literature revealed another human study in which plasma apoAII concentrations were highly correlated with plasma creatinine concentrations (Cerne *et al*., *Ren. Fail*., 22:799-808, 2000). In this study plasma apoAII concentrations also served as the best indicator of the degree of atherosclerotic lesion progression. The effect of apoAII plasma concentrations in the human studies described above, suggest that the apoAII transgenic mice could exhibit renal complications, in addition to the atherosclerosis and insulin resistance. We are presently collecting urine samples for analysis of creatinine and albumin in order to assess renal function/disease in these mice.

4. Most significant achievment:

We observed that the apoAII transgenic effects are much more pronounced on the apoEko background. This model appears to have more atherosclerosis than our original apoAII transgenic strain, and definitely has the potential to produce more severe, complex atherosclerotic lesions. During this year we will define the histology of the lesions and the mechanisms by which the lesions are accelerated.

Publications:

Castellani L.W., Gargalovic P., Febbraio M., Charugundla S., Jien M-L., and Lusis A.J. Mechanisms meditating insulin resistance in transgenic mice overexpressing mouse apolipoprotein A-II. *J. Lipid. Res*.,45:2377-2387, 2004.

Project 3: "The Elderly male LDLR-/- mice as a model of diabetes accelerated atherosclerosis"

Responsible Investigators: Willa A. Hsueh, Rajendra Tangirala, Alan Collins

1. Rationale and Relevance:

The majority of persons with diabetes-associated atherosclerosis are obese and present with pre-existing diabetes at diagnosis. The male LDLR-/- mouse administered a high fat diethas been shown to be a model of diet-induced obesity which develops diabetes similar to the progression of the disease in humans. The majority of patients also are diagnosed in their later life and have established atherosclerosis. The elderly model was developed in an attempt to more closely mimic the human disease. Previous studies in atherosclerosis were performed on young mice between 1-3 months of age at the beginning of treatement and 3-6 months of age at the end of treatment. These mice would be the equivalent of children and adolescence between the ages of 2-10 years of age and while useful in elucidating mechanisms in the development of atherosclerosis did not address the majority of patients who present with established and pathologically advanced lesions elderly $LDLR^{-/-}$ develop worse diabetes in response to high fat diet and as a consequence possibly more severe atherosclerosis.

2. Summary of Accomplishments:

We demostrated that elderly male LDLR-/- mouse at 15 months of age have more severe diabetes than younger control animals at 6 months of age after 3 months of western diet. The elderly mice were also more obese. Although their weight gain was similar to younger animals. Additionally, the elderly mice develop 4-5 times more lesions having equivalent plasma cholesterol levels with the lesions being more advanced and complex in histopathology, with necrotic lipid cores, fibrous caps, cholesterol clefts and proteoglycan-rich cores. These lesions represent the type found in the diabetic patient and are the form that various pharmacological treaments must take into account. Data is summarized in the accompanying tables.

3. Plans for the coming year:

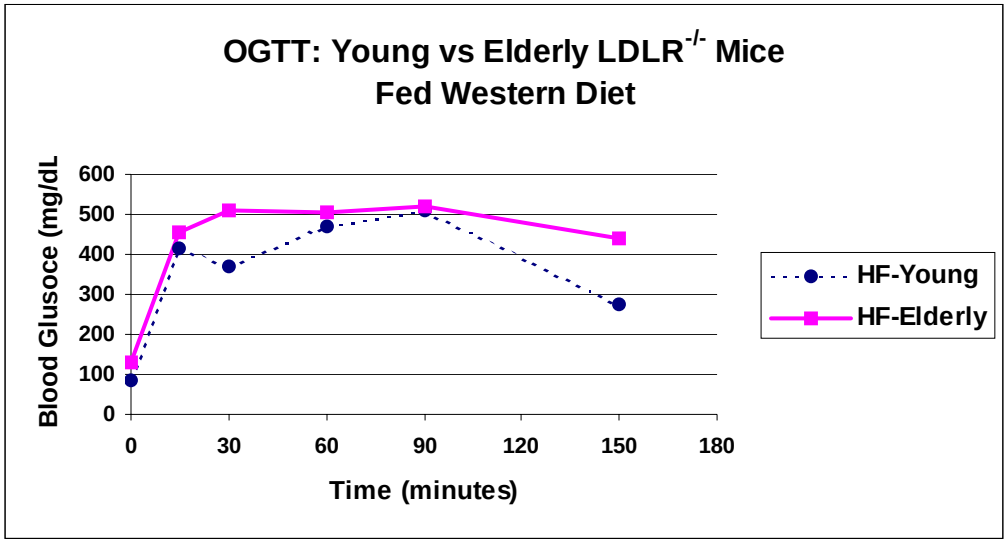
We will continue to more extensively phenotype these mice metabolically and determine the realtionship of the development of the atherosclerotic and diabetic pathologies. This includes disease development over time, collecting samples monthly beginning with baseline data on both the elderly and young control mice. We will assess glucose and insulin tolerance for the determination of the progression of insulin resistance and diabetes. Additionally, we will perform 24 hour urine colections to determine if microalbuminuria is present. At the monthly sacrifices, we will collect aorta, heart, liver, kidneys and any other tissues which may be of interest to other members of the AMDCC. We will be collaborating with another laboratory here at UCLA to look at the β-cell pathology and function in these mice.

4. Most significant achievement:

The development of a novel model of advanced atherosclerosis associated with more severe diabetes. The elderly male LDLR-/- mouse is a much better model of atherosclerosis associated with type 2 diabetes than those previously available as they are grossly obese with moderate to sevre diabetes and established lesions that are histologically advanced.

Data Summary

Time minutes	HF-Young glucose(mg/dl)	HF-Elderly glucose(mg/dl
0	86	130
15	413	455
30	371	510
60	472	506
90	511	518
150	277	440



Publications:

Abstracts

Collins, A.R., F. Blaschke, M.C. Fishbein and W.A. Hsueh. Inhibition of the renin-angiotensin system causes regression of complex pre-existing atherosclerotic lesions in elderly LDLR-/- mice. *Circulation* 2004

Collins, A.R., L. Castellani, and W.A. Hsueh. The Elderly LDLR-/- Mouse is a Model of Diabetes – Accelerated Atherosclerosis. *Diabetes*, 2004

Project 4: *"PPARy skeletal muscle KO."*

Responsible Investigators: Rajendra Tangirala, Jerrold Olefsky, Willa A. Hsueh,

1. ***Rationale and Relevance:***

The PPARγ skeletal muscle knockout mouse has been previously shown by Jerrold Olefsky to be a model of insulin resistance leading to type 2 diabetes. This condition develops spontaneously as the mice age and does not involve special diets or lead to hypercholesterolemia. We believe that the incorporation of this model of insulin resistance and type 2 diabetes will lead to increased atheroslerosis when bred into an atherosclerosis prone mouse model (LDLR-/-).

2. ***Summary of Accomplishments:***

We have obtained mice containing the desired genotype and are breeding sufficient numbers to begin experiments this year. The combination and screening for 3 separate genes proved to be time consuming but the mice are becoming available.

3. ***Plans for the coming year:***

We will be phenotyping these mice metabolically and determine the development of atherosclerotic pathologies. This includes disease development over time, collecting samples monthly beginning at 2 months of age and continuing to 1 year. We will assess glucose and insulin tolerance for the determination of the progression of insulin resistance and diabetes.

4. ***Most significant achievement:***

Devlopment of a murine model of atherosclerosis associated with skeletal muscle insulin resistance similar to found in the typical diabetic patient.