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# DIABETIC CARDIOMYOPATHY: PHYSIOPATOLOGIC CHARACTERIZATION IN RATS MODEL


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I dedicate thesis to my mother.

# ABSTRACT

**Background:** Diabetes mellitus is associated with an excessive cardiovascular morbidity and mortality. More recent data show that there may be a specific diabetic cardiomyopathy not associated to atherosclerotic coronaropathy. In fact, for reasons still not compltetaly understood, some diabetic subjects developed an earlier, more rapidly evolving, serious myocardiopathy with a worse prognosis than non-diabetic subjects, in the absence of atherosclerotic vascular lesions.

**Objective:** The aims of the study were , first, to obtain an adequate model of the most dangerous complications of diabetes: Cardiomyopathy. Second, to study alteration of vascular response in diabetes. Third, to characterize SHHF/Mcc-fa/fa strain, as congenital model of diabetic cardiomyopathy.

**Methods:** Initially, cardiac hypertrophy was obtained by placing a titanium clip (Ø 0.8 mm) around abdominal aorta proximal to renal arteries. The animals were studied 1 and 3 months after aortic coarctation. Cardiac contractility was evaluated through cannulation of the left ventricle (under anesthesia) to measure pressure and derived parameters. At the same time we investigated vascular reactivity in Wistar STZ rats after 11-and 18-weeks to induction of diabetes. The animals were randomized into four groups. Each group received cumulative escalating doses of: Endothelin-1, big-Endothelin-1,

Angiotensin I and Angiotensin II, respectively, and blood pressure was recorded.

Then, we prepared Wistar rats inducing cardiac hypertrophy with titanium clip method, and 1-month after aortic coarctation, the same rats were subjected to STZ 50 mg/kg i.v. administration. Unfortunately, we got a high rate mortality (> 80%). Thus we decided to change this model. It was recently available SHHF Mcc/fa-fa rats, recessive homozygotes for fa gene, and characterized by the expression of a Metabolic Syndrome (100%) associated to cardiomyopathy. So we decided to study the evolution of the Syndrome at different age, comparing this strain with WKY (age-matched rats), SHR (spontaneously hypertensive rats) and ZDF (Diabetic rats). We evaluated functional and humoral alterations and the nociceptive sensitivity to monitoring the evolution of neuropathy, another frequently diabetic complications.

**Results:** The left ventricular mass (obtained from ratio of left ventricular mass to body weight) of rats subjected to aortic banding increased greatly in both 1- and 3-month groups with respect to sham-operated rats ($p= 0.008$ and $p= 0.014$ respectively). On the contrary, hypertrophic rats did not show hemodynamic modifications with respect to sham-operated animals: left ventricular pressure, left ventricular end-diastolic pressure and heart rate seemed not to be modified by abdominal aortic coarctation in both groups (1- and 3-month coarcted rats).

Body weight in 11- and 18-weeks diabetic rats was markedly reduced ($p<0.0001$) and glycemia was strongly increased ($p<0.0001$).

The administering of Et-1 produced in both diabetic groups a typical significant biphasic effect : hypotension followed by hypertension significantly reduced both 11- and 18-weeks.

SHHF-Mcc-fa/fa strain was characterized by high basal pressure level at 6-months of age, hypercholesterolemia, hypertrigliceridemia, hyperinsulenemia in euglycemia conditions both 6-and10-months of age ($p<0.0001$). In addition, this strain had a reduction of nociceptive sensibility increasing times reaction at 6-months of age and a progression nociceptive function impairment at 10-months of age.

**Conclusions:** These results identify SHHF Mcc-fa/fa rat as a congenital model to better understand the physiopathology modifications that lead to diabetic cardiomiopathy.

# 1. INTRODUCTION

The conventional wisdom holds that diabetes causes myocardial contractile dysfunction through accelerated atherosclerosis and hypertension. Much less appreciated and more controversial is the notion that diabetes mellitus affects cardiac structure and function, independent of blood pressure or coronary artery disease[1-3]. The term *Diabetic Cardiomyopathy* (DCM) was first proposed by Rubler et al. in 1972, after clinical encounter of a specific type of cardiomyopathy that is closely associated with diabetes. An increasing body of evidence has independently confirmed the existence of such cardiac complications of diabetes[4]. DCM is characterized by a cascade of myocardial changes that occurs in diabetes mellitus with interstitial fibrosis, myocardial hypertrophy and microcirculatory abnormalities. These cardiovascular complications compromise cardiac performance ultimately resulting in cardiac failure[5]. The epidemiological link between diabetes mellitus and the development of heart failure, independent of atherosclerotic cardiovascular disease, has been evident for the better part of three decades. Several theories have been proposed, including Protein Kinase C (PKC) activation, enhanced oxidative stress, and accumulation of advanced glycation end products (AGEs). Among these, PKC activation no doubt plays a significant role[4]. The increased risk of heart failure

persists in the diabetic patients after considering age, blood pressure, weight, cholesterol, as well as history of coronary artery disease. Notably, there is a significant association between diabetes and diastolic dysfunction leading to congestive heart failure in the absence of impaired systolic function. The association between diabetes and cardiomyopathy is strongest among individuals with microvascular complications of diabetes that parallels the duration and the severity of hyperglycemia. However, it has been difficult to ascertain from these epidemiological correlations the causal relationship between the commonly observed metabolic abnormalities and the specific cardiac phenotype[6-9].

**1.1 Pathogenesis of Diabetic Cardiomyopathy:**

Cardiomyopathy is a complicated disorder, and several factors have been associated with its development. These include increased stiffness of the left ventricular wall associated with accumulation of connective tissue and insoluble collagen, depressed autonomic function, impaired endothelium function and sensitivity to various ligands (e.g. β-agonists), and abnormalities of various proteins that regulate ion flux, specifically intracellular calcium. More recently, there is a generalized view that diabetic cardiomyopathy also occurs as a consequence of altered fuel metabolism[10]. The 3 characteristic metabolic disturbances evident in diabetic states are *hyperlipidemia* (usually in the form of increased

triglycerides and nonesterified fatty acids [NEFAs]) and early *hyperinsulenemia* followed by pancreatic β-cell failure, leading eventually to *hyperglycemia.* Type 1 diabetes differs principally from type 2 diabetes in that it is unaccompanied by a period of hyperinsulenemia and is characterized by early- as opposed to late-onset *hyperglycemia.* Alterations in body mass (obesity) and adipocytokines (leptin, adiponectin) have also been implicated in the cardiovascular pathophysiology observed in diabetes. As such, the effects of increased NEFAs, altered insulin action, and hyperglycemia can be considered triggers to the cardiac phenotype in diabetes. An understanding of the cellular effects of these metabolic disturbances on cardiomyocytes should be useful in predicting the structural and functional cardiac consequences. Extensive cellular and molecular studies have identified putative mediators, effectors, and targets of these metabolic triggers in the pathogenesis of cardiac dysfunction in diabetes[11] (Fig. 1).

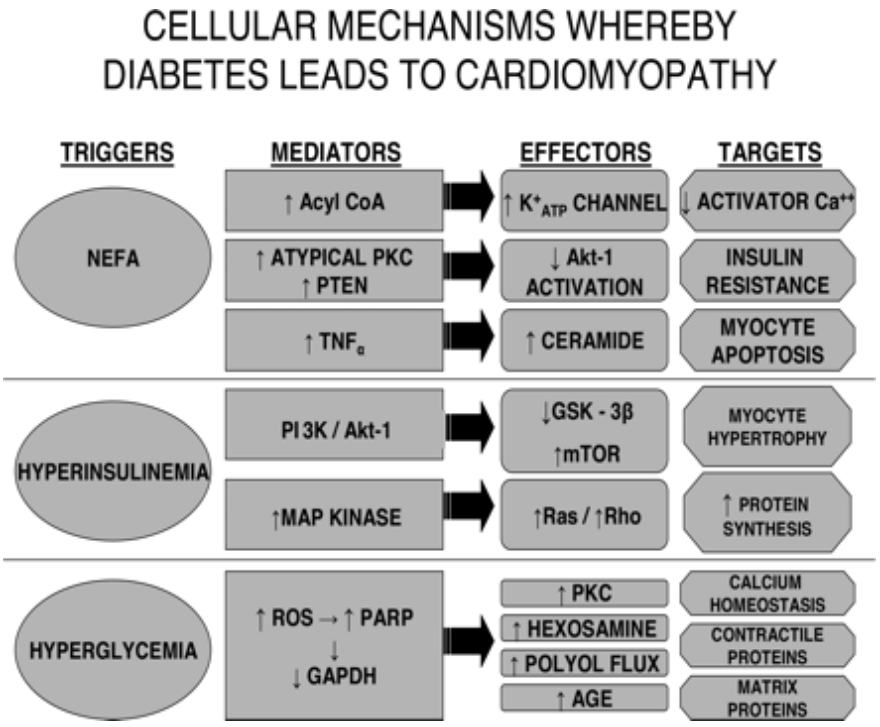


**Figure 1.** The relationship between diabetic metabolic disturbances (triggers) and the mediators, effectors, and intracellular targets that lead to a diabetic cardiomyopathic phenotype.

*1.1.1 Increased NEFAs*

NEFAs play a critical role in triggering the development of cellular insulin resistance, but also have been implicated in the development of myocardial contractile dysfunction. NEFAs play a central role in altering cellular insulin signalling through several mechanism leading to insulin resistance and compensatory hyperinsulenemia. In turn, hyperinsulenemia is an important trigger to the development of cardiac hypertrophy in diabetic

cardiomyopathy. NEFAs induce the activation of atypical protein kinase C (PKC) θ, a serine/threonine Kinase that phosphorylates and subsequently activates IkB kinase. IkB kinase phosphorylates serine residues on insulin receptor substrate-1 (IRS-1), inhibiting its ability to bind SH2 domains of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), impairing insulin signal transduction. Although this mechanism is active in skeletal muscle and adipose tissue, it has been less clear whether similar mechanisms are apparent in cardiac muscle. Increases in intracellular NEFAs can also alter insulin signalling without affecting IRS-1/PI3K activation (figure 2).

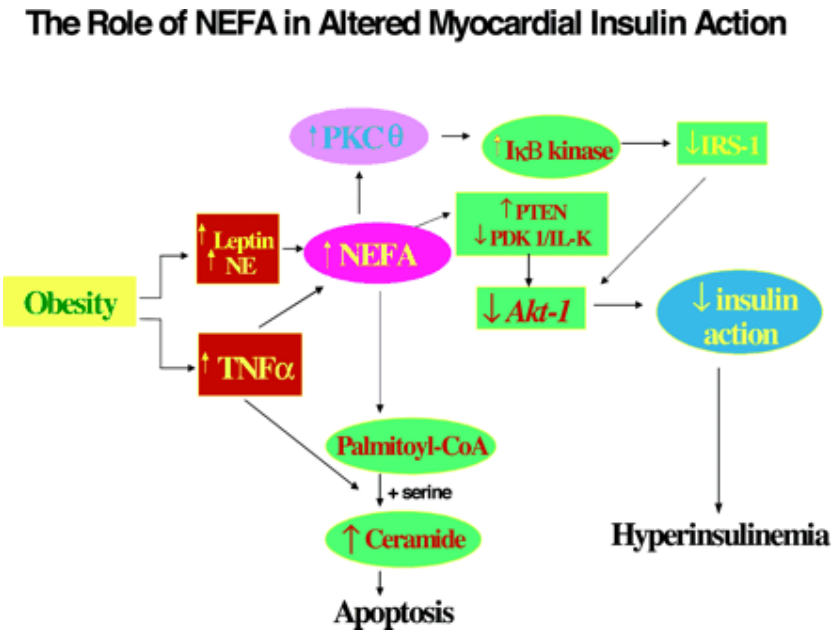


**Figure 2.** Role of increased NEFAs in the pathogenesis of cellular insulin resistance. There are several putative mechanisms including the induction of atypical PKC, phosphorylation, and activation of IκB kinase which serine phosphorylates and deactivates IRS-1. NEFAs may also activate the phosphatase PTEN, decreasing the tris-inositol phosphates to which the pleckstrin domain of Akt-1 binds. Finally, NEFAs can contribute to ceramide production, triggering cellular apoptosis.

Akt-1 activation is critically dependent on the generation of phosphatidylinositol 3,4,5-trifosphate (PtdIns(3,4,5)$P_3$) to bind the N-terminal pleckstrin domain and activate membrane bound kinase responsible for the phosphorylation of serine and threonine residues on Akt-1 that confer catalytic and regulatory properties. NEFAs are natural ligands for the nuclear receptor, peroxisome proliferator-activated receptor (PPAR) γ, and can induce the upregulation of the phophatase

and tesin homolog deleted on chromosome 10 (PTEN) which dephosphorylates PtdIns(3,4,5)P3, preventing the activation of Akt1.

NEFAs can directly alter myocardial contractility independent of altered insulin action by increasing NEFA flux into the myocardium. Recent evidences suggest that increases in fatty acyl coenzyme A (CoA) esters within cardiac myocites may modulate the contractile state of the myocardium by opening of the $K_{ATP}$ channel. Activation of the $K_{ATP}$ channel leads to shortening of the action potential and reduces transarcolemmal calcium flux and subsequently myocardium contractility. Finally, the increased intracellular accumulation of NEFAs may directly contribute to cell death under circumstances in which accumulating intracellular NEFAs do not undergo β-oxidation. The reaction between palmityol-CoA, an intracellular intermediate of NEFAs, and serine leads to the generation of the sphingolipid ceramid, and this reaction may be facilitated by the cytokine, tumor necrosis factor TNFα. Ceramide can induce cellular apoptosis through the induction of nuclear factor kB, caspase 3 activation, cytochrome c release, and can inhibit DNA repair by blocking poly ADP ribose polymerase. Under these circumstance, increased NEFAs seems to cause lipotoxicity. Although lipotoxicity has been implicated in the reduction in pancreatic β-cell reserves, the relevance of these findings in the myocardium remain controversial. Thus, NEFAs play a central role not only in inducing

cellular insulin resistance, but also indirectly affecting myocardial contractility and, under specific circumstances, in the promotion of cardiomyocyte cell death[12-22].

*1.1.2 Hyperinsulinemia*

Insulin resistance occurs as part of a cluster of cardiovascular-metabolic abnormalities commonly referred to as the "insulin resistance syndrome" or the "metabolic syndrome". Currently, insulin resistance is defined clinically as the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population. As stated previously, insulin action is the consequence of the binding of insulin to its plasma membrane receptor and the transmission of its signal throughout the cell by a series of protein-protein interactions and signalling cascades[4]. Cellular insulin resistance may precede frank diabetes by a decade or more and requires compensatory increases in plasma insulin levels to maintain glucose homeostasis in the face of impaired cellular insulin action, principally in skeletal muscle and liver.

The nature and extent of the cellular insulin resistance may be selective to certain organ systems and may vary in terms of the metabolic, mitogenic, prosurvival, and vascular actions of insulin. Systemic hyperinsulenemia may accentuate cellular insulin action in insulin

responsive tissues, such as the myocardium, that do not manifest cellular insulin resistence. In this regard, the mitogenic actions of insulin on myocardium during chronic systemic hyperinsulinemia bear directly the commonly observed finding of cardiac hypertrophy in diabetic cardiomyopathy. There are at least 3 cellular mechanisms whereby hyperinsulenemia mediates cardiomyocytes hypertrophy (figure 3).

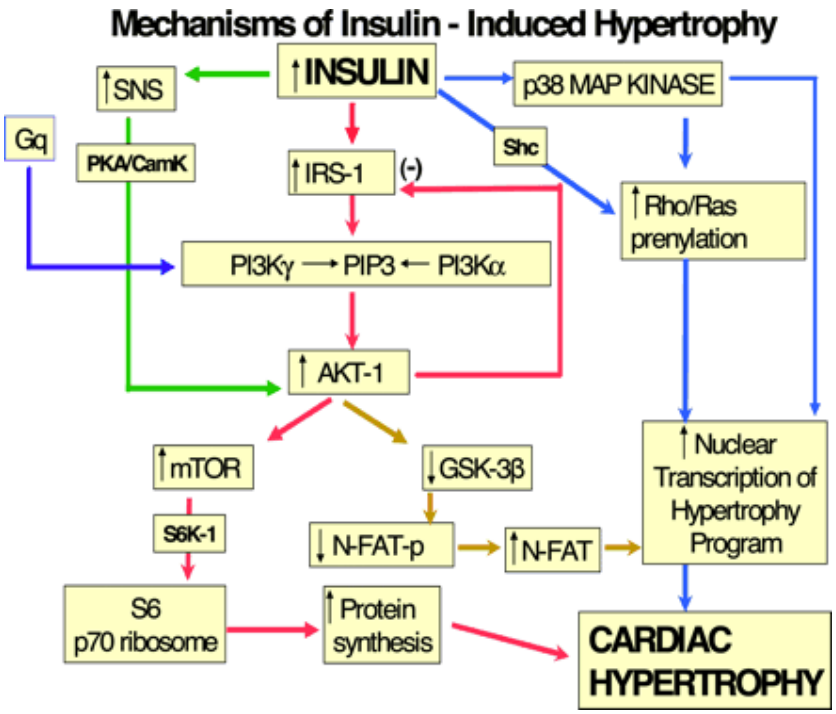


**Figure 3.** Alternative pathways whereby compensatory hyperinsulinemia contributes to myocyte hypertrophy through the sympathetic nervous system activation and MAP kinase/ERK pathways at a time when insulin receptor mediated Akt-1 activation is impaired.

Acutely, insulin stimulates growth through the same PI3Kα/Akt-1 pathway by which it mediates glucose uptake. Akt-1 phophorilatesand inactivates glycogen synthases kinase-3β (GSK-3β), a well –recognized inhibitor of nuclear transcription governing the hypertrofic processvia the nuclear factor in activated lymphocytes (NFAT-3). In addition, Akt-1 activates the mammalian target of rampamycin (mTOR) that activates the p70 ribosomal subunit S6kinase, leading to increased actions mediated through the insulin receptor may be mitigated when insulin signalling through the PI3Kα/Akt-1 pathway is impaired during chronic hyperinsulenemia. However, chronic hyperinsulenemia may augment myocardial Akt-1 activation indirectly through increased sympathetic nervous system activation. Recent evidence suggests that chronic Akt-1 activation in cardiac myocites is mediated through $\beta_2$-adrenergic receptors via protein chinase A and $Ca^{2+}$-calmodulin dependent kinase (CaMK) and these mechanisms may predominate when insulin signalling is attenuated through the PI3Kα pathway.

In addiction, there are other insulin-mediated, but Akt-1-indipendent, pathways that may be operative, most notably the extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase pathways. Significant cellular evidence exists for an insulin-induced activation of the p38 MAP kinase pathway, as well as prenylation of both Rho and Ras in the setting of hyperinsulenemia, leadind to myocyte

hypertrophy and expansion of the extracellular matrix (figure 3). These redundant pathways provide a strong mechanistic basis for the development of cardiac hypertrophy associated with chronic hyperinsulenemia as an early accompaniment of type 2 diabetes, even though the glucoregulatory effects of insulin are attenuated[23-26].

*1.1.3 Hyperglycemia*

Hyperglicemia plays a pivotal role in the development of both microvascular and macrovascular complications. The results of the Diabetes Complications and Control Trial (DCCT) indicate that hyperglycemia is a major contributor to microvascular disease (i.e. retinopathy and nefropathy) in type 1 diabetes. In addition, results from DCCT together with those of other smaller studies, suggest that intensive glycemic control can reduce cardiovascular disease. On the other hand, the actual effect of hyperglycemia on the development and progression of macrovascular complications remains unclear and even somewhat controversial, particularly in type 2 diabetes. The complicated nature of the metabolic abnormalities in type 2 diabetes and the relative roles of these associated conditions in the development of macrovascular disease make definite conclusions somewhat difficult. Nevertheless, certain aspects of postprandial glucose levels, are associated with macrovascular disease, and there are a number of basic mechanisms to explain these

associations that could lead to the development of cardiovascular disease. These abnormalities include activation of the sorbitol pathway, oxidative stress, and generation of reactive AGEs and AGE precursors. Results of these events include endothelial dysfunction, altered properties of key proteins (for example, due to glycation of structural proteins), and generation of proinflammatory cytokines. These considerations suggest that hyperglycemia plays an important, but as yet not clearly defined, role in clinical macrovascular disease[4].

The mechanism whereby hyperglycemia mediates tissue injury through the generation of reactive oxygen species has been elucidated largely through the work of the Brownlee and colleagues. Hyperglycemia leads to increase glucose oxidation and mithocondrial generation of superoxide. In turn, excess superoxide leads to DNA damage and activation of poly (ADP ribose) polymerase (PARP) as a reparative enzyme. PARP, through inhibition of glyceraldehyde phosphate dehydrogenase (GAPDH), diverts glucose from glycolytic pathways into alternative fates, including AGE formation, hexosamine and polyol pathway flux, protein kinase C activation, which are believed to mediate hyperglicemia-induced cardiac tissue damage. In this regard, increased formation of AGE forms irreversible cross-links with many macromolecules such as collagen, leading to tissue fibrosis, inactivation of SERCA2, and ryanodine receptor calcium-release channel, impaired

cardiac relaxation, contractility, and ventricular stiffness. Increased hexosamine influx reduces SERCA2a protein levels, resulting in prolonged calcium transients, QT duration increase and conseguetly, delayed myocardial relaxation. Increased hexosamine influx also impairs insulin signal transduction and contributes to insulin resistance. Increased polyol flux is associated with a decrease in intracellular glutathione and augmentation in cell apoptosis. Conversely, inhibition of polyol flux protects hearts from ischemic injury. Finally, activation of PKC-β2 leads to left ventricular hypertrophy, cardiac myocite necrosis, multifocal fibrosis, and decreased left ventricular performance, resulting in cardiomyopathy. Taken together, hyperglycemia, through multiple pathways, causes cardiac cellular and functional changes, possibly contributing to the development of cardiomyopathy[10].

*1.1.4 Diabetic cardiomyopathy: Unifying hypothesis*

Epidemiological, clinical and experimental evidence suggest the existence of a different form of cardiomyopathy related to pathogenic cellular and molecular changes that accompany diabetes mellitus. Figure 4 is a schematic illustration of a unifying hypothesis that links NEFAs, hyperinsulenemia, and hyperglicemia to the structural and functional phenotypes in cardiac cardiomyopathy. The magnitude of ventricular hypertrophy may depend on the magnitude and duration of

hyperinsulenemia that, in turn, may depend on magnitude and distribution of increasing NEFAs. In contrast, the extent of systolic dysfunction may depend more on the magnitude and duration of hyperglicemia and presence or absence of insulin. Diastolic abnormalities may occur as a consequence of either hypertrophy or hyperglycemia. As such, hypertrophy and diastolic abnormalities are observed most commonly and earlier than systolic abnormalities and thus dominate the clinical findings. The paradigm reconciles the distinctive cardiovascular feature attributable to type 1 (hypoinsulinemic/hyperglicemia→systolic dysfunction) and type 2 (hyperinsulemic/hyperglicemic→hypertrophy and diastolic dysfunction) diabetes in experimental models studied in vivo. In humans with type 1 diabetes, systolic dysfunction is less evident than in STZ-induced models because these patients receive exogenous insulin, making them metabolically similar to a type 2 diabetic from this mechanistic perspective. These findings are generally consistent with the established cellular consequence of increased NEFAs, hyperinsulenemia and hyperglycemia on cardiac structure and function. These insights afford the opportunity to design therapeutic approaches targeted at specific pathogenic mechanisms including suppression of lipolysis, maturation of adipocytes, and restoration of cellular insulin action, in addition to the traditional target of maintaining euglycemia[11].

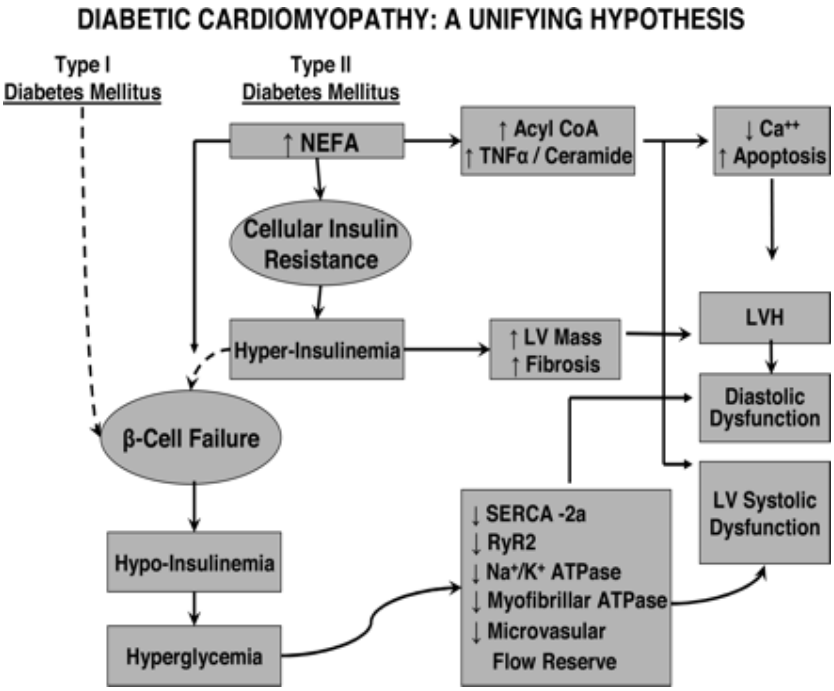


**Figure 4.** Schematic illustration of a unifying hypothesis that links NEFAs, hyperinsulinemia, and hyperglycemia to the structural and functional phenotypes in diabetic cardiomyopathy.

*1.1.5 Prevention and Treatment*

Treatment of congestive heart failure in the diabetic patient should follow the same guidelines as nondiabetic patients, with a few notable exceptions. Treatment of hypertension and hypercholesterolemia along with avoidance of excessive alcohol intake and cessation of smoking are advised for all patients. Diabetic patients requiring thiazide diuretics or β-blocking agents may run the risk of impaired insulin secretin or, conversely, profound or marked hypoglicemic reactions. The former

problem may be improved by the use of cardioselective β-blockers with intrinsic sympathomimetic activity. Similarly, arterial reflexes may be impaired in diabetic persons with autonomic dysfunction. Thus preload- and afterload-reducing agents should be used cautiously in diabetic patients. Although an altered myocardial sensitivity to glycoside has not been established clinically, isolated muscle from diabetic rats has shown a greater tendency to contracture when exposed to ouabain.

Finally, the role of intensive hypoglycemic therapy in the primary prevention or several of myocardial dysfunction remains to be elucidated. The results of clinical and experimental studies are equivocal, though encouraging results of metabolic control in improving myocardial function are presented. These studies suffer from inadequate sample size, varying patient populations, and variable institutions and duration of treatment. We agree that maintenance of metabolic control of diabetics is crucial, but we cannot support intensive insulin treatment with its attendant increased risk of hypoglycaemic and ketoacidosis in patients with myocardial dysfunction until the benefit-to-risk ratio for such treatment is better known[27].

**1.2 Diabetic Neuropathy**

Diabetic neuropathy is common in diabetic patient, with prevalence of more than 50%. The pathogenesis is multifactorial and is considered to

be rooted in both hyperglicemia-induced pathologic changes intrinsic to neurons and ischemia-induced neuronal damage resulting from decreased neurovascular blood flow. Because of the vascular elements, diabetic neuropathy is also considered to be a form of microvascular complications. Diabetes induces pathologic changes in the neurotrophic microvessels reduces blood flow via reduction in endothelial- and nitric oxide-dependent vasodilatation, and alters the expression and action of vasoconstrictors endotelina-1 (Et-1) and vascular endothelial growth factor (VEGF). This point of view is supported by studies in diabetic animal models. In vivo expression of VEGF restored blood flow in neurotrophic blood vessels as well as nerve functions in streptozotocin-induced diabetes. PKC activation has been known to cause similar changes in microvessels. Suppression of PKC activity streptozotocin-diabetic rats rectified diabetes-induced impairments in nerve blood flow, conduction velocity, the sodium-potassium pump ($Na^+$, $K^+$-ATPase), and glutathione deficits. Furthermore, inhibition of PKC-β II activity with the use of a selective inhibitors restored motor nerve conduction velocity and endoneuronal blood flow that were impaired by diabetes. All of this evidence supports a role of PKC, especially the PKC-βII isoform, in the pathogenesis of diabetic neuropathy[11].

### 1.3 Animal models for diabetic cardiomyopathy

The altered cardiac phenotypes associated with diabetic cardiomyopathy have been investigated in a wide array of experimental models. From a mechanicist perspective, the manifestations of diabetic cardiomyopathy should be predictable based on the duration and the severity of the abnormalities in NEFAs, insulin, and glucose homeostasis.

*Models of Hyperglicemia without hyperinsulenemia*

Streptozotocin (STZ) and alloxan are the 2 of the most common islet cell toxins that are used to induce type I diabetes in experimental animal models. The metabolic features include the prompt development of profound hyperglicemia (25 to 30 mmol/L), modest hypertriglyceridemia (200 to 400 μmol/L), ketosis, and markedly reduced plasma insulin levels (<20 pmol/L). As such, the model is particularly useful in examining the effects of hyperglycemia in the absence hyperinsulinemia.

STZ- and alloxan-induced diabetes have been associated with myocardial atrophy rather than hypertrophy. This is associated with loss of contractile proteins, myocyte dropout without reparative fibrosis, consistent with the absence of the mitogenic and prosurvival effects of insulin. Decreases in cardiac mass have also been noted with cardiac specific knockout of the insulin receptor associated with ventricular dilatation and impaired left ventricular (LV) systolic performance. There has been consistent evidence of intramyocardial lipid accumulation

reflecting a compensatory shift in myocardial preference for fatty acids in the absence of insulin mediated glucose uptake. Alterations in SERCA2 have been reported in association with the hyperglycemia in STZ induced diabetes leading to decreased SR calcium sequestration and intracellular calcium overload. Decreases in contractile proteins, such as α-actin and myosin ATPase activity, have been noted in association with shift in myosin heavy chain isoforms from α to β, contributing to decreased systolic tension development[28-31]. The functional consequences of these cellular effects have been studied in vivo or vitro measurements. For example, systolic and diastolic dysfunction have been detected by using echocardiography in STZ diabetic animals. With the use of in vivo catheterization of these animals, elevated left ventricular end-diastolic pressure and reduced left ventricular systolic pressure with diminished the maximal rate of rise (+dP/dt) or the maximal rate of decrease (-dP/dt) were observed. Reduced peak left ventricular pressure and ±dP/dt were confirmed in ex vivo perfused STZ hearts[10]. These studies suggest that experimental type 1 diabetes, characterized principally by hyperglicemia is associated with altered calcium handling, impaired diastolic function, altered contractile proteins, and progressive systolic dysfunction as magnitude and duration of the hyperglicemia progresses. These abnormalities occur in the absence of myocyte hypertrophy or fibrosis. The findings are consistent with a dominant influence of hyperglycemia

and the absence of the influence of insulin. Thus, hyperglicemia alone is sufficient to account for the functional, but not necessarily the structural, changes observed in diabetic cardiomyopathy.

*Models of Hyperinsulenemia with or without hyperglicemia*

Recently, evidence of cardiomyopathy has also been reported in animal models of insulin resistance and type 2 diabetes (ZDF rats and ob/ob or db/db mice)[3]. Models of type 2 diabetes are metabolically distinct from type 1 diabetes largely related to increased plasma insulin levels associated with altered cellular insulin action. Increased NEFAs and marked hyperinsulenemia are early accompaniments, whereas hyperglicemia is a later development attributable to exhaustion of pancreatic β-cell reserves. However, the cardiac manifestations in experimental models of type 2 diabetes vary considerably based on the onset and severity of these metabolic perturbations, as well as the complex interactions of intramyocardial lipids, insulin, and glucose on the cardiomyocyte structure and function.

Many of the commonly used experimental models of type 2 diabetes are associated with obesity caused by genetic perturbations in the Ob gene and its product, leptin. Leptin is a 16-kDa peptide produced by adipocytes that acts via specific receptors on the hypothalamus to inhibit food intake and increase energy expenditure. Notably, leptin receptors have been identified in the rat myocardium and regulate fatty acid

oxidation independent of effects on the acetyl CoA-malonyl CoA axis. Leptin has also been shown to stimulate myocyte hyperplasia in rats and humans through both PI3-K- and ERK1/2-dependent mechanisms, similar to insulin. The metabolic phenotype associated with both leptin-deficient ($ob^-/ob^-$) and leptin receptor-deficient ($db^-/db^-$) mice include obesity, insulin resistance, compensatory hyperinsulenemia, hypertriglyceridemia, and various degrees of hyperglycemia. The cardiac phenotype of the $ob^-/ob^-$ (leptin-deficient) mice has been studied both early and later in its development with varying results. LV hypertrophy and intramyocardial lipid accumulation are evidence both early and late, suggesting that hyperleptinemia is not necessary for the development of LV hypertrophy.

The hyperleptinemic $db^-/db^-$ (leptin receptor-deficient) mice have a similar metabolic phenotype, but more profound hyperglicemia (30 to 50 mmol/L) manifest earlier in development[31-33]. In db/db mice, systolic and diastolic dysfunction were detected by echocardiography. With the use of ex vivo perfused hearts from db/db mice, augmented left ventricular end-diastolic pressure and reduced cardiac output and cardiac power have also been observed. Given that rodents are resistant to atherosclerosis, these models provided strong evidence for the occurrence of diabetic cardiomyopathy[10].

A similar evolution in cardiac phenotype is evident in genetically engineered rat models involving the absence of a functional leptin receptor. The Zucker fatty (ZF) rat is characterized metabolically by hyperleptinemia and consequent obesity, hyperlipidemia, and marked hyperinsulenemia with the late onset of hyperglicemia. As such, the model is characteristic of obesity and insulin resistance early (3 to 6 months). In contrast, the Zucker diabetic fatty (ZDF) rat has a similar genetic background but demonstrates earlier hyperglicemia, consistent with metabolic feature of type 2 diabetes.

The morphological features of the ZF rat include increased intramyocardial lipid and increased myocardial mass. Hypertrophy is evident in individual myocytes and whole hearts where there is an associated expansion in the extracellular matrix. However, cardiac functional abnormalities have been observed inconsistently in isolated heart preparations with decreased cardiac power and systolic wall stress in some studies, whereas LV pressures and LV dP/dt and the rate-pressure product are maintained in others. In contrast to the ZF rats, the ZDF rats do not demonstrate consistent increase in cardiac mass. At 2 months, there is increased mass in the presence of marked hyperinsulenemia at a time when glucose levels are normal. However, by 3 months, there is no increase in mass when plasma glucose levels are high (27 mmol/L) and plasma insulin levels are reduced. Fredersdorf et al

estabilished the importance of the balance between hyperglycemia and hyperinsulenemia in 5-months old ZDF rats by controlling hyperglycemia with exogenous insulin and demonstrating increased cardiac mass, whereas ZDF rats that remained persistent hyperglycaemic had no increase in mass. Thus, hyperinsulinemic ZF rats have myocardial hypertrophy and variable degrees of diastolic abnormalities, whereas the diabetic ZDF rats have smaller increase in cardiac mass and impairments in LV systolic function in isolated hearts but not intact models. These findings are consistent with the cellular effects of hyperinsulenemia to induce cardiac hypertrophy in contrast to the effects of hyperglycemia that mitigate hypertrophy and affect systolic dysfunction to a greater extent[34-36]. SHHF Mcc-fa/fa is a genetic model of hypertension and end-stage congestive heart failure (10-14 months). SHHF rats that are homozygous for the corpulent ($fa^{cp}$) gene are leptin resistant due to failure to produce a functional leptin receptor. Consequently, they become markedly obese, secondary to leptin resistance. Obese SHHF rats exhibit anomalies similar to human Metabolic Syndrome, such as hyperinsulenemia, impaired glucose tolerance, hyperlipidemia, hypertension and cardiac hypertrophy. In addition, this rat model of leptin resistance and obesity shows alterations in neurohumoral activation that may modulate blood pressure control and progression to congestive heart failure[37]. SHHF Mcc-fa/fa rat represents a congenital model of diabetic cardiomyopathy,

which exhibits several hallmark signs of the human disease state[38]. However, this strain have not yet been extensively investigated in the evolution of their pathology.

*Models of increased intramiocardial lipid without Hyperinsulenemia or hyperglycemia*

Mice with cardiac specific overespression of PPARα is a model characterized by cardiac dysfunction that is mediate by increased NEFA uptake and intracellular accumulation. The model does not possess characteristic features of diabetes. However, this model is particularly useful in studying the contribution of excess intracellular lipid accumulation to cardiac contractile dysfunction, in the absence of hyperglycemia and hyperinsulenemia. Recent data using transgenic murine models of cardiac specific overespression of fatty acid transport protein (FATP) demonstrated a cardiac phenotype that feature diastolic dysfunction. Taken together, it is abundantly clear that increased circulating NEFAs, a ubiquitous feature of both type 1 e 2 diabetes, contribute fundamentally not only to the development of both insulin resistance and compensatory hyperinsulenemia but also can directly affect cardiac function.

*Cardiovascular manifestations in humans with type 1 and 2 diabetes*

In clinical studies of asymptomatic diabetics has been observed cardiac abnormalities such as LV hypertrophy and impairment in both

isovolumic relaxation and ventricular filling**,** particularly among diabetic women. In fact, estrogens induced activation of Akt-1 and its transcriptional effects in cardiomyocytes. In addition, clinical studies have demonstrated consistent abnormalities in coronary flow reserve in patients with diabetes in the absence of epicardial heart disease. Both findings correlate closely with macrovascular complications, particularly autonomic dysfunction. What is surprising that the clinical manifestation are similar in both type 1 and type 2 diabetics[36-43]. These clinical findings are different than animal models. Taken together, experimental data obtained using animals models of diabetes should be used with caution when extrapolating to the human condition[10]. Type 1 diabetic patients are treated with insulin and are therefore not truly hypoinsulemic as in the STZ models. Insulin has been shown to mitigate systolic dysfunction in STZ-induced diabetes in rats. Notably, systolic dysfunction at rest and during exercise in type 1 diabetes is observed most commonly in patients with microvascular complications, a reflection of poor glycemic control[44-45].

### 1.4 Aim of the study

The aims of the present study were, first, to obtain an adequate model to investigate physiopatological modifications that lead to one of the most dangerous complications of diabetes, cardiomyopathy. Second, to study

alteration of vascular response in diabetes. Third, we to characterize SHHF/Mcc-fa/fa rat, as a congenital model of diabetic cardiomyopathy, by exploring the pathological evolution of Metabolic Syndrome and cardiac complications, especially regarding functional and humoral alterations.

# 2. METHODS

## 2.1 Animals

Rats were housed in makrolon cages (Tecniplast Gazzada) with food (pellets, Mucedola, except for the ZDF/Gmi-fa/fa rats which were given a Purina 5008 diet, Charles River) and water ad libitum. The light cycle was automatically controlled (on at 7:00 AM and off at 7:00 PM), and the room temperature was thermostatically controlled to 22 ± 2 °C, relative humidity: 50 ± 15%. Before the experiment, the animals were housed in these conditions at least for 7 days to become acclimatized. Studies were performed in accordance with Italian and European regulations on protection of laboratory animals.

## 2.2 Hypertrophic myocardiopathy: induction and characterization

### *2.2.1 Cardiac hypertrophy induction*

Thirty six male Wistar rats (Harlan Italy S.p.A., Via Fermi 8, Correzzana (Mi), Italy) weighting 150-175 g were used.

Rats were numbered, randomized and assigned to sham or hypertrophied group. Each group was initially formed by ten animals. The animals were anesthetized (60 mg/2 mL/kg ip Pentobarbital) and positioned on their right side. Abdominal aorta was exposed through an ipsilateral incision and ventricular pressure overload hypertrophy was elicited by

constriction of a titanium clip (Ø 0.8 mm) around the vessel. Sham-operated rats underwent the same surgical procedure but without insertion of the clip. The cutaneous incision was sutured and animals kept alone in cages for the first 48 h. The animals that died within the first 48h were substituted. The study was divided into phases: the first provided for one-month survival of the rats after coarctation, the second, three month.

*2.2.2 Evaluation of cardiac contractility of hypertrophic rats*

Cardiac contractility was evaluated through cannulation of the left ventricle to measure pressure and derived parameters. Such procedure was performed utilizing a polyethylene catheter (PE50) connected to a pressure transducer (p23Db; Statham Wood Dale, Il. U.S.A.) attached to a preamplifier. The animals were stabilized for 15-20 minutes, after which basal values were recorded for at least five minutes. The monitored parameters included: left ventricular pressure (LVP) and its maximal rate of rise (LVdP/dt) and decrease (-LVdP/dt) and heart rate (HR), all recorded and analyzed via a computerized data acquisition system (IOX vers. 1.7.0, EMKA). At the end of homodynamic studies the animals were euthanized by a lethal dose of Nembutal the abdomen opened and the clip position checked. Then heart, lungs and liver were dried and carefully weighed.

## 2.3 Vascular reactivity in Streptozotocin-induced diabetic rats

### *2.3.1 Diabetes induction and animal preparation*

Male Wistar rats (Charles River – Calco CO Italy), weighting 280-320 g, were injected with *STZ* (50 mg/kg, iv; diabetic group) or vehicle sodium citrate buffer pH 4.5 (control group, "age-matched") after an overnight fast. After 10 days, rats with blood glucose values > 400 mg/100 mL (Reflomat S, Boehringer) were including in this study.

Rats were anesthetized by Pentobarbital sodium (Nembutal, 50 mg/kg ip), 11 and 18 weeks after induction of diabetes. Trachea was catheterized to improve breathing, right jugular vein for agonist injection, the left femoral vein for ganglion-blocking agent infusion and the left carotid artery to monitoring blood pressure by a pressure transducer (p23Db; Statham Wood Dale, Il. U.S.A.) connected to a preamplifier.

### *2.3.2 Experimental protocol*

The animals were stabilized for 15 minutes and after we administered a bolus of phenylephrine (Phe, 3 µg/kg iv) and after 15 minutes bolus of acetylcholine (Ach, 3 µg/kg iv). After recovery, we administered cumulative escalating doses of:

***a)*** Endothelin-1 (Et-1, 0.1 - 2 nmol/kg)

***b)*** Big Endothelin-1 (rbig-Et-1, 0.1 - 10 nmol/kg)

***c)*** Angiotensin II (Ang II, 0.001 - 10 nmol/kg)

***d)*** Angiotensin I (Ang I, 0.001 - 10 nmol/kg)

Each animal received only administration of escalating doses of one peptide. Blood pressure was recorded continuously along the experiments.

*2.3.3 Model of hypertrophic cardiomyopathy and diabetes*

Wistar rats were prepared, as describe in paragraph 2.2, to induce cardiac hypertrophy and 1-month after aortic coarctation were administered to the same rats, STZ 50 mg/kg i.v. Unfortunately, the 20 treated rats presented high rate mortality (> 80%). Thus we decided to abandon this model.

## 2.4 Genetic Diabetic Cardiomyopathy model: Functional and humoral evaluation

It was recently commercially available SHHF Mcc/fa-fa rat strain. The homozygote rats for recessive fa gene of this strain, was characterized by the expression of Metabolic Syndrome 100% associated to cardiomyopathy. SHHF rats originated from a mating between the Koletsky rat (an Sprague Dawley derivate) and inbred SHR from the Okamoto strain. This colony exhibits the following traits: hypertension (100 percent), obesity (25 percent; expressed as an autosomal recessive cp/cp trait) and congestive heart failure (100 percent)[46-47]. So we decided

to evaluate the evolution of Syndrome at different age to characterize the model[48-50]. The study was conducted in comparison with normal, diabetic and hypertensive rat strains. The animals used were 30 male of each strain (Charles River – Calco CO Italy), 10 weeks old on arrival: "age-matched" Wistar Kyoto (WKY), used as normotensive and euglycemic control, spontaneously hypertensive (SHR), Zucker diabetic fatty (ZDF/Gmi-fa/fa) and homozygote obese, spontaneously hypertensive heart failure-prone (SHHF Mcc-fa/fa) rats. All rats of each strain were enlisted at age of 6-10 months and randomly assigned to two age groups, based on arrival group.

*2.4.1 Nociception*

The exploitation of SHHF rats in comparison with WKY, SHR and ZDF started with the assessment of their nociceptive sensibility by means of a "Tail-Flick" test.

**Figure 5. "Tail Flick" apparatus**

The rats of each strain and same age were put into a small cage with opening for the tail at rear wall. The tail was held gently by the investigator. Through the opening of a shutter, a light beam exerting radiant heat was direct to the distal third of the tail (Figure 5). We have been observed the reaction of the animal for about 6 seconds. The rat tried to pull its tail away. The shutter has been switched closed as quickly as possible and the reaction time marked by instruments. The test has been repeated in triplicate and the values averaged.

*2.4.2 Humoral analysis*

Blood was sampled from tail vein of animals fasted for 6 hours at target ages two days after "Tail-Flick" test to measure plasma levels of glucose, triglyceride, cholesterol and insulin.

*2.4.3 Measurements of blood pressure, skin blood perfusion flow and aortic blood flow*

The hemodinamic was evaluated in anesthetized animals (1-2% Isofluorane in $N_2O/O_2$ mixture) fasted for 18 hours. Procedures: the right jugular vein was catheterized for ganglion-blocking agent infusion (Pentolinium, 0.075 mg/kg), the left jugular vein to peptide injection and the right carotid artery for blood pressure monitoring by a pressure transducer (p23Db; Statham Wood Dale, Il. U.S.A.) connected to a

preamplifier. Than, to monitor skin blood perfusion flow (PF), a laser Doppler probe was positioned on posterior left leg and data continuously recorded by a computerized acquisition system (Periflux 5000, Perimed, Italy). Blood flow microprobes, of a adequate internal diameter (1-1.5 mm), was placed around the upper abdominal aorta just below the diaphragm and connected to a pulsed Doppler flow meter (mod CB 9000, Crystal Biotech, USA). After instrumentation, the animals were placed in an incubator to maintain body temperature at 37 ± 1 °C and stabilized for 15-20 minutes and basal values recorded, after were ganglion-blocked (0.075 mg/kg/min pentolinium through the experiment).

*2.4.4 Vascular reactivity*

Seven animals, per strain and age group, were randomly assigned to experimental group and eight to control group. The rats of experimental group received iv bolus of cumulative escalating doses of endothelin-1 (Et-1; 0.03, 0.1, 0.3, 0.5, 1 and 1.5 nmol/kg). The animals assigned to the control group of each age and strain, received a similar sequences of administrations of physiological saline (1 ml/kg). The parameters monitored included: blood pressure (BP), heart rate, cardiac output (CO), systemic vascular resistance (SVR) all of which were recorded and analyzed continuously via a computerized data acquisition system (IOX version 1.7.0, EMKA). The animal were sacrificed by an overdose of

Pentobarbital (100 mg/kg i.p.). Hearts, lungs, brain and kidney were dried and carefully weighed, then hearts were frozen and stored at -80°C. Organs to brain weight ratio were calculated. Organ/brain weight ratios was used because the severe obesity makes organ/body weight ratios meaningless[49]. Brain weight reflects lean body mass, is easy to obtain, and is reproducible.

**2.5 Statistical Analysis**

Results were expressed as mean ± S.E.M. in the form of table and graph. Statistical evaluation were performed using Anova on ranks: Kruskal Wallis with Dunnet post test, Student *t* test or ANOVA with the Tuckey's post test for multiple groups as appropriate Graphpad Prism 4 (U.S.A.). A value < or = 0.05 was considered statistically significant.

# 3. RESULTS

## 3.1 Model of cardiac hypertrophy

### *3.1.1 One month coarctation*

Body weight was no statistically significant different between one month hypertrophied and sham operated rats (Fig. 1a: 294 ± 16.56 vs 326 ± 8.97 g; p=0.103 ), as well as lungs and liver weights. On the contrary the ratio left ventricular mass/body weight, was statistically significant higher in aortic banded rats with respect to sham animals (fig. 1b: 2.68 ± 0.28 vs 1.87 ± 0.03 mg/g; p= 0.008). At the schedule time, cardiac contractility was evaluated on nine of ten clipped animals because one rat died within the one-month period, and on eight of ten sham rats because 2 animals died during cannulation of the left ventricle.

Rats abdominal aortic banding did not produce significant changes in hemodynamic parameters compared to sham operated: left ventricular pressure showed a slight, but statistically non-significant tendency to increase (Fig.2a: 175 ± 16.53 vs 154 ± 5.13 mmHg, p=0.532), end-diastolic pressure (Fig.2b: 12.0 ± 6.38 vs 5.37 ± 5.01 mmHg, p=0.413) and heart rate (Fig. 4a: 418 ± 13.20 bpm vs 429 ± 8.37 bpm; p=0.496) did not show significant modifications when compared to sham rats.

The maximal dP/dt showed a slight, but not statistically significant decrease versus sham operated rats (Fig. 3a: 4241 ± 587.26 $mmHg^{-sec}$ vs

5168 ± 1003.15 $mmHg^{-sec}$; p=0.425). No significant change was observed in –dP/dt (Fig. 3b: -4420 ± 530.49 vs -4780 ± 749.01 $mmHg^{-sec}$; p=0.695). Also, ratio of +dP/dt to left ventricular mass, which can be translate as an indirect measure of the contractility efficiency of the heart, showed a non-statistically significant to a reduction in hypertrophied rats compared to shams (fig. 4b: 5.65 ± 0.73 vs 8.36 ± 1.66 $mmHg^{-sec}$/mg; p=0.140.

*3.1.2 Three-month coarctation*

The mortality of 3-months coarcted rats was higher with respect to those of 1 month; in fact, three animals out of ten died within the three-month period. No mortal episode occurred in sham operated animals. Body weight of three- months coarcted rats did not show statistically significant differences from sham animals (Fig. 1a: 326 ± 28.09 vs 417 ±11.55 g; p=0.189). Also lungs and liver weight were not modified by aortic coarctation. On the contrary, ratio of left ventricular mass to body weight was higher and statistically significant in hypertrophic rats with respect to sham animals (Fig. 1b: 2.49 ± 0.36 vs 1.64 ± 0.05 mg/g p=0.014). Left ventricular pressure on three-months banded rats showed a non statistically significant trend to increase compared to sham operated animals (Fig. 2a: 161 ± 10.44 vs 138 ± 5.64 mmHg). Left ventricular end-diastolic pressure also showed a non statistically significant trend to increase in hypertrophied rats with respect to sham

animals (Fig. 2b: 32 ± 8.95 vs 17 ± 5.04). Heart rate did not show statistically significant modifications in hypertrophic rats (Fig. 4a: 400 ± 9.15 vs 394 ± 14.53 bpm; p=0.717) nor did maximal dP/dt (Fig. 3a: 2641 ± 334.36 vs 2818 ± 333.01 $mmHg^{-sec}$; p=0.722) and - dP/dt (Fig. 3b: -3126 ± 360.41 vs -2836 ± 307.97 $mmHg^{-sec}$;p00.551). Measure of the contractile efficiency of the heart (ratio of +dP/dt to left ventricular mass) was not difference enough between the two groups to consider it statistically significant (Fig. 4b: 2.93 ± 0.33 vs 4.23 ± 0.58;p= 0.105).

| Parameter | Age, mo | SHAM | COARTED |
|---|---|---|---|
| LVP. mmHg | 1 | 154 ± 5.13 | 175 ± 16.53 |
| | 3 | 138 ± 5.64 | 161 ± 10.44 |
| LVEDP,mmHg | 1 | 5.37 ± 5.00 | 12 ± 6.38 |
| | 3 | 17 ± 5.04 | 32 ± 8.95 |
| HR,mmHg | 1 | 429 ± 8.37 | 418 ± 13.20 |
| | 3 | 394 ± 9.15 | 400 ± 14.53 |
| +dP/dt,$mmHg^{-sec}$ | 1 | 5168 ± 2837.33 | 4241 ± 1761.80 |
| | 3 | 2818 ± 1053.08 | 2641 ± 884.63 |
| -dP/dt,$mmHg^{-sec}$ | 1 | -4780 ± 749.01 | -4420 ± 530.49 |
| | 3 | -2836 ± 307.97 | -2641 ± 360.41 |
| $+dP/dt^{-LVW}$, $mmHg^{-sec}/mg$ | 1 | 8.36 ± 1.66 | 5.65 ± 0.73 |
| | 3 | 4.23 ± 0.58 | 2.93 ± 0.33 |

**Tab 1A. Basal parameters of cardiac contractility**
Values are means ± S.E.M. LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; +dP/dt, The maximal rate of increase; -dP/dt, the maximal rate of decrease; $+dP/dt^{-LVW}$, contractile efficiency of the heart .

## 3.2 STZ diabetic rats

*3.2.1 Eleven weeks to induction of diabetes*

3.2.1.1 Basal parameters

Body weight of 11-weeks STZ rats was markedly reduced (276.6 ± 9.7 vs 486.4 ± 6.0 g; p< 0.0001) and glycemia was significantly increased (492.8 ± 4.1 vs 79.1 ± 2.1 mg/dl; p<0.0001); only systolic blood pressure (SBP) showed a slight, but statistically significant reduction in diabetic rats with respect to controls before and after ganglion-blocking (Tab. 1B).

| | **BW g** | **Gly mg/dl** | | **SBP mmHg** | **DBP mmHg** | **MBP mmHg** |
|---|---|---|---|---|---|---|
| | | | | | ***Before*** | |
| **Controls** | 486.4 ± 6.0 | 79.1 ± 2.1 | | 158.3 ± 4.9 | 123.4 ± 3.9 | 131.4 ± 5.6 |
| **Diabetes** | 276.6 ± 9.7* | 492.8± 4.1* | | 144.6± 4.2* | 116.5 ± 3.3 | 125.8 ± 3.6 |
| | | | | | ***After*** | |
| **Controls** | | | | 89.7 ± 3.0 | 50.1 ± 1.5 | 63.3 ± 1.9 |
| **Diabetes** | | | | 75.6± 3.2* | 50.3 ± 2.6 | 58.7 ± 2.7 |

**Tab. 1B Body Weight, basal pressure levels and glycemia 11 weeks to STZ inducted diabetes**

Values are mean ± S.E.M. BW, body weight; Gly, glicemia; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. These are value of controls and diabetes anesthetized rats after 11 weeks of STZ administering before and after ganglion-blocking. * = p<0.05

*3.2.1.2 Agonist effects*

**Phe:**

Diabetic rats showed, after a bolus of Phe, a higher maximal hypertensive response with respect to controls (47.8 ± 2.0 vs 60.2 ± 1.7 mmHg; p<0.0001) (Fig. 5a).

**Ach:**

Bolus of Ach did not show significant difference for hypotensive response between two groups (Fig. 5b).

**Et-1:**

The administration of Et-1 induced a biphasic effects: hypotensive following hypertensive response, which were reduced significantly in diabetes rats compared with controls. The Dose/ response (D/R) curve of hypotensive response for diabetic rats was flat with Emax –22.8 ± 4.2 vs -47.7 ± 5.3 mmHg (p< 0.0001; Tab. 2B, Fig. 6a).

| | **Emax Δ mmHg ± sem** | | **ED50 nmol/kg (95 %)** | **Et-1/big Et-1 Ratio** |
|---|---|---|---|---|
| | **Hypo** | **Hy; NAper** | | |
| **Et-1 Controls** | -47.7 ± 5.3 | 102.9 ± 4.2 | 0.50 ( 0.44-0.58 ) | |
| **Diabetic** | -22.8 ± 4.2* | 67.5 ± 7.4* | 0.83 ( 0.63-1.09 )* | |
| **rbig Et-1 Controls** | **NA** | 103.1 ± 2.8 | 0.72 ( 0.62-0.84 ) | **0.69** |
| **Diabetic** | **NA** | 72.3± 4.0 * | 1.83 ( 1.48- 2.2 ) | **0.45** |

**Tab. 2B Pressure effects of Endothelin-1 (Et-1) or big-Endotelin-1 in controls and diabetic rats 11 weeks after STZ inducted diabetes.**
Values are mean ± S.E.M. Emax=maximal effect; hypo= hypotension; hyper= hypertension; NA= not applicable; Et-1/big-Et-1= potency ratio of two peptides; * = p<0.01.

The D/R of hypertensive response for diabetic rats showed a significant right shift with a statistically significant increase of ED50: 0.83 (0.63-1.09) vs 0.50 (0.44-0.58) nmol/kg and decreasing of the maximal response ( 67.5 ± 7.4 vs 102.9 ± 4.2 mmHg ; $p< 0.0001$; Tab. 2B, Fig 6b).

**rbig-endothelin:**

The administration of rbig-endothelin produced only hypertensive response. Diabetic rats showed a statistically significant reduction of the maximal effect exerted by peptide with respect to age-matched rats. The D/R curve also was shifted to right, and of ED50, was significantly higher in diabetic with respect to controls: 1.83 (1.48-2.2) vs 0.72 (0.62-0.84) nmol/kg; Tab. 2B, Fig.7a).

**Et-1/rbig Et-1:**

"Potency Ratio", ratio of ED50 of two peptides, considered as an indirect index of ECE activity, was reduced (0.45 vs 0.69) in diabetes rats with respect to controls (Tab. 2B).

**Ang II:**

The administration of escalating doses of Angiotensin II induced hypertensive dose-dependent effect in all experimental groups.

In diabetes rats D/R curve showed a modest right shift with respect to controls without significant modifications of Emax. Moreover, ED50 was only modestly and non statistically significant reduced in diabetes

rats with respect to controls: 33.8 (16-71) and 42.7 (31-59) pmol/kg, respectively; Tab. 3B, Fig. 7b).

| | Emax<br>Δ mmHg ± sem | ED50<br>pmol/kg (95 % C.I.) | Ang II/Ang I<br>Ratio |
|---|---|---|---|
| **Ang II** | | | |
| **Controls** | 109.6± 11 | 42.7 ( 31-59 ) | |
| **Diabetes** | 94.3 ± 3.4 | 33.8 ( 16.71 ) | |
| **Ang I** | | | |
| **Controls** | 121.7 ± 3.1 | 108.9 ( 90-131 ) | **0.39** |
| **Diabetes** | 89.8 ± 4.6* | 62.8 ( 37-107 ) | **0.54** |

**Tab. 3B Pressure effect of Angiotensin II (AngII) and Angiotensin I (AngI) in diabetes and controls rats**
Values are mean ± S.E.M. Emax=maximal effect; AngII/AngI Ratio= potency ratio of two peptides; * = $p<0.05$.

**Ang I:**

The administration of escalating doses of Ang I induced dose-dependent hypertensive effect in both experimental groups. In diabetes rats the curve showed a statistically significant decreasing of the maximal pressure effect. ED50 was not significantly different 62.8 (37-107) vs 108.9 (90-131) pmol/kg in both groups (Tab.3B, Fig 8).

**Ang II/Ang I:**

"Potency Ratio" (PR) index was 0.54 for diabetic rats and 0.39 for normal rats (Tab. 3B).

*3.2.2 Eighteen weeks to induction of diabetes*

3.2.2.1 Basal parameters

The body weight of diabetic rats was significantly decreased (292 ± 8 vs 554 ± 11 g ; p< 0.0001) and glycemia was strongly increased (479.5 ± 6.6 vs 71.8 ± 1.3 mg/dl; p< 0.0001). Basal pressure levels were significantly decreased in diabetes rats.

There was a decreasing of SBP after ganglion-blocking, while DBP and MBP were not statistically different in two groups (tab. 4B).

| | BW g | Gly mg/dl | SBP mmHg | DBP mmHg | MBP mmHg |
|---|---|---|---|---|---|
| | | | | **Before** | |
| **Controls** | 554 ± 11 | 71.8 ± 1.3 | 167.9 ± 2.3 | 128.8 ± 2.2 | 141.8 ±2.2 |
| **Diabetic** | 292 ± 8 | 479.5 ± 6.6* | 148.0± 4.4* | 118.0 ± 3.5* | 128.4± 3.7* |
| | | | | **After** | |
| **Controls** | | | 95.8 ± 4.6 | 53.4 ± 2.1 | 67.5 ± 2.7 |
| **Diabetic** | | | 79.6± 2.4* | 54.7 ± 3.1 | 63.0 ± 2.8 |

**Tab. 4B Body Weight, basal pressure levels and glycemia 18 weeks to induction diabetes**

Values are mean ± S.E.M. BW, body weight; Gly, glicemia; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. These are value of controls and diabetes anesthetized rats after 11 weeks of STZ administering before and after ganglion-blocking. * = p<0.05

3.2.2.2 Agonist effect

**Phe:**

Diabetes rats showed a significantly reduction of the maximal pressure response after bolus of Phe from controls (49.6 ± 2.2 vs 65.7 ± 2.1 mmHg; p< 0.0001; Tab. 4B, Fig. 9a).

**Ach:**

Bolus of Ach induced a not statistically significant reduction of the maximal hypotensive response in diabetic rats with respect to controls (-20.3 ± 1.5 vs -24. 0 ± 1.1 mmHg; Tab. 4B, Fig. 9b).

**Et-1:**

The administering of Et-1 induced the typical biphasic response: hypotension following hypertension, and both components of the response were significantly reduced in diabetic animals with respect to controls. The curve of the hypotensive response was significantly shifted on the right and Emax reduced in diabetic rats (-19. 9 ± 2.1 vs -60. 1 ± 8.3mmHg; p < 0.0001; Tab. 5B, Fig. 10a) .

| | Emax Δ mmHg ± sem | | ED50 nmol/kg (95 % C.I.) | Et-1/big Et-1 Ratio |
|---|---|---|---|---|
| | Hypo | Hyper | | |
| **Et-1** | | | | |
| **Controls** | -60.1 ± 8.3 | 87.4 ± 4.2 | 0.46 ( 0.39-0.54 ) | |
| **Diabetic** | -19.9 ± 2.1* | 73.2 ± 4.8* | 0.75 ( 0.66-0.85 )* | |
| **rbig Et-1** | | | | |
| **Controls** | **NA** | 99.6 ± 3.7 | 0.44 ( 0.32-0.60 ) | 1.08 |
| **Diabetic** | **NA** | 77.1 ± 4.7* | 1.06 (0.84-1.36 )* | 0.71 |

**Tab. 5B Pressure effect of Endothelin-1 (Et-1) or big-endothelin-1(bigEt-1) in controls and diabetes rats 18 weeks to induction diabetes.**
Values are mean ± S.E.M. Emax=maximal effect; hypo= hypotension; hyper= hypertension; NA= not applicable; Et-1/big-Et-1= potency ratio of two peptides; * = $p<0.05$.

Similarly, the hypertensive phases showed a marked shift to right with a significant increased of ED50: 0.75 (0.66-0.85) and 0.46(0.39-0.54) nmol/kg respectively, and a reduction of the Emax to the maximal experimental doses, (1.5 nmol/kg in controls and 2 nmol/kg in diabetes animal) (Tab. 5B, Fig. 10b). Higher doses were lethal.

**rbig-Et-1:**

The administration of rbig Et-1 only produced hypertensive phase in both groups. In diabetic animals the maximal response was significantly lower than in normal age-matched rats (Tab. 5B, Fig. 12a). Moreover, in diabetic animals, D/R curve was shifted to right and ED50 was increased significantly: 1.06 (0.84-1.36) nmol/kg vs 0.44 (0.32-0.60) nmol/kg (Tab. 5B, Fig. 11b).

**Et-1/rbig Et-1:**

Finally "Potency Ratio", index values continued to be lower in diabetic compared to age-matched normal rats : 0.71 vs 1.05 (Tab. 5B).

**Ang II:**

The administering of escalating doses of Ang II showed dose-dependent pressure effect for both experimental groups.

Ang II D/R curve was lightly right shifted and Emax reduced, without a statistically significant ED50 reduction [49.5 (34-70) vs 51.6 (37-72) nmol/kg Tab. 6B, Fig. 12a].

| | **Emax Δ mmHg ± sem** | **ED50 pmol/kg (95 % C.I.)** | **Ang II/Ang I Ratio** |
|---|---|---|---|
| **Ang II** | | | |
| **Controls** | 120.2 ± 7.8 | 51.6 ( 37-72 ) | |
| **Diabetic** | 99.6 ± 2.6* | 49.5 ( 34-70 ) | |
| **Ang I** | | | |
| **Controls** | 118.3 ± 4.0 | 97.9 ( 69-142 ) | **0.53** |
| **Diabetic** | 103.0 ± 9.4 | 99.8 ( 51-196 ) | **0.50** |

**Tab. 6B Pressure effect of Angiotensin II (AngII) and Angiotensin I (AngI) in diabetes and controls rats**
Values are mean ± S.E.M. Emax=maximal effect; AngII/AngI Ratio= potency ratio of two peptides; * = $p<0.05$.

**Ang I:**

Whereas, Ang I administration induced similar pressure response in both animal groups without significant variation in Emax or ED50: 99.8 851-196) vs 97. 9 (69-142) pmol/kg; (fig. 12b).

**Ang II/Ang I:**

Finally,“ Potency Ratio “ (PR) also was similar in both groups (Tab. 6B).

### 3.3 SHHF as a diabetic cardiomyopathy model

*3.3.1 Six months of age*

3.3.1.1 "Tail Flick" test

"Tail-Flick" test showed a significant reduction of nociceptive sensibility increasing times reaction (Tab. 1C) in SHHF 6.8 (5.0-11.0) sec and SHR 7.2 (2.8-12.7) sec strains with respect to WKY 2.9 (2.5-3.1) sec or ZDF 2,8 (2,3-4,2) sec, ($p<0.05$)

3.3.1.2 Body and organ weights

SHHF body and organ weights (liver, kidney e left ventricle; Tab. 2C), and in ZDF rats kidney and liver weight were statistically significant increased respect values found in SHR and WKY rats ($p<0.001$).

3.3.1.3 Humoral analysis

Experimental data confirmed metabolic disorders about SHHF and ZDF strains. In fact, in fasted animals of both strains has been found the typical hematochimical alteration of the Metabolic Syndrome (Tab. 3C) *ZDF*: Tri (889 ± 643 mg/dl); Cho (320±103 mg/dl); Glu (424 ± 94mg/dl); Ins (2 ± 1 ng/ml); *SHHF*: Tri (1052 ± 353 mg/dl); Cho (240 ±

46 mg/dl); Glu (116 ± 22 mg/dl); Ins (59 ± 17 ng/ml); respect to normal levels occurred in others two strains ***WKY***: Tri (83 ± 21 mg/dl); Cho (91 ± 10 mg/d); Glu (106 ± 7 mg/dl) and ***SHR***: Tri (78 ± 16 mg/dl); Cho (64 ± 6 mg/dl); Glu (102 ± 5 mg/dl); Ins (3 ± 1 ng/ml) ($p<0.001$).

3.3.1.4 Blood pressure, aortic blood flow and skin perfusion flow in anesthetized rat before and after ganglion-blocking

SBP was significantly elevated in SHHF and ZDF rats, before ($p<0.04$) and after ($p<0.001$) administering of ganglion-blocking agent, compared with WKY rats (tab4C-5C) and, significantly ($p<0.004$) lower compared with SHR rats (SBP: 165 ± 6.0 mmHg). In SHR and SHHF rats TPR showed values, statistically significantly, higher compared with WKY ($p=0.01$; $p= 0.03$) and ZDF ( $p=0.002$; $p=0.02$). In SHR rats ABF was significantly lower compared to WKY rats ($p=0.03$).

PF was significantly lower in SHHF rats with respect to ZDF and SHR rats ($p<0.001$ and $p<0.05$, respectively).

3.3.1.5 Vascular reactivity to Et-1 administration

Due to the high mortality in ZDF group, we excluded it of any consideration about Et-1-induced hypertensive responses.

Et-1 induced hypotension: were not statistically significant differently among the remaining all 3 rats strains. Whereas, the maximal hypertensive

response exerted by Et-1 in SHHF (21 ± 19 mmHg) was significant reduced with respect to SHR (75 ± 15 mmHg; p=0.05) (Tab. 6C).

In SHR and SHHF, Et-1-induced TPR increase were minor, although non statistically significant, compared with WKY response, in absence of ABF modification. Et-1 induced PF modification seemed to be greater in SHR and SHHF with respect to WKY.

*3.3.2 Ten months of age*

3.3.2.1 "Tail Flick" test

"Tail-Flick" test showed a progression nociceptive function impairment for SHHF and SHR rats 13.6 (7.8-16.8) (p< 0.05)(Tab. 1C).

| Parameter | Age, mo | Age, mo |
|---|---|---|
| | 6 | 10 |
| WKY | 2,9(2,5-3,1) | 3,0(2,8-3,2) |
| SHR | 7,2(2,8-12,7)* | 4,3(3,5-7,5)* |
| ZDF | 2,8(2,3-4,2) | 4,3(3,3-5,2) |
| SHHF | 6,8(5,0-11,0)* | 13,6(7,8-16,8)* |

**Tab 1C. Evaluation of nociceptive activity by " Tail Fick" test.**
Values are median (n=15, animals), in brackets there are ranges 25%-75%.
The adaptation was 2 minutes for each animals and current intensity 50 mÅ.
Anova on ranks: Kruskal- Wallis (6 mo) H=21,71; df=3; p<0.0001.
Dunnett-test;*p<0.05 SHR and SHHF vs WKY.
Anova on ranks: Kruskal- Wallis (10 mo) H=37,74; df=3; p<0.0001.
Dunnett-test;* p<0.05 SHR and SHHF vs WKY.

3.3.2.2 Body and organ weights

At 10-months of age, SHHF still showed cardiac, renal and hepatic hypertrophy (Tab. 2C), as demonstrating by remarkable increase of ratio

organ weight / brain weight OW/BrW with respect to all other strains ($p<0.001$).

| Parameter | Age, mo | WKY, n=15 | SHR, n=15 | ZDF, n=15 | SHHF, n=15 |
|---|---|---|---|---|---|
| BW, g | 6 | 381 ± 5 | 343 ± 5 | 355 ± 14 | 632 ± 14d |
| | 10 | 407 ± 5 | 368 ± 5 | 324 ± 11 | 610 ± 45d |
| LV/BrW, mg | 6 | 636 ± 15 | 718± 11a | 676± 18 | 876 ± 17d |
| | 10 | 690 ± 13 | 810 ± 25 | 691 ± 36 | 967 ± 55d |
| KW/BrW, mg | 6 | 1578 ±37 | 1766 ±42ab | 2866± 106ab | 2559 ± 65d |
| | 10 | 1587± 31 | 1723 ± 33ab | 2963 ±231ab | 2331 ± 171d |
| LW/BrW, mg | 6 | 6242 ± 111 | 7061± 12 | 13675± 853ab | 21222 ± 989d |
| | 10 | 6790 ± 164 | 8228 ±305 | 15789 ± 863ab | 15227± 1639d |

**Tab 2C. Body weights and organ weights/brain weights**
Values are means ± S.E.M. Both at 6- and 10-months-old n=15 animals WKY, Wistar Kyoto; SHR, Spontaneously hypertensive rats; ZDF, Zucker Diabetic Fatty rat; SHHF, SHHF/Mcc-fa/fa.
BW, Body Weight; LV/BrW, left ventricle/Brain weight; KW/BrW, Kidney weight/Brain weight; LW/BrW, Liver weight/Brain weight. a Significantly different from age-matched WKY; b significantly different from age matched SHR; c Significantly different from age-matched ZDF; d Significantly different from age-matched rats of other strains ( $P< 0.001$).

3.3.2.3 Humoral analysis

At ten-months of age SHHF rats confirmed the Metabolic Disorder characterized by obesity, high plasmatic level of triglycerides, cholesterol, and insulin ($p<0.0001$), maintaining euglycemic condition (Tab. 3C).

| Parameter | Age, mo | WKY | SHR | ZDF | SHHF |
|---|---|---|---|---|---|
| Tri, mg/dL | 6 | 83.7 ± 21.1 | 77.9 ± 16.4 | 889.2 ± 642.6ab | 1051.7 ± 353.4d |
| | 10 | 60.3 ± 9.1 | 66.4 ± 12.7 | 1503.6 ± 777.9ab | 715.7 ± 458.7d |
| Cho, mg/dL | 6 | 90.9 ± 10.2 | 63.6 ± 5.9 | 319.5 ± 103.4ab | 240.2 ± 45.6d |
| | 10 | 93.9 ± 11.5 | 64.0 ± 7.6 | 440.3 ± 142.6ab | 218.7 ± 101.9d |
| Ins, ng/mL | 6 | 4.7 ± 1.3 | 2.7 ± 1.0 | 1.4 ± 0.4 | 58.5 ± 17.1d |
| | 10 | 5.1 ± 1.4 | 3.5 ± 1.1 | 1.1 ± 0.3 | 23.5 ± 11.1d |
| Glu, mg/dL | 6 | 106.1 ± 6.5 | 101.4 ± 5.2 | 424.7 ± 94.3ab | 116.5 ± 21.7c |
| | 10 | 126.3 ± 6.4 | 119.8 ± 4.6 | 409.8 ± 122.1ab | 113.1 ± 10.9c |

**Tab 3C. Metabolic parameters**
Values are means ± S.E.M. Tri, triglyceride; Cho, cholesterol; Ins, insulin; Glu, glucose. See table 2C for definition of symbols.

3.3.2.4 Blood pressure, aortic blood flow and skin perfusion flow in anesthetized rat before and after ganglion-blocking

In SHHF strain, SBP was significantly lower compared with SHR rats (p=0.01). Instead, in SHR and ZDF rats blood pressure was still higher compared with WKY, normotensive control (p=0.005 and p=0.03; respectively). TPR and ABF were not statistically significant different with respect to all other strains. This suggested that SHHF rats showed a condition of heart failure. Then, in all strains PF did not show statistically significant modifications, but SHHF rats showed a slight, but statistically significant (p=0.02), reduction with respect to ZDF rats (Tab. 4C-5C).

| Parameter | Age, mo | WKY | SHR | ZDF | SHHF |
|---|---|---|---|---|---|
| | | 6 mo n=14<br>10 mo n=13 | 6 mo n=13<br>10 mo n=10 | 6 mo n=10<br>10 mo n=7 | 6 mo n=12<br>10 mo n=13 |
| SBP, mmHg | 6 | 114 ± 8 | 165 ± 6a | 136 ± 5ab | 137 ± 6ab |
| | 10 | 109 ± 3 | 134 ± 5a | 125 ± 7a | 107 ± 7b |
| TPR, AU | 6 | 46 ± 10 | 80 ± 9a | 40 ± 5b | 90 ± 17ac |
| | 10 | 33 ± 8 | 74 ± 32 | 31 ± 6 | 44 ± 12 |
| ABF, kHz | 6 | 9 ± 2 | 5 ± 0.3a | 6 ± 1 | 6 ± 1 |
| | 10 | 13 ± 1 | 10 ± 2 | 9 ± 1 | 11 ± 1 |
| PF, AU | 6 | 40 ± 3 | 55 ± 5a | 64 ± 6a | 33 ± 4bc |
| | 10 | 60 ± 6 | 54 ± 9 | 85 ± 15 | 45 ± 7c |

**Tab 4C. Hemodynamic basal parameters**
Values are means ± S.E.M. SBP, systolic blood pressure; TPR, tissutal pressure resistance; ABF, aortic blood flow; PF, perfusion flow. See table 2C for definition of symbols.

| Parameter | Age, mo | WKY | SHR | ZDF | SHHF |
|---|---|---|---|---|---|
| | | 6 mo n=14<br>10 mo n=13 | 6 mo n=13<br>10 mo n=10 | 6 mo n=10<br>10 mo n=7 | 6 mo n=12<br>10 mo n=13 |
| SBP, mmHg | 6 | 61 ± 2 | 97 ± 5a | 78 ± 3a | 80 ± 4ab |
| | 10 | 68 ± 3 | 77 ± 2a | 87 ± 2ab | 66 ± 4bc |
| TPR, AU | 6 | 17 ± 2 | 97 ± 5a | 78 ± 3ab | 41 ± 10d |
| | 10 | 11 ± 1 | 19 ± 5 | 19 ± 3a | 26 ± 9 |
| ABF, kHz | 6 | 10 ± 1 | 5 ± 0.6a | 8 ± 1 | 9 ± 1b |
| | 10 | 15 ± 1 | 13 ± 2 | 4 ± 1 | 12 ± 1 |
| PF, AU | 6 | 42 ± 6 | 97 ± 5a | 78 ± 3ab | 39 ± 5bc |
| | 10 | 62 ± 10 | 47 ± 7 | 88 ± 17b | 72 ± 26 |

**Tab 5C. Hemodynamic parameters after ganglion-blocking agent infusion (Pentolinium 0.075 mg/kg)**
Values are means ± S.E.M. SBP, systolic blood pressure; TPR, tissutal pressure resistance; ABF, aortic blood flow; PF, perfusion flow. See table 2C for definition of symbols.

3.3.2.5 Vascular reactivity of Et-1 administration

At 10-months of age, the administration of Et-1 induced the maximal SBP increase in SHR with respect to WKY and SHHF. The variations

induced by Et-1 on all other monitored parameters were non statistically significantly, although in SHR the TPR were tendentially higher than in SHHF and WKY; ABF less reduced in SHR with respect to SHHF and WKY and PF lower SHHF compared with SHR and WKY rats (Tab. 6C).

| Parameter | Age, mo | WKY | SHR | SHHF |
|---|---|---|---|---|
| | | 6 mo n=6<br>10 mo n=7 | 6 mo n=7<br>10 mo n=5 | 6 mo n=5<br>10 mo n=6 |
| ΔSBP, mmHg | 6 | 48 ± 24 | 75 ± 15 | 21 ± 19b |
| | 10 | 57 ± 18 | 88 ± 8 | 59 ± 10 |
| ΔTPR, mmHg | 6 | 39 ± 21 | 10 ± 46 | 16 ± 30 |
| | 10 | 38 ± 11 | 56 ± 23 | 28 ± 25 |
| ΔABF, kHz | 6 | -0.3 ± 2 | 0.5 ± 1 | -1.8 ± 2 |
| | 10 | -5.7 ± 1.7 | -3.1 ± 1.6 | -4.3 ± 2.3 |
| ΔPF, mmHg | 6 | 34 ± 16 | 77 ± 35 | 61 ± 56 |
| | 10 | 81 ± 46 | 54 ± 13 | 38 ± 38 |

**Tab 6C.The maximal responses after bolus Et-1(1.5 nmol/kg) in anesthetized and ganglion-blocked animals.**
Values are means ± S.E.M. SBP, systolic blood pressure; TPR, tissutal pressure resistance; ABF, aortic blood flow; PF, perfusion flow. See table 2C for definition of symbols.

# 4. DISCUSSION

It is well established that diabetic cardiomyopathy contributes to the heart failure and results in the high mortality rate observed in diabetic patients independent of coronary heart disease.

The purpose of the present study was to obtain an adequate model to investigate physiopatological modifications in diabetic cardiomyopathy. Initially we decided to associate the diabetes with cardiac hypertrophy model, that it is validated in our laboratories. To choose the level of cardiac hypertrophy before the induction of diabetes, we studied cardiac hypertrophy progression 1- and 3-months after abdominal aortic banding. The results obtained suggest that the cardiac hypertrophy occurring from pressure overload due to abdominal aortic banding in both 1- and 3-months coarcted rats, is hemodynamically compensated; in fact, the statistically significant increase of left ventricular mass occurs without compromising hemodynamic parameters such as the contractile and relaxation efficiency of the heart. Thus, for our purpose, we consider the hypertrophy induced by 1-month of aortic coarctation sufficient as background hypertrophic cardiomyopathy. Then, we induced diabetes in 1-month aortic coarctation rats.

Subsequently, to choose the duration of diabetes that produces cardiovascular modification, we valuated the vascular reactivity in STZ-induced diabetic rats after 11- and 18-weeks. STZ-induced diabetic rats

showed a remarkable homogeneity in body weight a glycemic plasma level. These findings strongly suggest that physiopathological alteration observed are strictly correlated to the diabetic condition. In diabetic animals basal systemic pressure occurs significantly lower compared with controls at 18-weeks after diabetes induction, while at 11-weeks after diabetes induction diabetic animals only shows a significantly reduction of the systemic blood pressure. The ganglion-blocking agent antagonized sympathetic tone in the diabetic animals to compensate for hypotension tendency. In fact, in diabetic animals there is a reduced responsiveness to Phe, α1-agonist; a chronic increased of basal sympathetic tone could take to a " down regulation" of the α-receptor with consequent reduction of the response to the exogenous stimulus. The experimental dates show an evident reduction of efficiency and potency of Et-1 to 11-week, reduction of the velocity of conversion rbig→Et-1 and reduction of the ratio Et-1/big Et-1 in diabetes rats compared with age-matched both 11-week and 18-week. In diabetic rats there is a great reduction of hypotensive phase with a normal response to Ach. This result suggests that population of Et-s receptor (ET-B) could be altered. The reduction of the maximal hypertensive response of Et-1 suggests an "alteration" also in the population of receptor of Et-1 of the vascular smooth muscle cells (VSMC). The ratio of potency of Et-1 among two groups (C/D ratio) is 0.60 at 11 weeks and 0.61 at 18 weeks.

This result suggest a reduction of the number of receptor for Et-1. ED50 Et-1/bigEt-1 showed at 11- and 18-weeks in diabetes rats (ratio = 0.45 and 0.71) with compared to controls with PR 0.69 and 1.08 (respectively), confirming the of hypothesis “alteration “ of ECE activity during diabetic pathology. The results about renin-angiotensin system suggest that in diabetes rats Ang II shows a reasonable reduction of efficiency and potency with respect to controls. The same result we have with pro-peptide, Ang I, that, after conversion in Ang II with ACE, induce pressure response. The ratio of potency Ang II/Ang I is not different in both groups at 11-and 18-weeks. Then, in experimental diabetes renin-angiotensin system shows an evident alteration of the vasoconstrictor function of the active peptide while the conversion AngI→AngII with ACE do not change. Then, in the similar pathologic conditions, renin-angiotensin and endothelin system react in different way. There is a selective alteration in vasal function during the development of diabetic pathology. The correlation between experimental dates and the physiopathology of the typical vascular complications of the diabetic pathology is not clear.

Afterwards, diabetes was induced to hypertophic rats, but high mortality showed under that conditions induced us to abandon this model, at that point was necessary to use a different model to study evolution of diabetic cardiomyopathy. The commercial availability of SHHF rat

induced as to characterize it. Thus a novel comprehensive evaluation of the functional and humoral expression of physiopathology of this rat strain was conducted in the obese male SHHF rat model compared with age-matched controls (WKY), hypertensive (SHR) and diabetic (ZDF) rats at 6- and 10-months of age. The results obtained showed that SHHF strain present the characteristic of Metabolic Syndrome: hypertrigliceridemia, hypercholesterolemia, hyperinsulinemia, in euglycemic condition with respect to all other strains both at 6- and at 10-months of age. Then, SHHF rats had significantly elevated body weight, elevated LvW/BrW with respect to WKY and SHR strains. This result confirmed that SHHF rats were characterized by metabolic syndrome and cardiac hypertrophy. Compared with age-matched WKY rats, resting blood pressure was elevated in all SHHF rats; compared with 10-month-old, blood pressure BF and PF were significantly decreased but not TPR, index of probably cardiac dysfunction. The results, obtained by tail flick test, showed that SHHF rats had a significantly elevated time of reaction especially at 10-month-old, index of progressive of diabetic neuropathy, another diabetic complication. Furthermore, SHHF strains showed a increased vascular reactivity to vasoconstrictor agent like Et-1 during different age (6- and 10- months-old). In conclusion, these result identify SHHF Mcc-fa/fa rat as a congenital model to better helpful to investigate the physiopathology modifications that lead to diabetic cardiomiopathy.

# 5. GRAPHS

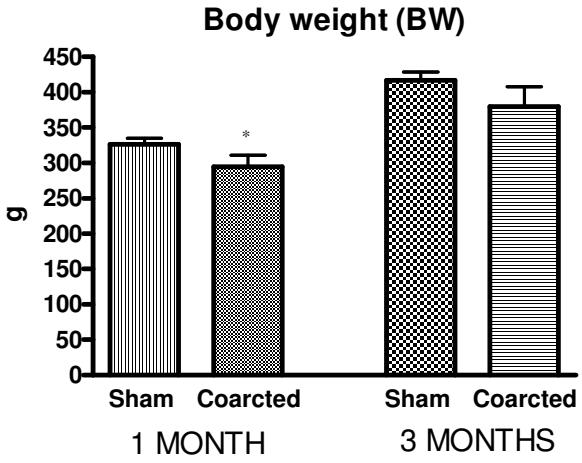


Fig. 1a

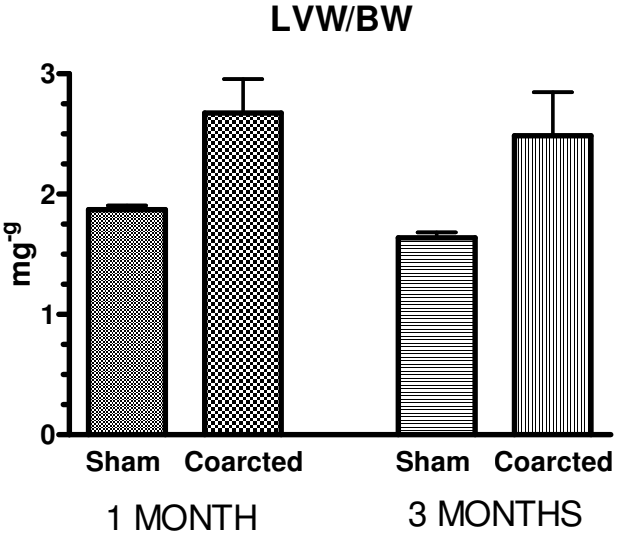


Fig. 1b

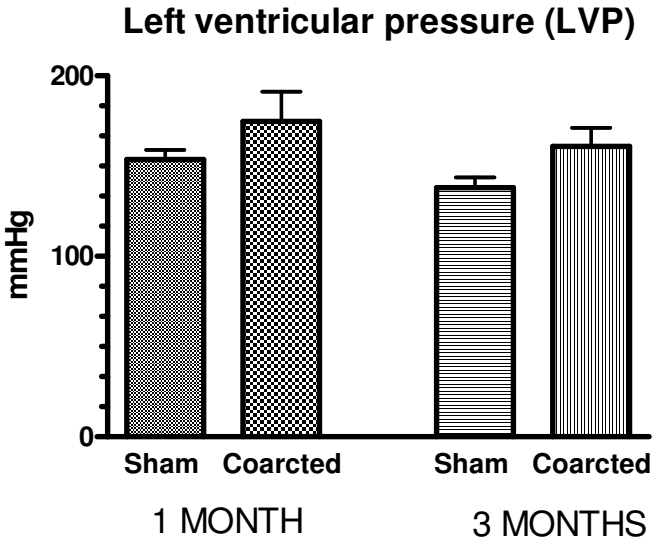
Left ventricular pressure (LVP)
200
100
0
mmHg
Sham
Coarcted
Sham
Coarcted
1 MONTH
3 MONTHS

Fig. 2a

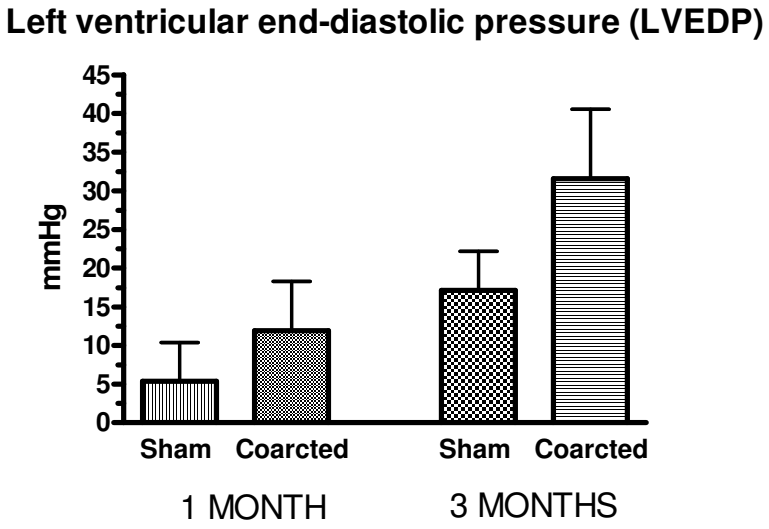
Left ventricular end-diastolic pressure (LVEDP)
45
40
35
30
25
20
15
10
5
0
mmHg
Sham
Coarcted
Sham
Coarcted
1 MONTH
3 MONTHS

Fig. 2b

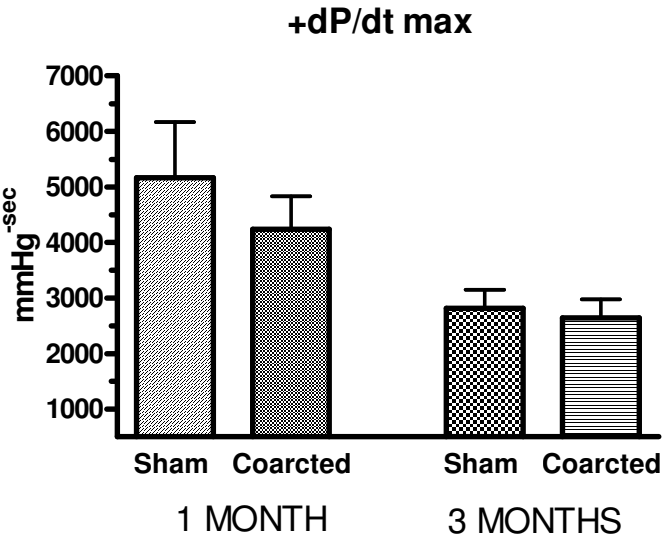
+dP/dt max
7000
6000
5000
4000
3000
2000
1000
mmHg-sec
Sham
Coarcted
Sham
Coarcted
1 MONTH
3 MONTHS

Fig.3a

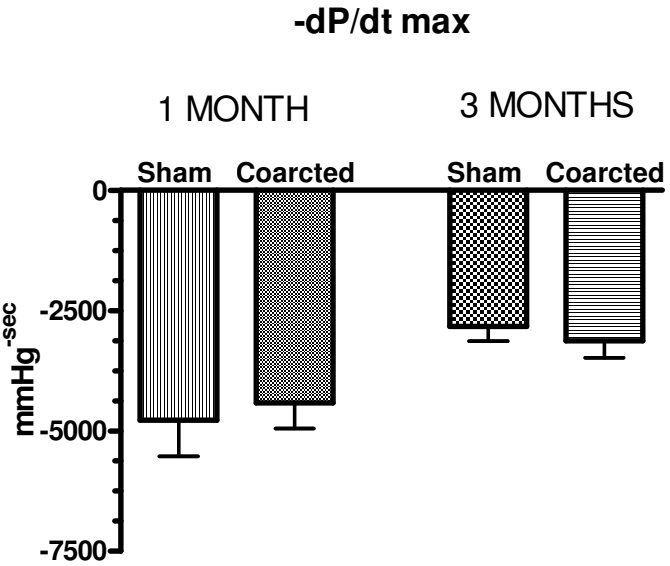
-dP/dt max
1 MONTH
3 MONTHS
Sham
Coarcted
Sham
Coarcted
0
-2500
-5000
-7500
mmHg-sec

Fig. 3b

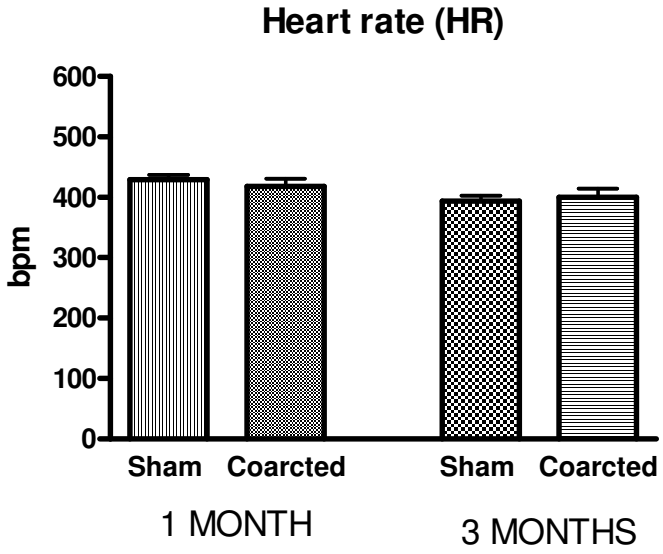
Heart rate (HR)
600
500
400
300
200
100
0
bpm
Sham
Coarcted
Sham
Coarcted
1 MONTH
3 MONTHS

Fig.4a

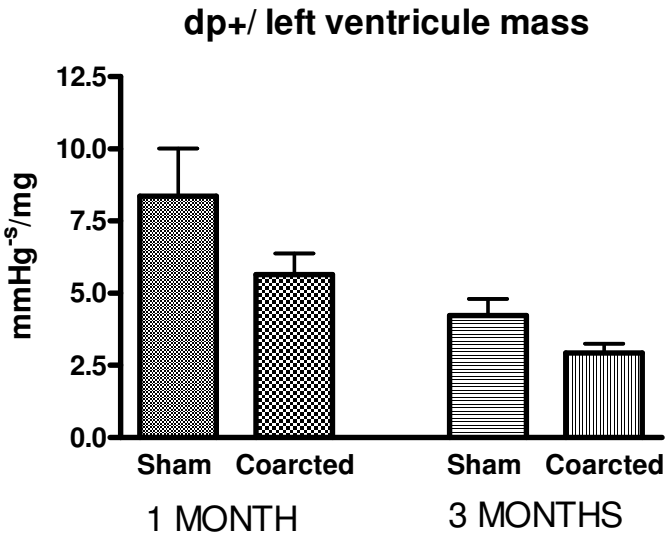
dp+/ left ventricule mass
12.5
10.0
7.5
5.0
2.5
0.0
mmHg-s/mg
Sham
Coarcted
Sham
Coarcted
1 MONTH
3 MONTHS

Fig.4b

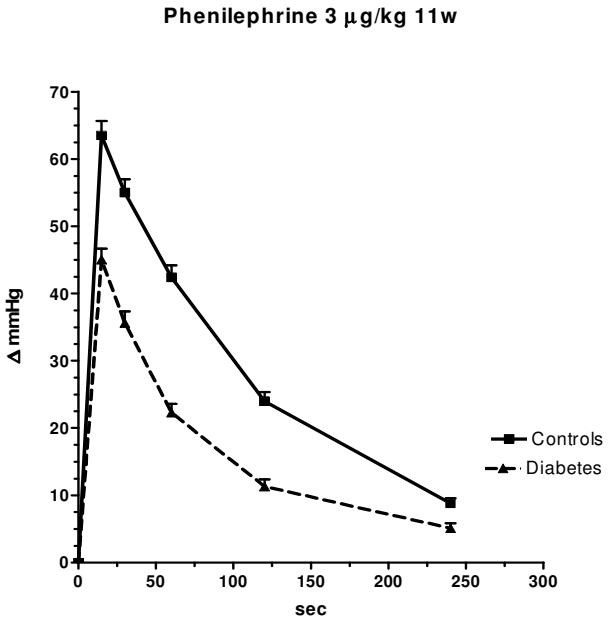
Phenilephrine 3 µg/kg 11w
Δ mmHg
sec
Controls
Diabetes

Fig. 5a

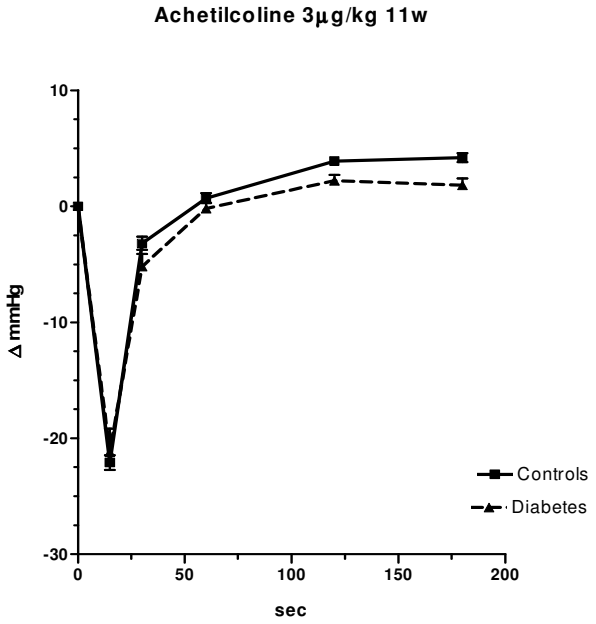
Achetilcoline 3µg/kg 11w
Δ mmHg
sec
Controls
Diabetes

Fig. 5b

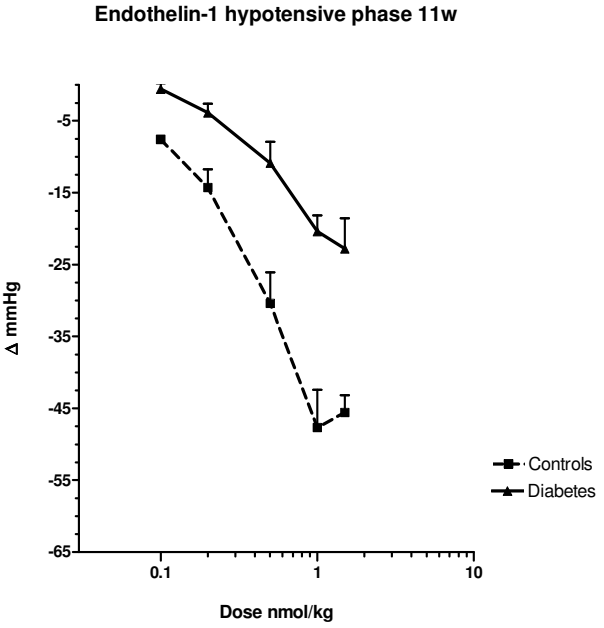
Endothelin-1 hypotensive phase 11w
Δ mmHg
-5
-15
-25
-35
-45
-55
-65
0.1
1
10
Dose nmol/kg
Controls
Diabetes

Fig. 6a

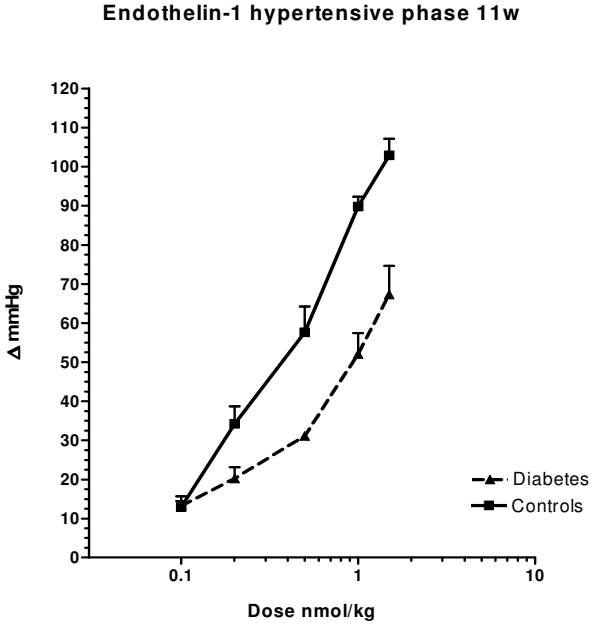
Endothelin-1 hypertensive phase 11w
Δ mmHg
120
110
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
Dose nmol/kg
Diabetes
Controls

Fig 6b

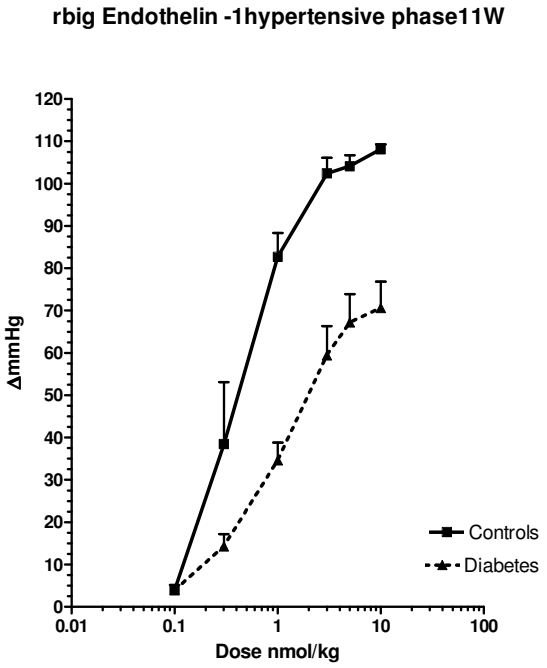
rbig Endothelin -1hypertensive phase11W
ΔmmHg
Dose nmol/kg
Controls
Diabetes

Fig 7a

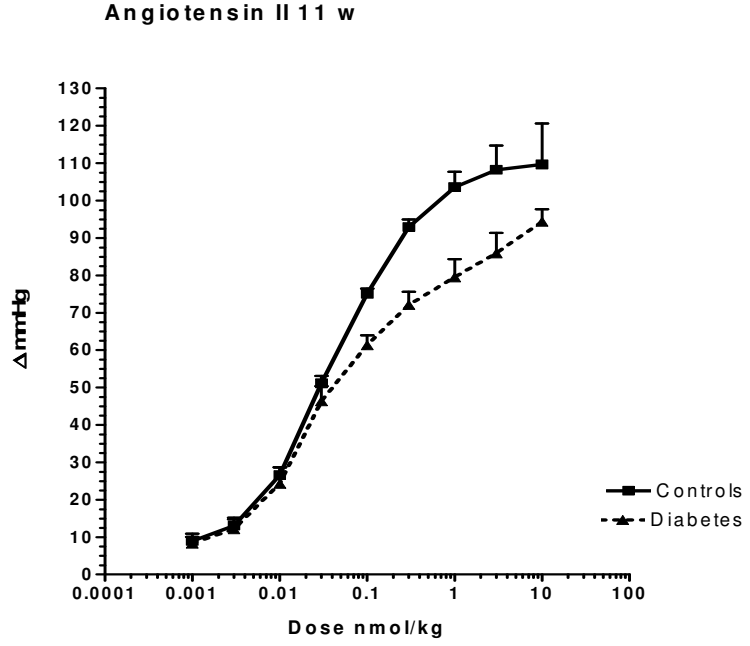
Angiotensin II 11 w
ΔmmHg
Dose nmol/kg
Controls
Diabetes

Fig.7b

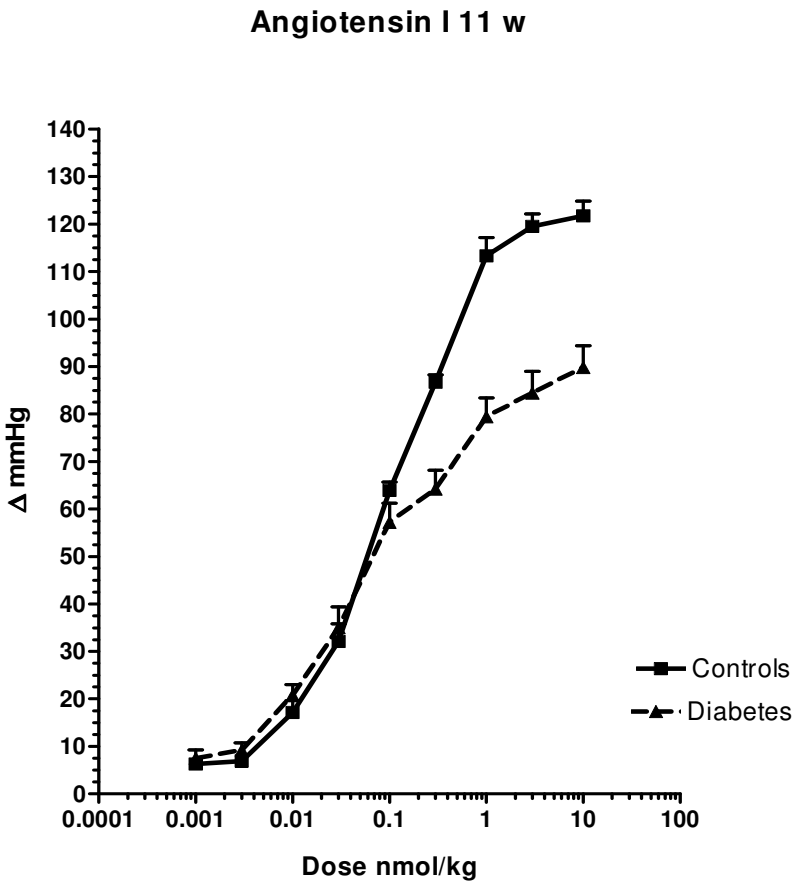
Angiotensin I 11 w
Δ mmHg
Dose nmol/kg
0.0001
0.001
0.01
0.1
1
10
100
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Controls
Diabetes

Fig. 8

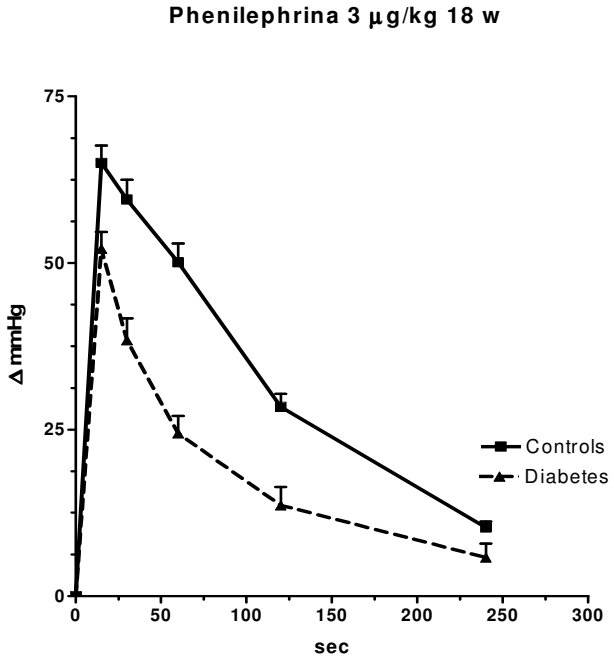
Phenilephrina 3 µg/kg 18 w
Δ mmHg
sec
Controls
Diabetes
75
50
25
0
0
50
100
150
200
250
300

Fig. 9a

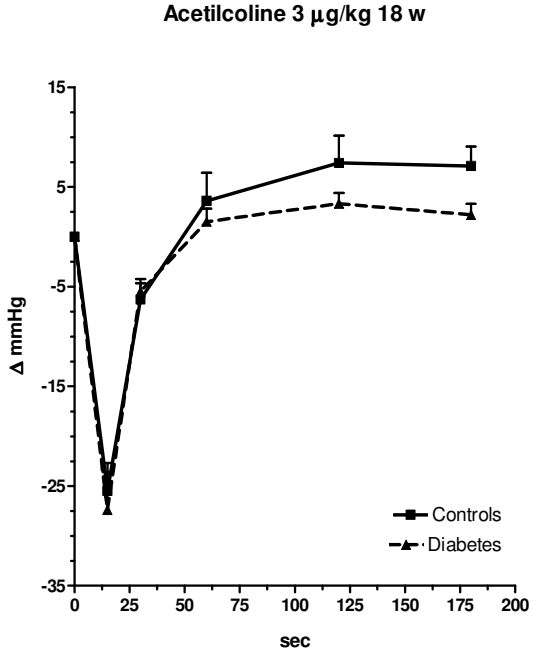
Acetilcoline 3 µg/kg 18 w
Δ mmHg
sec
Controls
Diabetes
15
5
-5
-15
-25
-35
0
25
50
75
100
125
150
175
200

Fig. 9b

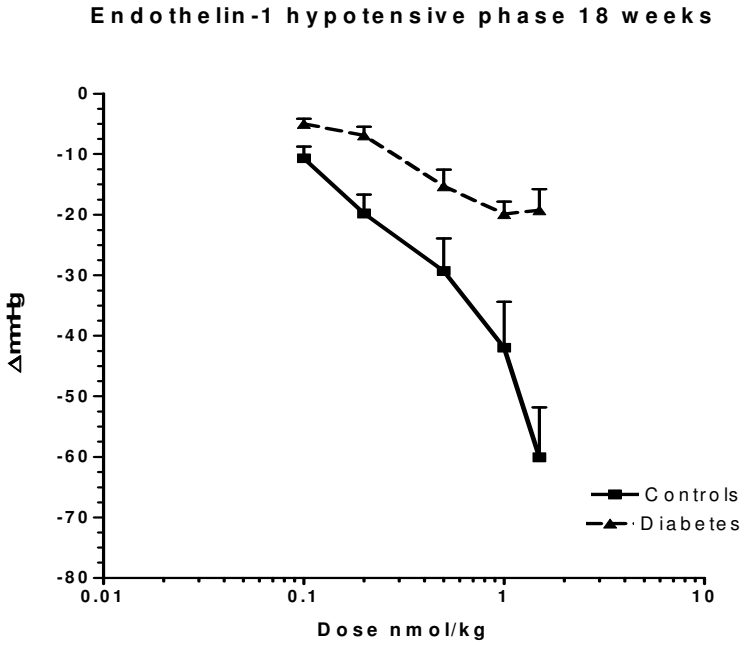


Fig. 10a

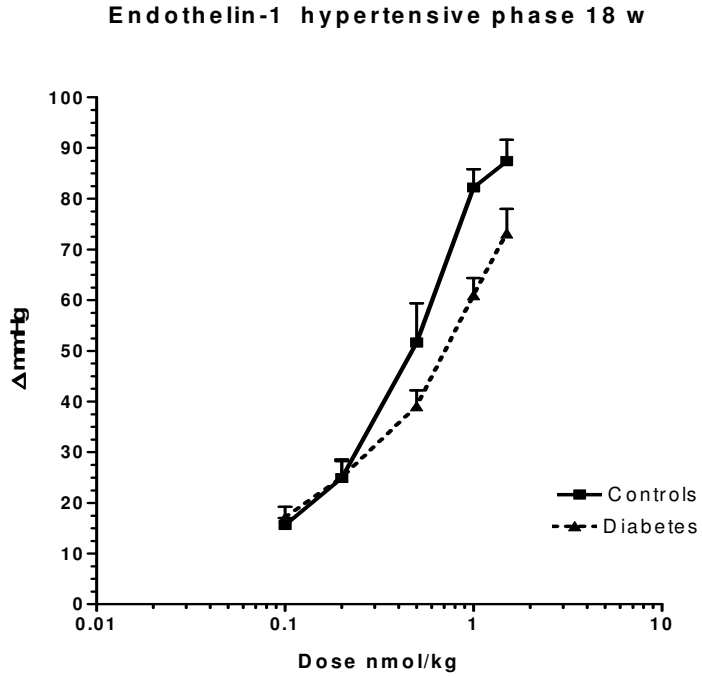


Fig. 10b

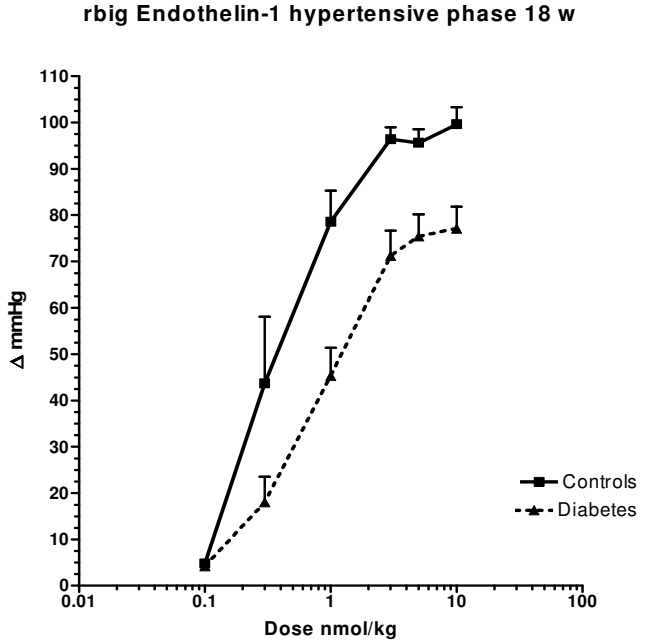


Fig. 11a

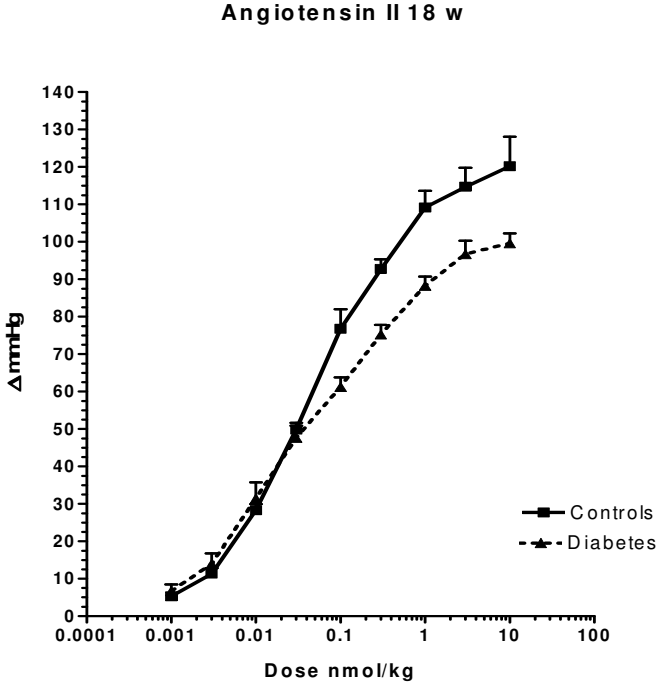


Fig. 11b

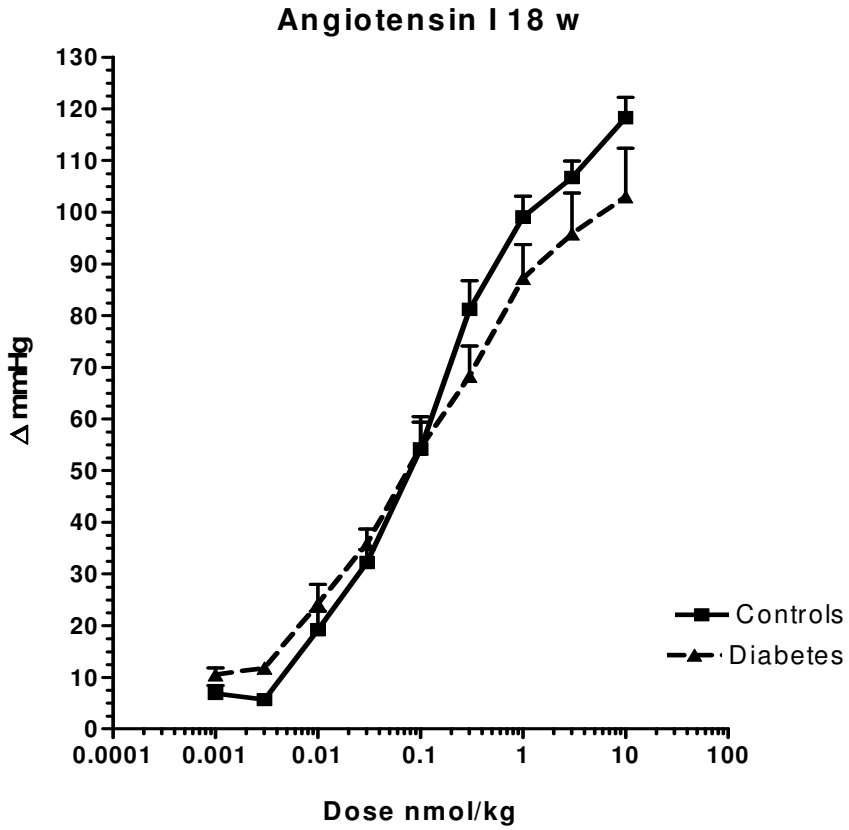
Angiotensin I 18 w
Δ mmHg
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0.0001
0.001
0.01
0.1
1
10
100
Dose nmol/kg
Controls
Diabetes

Fig. 12

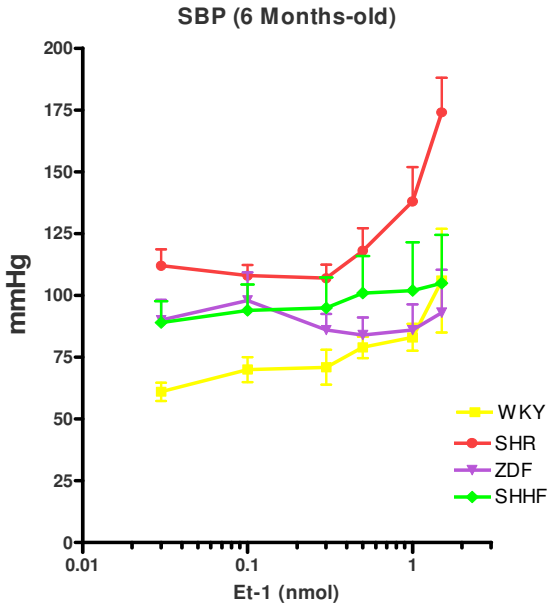
SBP (6 Months-old)
mmHg
200
175
150
125
100
75
50
25
0
0.01
0.1
1
Et-1 (nmol)
WKY
SHR
ZDF
SHHF

Fig. 13

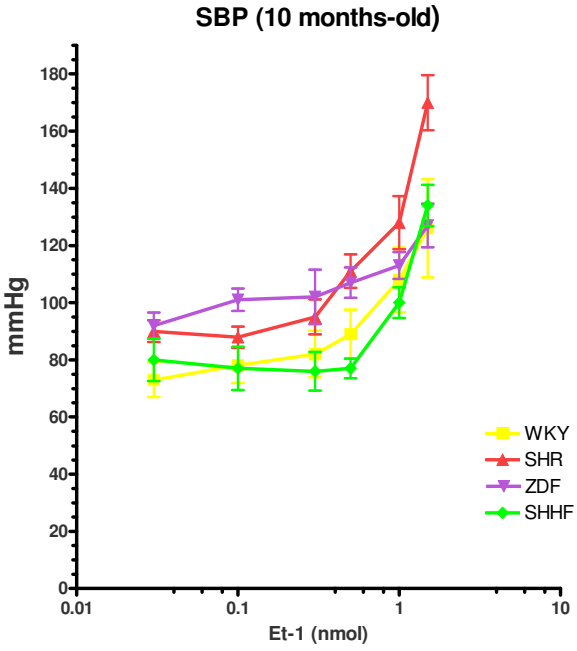
SBP (10 months-old)
mmHg
180
160
140
120
100
80
60
40
20
0
0.01
0.1
1
10
Et-1 (nmol)
WKY
SHR
ZDF
SHHF

Fig. 14

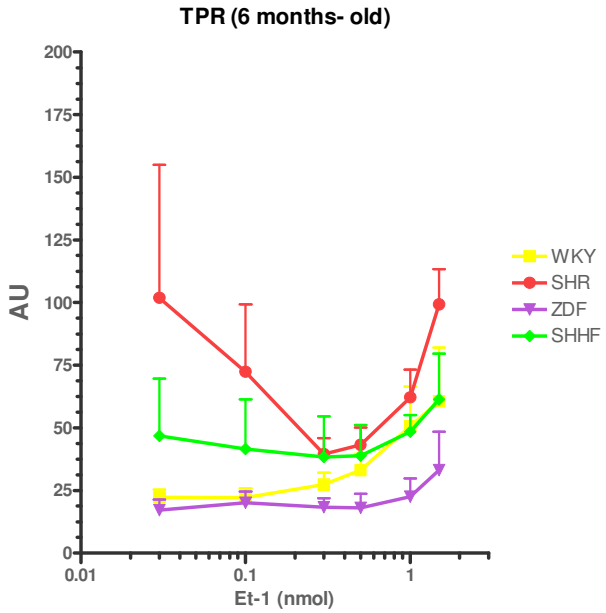
TPR (6 months- old)
AU
200
175
150
125
100
75
50
25
0
0.01
0.1
1
Et-1 (nmol)
WKY
SHR
ZDF
SHHF

Fig. 15

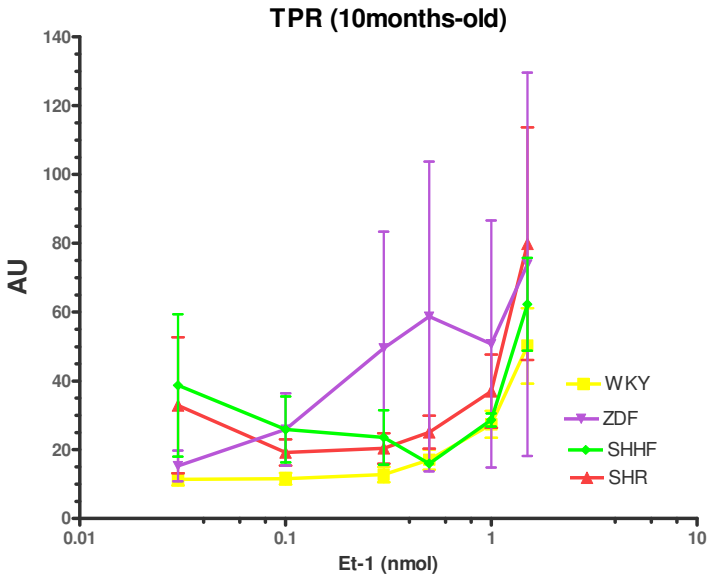
TPR (10months-old)
AU
140
120
100
80
60
40
20
0
0.01
0.1
1
10
Et-1 (nmol)
WKY
ZDF
SHHF
SHR

Fig. 16

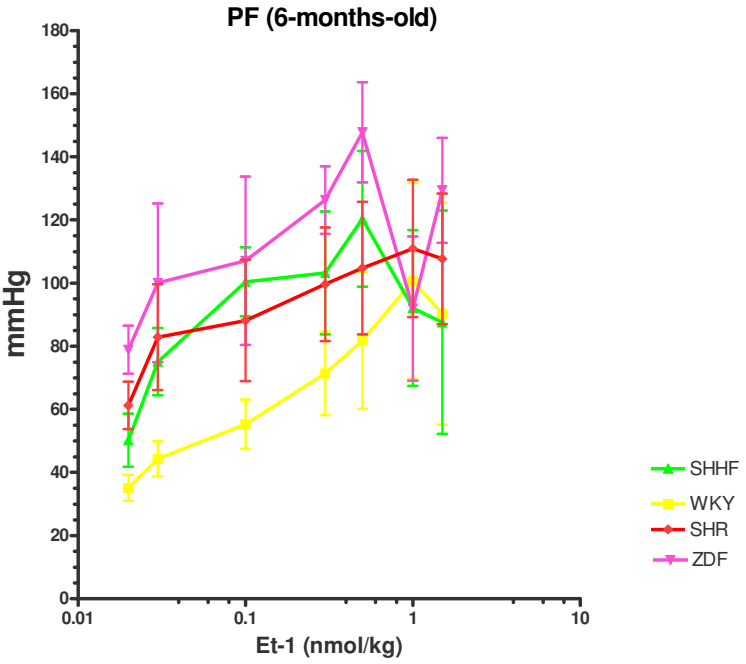
PF (6-months-old)
mmHg
Et-1 (nmol/kg)
SHHF
WKY
SHR
ZDF

Fig. 17

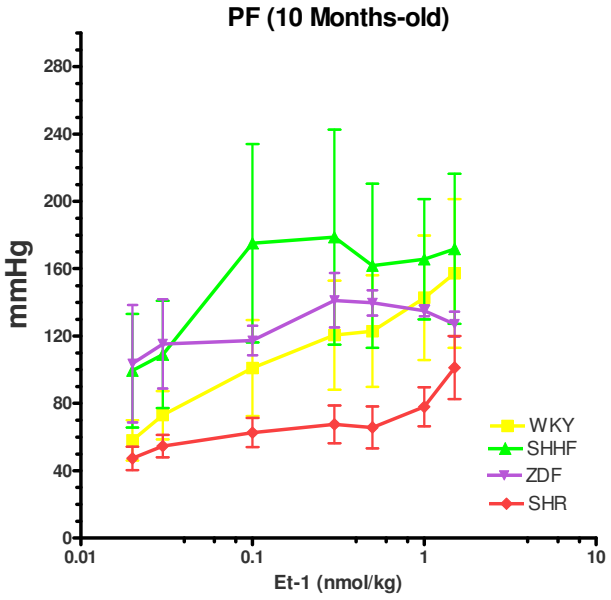
PF (10 Months-old)
mmHg
Et-1 (nmol/kg)
WKY
SHHF
ZDF
SHR

Fig. 18

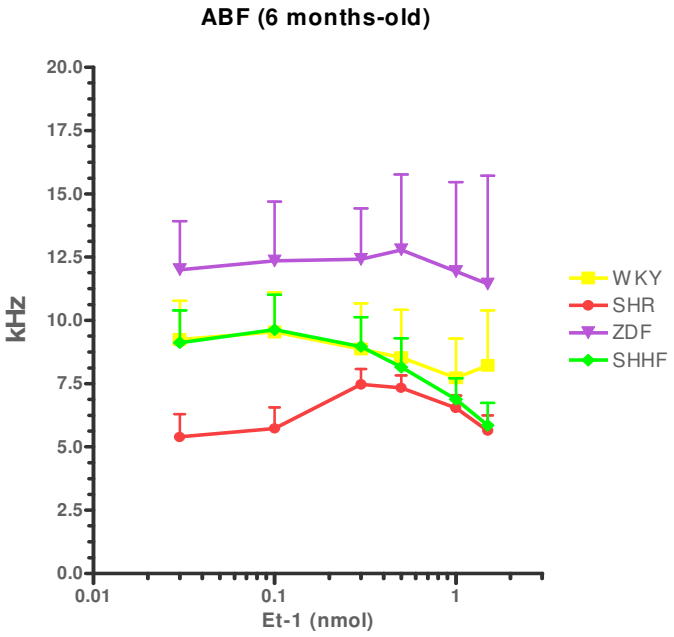
ABF (6 months-old)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
kHz
0.01
0.1
1
Et-1 (nmol)
WKY
SHR
ZDF
SHHF

Fig. 19

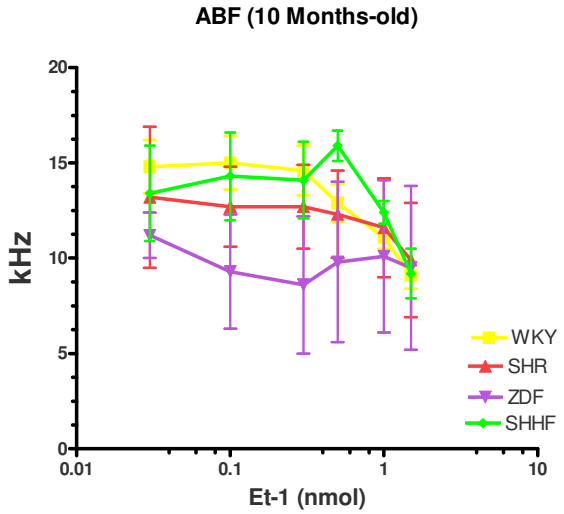
ABF (10 Months-old)
20
15
10
5
0
kHz
0.01
0.1
1
10
Et-1 (nmol)
WKY
SHR
ZDF
SHHF

Fig. 20

# 6. REFERENCES


1. Francis GS. *Diabetic cardiomyopathy : fact fiction?* Heart. 2001; 85 :247-248

2. Picano E. *Diabetic cardiomyopathy. The importance of being earliest.* J Am Coll Cardiol. 2003;42:454-457.

3. Avogaro A, Vigili dKS, Negut C, Tiengo A, Scognamiglio R. *Diabetic cardiomyopathy: a metabolic perspective.* Am J Cardiol. 2004;93:13 A – 16 A.

4. Steven P.Marso, David M.Stern. *Diabetes and Cardiovascular Disease, Integrating science and clinical medicine.* Lippincott Williams & Wilkins 2005.

5. Rajprasad Loganathan, Mehmet Bilgen, Baraa Al-Hafez. *Cardiac dysfunction in the diabetic rat: quantitative evaluation using high resolution magnetic resonance imaging.* 2006; 5:7.

6. Kannel WB, Hjortland M, Castelli WP. *Role of diabetes in congestive heart failure: the Framingham study.* Am J Cardiol. 1974;34:29-34.

7. Kitzman DW, Gardin JM, Gottdiener JS, Arnold A, Boineau R, Aurigemma G, *Cardiovascular Health Study Research Group. Importance of heart failure with preserved systolic function in patients > or = 65 years of age. CHS Research Group. Cardiovascular Health Study.* Am J Cardiol. 2001; 87:413-419.

8. McMurray JJ, Stewart S. *Epidemiology, aetiology, and prognosis of heart failure.* Heart. 2000;83:596-602.

9. Bertoni AG, Tsai A, Kasper EK, Brancati FL. *Diabetes and idiopathic cardiomyopathy: a nationwide case-control study.* Diabetes Care. 2003; 26: 2791-2795.

10. Ding An and Brian Rodrigues. *Role of changes in cardiac metabolism in development of diabetic cardiomyopathy.* Am J Physiol Heart Circ Physiol 2006;291:1489-1506.

11. Indu G. Poornima, Pratrik Parikh and Richard P. Shannon. *Diabetic Cardiomyopathy: The Search for a Unifying Hypothesis.* Circ Res. *2006;98;596-605.*

12. Shulman GI. *Cellular Mechanism of insulin resistance.* J Clin Invest. 2000;106:171-176.

13. Birnbaum MJ, *Turning down insulin resistance.* J Clin Invest. 2001;108:655-659.

14. Kim JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, Lee J, Yuan M. *Prevention of fat-induced insulin resistance by salicylate.* J Clin Invest. 2001;108:437-446.

15. Brazil DP, Hemmings BA. *Ten years of protein kinase B signalling: a hard Akt to follow.* Trends Biochem Sci. 2001;26:657-664.

16. Lawlor MA, Alessi DR. *PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?* J Cell Sci. 2001;114:2903-2910.

17. Morisco C, Zebrowski D, Condorelli G. *The Akt-glycogen synthase Kinase 3beta pathway regulates transcription of atrial natriuretic factor induced by bet-adrenergic receptor stimulation in cardiac myocytes. JBiol Chem. 2000,275:14466-14475*
.

18. Schwartzbauer G, Robbins J. The tumor suppressor gene PTEN can regulate cardiac hypertrophy and survival. J Biol Chem.2000;275:35786-35793.

19. Unger RH, Orci L. *Lipotoxic disease of nonadipose tissue in obesity. Int J Obes Relat Metab Disord. 2000;24(suppl 4):S28-S32.*

20. Liu GX, Hanley PJ, Ray J, Daut. *Long – chain acyl- coenzyme A esters and fatty acids directly link metabolism to K(ATP) channels in the heart.* Circ. Res. 2001;88:918-924.

21. Halse R, Pearson SL, McCormack JG. *Effects of tumor necrosis factor- alpha on insulin action in cultured human muscle cells.* Diabetes. 2001;50:1102-1109.

22. Zhang DX, Fryer RM, Hsu AK., Zou AP, Gross GJ. *Production and metabolism of ceramide in normal and ischemic reperfused myocardium of rats.* Basic Res Cardiol. 2001;96:267-274.

23. Iacobellis G, Ribaudo MC, Zappaterreno A, Vecci E, Tiberti C, Di Mario U, Legnetti F. 5. Rajprasad Loganathan, Mehmet Bilgen, Baraa Al-Hafez. *Cardiac dysfunction in the diabetic rat: quantitative evaluation using high resolution magnetic resonance imaging.* 2006; 5:7.

24. McNulty PH. Insulin resistance and cardiac mass: the end of the beginning? Obes Res. 2003; 11: 507-508.

25. Ueno M, Carvalheira JB, Tambascia RC, Bezzera RM. *Regulation of insulin signalling by hyperinsulinaemia: role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway. Diabetologia. 2005;4:506-518.*

26. Wang CC, Goalstone ML, Draznin B. *Molecular mechanism of insulin resistance that impact cardiovascular biology. Diabetes. 2004;53:2735-2740.*

27. Stuart W. Zarich, MD, and Richard W. Nesto, MD. *Diabetic Cardiomyopathy. Curriculum in cardiology.* 1989.

28. Fang Z, Prins JB, Marwich TH. Diabetic Cardiomyopathy: evidence, mechanisms, and therapeutic implications. Endocr Rev. 2004;25:543-567.

29. Depre c, Young ME, Ying J, Ahuja HS. Streptozotocin-induced changes in cardiac gene expression in the absence of severe contractile dysfunction. J Mol Cell Cardiol. 2000; 32:985-996.

30. Scognamiglio R, Avogaro A, Negut C. *Early myocardial dysfunction in the diabetic heart: current research and clinical applications.* Am J Cardiology.1995;27:169-179.

31. Mazumder PK, O'Neil BT, Rberts MW, Buchanan J, Yu UJ. *Impaired cardiac efficiency and increased fatty oxidation in insulin-resistant ob/ob mouse hearts.* Diabetes. 2004;53:2366-2374.

32. Barouch LA, Berkowitz DE, Hare JM. *Disruption of leptin signalling contributes to cardiac hypertrophy independently of body weight in mice*. Circulation. 2003; 108:754-759.

33. McDonagh PF, Hokama JY. *Microvascular perfusion and transport in the diabetic heart.* Microcirculation. 2000;7:163-181.

34. Chatham JC, Seymour AM. *Cardiac carbohydrate metabolism in Zucker diabetic fatty rats.* Cardiovasc Res. 2002;55:104-112.

35. Fredersdorf S, Thumann C, Ulucan C. *Myocardial hypertrophy and enhanced left ventricular contractility in Zucker diabetic fatty rats. Cardiovasc Pathol. 2004;13:11-19*

36. Abe T, Ohga Y. *Left ventricular diastolic dysfunction in type 2 diabetes mellitus model rats* .Am J Physiol Heart Circ Physiol. 2002;282:H138-H148.

37. Sonhee C. Park, Yiwen Liu-Stratton, Lydia C. Medeiros, Sylvia A. McCune and M. Judith Radin. *Effect of male sex and obesity on platelet arachidonic acid in spontaneous hypertensive heart failure. Exp Biol Med.* 2004;229:657-664.

38. Jonathan R. R. Heyen, Eileen R. Blasi, Kristen Nikula. *Structural, functional, and molecular characterization of the SHHF model of heart failure.* AJP-Heart. 2002;283:1775-1784.

39. Finck BN, Lehman JJ, Leone TC, Welch MJ. *The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus.* J Clin Invest. 2002;109:121-130.

40. Huss JM, Kelly DP. *Mitochondrial energy metabolism in heart failure: a question of balance.* J Clin Invest. 2002;109:121-130.

41. Chiu HC, Kovacs A, Blaton RM, Han X. *Transgenic expression of fatty acid transport protein 1 in the heart causes lipotoxic cardiomyopathy.* Circ Res. 2005;96:225-233.

42. Tenenbaum a, Fisman EZ,Schwammenthal E. *Increased prevalence of left ventricular hypertrophy in hypertensive women with type 2 diabetes mellitus.* Cardiovasc Diabetol. 2003;2:14.

43. Camper-Kirby D, Welch S, Walcer A, Shiraishi I. *Myocardial Akt activation and gender. Increased nuclear activity in females versus males.* Circ Res. 2001;88: 1020-1027.

44. Fein FS, Strobeck JE, Malhtra A, Scheuer J, Sonnenblick EH. *Reversibility of diabetic cardiomyopathywith insulin in rats.* Circ Res. 1981;49: 1251-1261.

45. Huikuri HV, Airaksinen JK, Lija M, Takkunen JT. *Echocardiographic evaluation of left ventricular response to isometric exercise in young insulin-dependent diabetics.* Acta Diabetol Lat. 1986;23: 193-200.

46. Xian Ping Yi, A. Martin Gerdes, Faqain Li. *Myocyte redistribution of GRK2 and GRK5 in hypertensive, heart failure-prone rats. Hypertension.* 2002; 39:1058-1063.

47. Mc Cune Sylvia A., Baker Peter B., Stills Harold F. *SHHF/Mcc-cp rat: model of obesity, non-insulin-dependent diabetes, and congestive heart failure.* Ilar news; 1990.

48. Bergaman MR., Kao RH., McCune SA, Holycross BJ. *Miocardial tumor necrosis factor-α secretion in hypertensive and heart failure-prone rats.* Am. J Physiol. 1999; 277 (Heart Circ. Physiol. 46): H543-H550.

49. Park S, McCune SA, Radin MJ. *Verapamil accelerates the transition to heart failure in obese, hypertensive, female SHHF/Mcc-fa/fa rats.* J Cardiovasc Pharmacol. 1997; 29:726-733

50. Radin MJ, Holycross BJ, McCune SA. *Gender modulates activation of rennin-angiotensin and ebdothelin system in hypertension and heart failure.* J Appl. Physiol 2002; 92:935-940.