ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM (U01 HL70525)

UPDATE REPORT (January 2004 –February 2005)

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PART A:

PRINCIPAL INVESTIGATOR'S SUMMARY

Program Accomplishments:

The University of Utah site of the Animal Models of Diabetic Complications Consortium has the aim of developing models for diabetic cardiomyopathy and other forms of diabetic cardiovascular disease. Within this theme we have focused on defining and phenotyping mice and defining the standards for diabetic cardiomyopathy using several different animal models of diabetes:

- Models of type 2 diabetes wherein the disease is largely triggered by excess nutrient delivery to tissues. Two of these models are ones in which the basic defect is in the regulation of appetite and energy expenditure based on the absence of functional leptin and leptin receptor (*ob/ob* and *db/db* mice). The other model is one with genetic ablation of brown fat, leading to decreased energy expenditure, insulin resistance, and subsequent diabetes.
- Models with impaired insulin signaling specifically targeted to the heart. This approach is based on the well accepted idea that insulin resistance is central to the development of type 2 diabetes, and that insulin resistance, even before the onset of overt diabetes, is a significant risk factor for cardiovascular disease. These specific models include: the CIRKO mouse with targeted deletion of the insulin receptor in the heart; and a mouse with a dominant negative PI3-kinase targeted to the heart, PI3-kinase being a central mediator of many aspects of downstream insulin signaling.
- Models with defective glucose transport, specifically a mouse with cardiac-specific knockout of the GLUT4 transporter (G4H-/-). This animal model mimics one of the central features of type 2 diabetes, namely the downregulation and insulin resistance of cellular glucose uptake.

Our approach has been to perform detailed phenotypic analysis of these models at the level of cardiac function in the intact animal, in the isolated heart, and in isolated cells and tissues to examine cell signaling, mitochondrial function, and gene expression profiling. These studies have determined that like the human disorder, the cardiac phenotypes of the diabetic heart may be subtle. However, the response of the heart to stress is usually impaired. Our observations to date support the paradigm that in the hearts of animals with insulin resistance, obesity and type 2 diabetes there is downregulation of insulin signaling and progressive mitochondrial dysfunction that develops in the heart. We believe that impaired insulin signaling and nutrient excess, particularly increased fatty acid uptake and metabolism play distinct roles in the pathogenesis of the diabetic cardiomyopathy. We are therefore now developing models that will allow us to test the interaction between altered insulin signaling and increased fatty acid flux into the myocyte.

SUMMARY OF ACHIEVEMENTS IN PRIOR REPORTING PERIOD (2001-2004):

(1) Development and validation of standardized criteria for diabetic cardiomyopathy
(2) Development of standardized definitions for determining glucose tolerance testing and the determination of insulin resistance and hyperinsulinemia in various strains of animals.

(3) We have extensively characterized ob/ob and db/db mice. We have shown that db/db mice are severely hyperglycemic by 6 weeks of age, while ob/ob mice develop equivalent degrees of hyperglycemia approximately 10 weeks later (age 15 weeks). Despite these differences, there are discernible differences in cardiac function as early as 4- weeks of age in both models at a time when they are hyperinsulinemic and insulin resistant and not yet diabetic. The major change is increased oxygen consumption, increased fatty acid utilization decreased glucose utilization and myocardial insulin resistance. These abnormalities become progressively more severe as a function of the duration of hyperglycemia. An important mechanism that accounts for these phenotypes is changes in mitochondrial function; specifically reduced activity of electron transport chain complexes and mitochondrial uncoupling. These changes result in decreased ATP production that limits myocardial energetic reserve.

(4) The association of insulin resistance with cardiac dysfunction in ob/ob mice provided in part the impetus to determine if myocardial insulin resistance per se could account for many of the defects observed in the hearts of mouse models of insulin resistance and type-2 diabetes. We therefore examined the cardiac phenotype of mice with cardiomyocyte-restricted deletion of insulin receptors (CIRKO). We found that these mice also developed a switch in mitochondrial substrate utilization (increased FA oxidation, decreased glucose oxidation) that was associated with progressive mitochondrial dysfunction that was due in part to increased production of superoxide. Moreover we also demonstrated that CIRKO mice exhibited increased injury following hemodynamic stressors such as hypertrophy and ischemia.

Thus our working model is that diabetic cardiomyopathy is due in part to abnormal insulin action and increased FA flux that precipitates/exacerbates mitochondrial dysfunction. Mitochondrial dysfunction limits ATP production, which limits myocardial energetic reserves. These abnormalities might not necessarily be sufficient to impair myocardial contractility under basal conditions but may account for the increased susceptibility to cardiac dysfunction in the face of hemodynamic stress such as ischemia and hypertrophy. Thus, future models will seek to determine the specific component of the insulin signaling pathway that is responsible for this as well as the interaction between impaired insulin signaling and increased fatty acid flux into the heart.

SUMMARY OF ACHIEVEMENTS IN THE PAST 12 MONTHS

We have extended our observations in ob/ob and db/db mice by determining glucose tolerance, insulin concentrations and in vivo cardiac function in a cohort of 40 week-old mice. Both groups of animals are severely hyperglycemic at this age. Cardiac function remains relatively preserved as measured by dP/dt. However, it should be noted that this represents a decline in function relative to younger animals, which actually exhibit higher dP/dt than controls at younger ages (<10 weeks of age). We have also further investigated the mechanism for mitochondrial dysfunction in ob/ob and db/db mouse hearts by examining protein expression of various components of the electron transport chain. We observed significant reductions in the protein content of various components of the mitochondrial electron transport chain of ob/ob mice.

We have further characterized the mechanism for increased myocardial injury in CIRKO hearts in response to hypertrophic stimuli. We speculated that the pattern of injury that we observed in CIRKO mice in response to hypertrophic stimuli was due in part to myocardial ischemia. We therefore examined capillary density in CIRKO mice following 5-day exposure to isoproterenol. There was a >60% reduction in capillary density in hypertrophied CIRKO hearts relative to hypertrophied controls. To investigate potential mechanisms for these changes we determined the expression levels of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS). Interestingly, hypertrophy was associated with an increase in VEGF and eNOS expression in wildtype hearts, but not in CIRKO hearts. We therefore conclude that one of the mechanisms by which insulin resistance leads to increased myocardial injury in response to hypertrophy is failure of release from cardiomyocytes of mediators that promote angiogenesis and maintain vascular integrity.

To begin to model the interaction between increased FA flux to the cardiomyocyte and impaired insulin signaling we have generated a colony of CIRKO mice with modest overexpression of Acyl CoA synthase. We are now expanding this colony and plan on characterizing this model further in the coming year. We have also decided to characterize in depth the cardiac phenotype of a mouse model of type-1 diabetes. The rationale for this decision is that: (a) many of the platforms that are being used to model other diabetic complications within the consortium, such as nephropathy are based on models of insulin deficiency as opposed to insulin resistance. (b) It is possible that although the underlying pathophysiology of diabetic cardiomyopathy in type-2 diabetes may share some similarities to the pathophysiology of type-1 diabetes, it is also likely that important differences may exist. Thus we have established a colony of Akita mice (a dominant model of type-1 diabetes on the C57BL6 background) and will characterize it in detail in a manner analogous to our efforts with ob/ob and db/db mice.

Interrelationships of projects:

All of the projects that are described in this report were conducted at the University of Utah in the laboratories of the PI, Dr. McClain and the co-investigators, Drs Abel and Litwin. Thus although the projects are described as separate projects in reality they represent a truly collaborative effort of all of the investigators involved.

Collaborations with other Groups (Including Core Facilities):

PRIOR: (1) Development of a mouse model with podocyte selective KO of the GLUT4 glucose transporter in collaboration with the Michigan group (PI Brosius). (2) Phenotyping of mice with cardiomyocyte-selective deletion of the PPARγ transcription factor in collaboration with the UCLA group (PI Hsueh).

CURRENT: (1) Phenotyping mice with altered expression (over-expression or KO) of lipoprotein lipase in the heart. We are specifically determining myocardial substrate utilization and oxygen consumption in these hearts. These animals were generated in

the laboratory of Dr. Ira Goldberg (Columbia University), who is a part of the Rockefeller/NYU/Columbia group. (2) Histological analysis of hearts obtained from pigs with type-2 diabetes and insulin resistance (UNC- Chapel Hill –Nichols).

Pertinent non-AMDCC Collaborations:

(1) We have collaborated with Daniel Kelly (Washington University in St. Louis) to characterize the mitochondrial phenotype of PGC-1 alpha KO mice. PGC-1 may play a role in the maturation/maintenance of cardiac mitochondria (2) We have collaborated with Robert Lane MD in the Department of Pediatrics (Neonatology) at the University of Utah to characterize the mitochondrial and myocardial phenotypes of rats with intrauterine growth retardation (IUGR). IUGR might be an important predisposing risk factor for the development of type-2 diabetes and the metabolic syndrome later in life.

Response to EAC Comments:

- The investigators provide a good platform to investigate other models for cardiac metabolism. Other models of diabetes should be sent for myocardial phenotyping. Of particular interest are the decorin -/- animals from Mt Sinai. Indeed, the Utah Center could offer a core service to perform these studies since it is not duplicated within the consortium. Alternatively, Utah members could travel to other sites to establish certain injury models and phenotyping measurements.

Response: We agree. We are willing to phenotype the hearts of other models as they become available, and are happy to provide a core cardiovascular phenotyping service for the consortium. Because the phenotyping involves specialized equipment, techniques and protocols, the most feasible approach will be to have mice sent to us. Because many of the other complications are based in type-1 diabetes models we have initiated a program to analyze the cardiac phenotype of the Akita mouse on the C57Bl6 background, as this represents a robust model of type-1 diabetes.

- Evaluation of myocardial function should continue with older animals, paying specific attention to insulin sensitivity and glucose measurements to correlate with function.

Response: We agree. We have now analyzed the cardiac phenotypes of 40-week-old ob/ob and db/db mice. These results are included in this report.

- Work by this group is exciting. However, reservations in using ob/ob and db/db mice remain, as these are such extreme examples of obesity and insulin resistance. Background strain is crucial, and long term studies of male db/db-C57BLKs mice will not be possible due to diabetes onset within about 3-4 months.

Response: We are cognizant of this concern. For this reason we will evaluate the mitochondrial phenotype of 2 models of type-2 diabetes that are less severe. These models are the UCP-DTA mouse, and high-fat diet induced obesity and diabetes. These studies are underway. The UCP-DTA mice are on the FVB background. The high-fat

diet studies are being performed on the C57BL6 background. In addition we believe that it is important to evaluate a model of type-1 diabetes. Studies are therefore underway to perform detailed phenotyping on Akita mice.

- Could mitochondrial function be altered in cardiomyocytes of the obese/insulin resistant animals?

Response: This is possible. We will address this question in the high-fat feeding model.

- Data shown indicates that capillary growth lagged behind. Was this also observed in the retina? Evaluate with Tim Kern.

Response: We have shown an impaired vascular response to cardiac hypertrophy in insulin resistant mouse hearts. Whether or not similar processes are present in the retina of these animals is not known. We will discuss with the Kern group the protocols for examining this question in the eyes of ob/ob and db/db mice. If this can be performed on mice that we currently have, then we will send the eyes to Dr. Kern. If procedures require live animals (e.g. fluorescein angiography), then it would be more feasible for db/db and ob/ob mice to be directly obtained by Dr. Kern.

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PART B:

UPDATE BY PROJECT LEADERS

Responsible Investigators: **Donald A. McClain, M.D., Ph.D.**

Project 1: Standardization of methods for determining insulin resistance and impaired glucose tolerance in mice, and characterization of glucose homeostasis in ob/ob and db/db mice as a function of age.

A. Rationale and Relevance:

Type-2 diabetes represents the major clinical burden in terms of cardiovascular complications of diabetes. Therefore relevant animal models need to be developed and or characterized that address the pathogenesis of heart disease in type 2 diabetes and insulin resistant states. Therefore, establishing standards for determining the presence of insulin resistance, diabetes and glucose intolerance are important pre-requisites that must precede the detailed analysis of relevant mouse models.

B. Summary of Accomplishments

In the 2001-2004 report we summarized our efforts to standardize glucose tolerance testing and presented our protocols for euglycemic hyperinsulinemic clamps to determine insulin sensitivity. We also presented data on glucose tolerance testing and insulin concentrations in ob/ob and db/db mice as a function of age from age 4 –15 weeks. We have now analyzed a cohort of mice at age 40-42 weeks. This was of importance because of the experience of other groups that noted an amelioration of diabetes in some lines of db/db mice.

Glucose Homeostasis in ob/ob and db/db mice - 2 Mouse Models of obesity insulin resistance and type 2 diabetes.

As shown in **Table1**, db/db mice remained severely hyperglycemic and hyperinsulinemic at 40-weeks of age. Glucose tolerance tests for 40-week old ob/ob mice are in progress and are not available as of the writing of this report**.**

C. Plans for the coming year

This analysis of ob/ob and db/db mice is close to being completed. The cardiac phenotype of these older animals will be described in another section.

D. Most significant achievement.

These studies are largely descriptive, but provide an important context for all studies that will be performed in these models

Publications

The glucose tolerance data and the gene expression studies are included in a manuscript that details the age dependent changes in cardiac function, substrate

metabolism and gene expression that develop in ob/ob and db/db mice. This manuscript is currently in revision and will be resubmitted shortly.

Aspects of this work have been presented at national meetings.

Chakrabarti G, **Abel ED**. Differential contributions of insulin resistance versus hyperglycemia to the transcriptional changes that characterize the diabetic heart. Diabetes 2003; 52, Suppl. 1 A285

Presented at 63rd annual scientific session of the American Diabetes Association 2003

Tables and Figures for Project 1.

TABLE 1: Glucose and insulin concentrations in 40-week-old db/db mice.

	BW	Glucose		Insulin	
		0 Min	30 Min	0 Min	30 Min
Db/db	59±3	564±7	>600*±0	1.6±0.2	2.1±0.3
Control	27±0.5	99±3	152±3	0.15±0.04	0.30±0.03

* Maximum Value of glucometer

Responsible Investigators: **Sheldon E. Litwin MD**

Project 2: Characterization of the in vivo cardiac function in ob/ob and db/db mice.

A. Rationale and relevance

Diabetic heart disease is an ill-defined entity sometimes referred to as "diabetic cardiomyopathy." Unfortunately, there has been little consistency amongst different investigators as to the criteria which constitute this disorder. Moreover, there has been significant variability in findings between different models of diabetes and confounding factors such as the degree of hyperglycemia, insulin resistance, obesity and hypertension have not routinely been controlled for. A number of groups have suggested that diabetes causes diastolic dysfunction and in later stages left ventricular hypertrophy and systolic impairment. However, agreement on these findings is weak. The lack of consensus and consistency probably hinges at least in part on the fact that cardiac changes in diabetes are dynamic and are probably related at least in part to the stage of the disease and the presence of other confounding conditions. To begin resolving some of these issues, we analyzed cardiac function by invasive LV catheterization and echocardiography in ob/ob and db/db mice at time points when they were either: hyperinsulinemic and insulin resistant, but not overtly diabetic (4 weeks); or, insulin resistant and more severely diabetic (8 weeks for db/db mice and 15 weeks for ob/ob mice. We have now extended these studies out to 40-week-old mice.

B. Summary of Accomplishments

Our methods that we use for in vivo hemodynamic monitoring of mice were summarized in the previous report and are also posted on the AMDCC web site.

<u>Findings</u>:

The results of invasive hemodynamic analyses are summarized in **Tables 2 and 3.**

Table 2

	ob/ob				WT			
Age	4 (10)	8 (9)	15 (3)	40 (5)	4 (9)	8 (9)	15 (4)	40 (5)
BW	30±1*	48±1*	59±1*	71±3*	19±0.3	24±0.6	30±1	29±1
HW	107±5	160±7*	155±3	158±8*	109±2	130±5	143±9	133±3
HR	531±18	460±39	500±40	396±31	505±34	413±47	525±29	432±22
LVSP	97±5*	95±4	112±6*	119±7*	84±1	88±3	94±3	103±6
LVEDP	8±1	13±2	10±3	5±2	8±1	8±1	9±3	5±1
+dP/dt	9834±1049*	8300±943*	11916±886*	6800±429	6727±381	6158±560	8023±578	6488±639
-dP/dt	-7697±765*	-6635±674	-8725±1031	-5328±447	-6141±341	-6297±425	-7286±283	-6380±605

Data are means±SE. *p<0.05 versus age-matched controls.. Age- Weeks, (number of animals are in parenthesis), BW – Body Wt. (g), HW- Heart Wt. (mg), HR- Heart Rate, LVSP- LV Systolic Pressure (mmHg), LVEDP- LV end diastolic pressure (mmHg), +dP/dt- First derivative of LV contraction (mmHg/s), -dP/dt- First derivative of LV relaxation (mmHg/s).

Table 3

	db/db				WT			
Age	4 (8)	8 (8)	15 (6)	40 (4)	4 (6)	8 (9)	15 (5)	40 (4)
BW	28±0.4*	44±1*	50±1*	61±3*	21±1	26±0.3	27±1	27±1
HW	108±2	142±4	142±2	156±6	114±2	136±3	137±5	145±2
HR	480±14	491±13	410±18	390±17	387±26	460±18	492±29	450±52
LVSP	99±4	104±4*	91±4	122±8	90±4	91±2	99±3	115±4
LVEDP	16±2*	14±2	6±3	4±2	9±1	6±2	10±4	4±1
+dP/dt	9760±694*	9241±513*	7226±686	8154±454	6339±715	7148±285	8691±1002	8013±1005
-dP/dt	7144±517*	6820±376	-5327±448*	-6654±425	-5572±637	-6626±307	-7815±445	-7857±958

Data are means±SE. *p<0.05 versus age-matched controls, (numbers of animals are in parenthesis). Abbreviations and units are the same as table 2.

The key observations are that ob/ob and db/db both mice exhibit increased dP/dt during the phase of insulin resistance. However, after hyperglycemia ensues a decline in dP/dt is observed. It should be noted that there is a time lag between the onset of hyperglycemia and the reduction in dP/dt, which is evident by 15-weeks of age in db/db mice and by 40 weeks of age in ob/ob mice. The increase in dP/dt in the younger ob/ob and db/db mice likely represents an adaptation to the increased plasma volume that occurs in obesity. Thus the reduction in dP/dt in older animals, when viewed in the context of the progressive increase in body weight is a likely manifestation of progressive LV dysfunction. Ob/ob mice tended to have higher systolic pressures and to exhibit cardiac hypertrophy while db/db mice did not. There was no consistent evidence to support diastolic dysfunction in these models.
The results of echocardiography are shown in **Tables 4 and 5**.

Table 4

	ob/ob				WT			
Age	4 (16)	8 (16)	15 (11)	40 (5)	4 (10)	8 (12)	15 (8)	40 (5)
LVDd	0.33±0.01*	0.37±0.01	0.43±0.01	0.43±0.05	0.26±0.01	0.35±0.01	0.41±0.02	0.42±0.02
LVDs	0.19±0.01*	0.23±0.01*	0.30±0.02	0.32±0.05	0.14±0.01	0.19±0.01	0.28±0.02	0.29±0.03
IVSd	0.09±0.00	0.1±0.00*	0.1±0.01*	0.08±0.01	0.1±0.01	0.09±0.00	0.07±0.00	0.08±0.01
LVPWd	0.08±0.01	0.09±0.01	0.07±0.01	0.07±0.01	0.1±0.01	0.08±0.00	0.07±0.00	0.08±0.01
%FS	43±2	40±1*	31±2	28±3	47±3	46±2	33±2	31±3
LVEF	0.81±0.02	0.78±0.02*	0.67±0.03	0.62±0.05	0.84±0.03	0.84±0.01	0.69±0.03	0.66±0.04
Mass	0.092±0.00	0.14±0.01*	0.142±0.01*	0.122±0.02	0.091±0.01	0.108±0.00	0.102±0.01	0.124±0.01
LVOTVi	3.5±0.2	3.9±0.2*	3.6±0.2	3.4±0.3	3.7±0.1	3.3±0.1	3.4±0.2	3.3±0.2
HR	511±19	525±17	444±15	449±35	540±18	541±28	461±12	394±15
Calc. CO	13.9±0.6	16±0.9	12.5±0.9	11.7±1.3	15.7±0.6	13.9±0.7	12.5±0.7	10.2±0.4

Data are means ±SE. SE are rounded to 2 decimal places thus a SE of <0.005 is entered as 0.00. * p<0.05 versus age matched controls. Age-weeks (numbers of animals are in parentheses). LVDd-LV diastolic diameter (cm), LVDs-LV systolic diameter (cm), IVSd-Interventricular septum thickness in diastole (cm), LVPWd-LV posterior wall thickness in diastole (cm), %FS-percent fractional shortening, LVEF-LV ejection fraction, Mass-LV mass (g), LVOTVi –LV stroke volume index (cm), HR- Heart Rate, Calc. CO – Cardiac Output (ml/min).

Table 5

	db/db				WT			
Age	4 (10)	8 (10)	15 (6)	40 (5)	4 (6)	8 (10)	15 (5)	40 (4)
LVDd	0.23±0.01	0.37±0.02	0.37±0.01	0.42±0.02	0.28±0.01	0.32±0.02	0.4±0.01	0.40±0.01
LVDs	0.13±0.02*	0.22±0.02	0.18±0.02	0.29±0.03	0.19±0.01	0.22±0.02	0.23±0.02	0.28±0.01
IVSd	0.13±0.01	0.15±0.08	0.09±0.00	0.09±0.01	0.13±0.01	0.12±0.01	0.08±0.01	0.09±0.01
LVPWd	0.13±0.01	0.06±0.00*	0.07±0.00	0.07±0.00	0.13±0.01	0.13±0.01	0.07±0.00	0.08±0.01
%FS	44±4	40±3	50±3	33±5	35±2	34±2	43±3	30±2
LVEF	0.81±0.04	0.77±0.04	0.87±0.02	0.68±0.06	0.72±0.03	0.7±0.03	0.81±0.03	0.66±0.03
Mass	0.112±0.01*	0.081±0.01*	0.095±0.01	0.132±0.01	0.15±0.01	0.167±0.01	0.110±0.01	0.13±0.01
LVOTVi	4.2±0.3	4.5±0.3	na	4.3±0.2*	4.5±0.4	4.4±0.3	3.5±0.2	3.6±0.2
HR	472±9	511±19*	na	430±27	480±17	376±25	446±14	445±13
Calc. CO	15.5±1.1	18.3±1.3*	na	14.3±0.6	17±1.8	12.6±0.8	12.3±0.7	12.5±0.7

Legend is the same as table 4. na data were lost for technical reasons. Repeat studies will be performed.

Overall, echocardiographic changes in ob/ob and db/db mice were not strikingly different from controls with the exception of increased LV mass (8 and 15-wk) and evidence for LV dilatation (4 and 8-wk). It should be noted that the relatively normal cardiac output and stroke volumes in ob/ob and db/db mice could be viewed as being abnormal in relation to the increased body mass and increased blood volume (on the basis of increased adipose tissue mass) in these animals.

C. Plans for the coming year

In light of the data obtained in these models indicating increased MVO_2 in isolated hearts as well as evidence of mitochondrial dysfunction we predict that differences in cardiac function in these models will be most striking in the presence of hemodynamic stress. Therefore, we are in the process of subjecting these hearts to pressure overload hypertrophy by transverse aortic ligation. The second goal is to characterize the in vivo cardiac phenotype of a model of type-1 diabetes, the Akita mouse, which is no confounded by the presence of obesity.

D. Significant Achievement

These studies represent the first detailed in vivo hemodynamic analysis of cardiac function in ob/ob and db/db mice in the evolution from insulin resistance to overt diabetes, and as a function of age. The studies are significant because they have defined early hemodynamic changes that may reflect the impact of obesity and insulin resistance on cardiac function. They also underscore the fact that the in vivo phenotypes of the hearts of diabetic rodents are subtle. We expect however to see more severe evidence of cardiac dysfunction in the context of stressors such as myocardial ischemia and hypertrophy.

Publications:

These in vivo hemodynamic data are included in a manuscript that details the age dependent changes in cardiac function, substrate metabolism and gene expression that

develop in ob/ob and db/db mice. This manuscript is currently in revision and will be resubmitted shortly.

An abstract of this work has been presented at national meetings.

Litwin SE, Hu P, Cooksey RC, Zhang D, Swenson L, **McClain DA**, **Abel ED.** Diastolic abnormalities in db/db mice precede the development of hyperglycemia. Diabetes 2003; 52, Suppl. 1 A389

Presented at 63rd annual scientific session of the American Diabetes Association 2003

Responsible Investigators:	**E. Dale Abel MD Ph.D.**

Project 3: Characterization of cardiac function, substrate metabolism and mitochondrial function in the hearts of ob/ob and db/db mice.

A. Rationale and Relevance

Cardiac dysfunction is present in many rodent models of diabetes. Most published studies have been performed in models of type 1 or insulin deficient diabetes. Less is known about the pathophysiology of cardiac dysfunction in the hearts of mouse models of insulin resistance, obesity and type 2 diabetes. The goal of these studies was to determine the mechanisms that are responsible for cardiac dysfunction in two mouse models of insulin resistance the ob/ob and the db/db mouse. Characterization of the molecular cardiac phenotypes of these mice will yield targets that will aid in the design of genetically manipulated mice that accurately model diabetic cardiomyopathy in insulin resistant states.

B. Summary of accomplishments

Most of these data were presented in the previous report (2001-2004) and can be summarized as follows:

Isolated Working Hearts: FA oxidation rates and MVO_2 are increased while cardiac efficiency and glucose utilization are reduced. These changes occur as early as 4-weeks of age and precede the onset of diabetes. Long chain Fatty Acyl CoA content is increased.

Langendorrf Perfused Hearts: The increase in MVO_2 in ob/ob and db/db hearts is FA dependent. Inotropic responses to increased perfusate calcium are reduced.

Permeabilized Fibers and Mitochondrial Studies: Mitochondrial electron transport flux is reduced. Mitochondria are uncoupled and ATP production is reduced. Mitochondrial uncoupling is exacerbated in hearts that are exposed to fatty acids. Mitochondrial proliferation occurs.

In the past year we have further characterized the mechanisms that are responsible for mitochondrial dysfunction in these models. We have determined the protein content of various electron chain complexes. As summarized in **Figure 1** the content of multiple electron transport chain complexes are reduced in mitochondria isolated from 8-week-old ob/ob mice. In contrast, these differences were not observed in db/db mice **Figure 2**. However, we believe that the major mechanism for mitochondrial dysfunction in db/db mice is increased mitochondrial uncoupling that occurs on the basis of overproduction of reactive oxygen species **Figure 3.** To further characterize the possibility that oxidative damage is occurring in the hearts of db/db mice we also assayed for the presence of the protein adduct hydroxy nonenal (HNE), which is generated by lipid peroxidation. We also observed a modest increase in the expression of MnSOD, which represents an adaptation to increased mitochondrial superoxide production **Figure 4**.

C. Plans for the coming year
We will now focus on the mitochondrial phenotypes of Akita mice, which is a model of type-1 diabetes, as well as mouse models with less severe insulin resistance and obesity such as mice with diet induced obesity and insulin resistance and the brown adipose tissue deficient, UCP-DTA mouse that develops insulin resistance and the metabolic syndrome, but in which diabetes is a late phenotype.

D. Significant achievement and its importance
These observations are significant because they elucidate the mechanisms that are responsible for mitochondrial dysfunction in the diabetic heart and provide a clear mechanism for cardiac dysfunction and an impaired ability to respond to stress that characterizes the hearts of diabetic animals.

Publications

Mazumder PK, O'Neill BT, Roberts MW, Buchanan J, Yun UJ, Cooksey RC, Boudina S, **Abel ED.** (2004) Impaired Cardiac Efficiency and Increased Fatty Acid Oxidation in Insulin Resistant *ob/ob* mouse hearts. 2004: Diabetes. 53: 2366-2374

Two additional manuscripts describing the mitochondrial phenotypes of ob/ob and db/db mice are in advanced states of preparation and will be submitted for publication this year.

Aspects of this work have been presented at National Meetings.
1. Mazumder PK, Graveleau C, Boudina S, Belke DD, **Abel ED**. Metabolic characterization of the insulin resistant mouse heart. Diabetes 2003; 52, Suppl. 1 A300

Presented at 63rd annual scientific session of the American Diabetes Association 2003

2. Boudina S, Mazumder PK, Cooksey RC, **McClain DA**, **Abel ED**. Mitochondrial impairment contributes to cardiac dysfunction in obese leptin-deficient (ob/ob) mice. Circulation 2003; 108 IV-75

Presented at the American Heart Association Scientific Sessions 2003

3. Boudina S, O'Neill BT, **Abel ED**. Mitochondrial uncoupling and decreased oxidative capacity contributes to cardiac dysfunction in db/db mice. Diabetes 2004; 53, Suppl. 1 A335

Presented at 64th annual scientific session of the American Diabetes Association 2003

4.Buchanan J, Mazumder PK, Chakrabarti G, Roberts MW, Cooksey RC, **Abel ED.** Decreased myocardial efficiency and increased fatty acid oxidation characterize ob/ob and db/db mouse hearts prior to the onset of hyperglycemia. Circulation 2004; 110: III-323

Presented at the American Heart Association Scientific Sessions 2004

Figures and Tables for Project 3

Figure 1: Levels of PGC-1, and various mitochondrial proteins in ob/ob mouse hearts. Data represent analysis of 4-5 hearts.

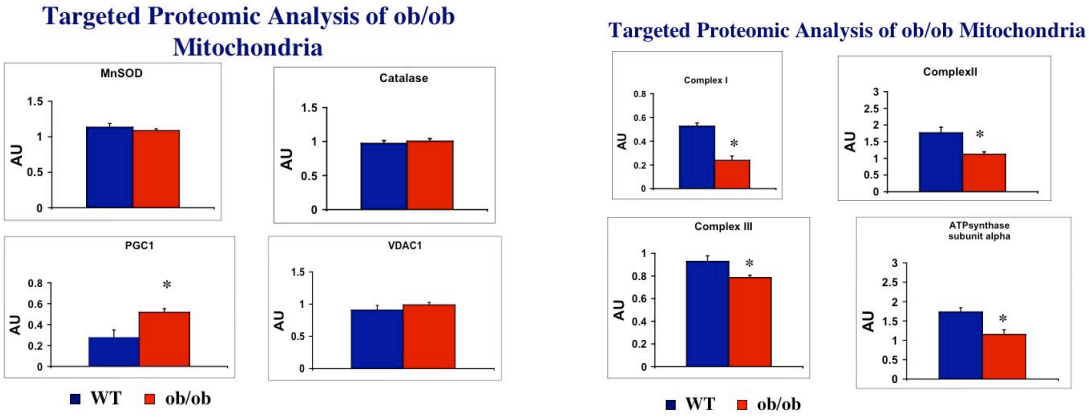


Figure 2: Levels of PGC-1, and various mitochondrial proteins in db/db mouse hearts. Data represent analysis of 4-5 hearts.

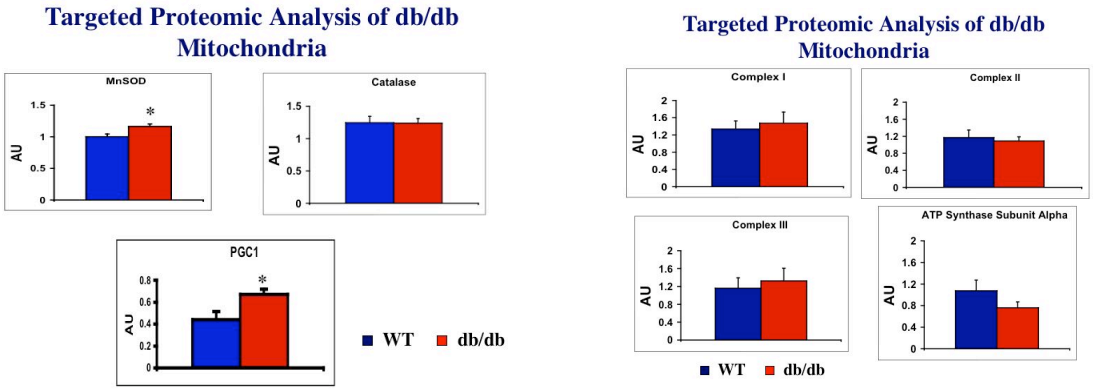


Figure 3: Mitochondrial ROS production is increased in mitochondrial isolated from db/db mice. Data were obtained from permeabilized fibers from 6 hearts per genotype. Mitochondrial respirations were stimulated with pyruvate. * p<0.01.

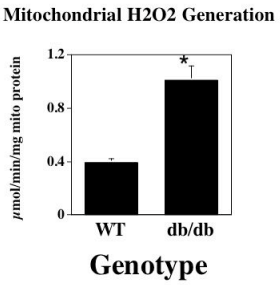
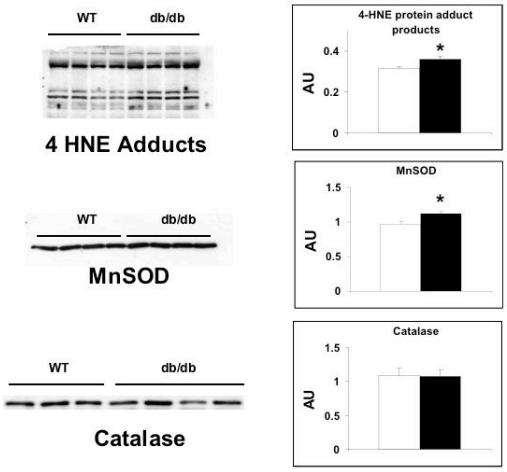


Figure 4: Evidence for oxidative damage in the hearts of db/db mice. Representative immunoblots are shown on the left and densitometry on the right. Controls are open bars and db/db mice are closed bars. * P<0.05 versus controls.



Responsible Investigators: **E. Dale Abel MD Ph.D., Sheldon E. Litwin MD, Donald A. McClain MD**

Project 4 Characterization of in vivo and ex vivo cardiac function and the response to stress of hearts of mice that lack insulin receptors in cardiomyocytes.

A. Rationale and Relevance

As outlined above, impaired insulin signaling is an early change that occurs in the hearts of mice with obesity and type 2 diabetes. The possibility therefore exists that myocardial insulin resistance may directly contribute to the pathogenesis of cardiac dysfunction in diabetes. To model this we generated mice with cardiomyocyte selective deletion of insulin receptors (CIRKO). These mice exhibit normal systemic glucose homeostasis, and so provide a unique opportunity to study the impact of impaired insulin signaling in the heart in the absence of systemic metabolic alterations such as hyperinsulinemia, hyperglycemia, and increased myocardial delivery of fatty acids.

B. Summary of accomplishments

We will summarize the results presented in the last report in a table that follows the criteria that were adopted by the consortium for the definition of diabetic cardiomyopathy. We will then present additional data that have been obtained in the current reporting period.

Minimal Criteria for Mouse Models of Diabetic Cardiomyopathy

In the context of insulin resistance and hyperglycemia:

- Decreased ejection fraction and/or evidence of diastolic dysfunction
- Interstitial or replacement fibrosis
- LV hypertrophy (models of type 2 diabetes)

Validation Criteria for Mouse Models of Diabetic Cardiomyopathy

- Invasive assessment of LV function in vivo to confirm systolic and diastolic dysfunction
- Evidence of LV dysfunction in isolated perfused hearts
- Evidence of abnormal cardiac metabolism and mitochondrial dysfunction
- Altered gene expression e.g. increased expression of beta-MHC, decreased expression of alpha-MHC, decreased expression of glucose transporters (GLUT4 and GLUT1)
- Impaired response to stress such as pressure overload hypertrophy and myocardial ischemia

Phenotype of mice with cardiomyocyte restricted deletion of insulin receptors (CIRKO)

Criteria/Validation	Results in CIRKO mice
Ejection Fraction	There is an age related decrease in ejection fraction in CIRKO mice, so that fractional shortening and ejection fractions (by echocardiography) is reduced by 30% and 12% respectively by 20 weeks of age.
Interstitial/replacement Fibrosis	There is a 2-fold ($p<0.05$) increase in interstitial fibrosis, quantified by point counting in picrosirius red stained sections in CIRKO hearts.
Cardiac Hypertrophy	CIRKO hears are initially reduced in size relative to age-matched controls. There is an age related increase LV mass relative to controls suggesting abnormal LV remodeling.
Invasive Assessment of LV function	In 12-week-old CIRKO mice LVEDP is 12±4 mmHg versus 6±3 mmHg, suggesting increased diastolic pressure.
Isolated Hearts	There is an age related decline in cardiac contractility in Langendorff perfused CIRKO hearts. The inotropic response of isolated CIRKO hearts to increasing calcium concentrations is blunted.
Cardiac Metabolism and Mitochondrial Dysfunction	CIRKO mice exhibit decreased rates of glucose oxidation at all ages studied(2), and increased rates of fatty acid oxidation in young mice < 8 weeks. As they age, they develop progressive mitochondrial dysfunction, which leads to a reduction in fatty acid oxidative capacity.
Altered Gene Expression	CIRKO hearts have increased expression of beta-MHC, decreased expression of alpha-MHC, increased expression of UCP3, and decreased expression of GLUT1.
Altered response to stress	CIRKO mice develop severe LV dysfunction following pressure overload hypertrophy induced by transverse aortic constriction. This is associated with a more exuberant fibrotic response than similarly treated control hearts. Similar observations have been observed in response to isoproterenol treatment. Following myocardial ischemia, CIRKO hearts develop abnormal LV remodeling and impaired LV function.
Abnormal action potential	We have recently published that CIRKO mice develop attenuated potassium currents and prolonged action potentials that are similar to changes observed in db/db mice.

Mechanisms Responsible for Increased Injury in CIRKO mice in Response to Hypertrophic Stimuli.

A consistent finding in CIRKO mice following stressors such as hypertrophy or ischemia is abnormal LV remodeling, characterized by decreased LV function and increased fibrosis. We speculated that one mechanism responsible was impaired expression of insulin responsive genes (eNOS and VEGF), which would play an important role in maintaining vascular integrity and promoting angiogenesis in myocardium that was undergoing hypertrophy. As summarized in **Figure 5**, exposure of hearts to the hypertrophic stimulus (isoproterenol), was associated with increased expression of eNOS and VEGF in wildtype hearts. This failed to occur in CIRKO hearts. Moreover, capillary density was markedly reduced in hypertrophied CIRKO hearts relative to controls (**Figure 6**). Thus impaired myocardial insulin signaling appears to induce a defect in the angiogenic response to cardiac hypertrophy. These observations may provide a novel mechanism for increased injury in diabetic hearts following hypertrophic or ischemic insults. We also explored the possibility that impaired endogenous myocardial insulin signaling would increase the susceptibility of developing apoptosis in response to ischemic and hypertrophic stress. **Figure 7** shows the results of TUNEL staining and cleaved caspace 3 immunohistochemistry, performed in isoproterenol treated hearts. These studies revealed increased apoptotic cell loss in CIRKO hearts.

C. Plans for the coming year

TO DETERMINE THE CONTRIBUTION OF IMPAIRED PI-3KINASE SIGNALING TO CARDIAC PHENOTYPES OF CIRKO MICE.

To address this question, we have begun to characterize mice with myocardial restricted expression of a dominant negative PI-3 Kinase transgene (dN-PI3K). These mice share many phenotypes of the CIRKO mice such as an initial reduction in cardiac size, and decreased rates of glucose metabolism. A recently published manuscript also revealed that in response to pressure overload hypertrophy, dN PI-3K transgenic mice develop increased injury and decreased LV function, that is similar to findings observed in CIRKO mice (McMullen,J.R., Shioi T et .al . *Proc Natl Acad Sci U S A* 100:12355-12360). These data support the hypothesis, that impaired myocardial insulin signaling contributes importantly to the pathogenesis of the diabetic cardiomyopathy. To date we have examined the mitochondrial phenotype of dN–PI3K mice. As shown in **Figure 8**, these mice exhibit defects in mitochondrial FA utilization as early as 5-weeks of age. These results are distinct from findings obtained in CIRKO mice of similar age, where an increase in mitochondrial FA utilization was observed. Thus the mitochondrial phenotypes of CIRKO hearts may involve PI-3 Kinase independent pathways. Alternatively, residual PI-3 Kinase signaling might be sufficient to modify the severity of the mitochondrial phenotypes observed in the CIRKO mouse. A role for PI-3 Kinase in impairing angiogenesis in response to LV remodeling remains to be elucidated. The previously published observation of increased injury in dN-PI3K mice following pressure overload hypertrophy suggests that this might be the case. We will therefore pursue these studies in the upcoming year, by measuring capillary density, VEGF and eNOS expression in dN-PI3K mice in response to hypertrophic stimuli.

TO DETERMINE THE CONSEQUENCE OF IMPAIRED MYOCARDIAL INSULIN SIGNALING AND INCREASED FATTY ACID DELIVERY TO THE PATHOGENESIS OF DIABETIC CARDIOMYOPATHY

Studies that we have performed in ob/ob and db/db mice indicate that they develop progressive mitochondrial dysfunction, which ultimately will limit their ability to oxidize fatty acids. This would therefore increase the likelihood of myocardial lipid accumulation and lipotoxicity. Given the progressive mitochondrial dysfunction that we have observed in CIRKO mice, we believe that generating a model in which lipid flux was increased into a heart that was already insulin resistant will lead to an accelerated lipotoxic phenotype. As such, we have crossed CIRKO mice with transgenic mice with myocardial overexpression of acetyl-CoA synthetase (ACS) that will increase fatty acyl CoA flux into the heart. This colony has now been established and is being expanded at the present time.

D. Significant achievement and its importance

These observations are significant because they establish a relationship between impaired myocardial insulin signaling and the development of mitochondrial dysfunction in the heart that is similar to the changes that we observed in ob/ob and db/db mice. We believe that if fatty acid delivery to these insulin resistant hearts is increased, myocardial dysfunction will be exacerbated, thus allowing us to model the respective contributions if lipotoxicity and insulin resistance to myocardial dysfunction in diabetic hearts.

Publications

1. Hu P, Zhang D, Swenson L, Chakrabarti G, **Abel ED**, Litwin SE. (2003) Minimally invasive aortic banding in mice: Effects of altered cardiomyocyte insulin signaling during pressure-overload. American Journal of Physiology. 285(3): H1261-1269

2. Shimoni Y, Chuang M, **Abel ED**, Severson DL. (2004) Gender dependent attenuation of cardiac potassium currents in type 2 diabetic db/db mice. J. Physiol. 2004; 555:345-354

3. Punske BB, Rossi S, Erschler P, Rasmussen I, **Abel ED.** (2004) Optical mapping of propagation changes induced by elevated extracellular potassium ion concentration in genetically altered mouse hearts. Journal of Electrocardiology. 37: Suppl. 128-134.

Abstracts of this work have been presented at national meetings.

1 Swenson L, Zhang D, Hu P, **Abel ED**, **Litwin SE**. Protective role of insulin signaling in isoproterenol-induced cardiac injury. Circulation 2002; 106 II-307

(1) Presented at American Heart Association Scientific Sessions 2002

2. Boudina S, O'Neil B, Belke DD, Rodnick KJ, **Abel ED**. Insulin

Resistance leads to multiple mitochondrial defects in the mouse heart. Diabetes 2003; 52, Suppl. 1 A284

(2) Presented at 63rd annual scientific session of the American Diabetes Association 2003

3. Boudina S, O'Neill B, Belke DD, Rodnick KJ, **Abel ED**. Insulin resistance leads to progressive mitochondrial dysfunction in the mouse heart. Circulation 2003; 108 IV-1404.

4. Mazumder PK, Hu P, Chakrabarti G, Zhang D, Avelar E, **Litwin SE**, **Abel ED**. Insulin signaling is required for the metabolic and functional adaptation of the heart to pressure overload hypertrophy. Circulation 2003; 108 IV-438

5. Hu P, Zhang D, Avelar E, **Abel ED**, **Litwin SE**. Insulin resistance in the heart increases the mortality and contractile dysfunction after myocardial infarction. Circulation 2003; 108 IV-894

(3-5) Presented at the American Heart Association Scientific Sessions 2003

6. Punske BB, Rossi S, Pappas AL, Ershler PR, Rasmussen I, **Abel ED**. Diminished propagation of electrical conductance in insulin receptor deficient mouse hearts under normoxic and ischemic conditions. Circulation 2004; 110: III-164

7. Boudina S, Sena S, Wright J, O'Neill BT, Mazumder PK, **Abel ED.** Postnatal deletion of insulin receptors augments myocardial fatty acid utilization and enhances mitochondrial biogenesis. Circulation 2004; 110: III-324

(6,7) Presented at the American Heart Association Scientific Sessions 2004

Figures and Tables for Project 4.

Figure5: eNOS and VEGF expression in response to isoproterenol

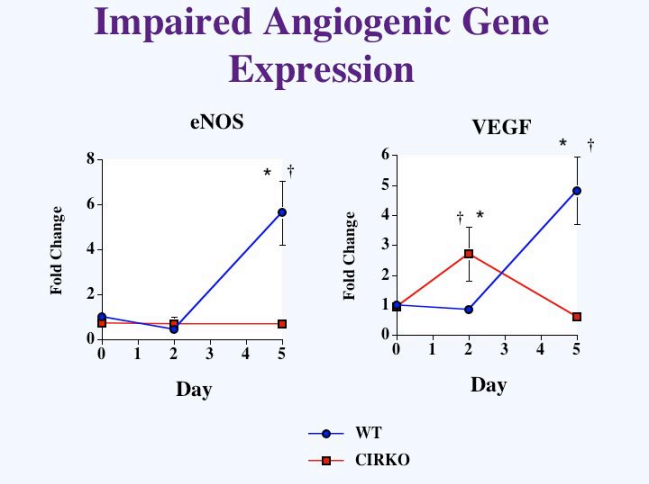


Figure 6: Capillary Density in WT and CIRKO hearts before and after Isoproterenol treatment.

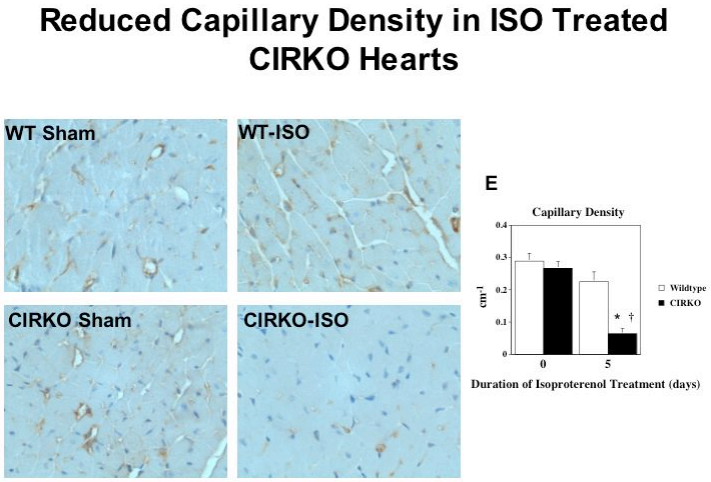


Figure 7: TUNEL: Evidence for increased apoptosis in Isoproterenol treated CIRKO Mice.

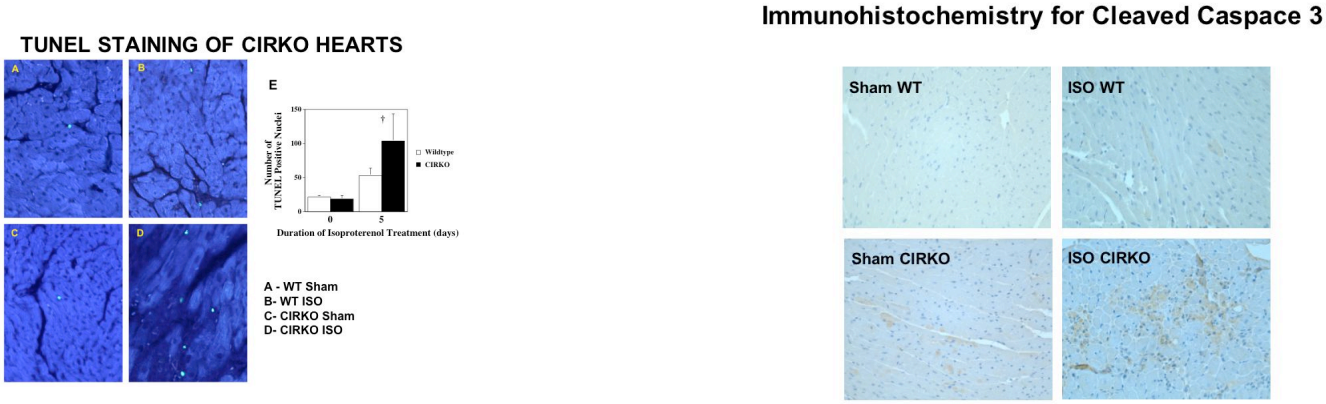
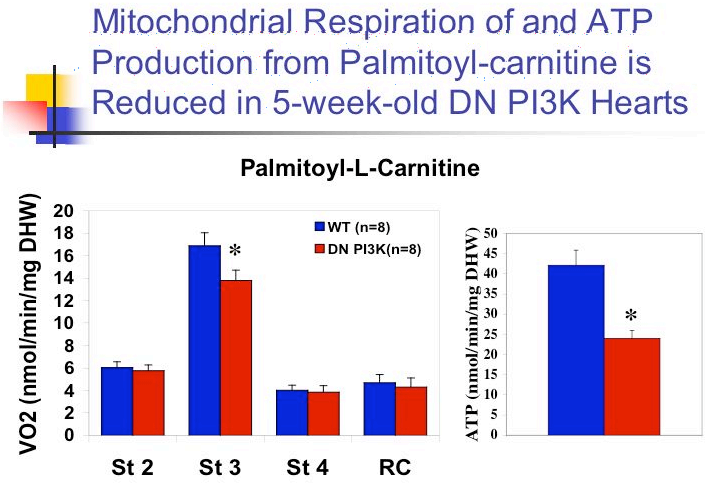


Figure 8: Evidence for mitochondrial dysfunction in mice that harbor a dominant negative PI-3 Kinase transgene.



University of Utah -Overview Summary of Current Models

Animal model	Background Strain	Current Status
Ob/ob	c57Bl/6J	Final/advanced phenotyping
Db/db	c57BlKS	Final/advanced phenotyping
Akita	C57Bl/6J	Early Phenotyping
CIRKO	Mixed	Final/advanced phenotyping
UCP-DTA	FVB	Ongoing phenotyping
Dominant Negative PI3Kinase	FVB	Phenotyping in Progress
Inducible CIRKO	Mixed	Early Phenotyping
ACS-CIRKO	Mixed	Colony Established
STZ	Various	CIRKO-STZ in progress
GLUT4 Heart Specific	Mixed	Ongoing phenotyping

Phenotyping to Date

Criteria/Validation	Db/db mice
Ejection Fraction	Reduced
Replacement/Interstitial Fibrosis	Trichrome pending. Increased myocardial lipid.
Cardiac Hypertrophy	No
Invasive Assessment of LV Function	↑LVSP, ↑LVDP as early as 4-weeks of age, ↑dP/dt (4 wks), contractility declines after 15 weeks of age.
Isolated Hearts	Decreased LV Function much worse than ob/ob.
Cardiac Metabolism/Mitochondrial Function	↓Glucose Ox, ↑FA OX & MVO_2 Mitochondrial Dysfunction is present. Precedes hyperglycemia and worsens thereafter
Gene Expression	MHC isoforms switched.
Response to Stress	Pending
Electrophysiology	Prolonged action potential.

Phenotyping to Date

Criteria/Validation	Ob/ob Mice
Ejection Fraction	Reduced
Replacement/Interstitial Fibrosis	Trichrome pending, Increased myocardial lipid.
Cardiac Hypertrophy	Yes
Invasive Assessment of LV Function	↑LVSP, ↑LVEDP as mice age, dp/dt↑ at 4 weeks, ↓at 8-weeks.
Isolated Hearts	Decreased LV Function.
Cardiac Metabolism/Mitochondrial Function	↓Glucose Ox, ↑FA- OX & MVO_2 Mitochondrial dysfunction is present. Insulin signaling in cardiomyocytes is impaired.Precedes Hyperglycemia
Gene Expression	MHC isoforms switched.
Response to Stress	Aortic Banding in Progress
Electrophysiology	NA

Phenotyping to Date

Criteria/Validation	CIRKO Mice
Ejection Fraction	Reduced
Replacement/Interstitial Fibrosis	Increased. Accelerated by Hypertrophy. May Reflect Impaired Angiogenesis
Cardiac Hypertrophy	Age dependent
Invasive Assessment of LV Function	LV Function reduced, ± Increased Diastolic Pressures.
Isolated Hearts	Decreased LV Function.
Cardiac Metabolism/Mitochondrial Function	↓Glucose Ox, ↑FA OX &MVO_2 (young)↓FA OX (Old). Mitochondrial dysfunction is present. ↑ROS
Gene Expression	MHC isoforms switched.
Response to Stress	Impaired response to ischemia and pressure overload.
Electrophysiology	Prolonged action potential

Phenotyping to Date

Criteria/Validation	dNPI3K
Ejection Fraction	Normal
Replacement/Interstitial Fibrosis	Increased following aortic banding
Cardiac Hypertrophy	Not at baseline
Invasive Assessment of LV Function	Pending
Isolated Hearts	Mild reduction in performance
Cardiac Metabolism/Mitochondrial Function	↓Glucose Ox, ↓FA- OX Mitochondral dysfunction is present. Insulin signaling in cardiomyocytes is impaired.
Gene Expression	MHC isoforms not switched, ANF increased.
Response to Stress	Decreased Function After Aortic Banding
Electrophysiology	NA

Phenotyping to Date

Criteria/Validation	CIRKO+ ACS Mice
Ejection Fraction	Pending
Replacement/Interstitial Fibrosis	Pending
Cardiac Hypertrophy	Pending
Invasive Assessment of LV Function	Pending
Isolated Hearts	Pending
Cardiac Metabolism/Mitochondrial Function	Pending.
Gene Expression	Pending
Response to Stress	Pending
Electrophysiology	Pending

The Colony is now Established and is Being Expanded for Phenoyping

Phenotyping to Date

THE UNIVERSITY OF UTAH

Criteria/Validation	Akita Mice
Ejection Fraction	Pending
Replacement/Interstitial Fibrosis	Pending
Cardiac Hypertrophy	Pending
Invasive Assessment of LV Function	Pending.
Isolated Hearts	Pending
Cardiac Metabolism/Mitochondrial Function	Pending
Gene Expression	Pending
Response to Stress	Pending
Electrophysiology	Pending

The Colony is now Established and is Being Expanded for Phenoyping