Improved Cardiac Glucose Uptake: A Potential Mechanism for Estrogens to Prevent the Development of Cardiac Hypertrophy

Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Vijayakumar Govindaraj

aus Vaniyambadi, Indien

Würzburg 2009

Eingereicht am:
.....

Mitglieder der Promotionskommission:

Vorsitzender: **Prof. Dr. Martin J. Müller**

Gutachter : **Prof. Dr. rer. nat. Thomas Brand**

Gutachter: **PD.Dr. Theo Pelzer**

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

I dedicate my work

to

my parents and sister

who made all of this possible,

for their endless encouragement and patience.

I would like to acknowledge the following people whether my appreciation is for intellectual supervision, or for making my time as an overseas PhD student was exciting and unforgettable, both are equally acknowledged.

I would like to thank **PD Dr. Theo Pelzer** for his guidance, understanding, patience and great support in many ways throughout my doctoral study. **Prof. Dr. Georg Ertl** who have initially considered my application and created an opportunity to join his department. **Dr. Marylin P Law, Dr. Kai Hu and Dr. Patric Diel** for their cooperation or collaboration and whose renowned expertise has greatly encouraged and contributed to my work. **Virginija Jazbutyte and Paula Anahi Arias-Loza** for their comments and suggestions in each step of my graduate life.

I also want to thank all of my present and former colleagues especially Natalie Bukhard**,** Matthias Hallhuber, Jenny Muck, Melanie Muelfelder and Tatjana Williams for their help and friendly environment in the lab.

I thank Amudhan, Anbu, Mahesh, Selvam, Samuel, Venkat and Kupps for their constant support and encouragement. I also express my gratitude to my friends in Wuerzburg particularly Narayan, Jaya, Naresh, Padma, Jaggi, Palanichamy and Neelam.

More importantly, none of this would have been possible without the love and patience of my family and it has been constant source of love, concern, support and strength all these years.

I gratefully acknowledge the “Interdisciplinary Center for Clinical Research (*IZKF*, *Wuerzburg*)” for their financial support.

1. Summary

The incidence of cardiovascular diseases including cardiac hypertrophy and failure in pre-menopausal women is lower compared to age-matched men but the risk of heart disease increases substantially after the onset of menopause. It has been postulated that female sex hormones play an important role in cardiovascular health in pre-menopausal women. In animal studies including spontaneously hypertensive (SHR) rats, the development of cardiac hypertrophy is attenuated by 17β-estradiol treatment. Cardiac energy metabolism is crucial for normal function of the heart. In cardiac hypertrophy and heart failure, the myocardium undergoes a metabolic shift from fatty acid as primary cardiac energy source to glucose, which re-introduces the fetal type of metabolism that representing the glucose as a major source of energy. Many studies have reported that the disruption of the balance between glucose and fatty acid metabolism plays an important role in cardiac pathologies including hypertrophy, heart failure, diabetes, dilative cardiomyopathy and myocardial infarction. Glucose enters cardiomyocytes via GLUT1 and GLUT4 glucose transporters and GLUT4 is the major glucose transporter which is insulin-dependent. Cardiac-selective GLUT4 deficiency leads to cardiac hypertrophy. This shows that the decrease in cardiac glucose uptake may play a direct role in the pathogenesis of cardiac hypertrophy. Estrogens modulate glucose homeostasis in the liver and the skeletal muscle. But it is not known whether estrogens affect also cardiac glucose uptake which could provide another mechanism to explain the prevention of cardiac hypertrophy by female sex hormones. In the present study, SHR Rats were ovariectomized (OVX), not ovariectomized (sham) or ovariectomized and treated with subcutaneous 17β-estradiol. After 6 weeks of treatment, body weight, the serum levels of estrogen, insulin, intra-peritoneal glucose tolerance test (IP-GTT), myocardial glucose uptake

by FDG-PET (2-(^{18}F)-fluoro-deoxyglucose (^{18}FDG) and Positron Emission Tomography), cardiac glucose transporter expression and localization and cardiac hexokinase activity were analyzed. As results of this study, PET analysis of female SHR revealed decreased cardiac glucose uptake in OVX animals compared to intact that was normalized by estrogen supplementation. Interestingly, there was no change in global glucose tolerance among the treatment groups. Serum insulin levels and cardiac hexokinase activity were elevated by E2 substitution. The protein content of cardiac glucose transporters GLUT-4 and GLUT-1, and their translocation as determined by fractionation studies and immuno-staining did not show any significant change by ovariectomy and estrogen replacement. Also levels of insulin receptor substrate-1 (IRS-1) and its tyrosine phosphorylation, which is required for activation and translocation of GLUT4, was un-affected in all groups of SHR. Cardiac gene expression analysis in SHR heart showed that ei4Ebp1 and Frap1 genes which are involved in the mTOR signaling pathway, were differentially expressed upon estrogen treatment. These genes are known to be activated in presence of glucose in the heart. As a conclusion of this study, reduced myocardial FDG uptake in ovariectomized spontaneously hypertensive rat is normalized by 17β-estradiol treatment. Increased myocardial hexokinase appears as a potential mechanism to explain increased myocardial glucose uptake by 17β-estradiol. Increased cardiac glucose uptake in response to 17β-estradiol in ovariectomized SHR may provide a novel mechanism to explain the reduction of cardiac hypertrophy in E2 treated SHR. Therefore, 17β-estradiol improves cardiac glucose utilization in ovariectomized SHR which may give rise to possible mechanism for its protective effects against cardiac hypertrophy.

1(a). Zusamanfassung

Erkrankungen des kardiovaskulären Systems, wie beispielsweise Herzhypertrophie oder Herzinsuffizienz treten bei Frauen vor der Menopause im Vergleich zu gleichaltrigen Männern seltener auf. Das Risiko für eine solche kardiovaskuläre Erkrankung steigt jedoch drastisch mit dem Beginn der Menopause an. Aus diesem Grund wird angenommen, dass weibliche Geschlechtshormone kardioprotektive Wirkungen besitzen. Tierstudien an spontan hypertensiven Ratten (SHR) haben belegt, dass eine Herzhypertrophie durch die Behandlung der Tiere mit 17β-Estradiol abgemildert werden kann. Entscheidend für die Funktion des Myokards ist sein Energiemetabolimus, der sich im Verlauf einer Hypertrophie oder Herzinsuffizienz vom primären Fettsäurestoffwechsel auf Glucosemetabolismus umschaltet. Diese Situation entspricht der des fetalen Herzens. Viele Studien haben belegt, dass eine Störung der Balance zwischen Glucose- und Fettsäurestoffwechsel oftmals ein erstes Anzeichen für einen pathologischen Zustand des Herzens, wie z.B. Hypertrophie, Herzinsuffizienz, Diabetes, dilative Kardiomyopathie und Myokardinfarkt ist. Im gesunden Herzen gelangt Glucose über die zwei Glucosetransporter GLUT1 und GLUT4 in die Zellen des Myokards, wobei der insulinabhängige Glut4-Transporter der Hauptglucosetransporter ist. Eine GLUT4-Defizienz führt daher ebenfalls zu einer Herzhypertrophie was wiederum zeigt, dass eine verminderte Glucoseaufnahme im direkten Zusammenhang mit pathologischen Zuständen des Herzens steht. Bisherige Studien haben gezeigt, dass Östrogen an der Glucosehomöostase in Leber und Skelettmuskeln beteiligt ist. Jedoch ist wenig darüber bekannt, ob Östrogen ebenfalls in die kardiale Glucosehomöostase eingreift und inwiefern die kardioprotektive Wirkung des Östrogens in diesem Zusammenhang steht.In der vorliegenden Arbeit wurden weibliche SH-Ratten ovariektomiert (OVX), nicht ovariektomiert (sham) oder ovariektomiert und zusätzlich subkutan mit 17β-Estradiol behandelt. Nach einer Behandlungszeit von 6 Wochen wurden dann das Körpergewicht, die Serumspiegel von Östrogen, Insulin und IPGTT bestimmt, und die Glucoseaufnahme des Myokards mittels FDG-PET analysiert. Zusätzlich wurden Expression und zelluläre Lokalisation der kardialen Glucosetransporter sowie die kardiale Hexokinaseaktivität untersucht. Es konnte gezeigt werden, dass sich eine verminderte Glucoseaufnahme des Herzens bei ovariektomierten Tieren durch Östrogen-Supplementation normalisieren lässt.

Eine Abweichung bezüglich der Glucosetoleranz der einzelnen Gruppen konnte nicht beobachtet werden. Jedoch konnte ein erhöhter Insulinspiegel des Serums und eine erhöhte kardiale Aktivität des Enzyms Hexokinase durch die Behandlung mit Östrogen bei den ovariektomierten Tieren beschrieben werden. Durch Fraktionierungen und immunhistologische Untersuchungen konnte kein signifikanter Unterschied in Bezug auf die Menge sowie die Translokation der Glucosetransporter GLUT1 und GLUT4 im Myokard zwischen den einzelnen Behandlungen der Tiere beschrieben werden. Ferner konnte zwischen den einzelnen Tiergruppen auch kein Unterschied zwischen dem Insulin Rezeptor Substrat-1 (IRS-1) und seiner Tyrosin-phosphorylierten Form festgestellt werden, die für die Aktivierung und Translokation des GLUT4 benötigt werden. Analysen der Genexpression in den Herzen der SH-Ratten konnten allerdings zeigen, dass die Gene *ei4Ebp1* und *Frap1*, die im mTOR Signalweg involviert sind, bei den Östrogen-supplementierten Tieren ein abweichendes Expressionsmuster aufweisen. Über diese Gene ist bekannt, dass sie in der Gegenwart von Glucose im Herzen aktiviert werden und bei der Entstehung einer Herzhypertrophie mitwirken. Basierend auf den PET-Analysen und der Hexokinaseaktivität lässt sich als Resultat dieser Arbeit aussagen, dass Östrogen die kardiale Glucoseaufnahme in SH-Ratten fördert. Diese Ergebnisse könnten einen Hinweis auf einen noch unbekannten Mechanismus geben, um die protektive Wirkung des Östrogens im Hinblick auf die Herzhypertrophie zu erklären. Hinsichtlich der Tatsache, dass keine Veränderungen in der Translokation der GLUT4-Transporter in der Plasmamembran bei den einzelnen Behandlungen der Tiere zu verzeichnen sind, jedoch Veränderungen der Glucoseaufnahme durch die PET-Analysen dargestellt werden konnten, besteht jedoch noch Erklärungsbedarf. Es liegen diverse Studien vor, die diesen Unterschied damit erklären könnten, dass der GLUT4-Transporter in einer inaktiven Form in der Plasmamembran vorliegt bis die Glucoseaufnahme durch den GLUT4-Transporter mittels der Insulin Signaltransduktionskaskade reguliert wird.

2. Introduction

Cardiac hypertrophy, clinically defined as an increase in ventricular mass is an independent risk factor for cardiovascular disease which is a major cause of morbidity and mortality (Levy, Garrison *et al.* 1990). Women have a lesser prevalence for left ventricular hypertrophy (LVH) than men but heart disease increases in postmenopausal women compared to age matched males (Agabiti-Rosei and Muiesan 2002). In animal studies, pressure over-load hypertrophy in ovariectomized mice was reduced by 17β-estradiol substitution (van Eickels, Grohe *et al.* 2001). Cardiac hypertrophy is associated with altered cardiac energy demand which promotes a shift in energy substrate utilization from fatty acid to glucose. Matching of energy metabolism to cardiac demand is very important for heart function and fine tuning of the balance between fatty acid and glucose utilization leveled under physiological condition. The healthy adult heart makes mainly use of long chain fatty acids for its energy requirements (65–90%); the remaining energy is provided by glucose and lactate oxidation. Interruption of the balance between fatty acid and glucose metabolism is frequently noticed in cardiac pathologies (Carvajal and Moreno-Sanchez 2003; Davidoff 2006). Myocardium subjected to a metabolic switch from fatty acid to glucose, reintroduces the fetal substrate metabolic design (Bishop and Altschuld 1970; Taegtmeyer and Overturf 1988; Yonekura, Brill *et al.* 1985). Changes in energy metabolism decrease cardiac ATP levels and cause a shift in contractile protein isoforms expression (α & β MHC). The re-entry of fetal metabolism and metabolic adaptation toward glucose utilization occurs early not before any change in cardiac mass in hypertensive animals (Taegtmeyer and Overturf 1988). This advocates that the change in metabolic substrate utilization to cardiac overload precedes cardiac hypertrophy. The foetal switch appears to be opposite to

systemic insulin resistance (Kemppainen, Tsuchida *et al.* 2003) and decreased myocardial FDG uptake in failing human heart (Razeghi, Young *et al.* 2001). The occurrence of hypertension, insulin resistance and type II diabetes is increased in postmenopausal women which have been shown to have decreased myocardial glucose uptake. While the development of hypertrophy and cardiac failure is characterized by a gradual decrease in fatty acid utilization, compensated by increased glucose utilization, the diabetic heart suffers from impaired glucose uptake, and relies almost completely on fatty acid oxidation. Estrogen is known to maintain glucose metabolism in liver and skeletal muscle. Estrogen controls insulin secretion and release and also expression of glucose transporters in non-cardiac tissues. Genetic alterations in cardiac glucose uptake also adversely affect cardiac function, for instance the disruption of glucose transport by deletion of cardiac specific insulin responsive GLUT4, which is a major glucose transporter, leads to cardiac hypertrophy, myocardial insulin resistance and compensatory expression of the basal glucose transporter GLUT1. Therefore, the present study is mainly to determine whether alteration in cardiac glucose uptake and utilization might contribute to the role of estrogen in preventing development of cardiac hypertrophy in female spontaneously hypertensive rats.

2.1. Estrogen and Estrogen Receptors

Estrogens are steroid hormones produced primarily in ovary, testis, and adrenal cortex. Estrogens regulate the oestrous cycle, promote the development of secondary sexual characters in females and are important for female and male reproduction. Estrogens are derived from cholesterol (fig.1.). There are three naturally occurring estrogens namely estradiol, which is the main ovarian estrogen and has greater

affinity to estrogen receptors, estriol and esterone (Ackerman and Carr 2002; Kuiper, Carlsson *et al.* 1997).

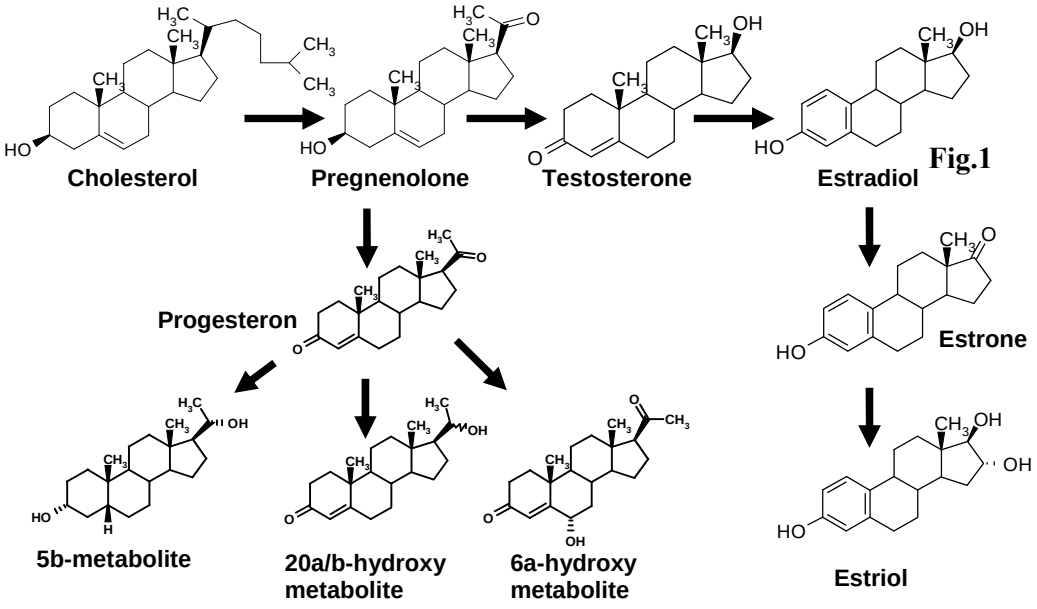


Fig.1. Illustration of Estrogen Biosynthetic Pathway.

Estrogen mediates its effects through estrogen receptors (ER) to activate specific target genes. There are two different isoforms of ER namely ER-α and ER-β (fig.2). ER-β homologues to ER-α in the DNA binding domain (97% amino acid identity) and in ligand binding domain (55% amino acid identity) (Enmark and Gustafsson 1999; Kuiper, Enmark *et al.* 1996). The structural features of the ER's share homologies with other member of nuclear receptor super family in having 5 different domains A/B, C, D, E and F. A/B participates in activation of transcription of target genes, C is the DNA binding domain. The D domain functions as hinge between DNA binding domain and E-domain and the E domain, which is important for ligand binding, receptor dimerization and transcription activation. The F domain is not

present in all nuclear receptor family members and its role is not yet revealed. Both ERs have different ligand binding properties.

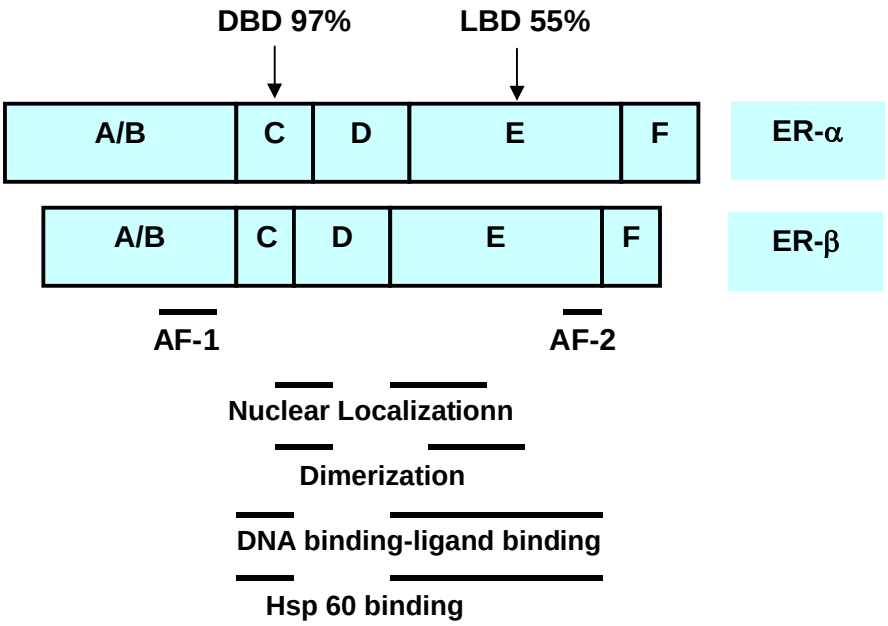


Fig.2. Illustration of the structure of the Estrogen Receptor(ER).

SERMs (Selective Estrogen Receptor Modulators) are compounds which function as ER agonists in some tissues and block estrogen action in other tissues (Dutertre and Smith 2000; Kuiper, Enmark *et al.* 1996). Distribution of estrogen receptors may also account for tissue specific effect of estrogen (Couse and Korach 1999; Kuiper, Carlsson *et al.* 1997)

2.2. Mechanism of Estrogen Signalling

The classical confer actions of estrogen are genomic effects which arise from estrogen receptor by interaction with DNA and activate or repress the specific set of gene

expression. Estrogen confers also non-genomic effects which account for transcriptional activation or repression of target genes at apace manner (fig.3).

2.2.1. Genomic Effects of Estrogen

Estrogen diffuses through the plasma membrane and the nuclear membrane of the cell. Intracellular estrogen binds to estrogen receptors which are present in a complex with proteins like heat shock proteins. The dissociation of estrogen receptor from heat shock proteins promotes dimerization of receptor protein (Auricchio, Migliaccio *et al.* 1990; Landers and Spelsberg 1992; Pratt and Toft 1997). The activated ER bind directly to Estrogen Response Elements (ERE) as homodimer or heterodimers and facilitates the transcription of target mRNA synthesis (Klein-Hitpass, Schorpp *et al.* 1986; Nilsson and Gustafsson 2002; Peale, Ludwig *et al.* 1988) or indirectly through transcription factors (Nilsson, Makela *et al.* 2001). Post translational modifications such as phosphorylation, acetylation, sumoylation etc., also modulate transcriptional activation of nuclear hormone receptors (Germain, Staels *et al.* 2006).

2.2.2. Non-Genomic Effects of Estrogen

Non-genomic effects of estrogen have been seen observed many tissues like uterus and neurons (Falkenstein and Wehling 2000; Nilsson, Makela *et al.* 2001; Sak and Everaus 2004). These occur quickly after estrogen treatment and cannot be blocked by inhibitors of transcription. Studies have proposed that the non-genomic effects by estrogen may stimulate mitogen-activated protein kinase (MAPK) (Pedram, Razandi

et al. 2006) and extracellular regulated kinase signalling or intracellular Ca2+ release (Mermelstein, Becker *et al.* 1996), due to specific plasma membrane estrogen

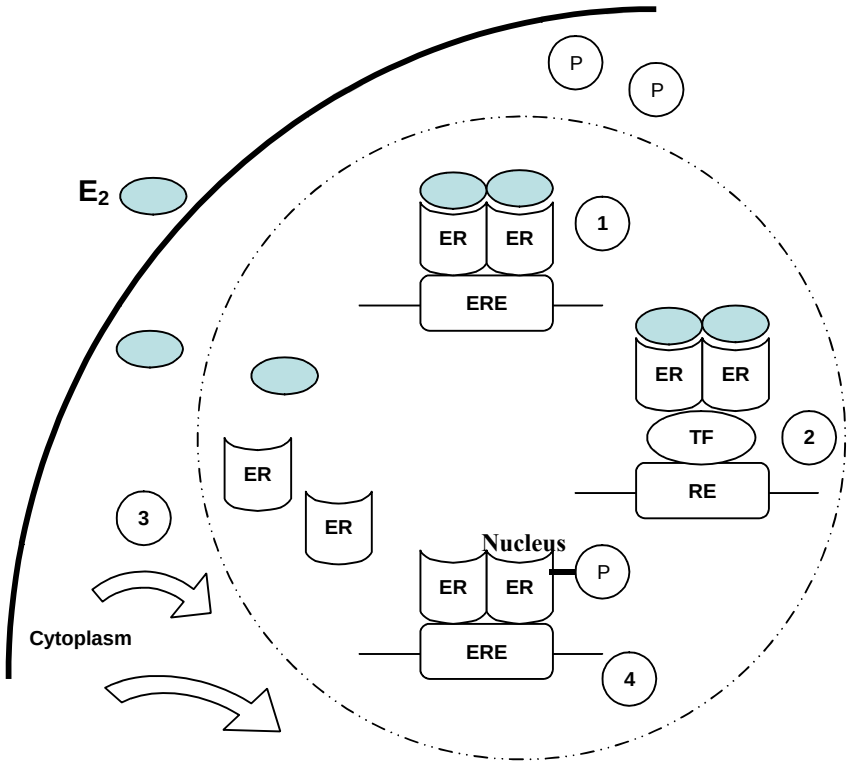


Fig.3. Mechanisms of Estrogen Signalling: 17-β Estradiol binds to ER which associate with and Estrogen Response Element (ERE) (1); E2 binds to ER which associate with Response Element (RE) through transcription Factor (TF)(2); E2 mediates non-genomic effects(3); phosphorylation activates ERs and bind to ERE(4).

receptors. Nevertheless, the molecular mechanism for non-genomic effect is still under debate (Warner and Gustafsson 2006).

2.3. Estrogen and Cardiovascular System

Estrogen is thought to protect against the cardiovascular disease by reducing plasma LDL cholesterol and increasing HDL cholesterol, and improving vasodilatation. It is

suggested that estrogen may also have direct cardiac protective effects (Mendelsohn 2002). Estrogens have been shown to avert vascular dysfunction, promote vasodilatation in coronary arteries by increasing nitric oxide production through PI3K/Akt signalling and inhibits atherosclerosis (Haynes, Sinha *et al.* 2000; Krasinski, Spyridopoulos *et al.* 1997; Thompson, Pinkas *et al.* 2000). Estrogen receptors have direct anti-hypertrophic effect on the myocardium and estrogens prevent also the development of hypertension in animal models. Estrogen (17β-Estradiol) reverses left ventricular hypertrophy (LVH) in postmenopausal women by hormone replacement therapy (Miya, Sumino *et al.* 2002). 17β-Estradiol (E2) effectively decreased angiotensin II (AngII) or endothelin ET1 induced hypertrophy and found hypertrophic signalling partially through PI3K (Pedram, Razandi *et al.* 2005). These animal studies suggest that estrogen replacement could reduce the risk of coronary artery disease in post menopausal women. However the data from two big clinical trials the heart and estrogen/progestin replacement study (HERS) and the world health initiative (WHI) indicate the therapy with combined estrogen and progestin replacement therapy is not effective in prevention of coronary heart disease (CHD) and might actually increase the risk of CHD in the first year (Hulley, Grady *et al.* 1998; LaCroix 2005; Mendelsohn and Karas 2007; Rossouw, Anderson *et al.* 2002). On the other hand, several authors have criticized in particular the women's health initiative studies claiming that the conclusions drown were not warranted due to the flawed design of this study. Irrespective of the importance of estrogen in protecting against cardiovascular disease, the hormone will remain therapeutic for other indications. Its effects on the cardiovascular system need to be further characterized.

2.4. Gender Difference in Cardiac Hypertrophy

Clinical studies have shown gender differences in the model of adaptive left ventricular hypertrophy (LVH) in response to increased overload, such occurs in aortic stenosis and hypertension. Compared to male, female patients with similar conditions like aortic stenosis, cardiac performance is more commonly preserved and associated with smaller LV diameters. Gender differences occur also in hypertensive patients with LVH (Aurigemma and Gaasch 1995; Carroll, Carroll *et al.* 1992) In mice, pressure overload hypertrophy is attenuated by 17β-estradiol (van Eickels, Grohe *et al.* 2001). Also it has been reported that cardiac contractile function is improved in many animal models (Dash, Schmidt *et al.* 2003; Kadokami, McTiernan *et al.* 2000). High dose of estradiol prevented development of post-MI remodeling as assessed LV dysfunction (Beer, Reincke *et al.* 2007).

2.5. Estrogen Receptors in Cardiac Hypertrophy

Estrogens exert their function through estrogen receptors ER-α and ER-β which are known to be expressed in the cardiovascular system. Studies employing transverse aortic constriction (TAC) in estrogen receptor-α knockout (ERKO) and estrogen receptor-β knockout (BERKO) mice showed that the heart to body weight ratio is increased significantly in BERKO compared to ERKO and wild type littermate females indicating ERβ is attenuating the hypertrophic response to pressure overload (Skavdahl, Steenbergen *et al.* 2005). BERKO females showed increased mortality following myocardial infarction (Pelzer, Loza *et al.* 2005). Estradiol protects the

murine heart against LVH via ERß (Babiker, Lips *et al.* 2006). The protective effects of estrogen with respect to cardiac hypertrophy in young and senescent ovariectomized SHR rats showed the attenuation of cardiac hypertrophy only in young rats not in senescent SHR due to aging which is associated with increased cardiac hypertrophy in post menopausal women.

2.6. Cardiac Hypertrophy

The thickening of heart muscle is called cardiac hypertrophy. Continued increase in hemodynamic overload paves the route to cardiac hypertrophy. Which is considered as an adaptive response in order to maintain cardiac function (Taegtmeyer 2000a; Young, Laws *et al.* 2001) and normalize cardiac oxygen consumption (Grossman, Jones *et al.* 1975). It is known that continued pressure overload because of pathologic stimuli such as hypertension causes cardiac hypertrophy and heart failure. This pathologic cardiac hypertrophy is considered a maladaptive response. Cardiac hypertrophy also occurs due to physiologic stimuli like exercise training and it is termed as physiologic cardiac hypertrophy (Richey and Brown 1998b). Physiologic hypertrophy is not concomitant with adverse long term out comes (Burelle, Wambolt *et al.* 2004; Frey and Olson 2003; Ritchie and Delbridge 2006). Pathological and physiological hypertrophic response to metabolic stress such as ischemia and reperfusion differ from each other (Allard, Schonekess *et al.* 1994; Bowles, Farrar *et al.* 1992; Richey and Brown 1998b). Physiologic cardiac hypertrophy is considered as adaptive in nature because post ischemic recovery is improved relatively to non-hypertrophied hearts (Moore and Korzick 1995; Richey and Brown 1998b). The energy substrate metabolism is altered in cardiomyocytes due to pathological or physiological hypertrophic responses (Fig.4). There is ample amount of evidence that

cardiac hypertrophy (both adaptive and maladaptive) is associated with alteration in energy substrate metabolism which may influence outcomes from ischemia and reperfusion (Anderson, Allard *et al.* 1990; Richey and Brown 1998b; Taegtmeyer 2000a; Young, Laws *et al.* 2001).

2.7. Myocardial Energy Metabolism

For normal cardiac function, energy is required in the form of adenosine tri phosphate (ATP) which is produced from fatty acids, glucose and ketone bodies (Bing, Siegel *et al.* 1953). In the adult myocardium, fatty acids are the major source of energy and account for 60-90% of total energy production (Shipp, Opie *et al.* 1961; Wisneski, Gertz *et al.* 1987). During fetal development, the main source of energy is glucose (Ascuitto and Ross-Ascuitto 1996; Fisher 1984; Hoerter and Opie 1978). Acute cardiac work results in stimulation of metabolic process in an co-ordinated way and when the workload of the heart is two fold, oxygen consumption rate doubles; at the same time there is an immediate increase in the oxidation of glucose (Goodwin, Taylor *et al.* 1998).

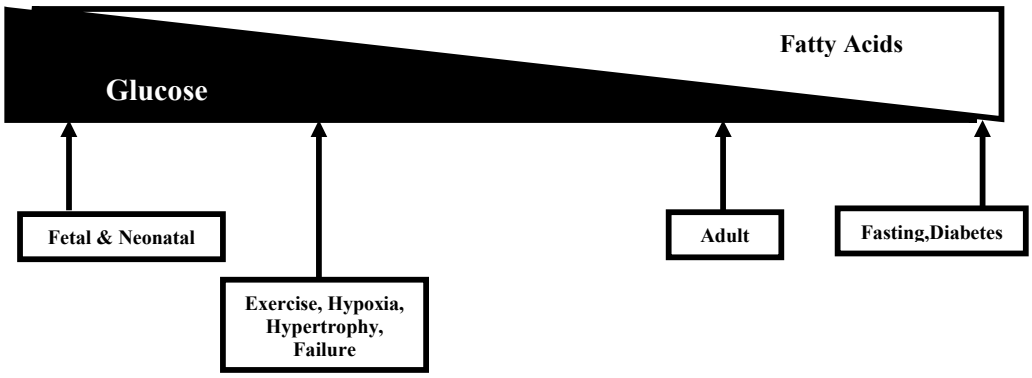


Fig.4. Substrate preference of the heart.

2.7.1. Cardiac Glucose Metabolism

In cardiomyocytes, glucose is transported via a family of facilitative glucose transporters (GLUTs) (Fig.5). The heart expresses predominantly two glucose transporters namely GLUT4 and GLUT1 (Gould and Holman 1993; Pessin and Bell 1992). GLUT1 is highly expressed during fetal life and decreases after birth (Castello, Rodriguez-Manzaneque *et al.* 1994). GLUT4 expression increases postnatally and reaches maximum in adulthood (Castello, Rodriguez-Manzaneque *et al.* 1994; Santalucia, Boheler *et al.* 1999; Santalucia, Camps *et al.* 1992). Insulin and contraction are the two main stimuli for cardiac glucose uptake at physiological conditions. Ischemia, hypoxia and increased cardiac workload stimulate translocation of GLUT4 on to the plasma membrane (Egert, Nguyen *et al.* 1997; Sun, Nguyen *et al.* 1994; Till, Kolter *et al.* 1997).

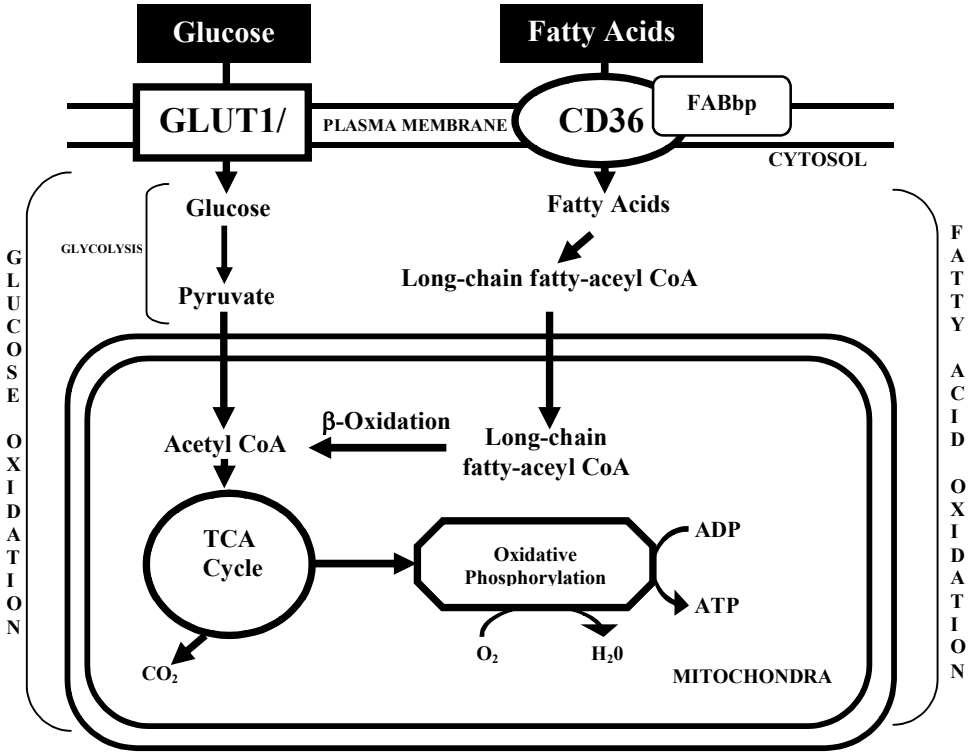


Fig.5. Cardiac Substrate Metabolism

2.7.1.1. Glucose Transporters

2.7.1.1.1. GLUT4

GLUT4 is predominantly expressed and highly regulated by insulin in the heart. Insulin increases glucose uptake facilitative via the glucose transporter GLUT4. Insulin stimulates translocation and activation of both GLUT4 and GLUT1 in heart

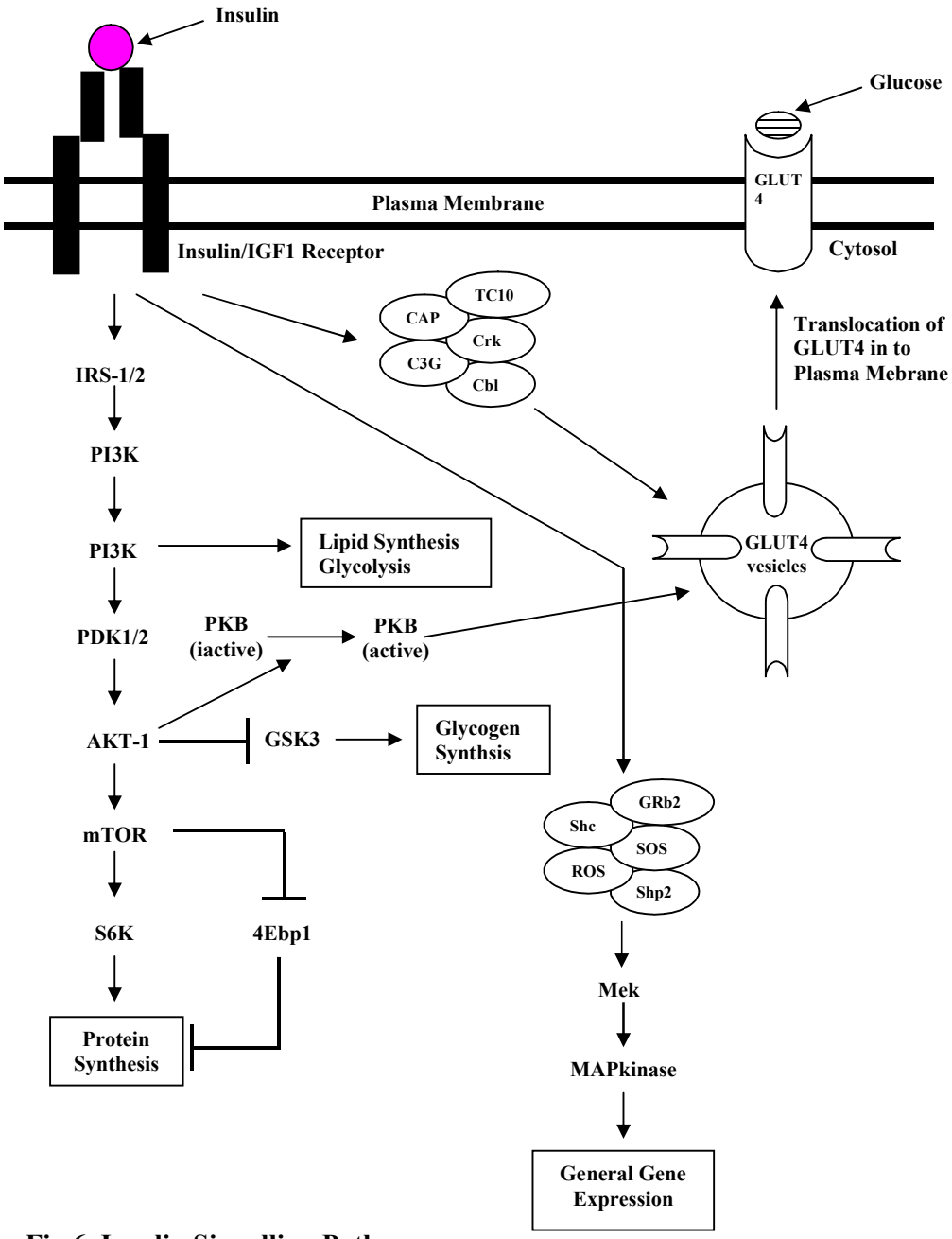


Fig.6. Insulin Signalling Pathway.

(Egert, Nguyen *et al.* 1999b; Zaninetti, Greco-Perotto *et al.* 1988). GLUT4 regulates insulin dependent glucose uptake in heart (Abel 2004; Zorzano, Sevilla *et al.* 1997). Among these transporters, insulin mediated GLUT4 translocation has been investigated immensely (Fig.6).

2.7.1.1.2. GLUT1

GLUT1 is highly expressed in during fetal growth but down-regulate rapidly after birth. GLUT1 has a role in basal glucose uptake (Smoak and Branch 2000). GLUT1 undergoes modest translocation to the sarcolemma with insulin and ischemia. During fasting, glucose and insulin levels are decreased with increased levels of free fatty acids that are associated with increased cardiac fatty acid uptake and decreased glucose uptake and GLUT1 levels (Kraegen, Sowden *et al.* 1993). Hyperinsulinemia causes increased levels of GLUT1 due to either increased glucose concentration or free fatty acids (Laybutt, Thompson *et al.* 1997). Studies with cardiomyocyte specific deletion of insulin receptor show reduced GLUT1 level and basal glucose uptake providing a role for insulin on regulation of cardiac GLUT1 (Belke, Betuing *et al.* 2002). Left ventricular hypertrophy is associated with increased total GLUT1 levels and reduced GLUT4 levels but the levels of both transporters at plasma membrane are increased in the hypertrophied heart (Tian, Musi *et al.* 2001).

2.7.2. Metabolic Adaptation in Cardiac Hypertrophy

During cardiac hypertrophy, the myocardium undergoes a metabolic shift from fatty acid to glucose utilization, which reintroduces the fetal substrate metabolic design

(Bishop and Altschuld 1970; Taegtmeyer and Overturf 1988; Yonekura, Brill *et al.* 1985). Increased energy production is seen through glycolytic pathway compared to energy from fatty acid in hypertrophic heart and this shift causes lower oxygen consumption since 10% more ATP is generated from glucose than fatty acids per mole of oxygen (Allard, Schonekess *et al.* 1994). Myocardial energy utilization pathways also undergo alterations during cardiac hypertrophy. The re-entry of fetal metabolism and metabolic adaptation toward glucose occur before any change in cardiac mass in hypertensive animals (Taegtmeyer and Overturf 1988) and suggest that the change in metabolic substrate utilization to cardiac overload precedes cardiac hypertrophy.

2.7.3. Cardiac Glucose Uptake in Insulin Resistance and Type II Diabetes

Hyperglycemia defines both types of diabetes and results from an absolute insulin deficiency in type 1 diabetes and tissue insulin resistance in type 2 diabetes (American 1997; Association 1997) High circulating levels of glucose cause accelerated micro- and macro-vascular diseases (such as ischemic heart diseases, stroke, retinopathy, neuropathy and nephropathy) and increase morbidity and mortality in diabetic patients (Klein 1995). Diabetes is a strong independent cardiovascular risk factor, and the likelihood of death from cardiovascular causes is two to five folds higher in diabetics (Kannel and McGee 1979; Stamler, Vaccaro *et al.* 1993). Clinically, diabetes mellitus associated with a diabetic cardiomyopathy which is not directly attributable to microvascular disease, hypertension or obesity (Grundy, Benjamin *et al.* 1999; Hayat, Patel *et al.* 2004)

Insulin resistance is a principal feature of type 2 diabetes and explained as inefficiency of insulin to stimulate glucose transport into peripheral target tissues (Petersen and Shulman 2002). There is a robust correlation between GLUT4 protein amount and the rate of glucose disposal. In diabetes, the insulin action and GLUT4 activity are impaired, GLUT4 deficiency leads to insulin resistance, hypertension, left ventricular hypertrophy and chronic heart failure (Anker, Ponikowski *et al.* 1997; Paolisso, De Riu *et al.* 1991; Reaven 1991).

Insulin resistance is responsible for metabolically induced cardiac remodeling and caused by impaired glucose uptake relative to cardiac workload (Belke, Larsen *et al.* 2000; Desrois, Sidell *et al.* 2004). As already discussed in the previous section, glucose is supplied through GLUT4 and GLUT1 in cardiomyocytes and GLUT4 is most the abundant glucose transporter in the adult heart. GLUT4 translocates in to plasma membrane, in response to insulin, ischemia, hypoxia, and contraction (Till, Kolter *et al.* 1997). Decreased GLUT4 activity and expression is suggested as one of the factors responsible for metabolic and contractile dysfunction in the diabetic heart, where glucose uptake is compromised (Desrois, Sidell *et al.* 2004; Eckel and Reinauer 1990).

2.7.4. Estrogen in Regulation of Glucose Homeostasis

Estrogen treatment in postmenopausal women had showed decreased blood glucose and improved insulin sensitivity (Crespo, Smit *et al.* 2002; Espeland, Hogan *et al.* 1998; Saglam, Polat *et al.* 2002). Estrogen reverses the effects of menopause on glucose and insulin metabolism by increasing insulin secretion from pancreas as well

as insulin sensitivity (Brussaard, Gevers Leuven *et al.* 1997; Stevenson, Crook *et al.* 1994). In mice, both ERKO and ArKO exhibit reduced glucose tolerance, insulin resistance and obesity (Heine, Taylor *et al.* 2000; Jones, Thorburn *et al.* 2001). ERKO mice also had shown decreased GLUT4 level in skeletal muscle that could account for impaired glucose uptake (Barros, Machado *et al.* 2006). The silencing of ER alpha in brain causes obesity, impaired glucose tolerance and decreased energy expenditure (Musatov, Chen *et al.* 2007). Estrogen treatment enhances insulin stimulated glucose uptake diabetic animals and reduced by ovariectomized (Louet, LeMay *et al.* 2004). However, the role of estrogen on cardiac glucose uptake is not kown.

3. Aim of the Study

The main aim of this study is to determine whether modulation in cardiac glucose uptake might contribute to the role of estrogens to prevent against development of cardiac hypertrophy. For this, female SHR animals at 12 weeks of age were either sham operated or ovariectomized (placebo) or ovariectomized treating 17β-estradiol over 6 weeks of period, then the following analyses was carried out.

1. Morphological analysis; Analyzing body weight, heart weight, tibia length
2. Metabolic and Biochemical analysis,
    - Intra-peritoneal glucose tolerance test (IP-GTT)
    - Measurement of myocardial uptake of the positron-emitting glucose analogue 18F-2-deoxy-2-fluoro-D-glucose (FDG) and *in-vivo* positron emission tomography (PET) in SHR
    - Measurements of serum estrogen and insulin levels
3. Molecular analysis of cardiac glucose transporters (GLUT4 & GLUT1) expression and distribution in SHR rat, analyzed by using western blot and immunofluorescence staining.
4. Examination of effects of estrogen on modulation of insulin signalling leading to cardiac glucose uptake.
    - Analysis of early insulin signaling molecule insulin receptor substrate (IRS-1) and its tyrosine phosphorylation were evaluated by western blot.
    - Gene microarray analysis of SHR hearts using insulin signaling pathway specific genes.

4. Material & Methods

4.1. Materials

Antibodies:

Primary Antibodies	Provide/ Cat. No.	Mol Wt	Dilution
Anti-GLUT1 (Rabbit Polyclonal)	Chemicon #AB1340	~42-45 kDa	1 in 4000 (WB) 1 in 250 (IF)
Anti-GLUT4 (Rabbit Polyclonal)	Abcam #ab654	~45 kDa	1 in 5000 (WB) 1 in 500 (IF)
Anti- Na+/K+ ATPase α-1 (Rabbit Polycloneal)	Upstate #06-520	110 kDa	1 in 500 (WB)
Anti-IRS-1 (Rabbit Polyclonal)	Upstate #06-248	~160 kDa	1 in 500 (WB)
Anti-phospho IRS-1 (tyr612) (Rabbit Polyclonal)	Upstate #07-846	~160 kDa	1 in 250 (WB)
Anti-4Ebp1 (Rabbit Polyclonal)	Cell Signalling #9452	~15 to 20 kDa	1:500 (WB)
Glyceraldehyde-3-phosphate (Rabbit Polyclonal)	Upstate #06-248	~37kDa	1 in 3000(WB)

Solutions and Buffers:

For DNA electrophoresis

Agarose gel Agarose Ultrapure 2%

1x TAE

Ethidum bromide (0.5 µg/ml)

6x loading Dye (Agarose gel) 0.25 % bromphenol blue

30 % glycerol in water

0.25 % xylen cyanol

TAE (50x) 242 g Tris base

57.1 ml acetic acid

100ml 0.5M EDTA

Add H_2O to 1 liter, adjust pH to 8.5.

For RNA electrophoresis:

MOPS running buffer (10x) 41.86 g MOPS

6.8 g $NaOAc.3H_2O$

3.8 g Na_4EDTA

H_2O to 1L

RNA electrophoresis 1 g Agarose

88 µml H_2O DEPC

10 ml 10x MOPS buffer

2.3 µl EtBr

1.87 ml Formaldehyde

RNA loading dye 720 µl Formamide

160 µl 10xMOPS buffer

260 µl Formaldehyde

193 µl H2O

267 µl 6x Bromophenol blue DNA loading dye

Buffers:

1X PBS (Phosphate Buffered Saline) pH 7.4

NaCl	137.0 mM
KCl	2.7 mM
Na_2HPO_4 $2H_2O$	1 mM
KH_2PO_4	1.5 mM

RIPA (Radio Immuno Precipitation Assay) Buffer (for 100ml)

NaCl	150.0 mM
Tris	50.0 mM
PMSF	1.0 mM
IGEPAL CA-630	1.0 %
Sodium deoxycholate	0.5 %
Sodium dodecyl sulfate	0.1 %

5x Electrophoresis Buffer pH 8.3 (for 1000ml)

Tris	124.0 mM
Glycin	960.0 mM

SDS 0.1 %

Transfer Buffer (Western Blotting)

200 ml of 5x Electrophoresis + 200ml of Methanol (absolute) + 800 ml distilled H_2O

Washing solution (Western Blotting)

1x PBS pH 7.4 5
Tween 20 (0.05 %)

Blocking solution (Western Blotting)

1x PBS pH 7.4
Non-fat dried milk powder (5.0 %)

SDS-PAGE:

Separating Gel Component	**10 %** (ml)	**12 %** (ml)
1,5 M Tris pH 8,8	5.0	5.0
10 % SDS	l 0.2	0.2
Acrylamide+Bis acrylamide (30 % - Stock)	6.67	8.0
10 % APS	0.1	0.1
TEMED	0.01	0.01
dH_2O	8.02	6.69

Stacking Gel Component	**5 %** (ml)
1,5 M Tris pH 8,8	5.0
10 % SDS	l 0.2
Acrylamide+Bis acrylamide (30 % - Stock)	6.67
10 % APS	0.1
TEMED	0.01
dH_2O	8.02

4.2. Methods

4.2.1. Animals and Treatment

The total of 30 female spontaneously hypertensive (SHR) rats with 12 Weeks of age (with body weight of approximately 180-190 grams upon arrival) were obtained from Charles River Laboratories (Sulzfeld, Germany) and housed communally (5 animals per cage) under controlled temperature and lighting (12 hr light/dark cycle), fed with free food and water. Arbitrarily, 20 animals were ovariectomized and the other 10 animals were sham-operated. The experimental groups consist of Sham-operated ('Sham', n=10); Ovariectomized ('Ovx', n=10) placebo received; Ovariectomized + 17β-estradiol ('Ovx+E_2', n=10) which administered with 17β- estradiol at a concentration of 2 μg/kg of body weight/day. Initially, 17β- Estradiol compound was dissolved in absolute ethanol and the required final concentration has been made with peanut oil. For placebo, only peanut oil was given without any medicament. The animals were undergone 4 weeks of treatment and subcutaneous mode of injection was chosen.

4.2.2. Positron Emission Tomography (PET)

The glucose uptake in female SHR rats (five animals from each group, 1.Sham, 2.Ovariectomized and 3.Ovx+E2) was evaluated in-vivo by means of radio labelled tracer 2-(^{18}F)-fluoro-deoxyglucose (^{18}FDG) and Positron Emission Tomography scanner *(Oxford Positron Systems, Oxford, UK)*. ^{18}FDG is positron emitting glucose analogue which is injected to the animals and positron the observation of glucose metabolism with a positron emitting glucose analogue can show the uptake of this

analogue displays in myocardium. After entering a cell it is phosphorylated by the hexokinase ((Phelps, Schelbert *et al.* 1983; Ratib, Phelps *et al.* 1982). Further breakdown of FDG-6-phosphate is inhibited in contrast to Glucose-6-phosphate. Metabolism back to FDG is improbable because the required enzyme glucose-6-phosphatase is not highly expressed in heart. FDG-6-phosphate is trapped in the cell since this molecule is too polar to pass the sarcolemma. The intracellular concentration of FDG is proportional to glucose utilisation of the tissue (Gallagher et al., 1978). Under fasting conditions FDG uptake is markedly suppressed by fatty acids.

After 12 weeks of treatment, the animals were overnight fasted and the each rat were anesthetised with isofluorane / O_2 for insertion of Insyte® 24G catheters (~10min) and placed in Bollman's restraining cage. Later, the rat was infused with glucose (G20) according to body weight at a concentration of 25mg/Kg/min for 15 minutes. One minute after end of glucose infusion, ^{18}FDG (~15MBq) was injected in a bolus. The tissue paper was placed under the rat to collect any urine. Then rats were anaesthetized with isofluorane/O_2 and placed in HIDAC for scanner. After 60 minutes of (^{18}F) FDG injection, HIDAC scan was started and scanned for a during of 15 minutes. The glucose levels were measured using Ascensia Elite Test Strips when the catheters were inserted and by tail vein puncture after injection (^{18}F) FDG, 15 minutes before and after the PET scan.

4.2.3. Morphometric Assessment:

The weight of whole body, heart, uterus, liver, kidney and the length of tibia were measured. These dissected wet tissues were blotted on paper towels before weighing.

The length of the tibia devoid of all soft tissue was measured. The hear:body weight ratio was calculated by subtracting the heart weight by the body weight in grams. In the same way, the heart:tibia length ration also was calculated by diving the values of heart weight in grams by the values of tibia length in millimetres.

4.2.4. IP-GTT (Intra Peritoneal Glucose Tolerance Test)

After 4 weeks of treatment, the alteration of glucose excursion in Intra-peritoneal Glucose Tolerance Test (IGTT) was performed on all three different treatment groups. First, the rats were fasted overnight and glucose injected at a concentration of 1.5g per kilo gram of body weight by intra-peritoneally. The blood samples were withdrawn from venous blood from a small tail clip before and 10, 20, 30, 40, 50, and 60 minutes after glucose infusion for the measurement of blood glucose levels. And also the blood has been collected for serum Insulin assay.

4.2.5. Measure of Serum Insulin

Insulin was assayed by using specifically synthesized antibodies against rat Insulin Radioimmunoassay (RIA) kit which obtained from DRG Systems, Marburg, Germany. This kit uses specifically synthesized antibodies against rat Insulin.

4.2.6. Total Protein Isolation:

Approximately 50-100mg of tissue was taken in 200µl of RIPA buffer (including

protease and phosphatise inhibitors) and incubated for 15 minutes on ice. Following homogenized (motor driven homogenizer) the tissue and centrifuged at 14000 *rpm* for 10 minutes at 4°C. The resultant supernatant was considered as total protein. The protein concentration was measure by using BCA method.

4.2.7. Subcellular Plasma Membrane Fractionation

The plasma membrane fraction was prepared as previously described (Fuller, Eaton *et al.* 2001). In detail, the rat heart tissue was first incubated in high salt buffer (2 M NaCl, 20 mM Hepes, pH 7.4) on ice for 30 minutes, and then the tissue was retrieved by centrifugation at 1000g for 5 minutes, afterwards washed with Buffer A (10mM Hepes, pH 7.4, 2 mM EDTA, pH 8.0, 1 mM MgCl2, 250 mM sucrose) and centrifuged once again. Using hand-held ground glass tissue grinder, the tissue was homogenized with buffer A (10ml Buffer A per 1g of tissue). Then the homogenates were centrifuged at 1000g for 5 minutes. The supernatants were centrifuged in a Beckman bench top optima TL ultracentrifuge using TLA 100.4 rotors. The resultant pellet was considered as plasma membrane which was resuspended with buffer A and supernatant was cytosolic fraction.

4.2.8. Western Blot

SDS-PAGE*, sodium dodecyl sulfate polyacrylamide gel electrophoresis:*

Prior to SDS-PAGE analysis, the protein samples were denatured by adding 2X Sample buffer (Lammeli Loading buffer) at a ratio of 1:1 and were boiled at 95°C for

10 minutes. Then the samples were run on 8-10% SDS- PAGE gel using 1X Tris-Glycine running buffer. (manniatis). After resolving the proteins on the gel, immediately proceeded with wet transfer by submerging the sandwich (sponge/paper/gel/membrane/paper/sponge) in 1X transfer buffer and applying electrical field allows the proteins from the gel blotting on the nitrocellulose membrane. Then the membrane was blocked with blocking solution (5% non- fat milk powder prepared in PBS/Tween 20 buffer) for 1 hour incubation under agitation. This blocking step prevents the non-specific binding of primary/secondary antibody to the membrane. After incubation, the membrane was washed with PBST buffer for 3 times with 10 minutes interval. The primary antibodies were diluted in blocking solution. Then, the membrane was incubated with the primary antibody solution for overnight at +4°C with gentle agitation. The membranes were washed 4-5 times with PBST while agitating for overall of 30-40 minutes. The membranes were incubated with HRP-conjugated secondary antibodies (diluted with blocking solution) for 1 hour at room temperature. Finally the membranes were again washed 4-5 times with PBST while agitating for overall of 30-40 minutes. Then the membranes were soaked in Enhanced Chemiluminescence Reagent (ECL obtained from GE Bioscience) that elicits a peroxidase-catalyzed oxidation of luminol and subsequently enhanced chemiluminescence, where the HRP labeled protein is bound to the antigen on the membrane. The resulting light was detected on an X-ray film.

4.2.9. Immunofluorescence Staining

The frozen rat hearts which embedded in tissue tek (an OCT compound obtained from *Sakura* Finetek Germany GmbH) were cut in to 3 micron sections in a cryostat and

mounted on the slides then stored at −80°C until use. For the immunofluorescence staining, the sections were first washed (Throughout the protocol, washing step involves three times of wash using PBS buffer at 10 minutes interval) and then fixed with 4% paraformaldehyde (PFA) for 15 min. Again wash with PBS buffer, the section slides were incubated at room temperature for 1hr with the goat serum (diluted in PBS) in order to prevent non specific binding of primary or secondary antibodies. Then serum was removed and the sections were incubated with primary antibodies with either GLUT4 (1 in 250) or GLUT1 (1 in 200) overnight at 4°C by placing the slides in a humid chamber to prevent dehydration. Some slides were treated in the similar way without primary antibody and considered as a negative control. After overnight incubation, let the slides come down to room temperature and washed. Then the slides were incubated with goat anti- rabbit AlexaFluor 594 (Molecular Probes, Invitrogen) secondary antibody including negative control slides. After a subsequent time, all the sections were washed and stained with Wheat Germ agglutinin which was used as a marker for plasma membrane (requires 10mins of incubation with Fluorescent Wheat germ agglutinin- Alexa Fluor 488 which obtained from Molecular Probes, Invitrogen at a dilution of 1 in 200 from 1ug/ul concentration). Followed by agglutinin staining, the slides were washed and incubated with DAPI for 30min and final wash was performed. At the end, the slides were mounted in Vectashield® mounting medium (Vector Labs) and covered with cover slips and the cover slip edges were sealed with nail polish.

4.2.10. RNA Isolation

The total RNA from rat heart was extracted according to manufacture's instructions in Trizol reagent (Invitrogen). In brief, the frozen rat myocardial tissue (~50mg) was homogenized in 1ml Trizol reagent until the suspension becomes homogenous and subsequently centrifuged at 15000*rpm* for 10 minutes at 4°C in order to eliminate the insoluble materials (extracellular membranes, polysaccharides and high molecular weight DNA). Then supernatant (containing RNA and protein) was recovered and incubated at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes and then 200µl of Chloroform was added and incubated at room temperature for 15 minuets and the separation of three layers (Colourless upper phase containing RNA, interphase containing DNA, red organic phase containing protein) has been achieved by centrifugation at 15000 *rpm* for 15 minutes at 4°C. The colourless aqueous phase has been taken separately and 1 volume of isopropanol has been added and mixed and allowed it to stand at room temperature for 8minutes. After centrifugation at 15000rpm for 20 minutes at 4°C, RNA precipitates to form a pellet on the side and bottom of the tube and the RNA pellet was washed by adding 1ml of 75% ethanol centrifugation at 15000 *rpm* for 20 minutes at 4°C. After complete removal of ethanol, the RNA pellet has been dissolved with 25ul of 0.1% DEPC treated H_20 and store it at –80°C until further use. The concentration and purity of the isolated total RNA was determined by absorbance measurement, and the integrity of ribosomal RNAs that were demonstrated by running it on 1% agarose gel electrophoresis.

4.2.11. Hexokinase Assay

Hexokinase is an enzyme that catalyses glucose to glucose phosphate was evaluated by measuring the rate of reduction of NADP+ as previously described (Swislocki, Burgie *et al.* 2002). First, 100µg of protein was dissolved in 2ml of reaction buffer (40mM HEPES, 0.8mM EDTA, 7.5mM $MgCl_2$, 1.5mM KCl, 2.5mM ATP (2Na), 10mM Creatine Phosphate (2Na), 0.9 IU/ml Creatine Phosphokinase, 0.7 IU/ml glucose-6-phosphate dehydrogenase, and 0.4mM NADP+, pH 7.4). Then 100µl of this reaction buffer containing sample was taken in 96well plate and assay was initiated by adding D-glucose (at a final concentration of 1.0mM) and the production of NADPH were measured at 340 nm per minute at 25°C. The samples with out glucose and reaction buffer alone were considered as a negative control.

4.2.12. Oligo GEArray® Rat Insulin Signaling Pathway Microarray

Oligo GEArray® Rat Insulin Signaling Pathway Microarray kit was obtained from SuperArray Bioscience Inc (Bethesda, MD, USA). This Oligo GEArray is a pathway-focused DNA microarray. The nylon membrane array matrix is a permeable support with a high DNA binding capacity. The designed 60-mer oligonucleotide probes printed on each microarray minimizes any cross-hybridization between spots on the same array despite the representation of closely related members of the same gene families. The target synthesis and labelling protocol provides un-biased linear RNA amplification in a simple one-tube protocol. The biotinylated cRNA target and the carefully designed oligonucleotides on the array correspond to the same 3'-biased gene-specific sequences permitting efficient and specific hybridization. The oligo GEArray has been optimized for chemiluminescence detection method, which allows

the use of either X-ray film or a CCD-camera imaging system for image acquisition. This Oligo GEArray® Rat Insulin Signalling Pathway Microarray contains 127 oligonucleotide probes (Detailed array layout, fig.18) representing genes associated with insulin receptor and target genes in the insulin signalling pathway. And the genes involved in carbohydrate, protein and lipid metabolism and members of the PI3K and MAPK Pathways and other related biological responses are also embedded. The total RNA was isolated from the SHR rat heart tissues by agen Inc., Valencia, CA, USA), and 3g RNA was used as a template to generate Biotin-16-dUTP-labeled cDNA probes according to the manufacturer's instructions. The cDNA probes were denatured and hybridized at 60°C with the SuperArray membrane, which was washed and exposed with the use of a chemiluminescent substrate. To analyze the SuperArray membrane, we scanned the membranes via a CCD camera and imported it into Adobe Photoshop as a TIFF file. The image file was inverted, and the spots were digitized with the use of GEarray analyzer program (SuperArray Corp.), and normalized by subtraction of the background as the average intensity value of 2 blank spots. The averages of two GAPDH spots were used as positive controls and set as baseline values with which the signal intensity of other spots was compared.

4.2.13. Statistics

Statistical analysis was performed using one way ANOVA, Bonferroni t-test. The results are presented as mean values of each treatment group ± SEM. The $p<0.05$ values were considered as statistically significant. The statistical analysis was per perfomed using *sigmastate 32 software (SPSS Inc)*

5. RESULTS

5.1. Measurement of Myocardial Glucose Uptake

The effect of estrogen on myocardial glucose uptake in SHR rats was measured by *[18]F*-fluoro -2-deoxy-d-glucose positron emission tomography (FDG-PET). After two hours of food withdrawal with free access to water, the rats were anaesthetized with isofluorane / oxygen for insertion of Insyte 24 G catheters about 10mins and placed in bollman's restraining cage and infused with glucose at concentration of 25mg/kg/min for 15 minutes for a total of 16 to 17mins. One minute after the end of glucose infusion [^{18}F] FDG (approximately 15MBq) was injected via tail vein

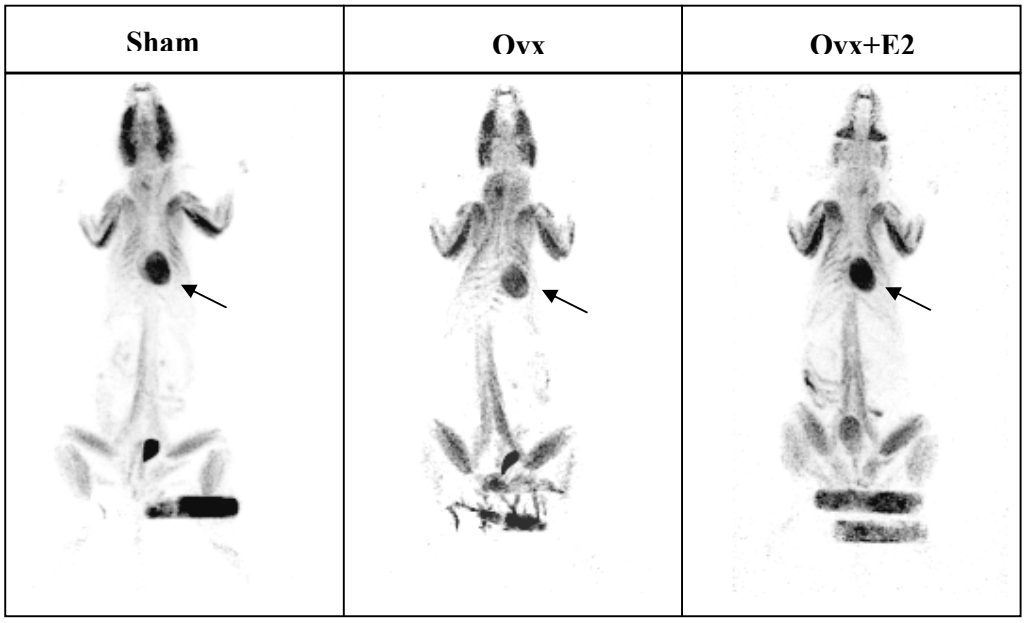


Fig.7. PET images obtained 60mins after injection of 15 MBq [^{18}F] FDG to sham (A), ovx (B) and E2 treated (C) SHR rats via a tail-vein catheter. Cardiac [^{18}F] fluorodeoxyglucose uptake in SHR rats was visualized by a quadHIDAC PET scanner following. Representative images of sham, ovx and ovx+E2 are shown. (PET analysis were performed in collaboration with Dr.Marilyn P. Law, Department of Nuclear Medicine, University Hospital, Muenster, Germany)

catheter and urine was collected in tissue papers before PET scanning. Sixty minutes after [^{18}F] FDG injection the HIDAC scan was started and scans were performed for 15 minutes. Figure.7 shows PET images from sham operated, ovx and ovx+E2 SHR animals. FDG-PET revealed a significant difference in myocardial glucose uptake between sham, ovx and ovx+E2 SHR animals (Fig.7). Myocardial FDG uptake was substantially enhanced by E2 treatment compared to ovariectomized SHR animals. In house software was used to reconstruct the HIDAC data and to compute the total radioactivity in the left ventricular wall (HIDAC counts per second (cps) (A) cps), and septum (HIDAC counts per second (cps) (A) cps) and the volume of the left ventricle wall and septum [V] ml). The difference in the radioactivity injected, the activity cube drawn round the rat [R] cps) and round the urine tubes ([U] cps) were calculated for normalization of the data and the sum of the cps in these cubes ((R) + (U)) were calculated.

Fig.8(a)

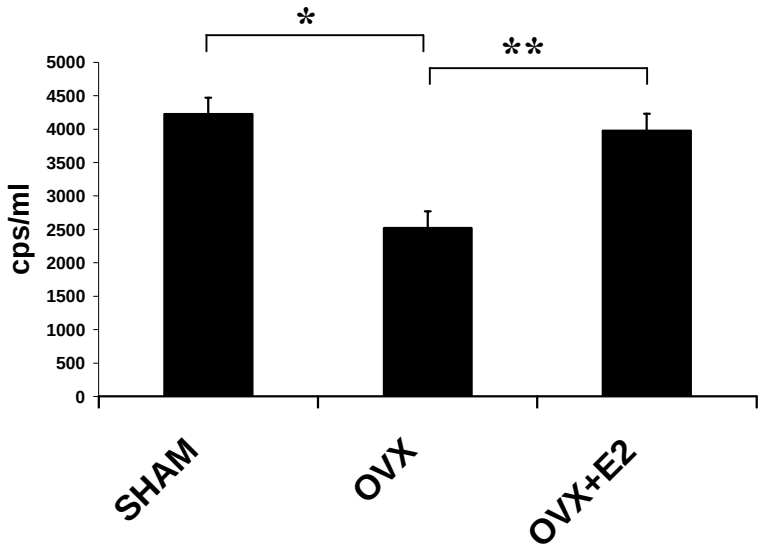


Fig.8(b)

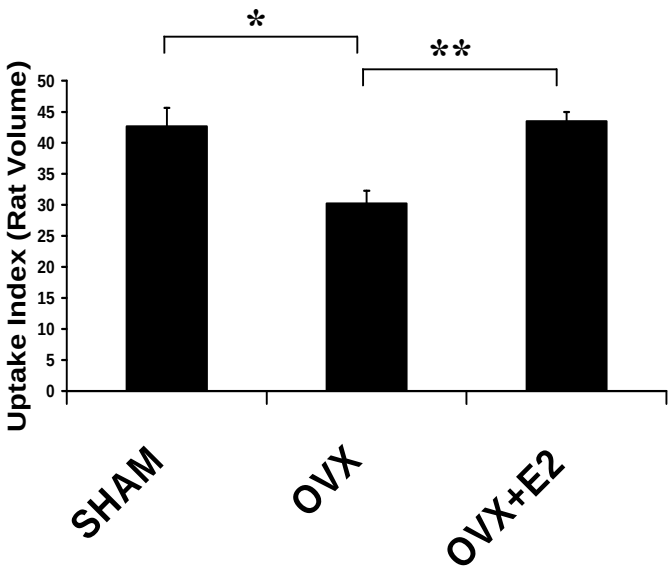


Fig.8. Myocardial FDG uptake in SHR. (a). The myocardial FDG uptake (counts per second per ml) indicates the improved glucose uptake by E2 treatment. (b). Indicate the FDG uptake index, expressed as %dose/l00 ml tissue of myocardium in SHR. Myocardial [^{18}F] FDG uptake in ovariectomized SHR animals was lower than sham and E2 treated animals (P>0.05).

5.2. Influence of E2 treatment on morphology in SHR animal

The increased body weight due to ovariectomy in SHR was abolished by 17β-estradiol treatment effectively which was comparable to sham operated rats. As shown in the figure 9, there was significant difference among the following treatment groups. Sham vs Ovx ($p<0.001$), ovx vs ovx+ E2 ($p<0.001$) and Sham vs Ovx+E2 ($p<0.001$). The number of the animals from each group were 8 (Sham), 9 (Ovx) and 10 (Ovx+E2).

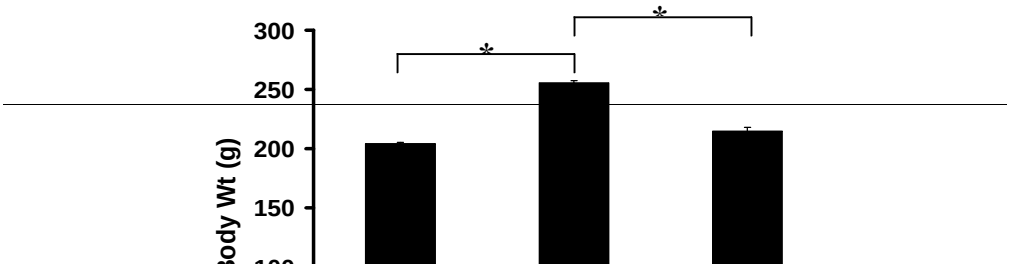


Fig.9. Body weight of SHR rats. The solid bar represents body weight of SHR rat groups of sham operated, ovariectomized SHR treated with placebo received, and 17β- estradiol (E2). Each bar represents mean ± SEM * $p<0.001$ and **$p<0.001$.

Uterus weight was measured to evaluate the efficacy of estrogen treatment *in-vivo.* Uterus weight (fig.10) was decreased significantly in ovariectomized rats due to endogenous estrogen deprivation when compared to sham operated and 17β- estradiol substituted ovariectomized SHR

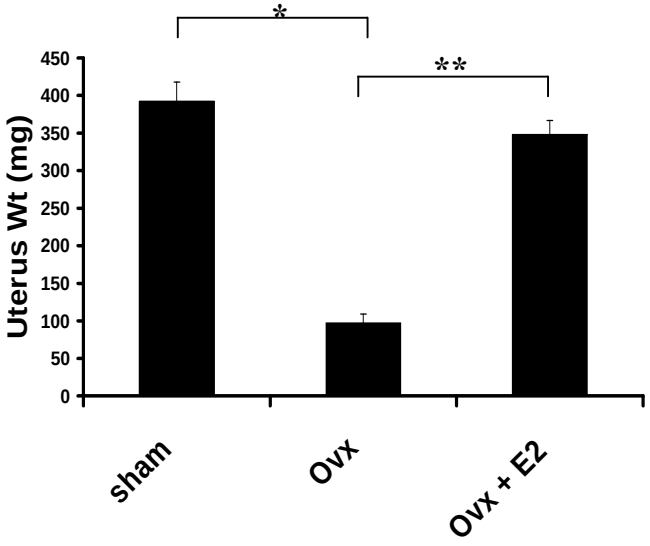


Fig.10. Uterus weight of Ovariectomized and E2 treated ovariectomized animals. SHR rats were sham operated (sham), ovariectomized (ovx) placebo received, ovariectomized with 17β-estradiol treated. Ovariectomy resulted in reduced uterus

weight, which was blocked by estrogen treatment. Each bar represent uterus weight mean ± SEM. *P< 0.001 and ** P< 0.001.

Increased heart weight is the hallmark of cardiac hypertrophy. Previous animal studies supported that E2 has anti hypertrophic action, so the degree of cardiac hypertrophy in the present study was calculated by normalizing heart weight to tibia length. As seen from figure 3, cardiac mass was increased in ovariectomized SHR rats compared to sham operated controls. After E2 treatment, heart weight was significantly reduced.

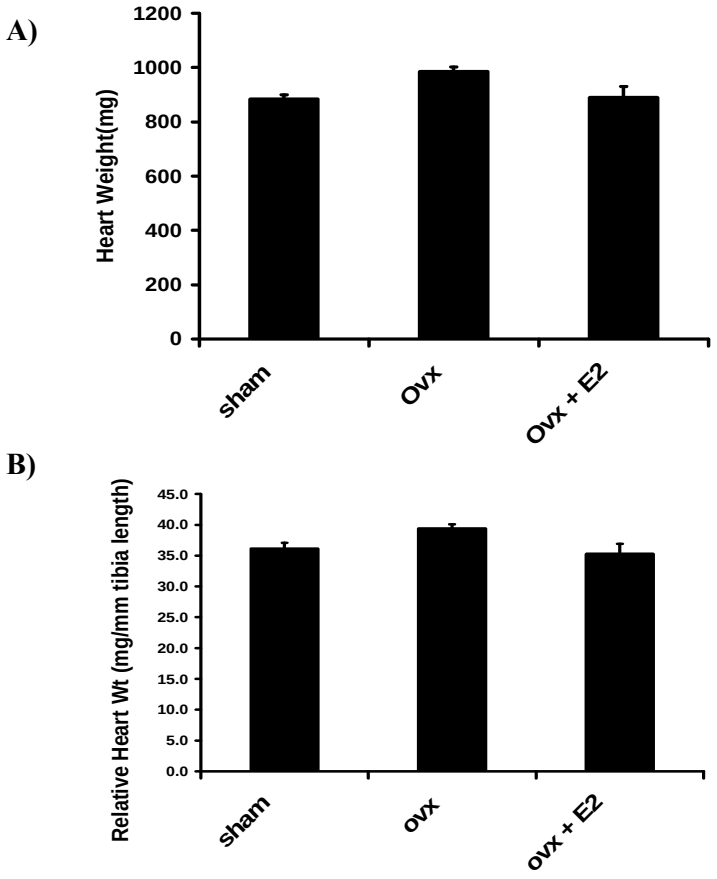


Fig.11. Absolute and relative heart weight of SHR rats. Absolute heart weight of SHR rats (A) and the relative heart weight (B). The absolute and relative heart weights were increased in ovx SHR. Each solid bar represents mean mean ± SEM (n=8 (sham), n=9 (ovx) & n=10(ovx+E2).

5.3. Hormone Measurements

Serum estrogen and insulin level in ovariectomized and estrogen- treated female SHR rats were measured by radioimmunoassay as mentioned in method section. Ovariectomy of SHR rats resulted in significant reduction of endogenous estrogens compared to sham operated animals. Estrogen levels in ovariectomized rats were lower comparing to sham operated rats (1.3±0.10 vs. 12.2±2.6 pg/ml, $p<0.05$) (fig.12).

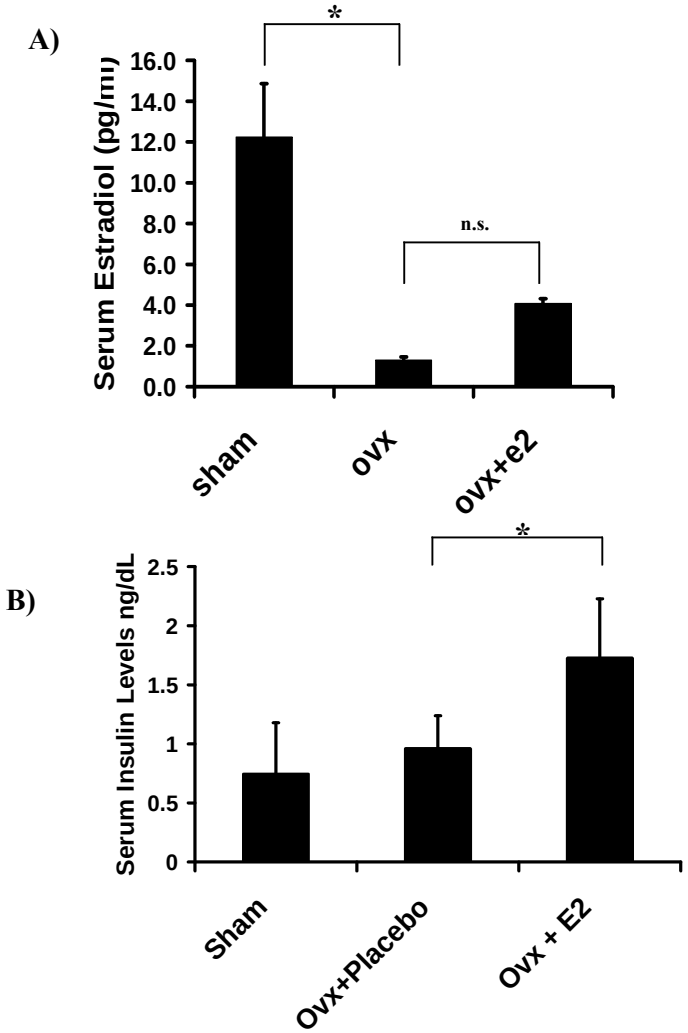


Fig.12. Serum Estrogen and Insulin levels in SHR rats. Serum estradiol (A) and insulin (B) levels from sham, ovariectomized (placebo) and E2 treated SHR rats. Each bar represents mean ± SEM, * $p < 0.05$.

17β- estradiol treatment resulted in increased serum estrogen concentrations compared to ovariectomized animals. Insulin levels were increased 5 fold in E2 treated SHR rats compared to ovariectomized (0.31±0.08 Vs 1.64±0.35ng/dL, $p<0.05$). There were no significant difference in serum insulin levels between sham operated group and ovariectomized SHR.

5.4. IP-GTT (Intra Peritoneal Glucose Tolerance Test)

To investigate the effect of ovariectomy and E2 treatment on insulin sensitivity in SHR rats, intra-peritoneal glucose tolerance test (IP-GTT) was performed on all three different treatment groups after 12 weeks of treatment. As shown in the fig.13, blood glucose levels in the all the three groups increased to a maximum at around 25 minutes and declined slowly thereafter. There was no significant change in blood glucose levels in all the three groups.

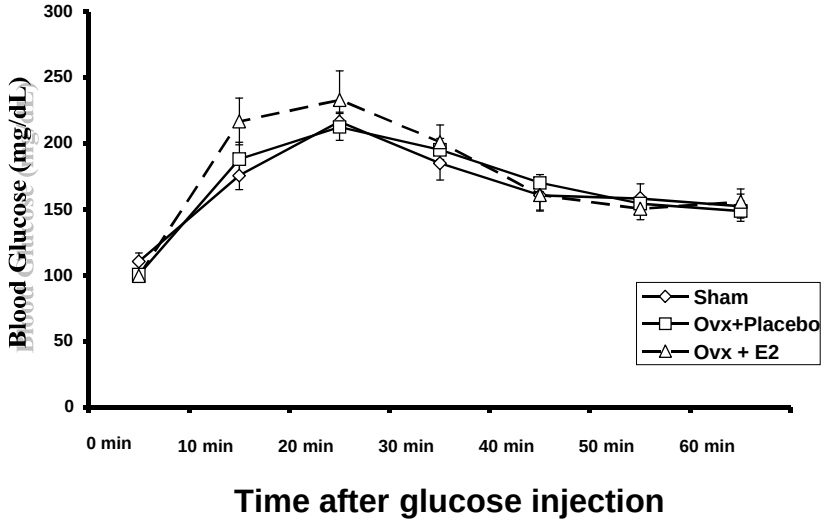
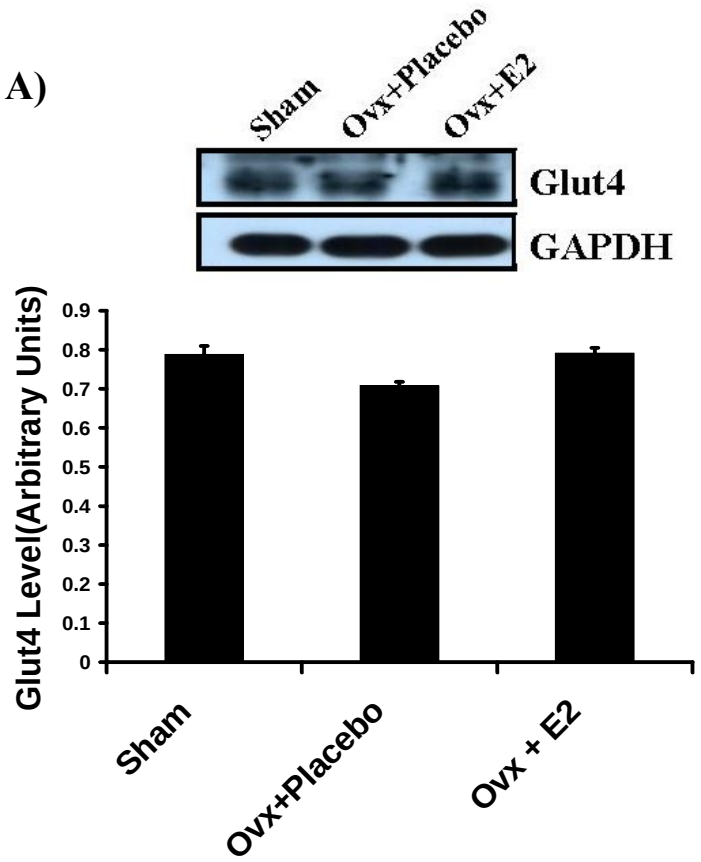


Fig.13. Intra-peritoneal glucose tolerance test (IP-GTT) in sham, ovx+placebo and E2 treated SHR animals. Values are mean ± S.E.M.

5.5. Effect of E2 on Expression Levels of Total Content of Glucose Transporters (GLUT4 and GLUT1) in SHR animals

The translocation of glucose transporters from the perinuclear compartment to the plasma membrane is a prerequisite for glucose uptake. The total content of glucose transporters and subcellular distribution was assessed by western blot and immunofluorescence staining. As seen in the figure 14 (A) and 5(B), 17ß-estradiol treatment did not alter total content of either GLUT4 or GLUT1 in SHR rat hearts.



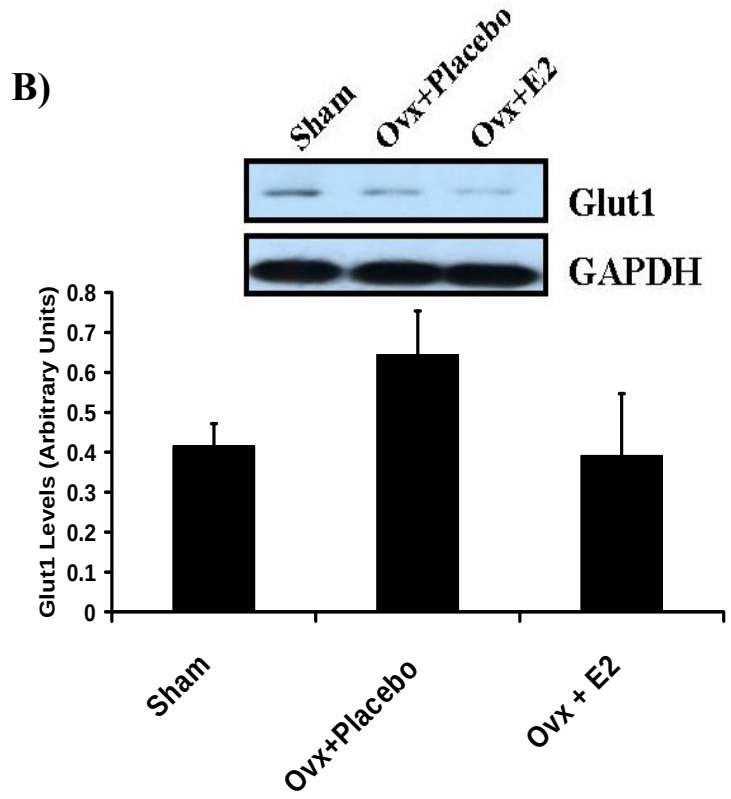
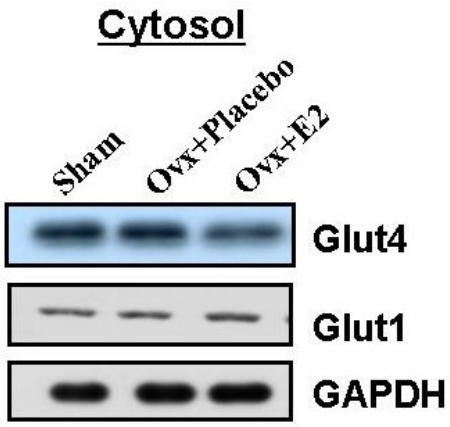


Fig. 14. Western blot analysis for total GLUT4 and GLUT1. A) The total protein from heart tissues of SHR animals of different treatment groups sham operated ovariectomized (ovx) and ovariectomized plus E2 treated (ovx+e2) groups were resolved in SDS-PAGE and consequently subjected to immunobloting with anti-GLUT4 and GLUT1. GAPDH was used as a loading control. A) GLUT4 levels and B) GLUT1 Levels. The values are mean ± SEM expressed as arbitrary densitometric units (ADU).

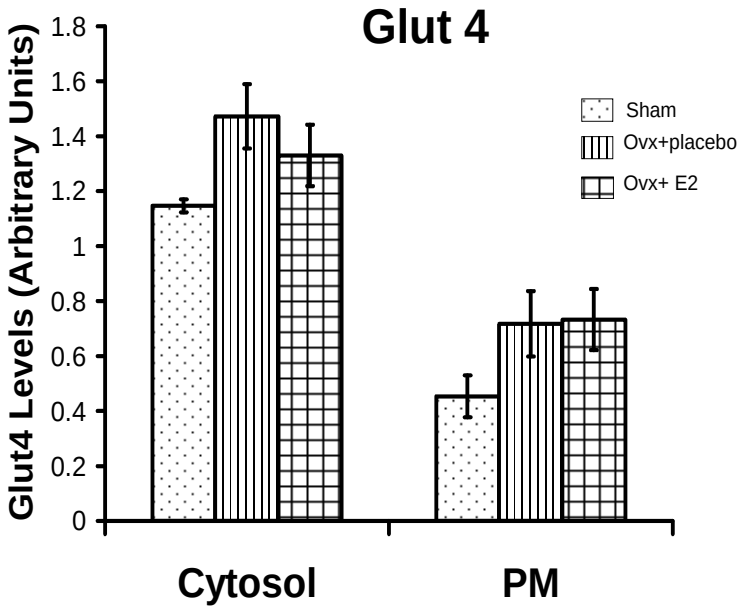
5.6. Effect of Estrogen on Sub-cellular Translocation of GLUT4 and GLUT1 in SHR rat hearts

To investigate whether estrogen influences GLUT4 and GLUT1 translocation to plasma membrane, the cytosol and plasma membrane fractions from SHR hearts were analyzed by western blot. There was no difference of either GLUT4 or GLUT1 translocation among the treatment groups (Fig. 15(a-d)).

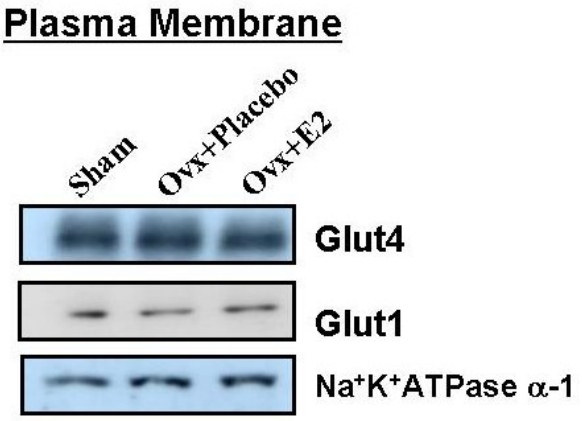
15 (a)


Cytosol
Sham
Ovx+Placebo
Ovx+E2
Glut4
Glut1
GAPDH

15 (b)


Glut 4
Glut4 Levels (Arbitrary Units)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sham
Ovx+placebo
Ovx+ E2
Cytosol
PM

15 (c)



15 (d)

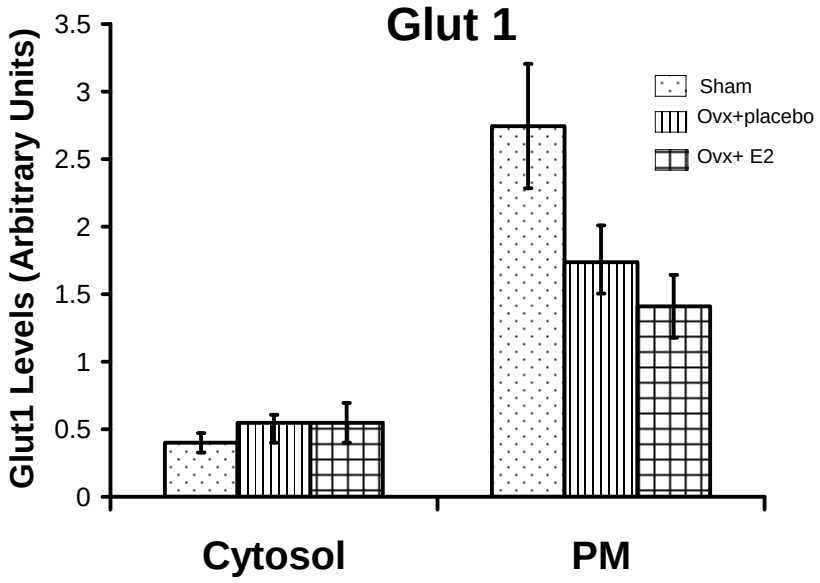


Fig.15. Western blot analysis of GLUT: Cytosolic and plasma membrane GLUT4 and GLUT1 protein levels (A-D) were examined in the SHR heart. Equal amounts of proteins were resolved on 10% SDS-PAGE and blotted with respective GLUT4 and GLUT1 antibodies. All the blots were also blotted with respective control antibody to GAPDH (Cytosolic) or Na^+K^+ATPase-α-1 (Plasma membrane) CDEF show densitometry measurements of protein bands in A-D respectively.

5.7. Immunofluorescence staining of GLUT4 and GLUT1 in SHR rat heart

Immunostaining for GLUT4 and GLUT1 on SHR heart tissue sections from sham, ovariectomized and E2 treated groups were analyzed as described in methods section. As seen from fig 16 (a), GLUT4 fluorescence signal (Green) was observed throughout the cytoplasm and strong in the plasma membrane in all three experimental groups which indicates no change in translocation pattern of GLUT4 by estrogen. Agglutinin

Fig.16(a)

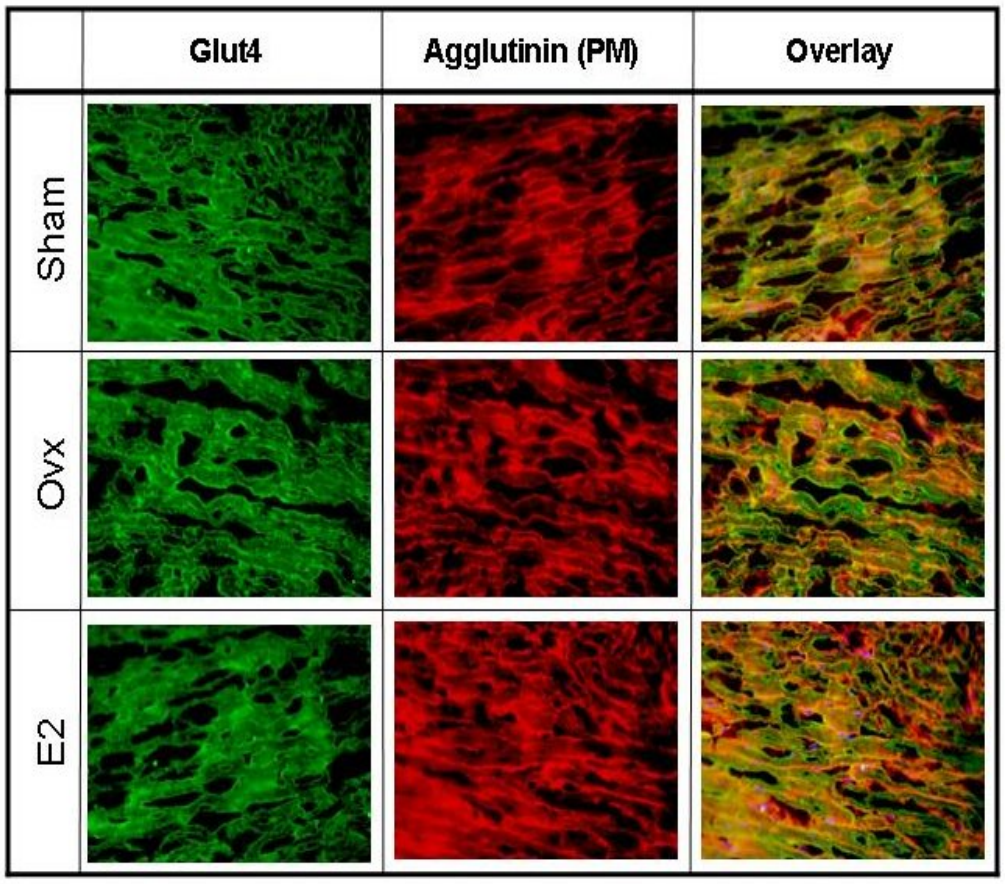


Fig.16(b)

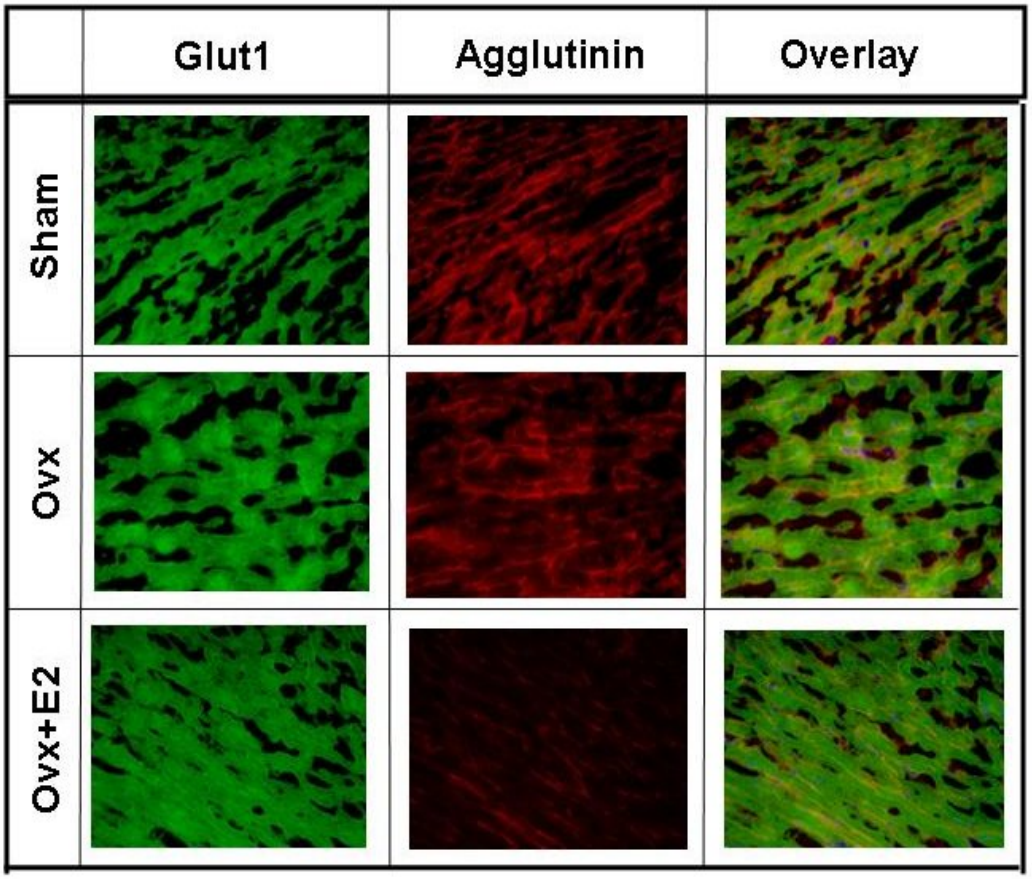


Fig. 16. Immunofluorescence staining of GLUT4 and GLUT1 in SHR heart. The distribution of GLUT4 (a) and GLUT1(b) in the heart tissue from Sham operated, ovariectomized and E2 treated SHR was analyzed by immunofluorescence staining and fluorescence microscopy using specific antibodies directed against GLUT4 and GLUT1 (*in green*), agglutinin (*in red).*

(red) was used as a plasma membrane marker. Slides without primary antibodies were employed as a negative control. Agglutinin was used as a plasma membrane marker. GLUT1 staining (fig.16 (b)) showed its distribution throughout the intracellular space. In overall, sub-cellular distribution of GLUT4 and GLUT1 was not changed by estrogen.

5.8. Effect of E2 on IRS-1 and Tyrosine Phosphorylation

To investigate whether estrogen treatment affects cardiac insulin signalling molecules in SHRs, insulin receptor substrate (IRS-1) and its tyrosine phosphorylation were examined. Total protein from each group was subjected to western blot analysis with specific antibodies to total IRS-1 and phosphor IRS-1 (aa612).

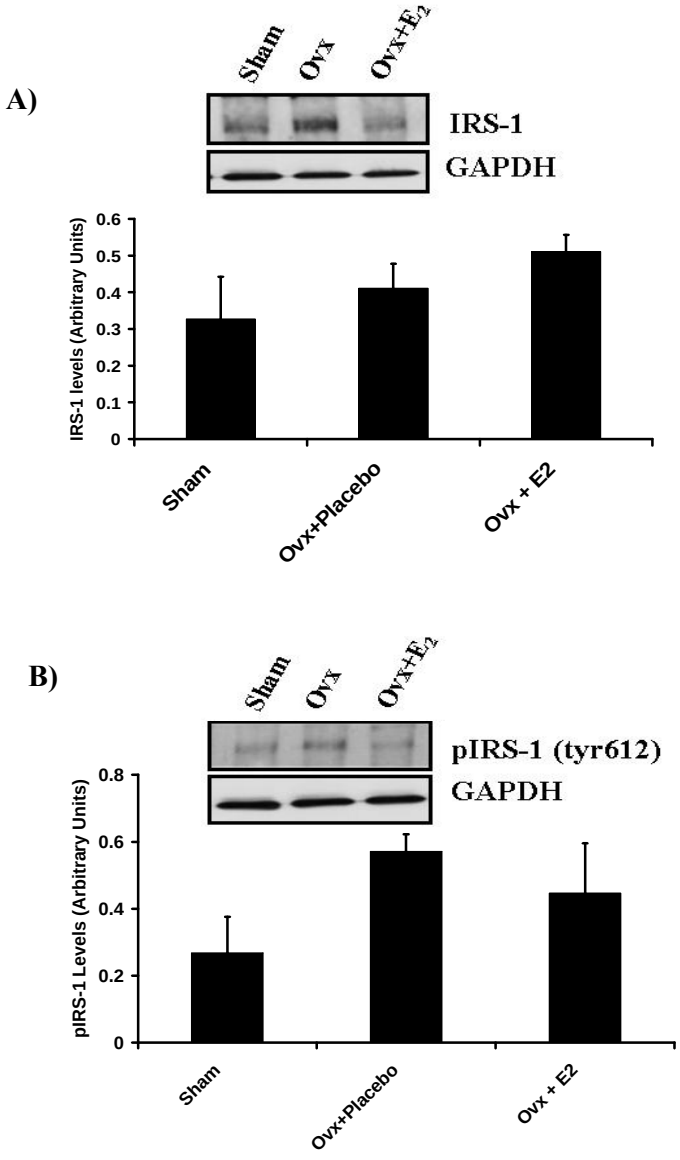


Fig.17. Effect of E2 on IRS-1 and tyrosine phosphorylation of IRS-1. The total protein from heart tissues of SHR animals with different treatment groups Sham

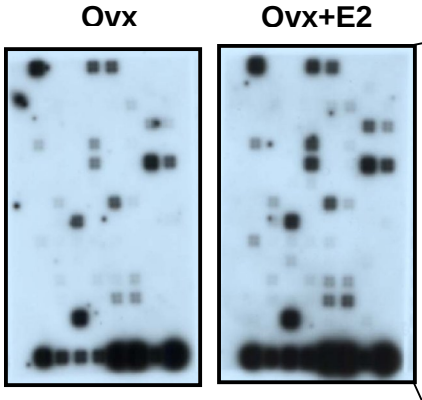
operated ovariectomized (ovx) and ovariectomized plus E2 treated (Ovx+e2) groups were resolved in SDS-PAGE and consequently subjected to immunobloting with anti-IRS-1 and pIRS-1 (tyr 612). GAPDH was used as a loading control. 7(A) represents IRS-1 levels and 7(B) Levels of pIRS-1. The values are mean ± SEM expressed as arbitrary densitometric units (ADU) plotted.

As shown in the fig 17(a) & (b), there was no significant change was seen in either the total IRS-1 protein or tyrosine phosphorylation levels in SHR rats in all treatment groups.

5.9. Microarray analysis:

OligoGE microarray analysis was performed using group wise pooled RNA (sham, ovx, and ovx+E2; n=6) as described in the methods section using insulin signaling pathway specific array (Fig 18 (A) - Gene Layout and Gene Group) to assess changes byE2 substitution on Insulin signaling pathway genes in SHR rat heart. Seven genes were up-regulated by E2 treatment which has more than 1.5 fold differences. Genes up-regulated by E2 treatment are involved in protein biosynthesis, *Eif4ebp1*; PI-3K pathway, *Frap1*; MAPK pathway, *Mapk21*,*Raf1 & Ercc1*; SREBP1 component, *Fbp1*; Glucose Metabolism, *Gpd1*.

18(A)



Array Layout
Oligo GEArray Rat Insulin Signaling Pathway Microarray

Ppia	(Acaca)	(Acacb)	Acox1	Cfd	(Adra1d)	(Adrb3)	(Aebp1_predicted)
(Ahsg)	(Akt1)	(Akt2)	(Akt3)	(Araf)	(Bcl2l1)	(Braf)	(Cap1)
(Cebpa)	(Cebpb)	(Cebpd)	(Crk)	(Csn2)	(Csnk2a1)	(Csnk2a2_predicted)	*(Dok1) (183 pixels)*
(Dok2_predicted)	(Dok3_predicted)	(Dok4_predicted)	(Dusp14_predicted)	(Eif2b1)	(Eif4e)	Eif4ebp1	Ercc1_predicted
Fasn	(Fbp1)	(Fos)	Frap1	(Frs2_predicted)	(Frs3)	(G6pc)	(G6pdx)
(Gab1_predicted)	(Gcg)	(Gck)	Gpd1	(Gpd2)	(Grb2)	Gsk3a	Gsk3b
(Hint1)	(Hk2)	(Hras)	(Igf1r)	(Igf2)	(Igfbp1)	(Ins2)	(Insl3)
(Insr)	(Irs1)	(Irs2)	(Irs4_predicted)	Jun	(Kras)	Ldlr	(Lep)
(RGD1564964_predicted)	(Map2k1)	Map2k2	(Mapk1)	(Mapk3)	(Mapk8)	(Nck2_predicted)	Nos2
(Npy)	(Orm1)	(Pck2_predicted)	(Pdpk1)	(Pik3ca)	(Pik3cb)	Pik3r1	(Pik3r2)
(Pik3r3)	(Pklr)	(Pkm2)	(Pparg)	(Ppp1ca)	(Prkcb1)	Prkcc	(Prkcz)
(Prl)	*(Ptpn1) (189 pixels)*	(Ptpn11)	(Ptprf)	*Raf1 (188 pixels)*	Rasa1	(Retn)	(RGD1562846_predicted)
(RGD1566234_predicted)	*(Rps6ka1) (186 pixels)*	(Rps6kb1)	(Rras_predicted)	*Rras2 (189 pixels)*	*Serpine1 (188 pixels)*	(Shc1)	(Shc3)
(Slc27a4)	(Slc2a1)	Slc2a4	(Sos1)	(Srebf1)	(Tg)	*(Klf10) (184 pixels)*	(Ucp1)
(Ucp2)	(Vegfa)	(PUC18)	*(Blank) (188 pixels)*	*(Blank) (192 pixels)*	*(AS1R2) (192 pixels)*	*(AS1R1) (188 pixels)*	*(AS1) (192 pixels)*
Rpl32	Ldha	Aldoa	Aldoa	Gapdh	Gapdh	BAS2C	BAS2C

18(B)

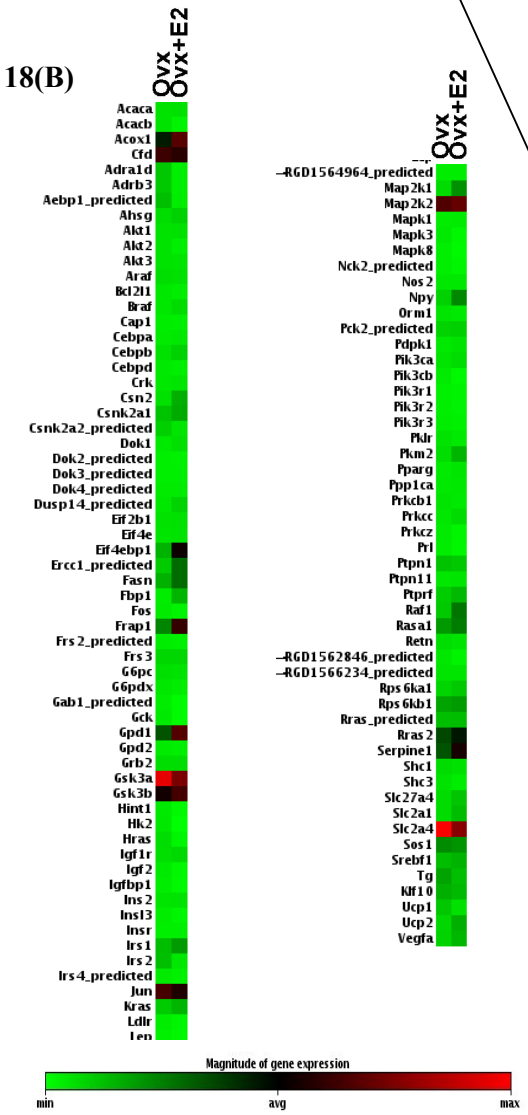


Fig.18.Gene Expression Profile in SHRs. RNA from ovariectomized and E2 treated rat heart were tagged with biotin, amplified, and hybridized with insulin signaling pathway specific microarray as explained in methods section. The autoradiograph images of the microarray are shown here as ovariectomized and E2 treated (fig.18(A)). The autoradiograph images were analyzed using web-based GEArray Expression Analysis Suite software. Qualitative analysis of gene expression changes comparing ovariectomized and E2 treated group. Color representation (fig. 18(B) of relative expression levels of genes encoding insulin signaling pathway genes in the SHR rat heart from ovariectomized and E2 treated animals. A color code key for the magnitude of gene expression is shown at the *bottom* (B).The differentially gens expressed are listed in table 2.

Table 2. Genes up-regulated by E2 treatment in SHR heart

Gene Name	GenBank™ accession number	Fold Difference	Function
Eif4ebp1 (Eukaryotic translation nitiation factor 4E binding protein 1)	NM_053857	+2.29	Forms a complex with eukaryotic initiation factor-4E (eIF-4E) which responsible for protein synthesis and also an intracellular target for insulin and growth factors
Frap1 (FK506 binding protein 12-rapamycin associated protein 1)	NM_019906	+1.97	Binds the complex formed by the immunosuppressive drug rapamycin and its receptor FKBP12; may play a role in the cell cycle G1 to S transition.
Map2k1 (Mitogen activated protein kinase kinase 1)	NM_031643	+1.71	A kinase that activates Mapk3 (Erk1) and Mapk1 (Erk2) kinases
Raf1 (V-raf-1 murine leukemia viral oncogene homolog 1)	NM_012639	+1.68	Acts as a mitogenic protein kinase; mutant forms may play a role in transformation
Fbp1 Fructose-1,6- biphosphatase 1	NM_012558	+1.64	Catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate in gluconeogenesis
Gpd1 Glycerol-3-phosphate dehydrogenase 1 (soluble)	NM_022215	+1.68	Target gene of PPARγ, involved in glycolysis
Ercc1_predicted Excision repair cross-complementing rodent repair deficiency, complementation group 1 (predicted)	XM_214833	+1.78	Target gene in MAPK pathway

5.10. Hexokinase Activity

Hexokinase (HK; EC 2.7.1.1) is required for glucose metabolism in SHR hearts was measured as described in the methods section. As shown in the figure.19, the reduced hexokinase activity (the amount of production of NADPH) in ovariectomized SHR was significantly increased by E2 treatment (*p<0.010).

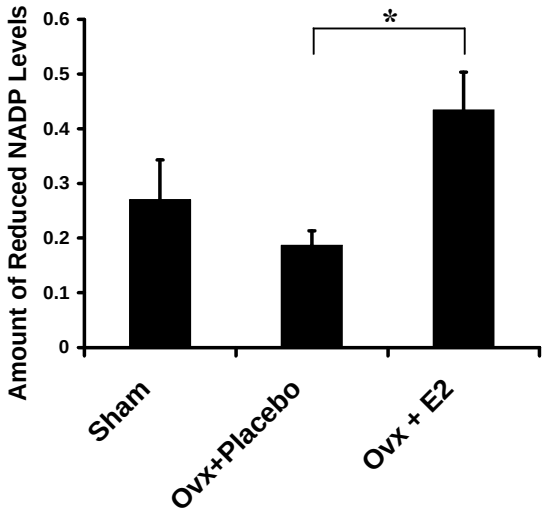


Fig.9. Total activity of hexokinase in heart homogenates from SHR rats of different treatment groups were measured spectrophotometrically by measuring the amount of NADPH formed per minute at 340nm. The solid bar represents the amount of reduced NADP+ levels. Each bar represents mean ± SEM.

5.11. Western blot analysis of 4Ebp1:

The differentially expressed genes Frap1 (mTOR), and Eif4bp1 were up-regulated in E2 treated group. These two genes are belonging to mTOR signalling pathways which

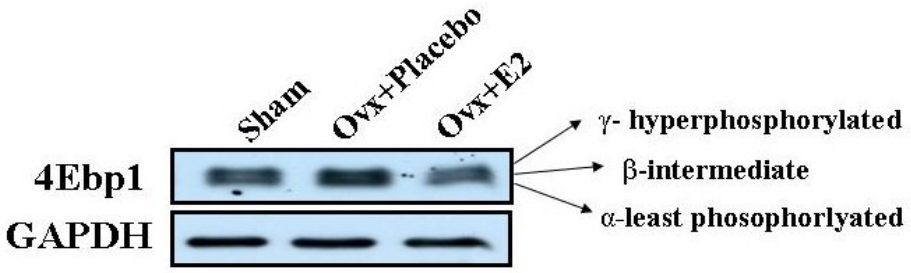


Fig. 10. Western blot analysis of 4E-BP1. The total protein from SHR (sham, ovx and ovx+E2) was subjected to immunoblotting with anti-4Ebp1 antibody. The position of the 3 forms of 4E-BP1 from unphosphorylated (α) to hyperphosphorylated (γ) is indicated.

regulates protein synthesis. One of the effectors of mTOR is 4E-BP1. On phosphorylation of 4E-BP1 results in release of eIF4E, allowing increased formation of the eIF4F translation factor complexes leads to increased protein synthesis. The increased level of 4E-BP1 phosphorylation in ovariectomized SHR was reduced by E2 treatment. The phosphorylation of 4E-BP1 is known to be critical for the development of cardiac hypertrophy in response to pressure overload (S Sharma *et al,* 2007).

6. Discussion

This study primarily demonstrates that estrogen modulates myocardial glucose utilization in female spontaneously hypertensive rat (SHR). The myocardial glucose uptake in intact versus ovariectomized female SHR with non-selective estrogen 17β-estradiol and without (placebo) was evaluated by using 2-[^{18}F-fluorodeoxyglucose and positron emission tomography; the data indicated that the 17β-estradiol exert significant increase of myocardial glucose uptake. Estrogen deficient ovariectomized SHR demonstrated reduced myocardial FDG accumulation compared to intact SHAM controls and it was normalized by 17β-estradiol treatment.

Cardiac hypertrophy induced by pressure overload such as hypertension is a characteristic increase in cardiac muscle mass and alterations in the structure of the heart and is an independent risk factor for cardiovascular diseases. Cardiac hypertrophy is also associated with abnormalities in energy metabolism, the chief myocardial energy source switches from fatty acid to glucose, a regression to the fetal energy substrate preference pattern (Panidis, Kotler *et al.* 1984; Richey and Brown 1998a; Takano, Zou *et al.* 2002). The rate of glucose oxidation are reduced and also the amount of ATP or high-energy phosphates in the myocardium in cardiac hypertrophy and failure and predicted that it might be very important contributors to contractile dysfunction, and it is consider that efficiency of the heart is improved as long as glucose can be oxidized (Bishop and Altschuld 1970; Depre, Vanoverschelde *et al.* 1999; Opie 1968; Taegtmeyer 2000b; van der Vusse, Glatz *et al.* 1992);(Zhang, Merkle *et al.* 1993). In hypertrophied heart, insulin dependent glucose uptake is impaired but with increased basal glucose uptake (Bishop and Altschuld 1970; Christe and Rodgers 1994; Kagaya, Kanno *et al.* 1990). It has been shown that cardiac

specific ablation of major cardiac glucose transporter insulin-dependent GLUT4 causes development of cardiac hypertrophy. Collectively, these observations indicate that the disturbance in cardiac glucose utilization and oxidation is critical for cardiac function which causes cardiac hypertrophy. Women have lesser prevalence for cardiac hypertrophy than men but risk of heart disease increases in postmenopausal women compared to age matched males (Agabiti-Rosei and Muiesan 2002). In animal studies already have shown that estrogen prevents development of cardiac hypertrophy and 17β-estradiol or selective estrogen receptor agonist (16α LE2) in ovariectomized SHR efficiently attenuate cardiac hypertrophy by reducing cardiac mass and increased cardiac output and contractility (Jazbutyte, Arias-Loza *et al.* 2008; Pelzer, Jazbutyte *et al.* 2005). For this improvement of cardiac performance energy metabolism must be maintained. In the present study myocardial FDG accumulation showed that the reduced glucose uptake by ovariectomy was normalized by 17β-estradiol treatment in SHR. With the beneficial effects of 17β-estradiol in preventing development of cardiac hypertrophy in female SHR, the present data provide a novel mechanism to explain for direct protective role of estrogen in cardiac hypertrophy. In order to explain whether this difference in myocardial glucose accumulation is due to changes in blood glucose levels or involvement of intrinsic changes in cardiac metabolism, intra-peritoneal glucose tolerance tests were performed. Glucose responses during IP-GTT were similar between the treatment groups. Ovariectomy did not alter in vivo glucose tolerance in SHR. However, the reduced serum insulin levels were observed in ovariectomized SHR and were normalized by E2 treatment. Previous studies have stated that any disturbances in female gonad hormone levels that occur with gestational diabetes mellitus and polycystic ovarian syndrome result in impaired glucose tolerance and insulin resistance. Estrogen treatment in

postmenopausal women showed improved insulin sensitivity (Crespo, Smit *et al.* 2002; Espeland, Hogan *et al.* 1998; Saglam, Polat *et al.* 2002). Estrogen reverses the effect of menopause on glucose and insulin metabolism by increasing insulin secretion from pancreas as well as insulin sensitivity (Brussaard, Gevers Leuven *et al.* 1997; Stevenson, Crook *et al.* 1994). In mice, Both ERKO and ArKO exhibit reduced glucose tolerance, insulin resistance and obesity (Heine, Taylor *et al.* 2000; Jones, Thorburn *et al.* 2001). ERKO mice also showed decreased GLUT4 in skeletal muscle level that could account for impaired glucose uptake (Barros, Machado *et al.* 2006). Estrogen treatment enhances insulin stimulated glucose uptake in diabetic animals and reduced by ovariectomy (Louet, LeMay *et al.* 2004). The alteration in insulin levels may account for increased myocardial glucose uptake by estrogen in SHR hearts. Further it raises the question whether the difference in glucose transporters are responsible for the increased FDG uptake by E2. Many studies revealed that glucose transport through the plasma membrane is a rate-limiting step of myocardial glucose utilization and glycolytic flux (King and Opie 1998; Manchester, Kong *et al.* 1994). Glucose is an important metabolic substrate for the heart and assumes increased importance in the response of the heart to ischemia and in the adaptation of the heart to cardiac hypertrophy. Secondly, the heart demonstrates a unique ability to alter its substrate utilization on the basis of changes in substrate supply and cardiac work. Thus understanding the factors that regulate glucose entry into the heart will increase our understanding of cardiac physiology and pathophysiology. Glucose transported in to cardiomyocytes through glucose transporters GLUT 4 and GLUT 1 (Mueckler 1990). GLUT4 is major glucose transporter localized in the cytosol and translocation in to plasma membrane to facilitate the glucose in response to insulin (Slot, Geuze *et al.* 1991), workload, ischemia, and hypoxia (Sun, Nguyen *et al.* 1994). Insulin bind

with its receptor stimulate the tyrosine phosophorylation of beta subunit of insulin receptor (Lee and Pilch 1994) This inturn activates tyrosine phosphorylation of many other insulin receptors substrates and interact with phosphatidylinositol 3-kinase (PI3K), in turn activates Akt, a downstream serine/threoninekinase that stimulates uptake of glucose through glucose transporter GLUT4 translocation in to the plasma membrane (Cong, Chen *et al.* 1997). The activity of GLUT4 is controlled by insulin in cardiac muscle in which glucose transport needs to be rapidly and markedly enhanced (Kahn 1992; Slot, Geuze *et al.* 1991). GLUT-1 is evenly distributed between the plasma membrane, whereas GLUT-4 is almost entirely stored in an intracellular pool (James, Strube *et al.* 1989; Kahn, Charron *et al.* 1989; Slot, Geuze *et al.* 1991). When insulin levels are less, GLUT-4 is re-sequestered in intracellular vesicles (Holman and Cushman 1994). A reduction in GLUT-4 mRNA and protein is thought to be a mechanism for insulin resistance in various models of diabetes, and it has been associated with lower myocardial glucose uptake (Garvey, Hardin *et al.* 1993; Garvey, Maianu *et al.* 1991; Sinha, Raineri-Maldonado *et al.* 1991). In the present study, there is no change on GLUT4 and GLUT1 protein expression or translocation to the sarcolemma in SHR. A large amount of GLUT1 in the myocardium is localized to the sarcolemma and is considered to be responsible for basal glucose transport, even though its translocation can also be induced by stimuli that provoke GLUT4 accumulation in the sarcolemma (Egert, Nguyen *et al.* 1999a; Fischer, Thomas *et al.* 1997; Young, Renfu *et al.* 1997). Any defect in insulin signaling will lead to insulin resistance. Insulin resistance is a major complication for the development of hypertension, LVH and dysfunction, and HF, will causes the defect in glucose metabolism and will cause sever metabolic changes cardiac muscles. In present study there was no difference between intracellular and plasma membrane

localization or protein levels of GLUT4 or GLUT1 levels SHR myocardial tissue was not altered by estrogen. There are some studies which could explain this difference, the inactive GLUT4 localized on plasma membrane without any glucose transport activity since mechanism of glucose uptake through GLUT4 is precisely controlled in time and space in insulin signal transduction (Calderhead, Kitagawa *et al.* 1990; Holman, Kozka *et al.* 1990; Joost, Weber *et al.* 1987; Palfreyman, Clark *et al.* 1992). And also there were no changes on initial step insulin signaling molecules like IRS-1 and its phosphorylation in insulin induced glucose uptake in SHR hearts. These findings suggest that that GLUT4 is not rate limiting for increased glucose uptake observed in E2 treatment.

The efficiency of ovariectomy and estrogen treatment in Spontaneously Hypertensive rat has been assessed. In ovariectomized SHR rats, serum levels of estrogen were significantly decreased in contrast to sham operated animals and serum estrogen levels brought back by estrogen treatment in SHR animals. And uterus weight provides valuable information not only about estrogen substitution efficacy but also about the quality of ovariectomy. Systemic estrogen levels have long been recognized to modulate body mass and body mass composition in humans and in whole variety of animal models; decreased estrogen prevented by substituting physiological estrogen levels. The degree of cardiac hypertrophy also was measured by normalizing absolute heart weight versus tibia length. The ovariectomy of SHR females had showed a trend to increase cardiac hypertrophy which was attenuated by estrogen treatment. These observations of estrogen efficacy, morphological changes and degree of cardiac hypertrophy in female SHR animals, was in agreement with the data reported previously (Jazbutyte, Arias-Loza *et al.* 2008; Pelzer, Jazbutyte *et al.* 2005)

After glucose transporters, hexokinase activity have been implicated as the rate limiting step in myocardial ^{18}FDG uptake (Hariharan, Bray *et al.* 1995; Ratib, Phelps *et al.* 1982; Young, Russell *et al.* 1999). Since intracellular glucose must first be phosphorylated for further metabolism, hexokinase is implicated as a critical step for control of glucose utilization. Hexokinase activity had showed a significant increase by E2 treatment that was reduced in ovariectomized SHR cardiac muscle confirms ability of estrogen treatment increasing the glucose utilization and metabolism in SHR animals.

Microarray analysis in SHR heart showed that ei4Ebp1 and Frap1 genes which are involved in mTOR signaling pathway were up-regulated in E2 treated group. These two genes are regulating the protein synthesis and activated in presence of glucose in the heart contributing to cardiac hypertrophy (Sharma, Guthrie *et al.* 2007). Since phosphorylation of Eif4bp1 is important step in protein synthesis, the reduced levels of 4Ebp1 in ovariectomized SHR rat hearts were increased E2 treatment. In contrast to up-regulation of Eif4Ebp1 gene by E2 treatment from microarray analysis did not show similar pattern on its protein expression instead increased levels of its level in ovariectomized SHR heart. These findings suggest a possible link between estrogen and well established intermediately metabolism involved mTOR signalling pathway with respect to cardiac hypertrophy.

7. Conclusion

In the present study, the reduced myocardial FDG uptake in ovariectomized spontaneously hypertensive rat is normalized by 17β-estradiol treatment. The

increased serum insulin level and myocardial hexokinase activity warranted increased myocardial glucose uptake. The present work showing increased glucose uptake in response to 17β-estradiol in ovariectomized SHR may provide a novel mechanism to explain known function of estradiol in reduction of cardiac hypertrophy in SHR. Unaltered glucose transporter (GLUT4) expression/localization or IRS-1 and its phosphorylation suggesting not a rate limiting for changes in myocardial FDG uptake that observed in SHR.

8. Clinical Implications

Decreased cardiac glucose uptake directly responsible for diminish of cardiac energy production (Abel 2004; Depre, Vanoverschelde *et al.* 1999). The lack of energy yield not only damage the contractile function, also responsible for inefficiency of myocardium manage with ischemia/reperfusion stress (Bugger and Abel 2008; Russell, Li *et al.* 2004). Formation of O2 free radicals and flow of calcium are known outcome of ischemia-reperfusion which can be amplified by decline in energy formation due to reduced glucose utilization (Bugger and Abel 2008; Ferdinandy, Schulz *et al.* 2007). Post-ischemic contractile dysfunction due to impaired glucose uptake and oxidation can improve contractile function by the agents that enhance (Depre, Vanoverschelde *et al.* 1999; Ferdinandy, Schulz *et al.* 2007; Russell, Li *et al.* 2004). Therapeutic strategies to agument glucose uptake may beneficial for ischemic heart (Wang and Lopaschuk 2007). The improved cardiac glucose metabolism by 17β-estradiol treatment contributes to its cardio-protective effects.

9. References

Abel ED (2004) Glucose transport in the heart. *Front Biosci* **9**, 201-15.

Ackerman GE, Carr BR (2002) Estrogens. *Rev Endocr Metab Disord* **3**, 225-30.

Agabiti-Rosei E, Muiesan ML (2002) Left ventricular hypertrophy and heart failure in women. *J Hypertens Suppl* **20**, S34-8.

Allard MF, Schonekess BO, Henning SL, English DR, Lopaschuk GD (1994) Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *American Journal of Physiology- Heart and Circulatory Physiology* **267**, 742-750.

American (1997) American Diabetes Association: clinical practice recommendations 1997. *Diabetes Care* **20 Suppl 1**, S1-70.

Anderson PG, Allard MF, Thomas GD, Bishop SP, Digerness SB (1990) Increased ischemic injury but decreased hypoxic injury in hypertrophied rat hearts. *Circulation Research* **67**, 948-959.

Anker SD, Ponikowski P, Varney S, Clark AL, Webb-Peploe KM, Harrington D, Kox WJ, Poole-Wilson PA, Coats AJS (1997) Wasting as independent risk factor for mortality in chronic heart failure. *LANCET-LONDON-*, 1050-1053.

Ascuitto RJ, Ross-Ascuitto NT (1996) Substrate metabolism in the developing heart. *Semin Perinatol* **20**, 542-63.

Association AD (1997) American Diabetes Association: clinical practice recommendations 1997. *Diabetes Care* **20 Suppl 1**, S1-70.

Auricchio F, Migliaccio A, Castoria G, Di Domenico M, Pagano M (1990) Phosphorylation of uterus estradiol receptor on tyrosine. *Prog Clin Biol Res* **322**, 133-55.

Aurigemma GP, Gaasch WH (1995) Gender differences in older patients with pressure-overload hypertrophy of the left ventricle. *Cardiology* **86**, 310-317.

Babiker FA, Lips D, Meyer R, Delvaux E, Zandberg P, Janssen B, van Eys G, Grohe C, Doevendans PA (2006) Estrogen Receptor {beta} Protects the Murine Heart Against Left Ventricular Hypertrophy. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**, 1524.

Barros RP, Machado UF, Warner M, Gustafsson JA (2006) Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. *Proc Natl Acad Sci U S A* **103**, 1605-8.

Beer S, Reincke M*, et al.* (2007) High-dose 17ß–estradiol treatment prevents development of heart failure post–myocardial infarction in the rat. *Basic Research in Cardiology* **102**, 9-18.

Belke DD, Betuing S*, et al.* (2002) Insulin signaling coordinately regulates cardiac size, metabolism, and contractile protein isoform expression. *Journal of Clinical Investigation* **109**, 629.

Belke DD, Larsen TS, Gibbs EM, Severson DL (2000) Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. *American Journal of Physiology- Endocrinology And Metabolism* **279**, 1104-1113.

Bing RJ, Siegel A, Vitale A, Balboni F, Sparks E, Taeschler M, Klapper M, Edwards S (1953) Metabolic studies on the human heart in vivo. I. Studies on carbohydrate metabolism of the human heart. *Am J Med* **15**, 284-96.

Bishop SP, Altschuld RA (1970) Increased glycolytic metabolism in cardiac hypertrophy and congestive failure. *Am J Physiol* **218**, 153-9.

Bowles DK, Farrar RP, Starnes JW (1992) Exercise training improves cardiac function after ischemia in the isolated, working rat heart. *American Journal of Physiology- Heart and Circulatory Physiology* **263**, 804-809.

Brussaard HE, Gevers Leuven JA, Frolich M, Kluft C, Krans HM (1997) Short-term oestrogen replacement therapy improves insulin resistance, lipids and fibrinolysis in postmenopausal women with NIDDM. *Diabetologia* **40**, 843-9.

Bugger H, Abel ED (2008) Molecular mechanisms for myocardial mitochondrial dysfunction in the metabolic syndrome. *Clin Sci (Lond)* **114**, 195-210.

Burelle Y, Wambolt RB*, et al.* (2004) Regular exercise is associated with a protective metabolic phenotype in the rat heart. *American Journal of Physiology- Heart and Circulatory Physiology* **287**, 1055-1063.

Calderhead DM, Kitagawa K, Tanner LI, Holman GD, Lienhard GE (1990) Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. *J Biol Chem* **265**, 13801-8.

Carroll JD, Carroll EP, Feldman T, Ward DM, Lang RM, McGaughey D, Karp RB (1992) Sex-associated differences in left ventricular function in aortic stenosis of the elderly. *Circulation* **86**, 1099-1107.

Carvajal K, Moreno-Sanchez R (2003) Heart metabolic disturbances in cardiovascular diseases. *Arch Med Res* **34**, 89-99.

Castello A, Rodriguez-Manzaneque JC, Camps M, Perez-Castillo A, Testar X, Palacin M, Santos A, Zorzano A (1994) Perinatal hypothyroidism impairs the normal transition of GLUT4 and GLUT1 glucose transporters from fetal to neonatal levels in heart and brown adipose tissue. Evidence for tissue-specific regulation of GLUT4 expression by thyroid hormone. *J Biol Chem* **269**, 5905-12.

Christe ME, Rodgers RL (1994) Altered glucose and fatty acid oxidation in hearts of the spontaneously hypertensive rat. *J Mol Cell Cardiol* **26**, 1371-5.

Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI, Quon MJ (1997) Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol Endocrinol* **11**, 1881-90.

Couse JF, Korach KS (1999) Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* **20**, 358-417.

Crespo CJ, Smit E, Snelling A, Sempos CT, Andersen RE (2002) Hormone replacement therapy and its relationship to lipid and glucose metabolism in diabetic and nondiabetic postmenopausal women: results from the Third National Health and Nutrition Examination Survey (NHANES III). *Diabetes Care* **25**, 1675-80.

Dash R, Schmidt AG, Pathak A, Gerst MJ, Biniakiewicz D, Kadambi VJ, Hoit BD, Abraham WT, Kranias EG (2003) Differential regulation of p38 mitogen-activated protein kinase mediates gender-dependent catecholamine-induced hypertrophy. *Cardiovascular Research* **57**, 704-714.

Davidoff AJ (2006) Convergence of glucose- and fatty acid-induced abnormal myocardial excitation-contraction coupling and insulin signalling. *Clin Exp Pharmacol Physiol* **33**, 152-8.

Depre C, Vanoverschelde JL, Taegtmeyer H (1999) Glucose for the heart. *Circulation* **99**, 578-88.

Desrois M, Sidell RJ, Gauguier D, King LM, Radda GK, Clarke K (2004) Initial steps of insulin signaling and glucose transport are defective in the type 2 diabetic rat heart. *Cardiovascular Research* **61**, 288-296.

Dutertre M, Smith CL (2000) Molecular mechanisms of selective estrogen receptor modulator (SERM) action. *J Pharmacol Exp Ther* **295**, 431-7.

Eckel J, Reinauer H (1990) Insulin action on glucose transport in isolated cardiac myocytes: signalling pathways and diabetes-induced alterations. *Biochem Soc Trans* **18**, 1125-7.

Egert S, Nguyen N, Brosius FC, 3rd, Schwaiger M (1997) Effects of wortmannin on insulin- and ischemia-induced stimulation of GLUT4 translocation and FDG uptake in perfused rat hearts. *Cardiovasc Res* **35**, 283-93.

Egert S, Nguyen N, Schwaiger M (1999a) Contribution of alpha-adrenergic and beta-adrenergic stimulation to ischemia-induced glucose transporter (GLUT) 4 and GLUT1 translocation in the isolated perfused rat heart. *Circ Res* **84**, 1407-15.

Egert S, Nguyen N, Schwaiger M (1999b) Myocardial glucose transporter GLUT1: translocation induced by insulin and ischemia. *J Mol Cell Cardiol* **31**, 1337-44.

Enmark E, Gustafsson JA (1999) Oestrogen receptors - an overview. *J Intern Med* **246**, 133-8.

Espeland MA, Hogan PE, Fineberg SE, Howard G, Schrott H, Waclawiw MA, Bush TL (1998) Effect of postmenopausal hormone therapy on glucose and insulin concentrations. PEPI Investigators. Postmenopausal Estrogen/Progestin Interventions. *Diabetes Care* **21**, 1589-95.

Falkenstein E, Wehling M (2000) Nongenomically initiated steroid actions. *Eur J Clin Invest* **30 Suppl 3**, 51-4.

Ferdinandy P, Schulz R, Baxter GF (2007) Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev* **59**, 418-58.

Fischer Y, Thomas J*, et al.* (1997) Insulin-induced recruitment of glucose transporter 4 (GLUT4) and GLUT1 in isolated rat cardiac myocytes. Evidence of the existence of different intracellular GLUT4 vesicle populations. *J Biol Chem* **272**, 7085-92.

Fisher DJ (1984) Oxygenation and metabolism in the developing heart. *Semin Perinatol* **8**, 217-25.

Frey N, Olson EN (2003) C ARDIAC H YPERTROPHY: The Good, the Bad, and the Ugly. *Annual Reviews in Physiology* **65**, 45-79.

Fuller W, Eaton P, Medina RA, Bell J, Shattock MJ (2001) Differential centrifugation separates cardiac sarcolemmal and endosomal membranes from Langendorff-perfused rat hearts. *Anal Biochem* **293**, 216-23.

Garvey WT, Hardin D, Juhaszova M, Dominguez JH (1993) Effects of diabetes on myocardial glucose transport system in rats: implications for diabetic cardiomyopathy. *Am J Physiol* **264**, H837-44.

Garvey WT, Maianu L, Huecksteadt TP, Birnbaum MJ, Molina JM, Ciaraldi TP (1991) Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. *J Clin Invest* **87**, 1072-81.

Germain P, Staels B, Dacquet C, Spedding M, Laudet V (2006) Overview of nomenclature of nuclear receptors. *Pharmacol Rev* **58**, 685-704.

Goodwin GW, Taylor CS, Taegtmeyer H (1998) Regulation of energy metabolism of the heart during acute increase in heart work. *J Biol Chem* **273**, 29530-9.

Gould GW, Holman GD (1993) The glucose transporter family: structure, function and tissue-specific expression. *Biochem J* **295 (Pt 2)**, 329-41.

Grossman W, Jones D, McLaurin LP (1975) Wall stress and patterns of hypertrophy in the human left ventricle. *Journal of Clinical Investigation* **56**, 56.

Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC, Jr., Sowers JR (1999) Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. *Circulation* **100**, 1134-46.

Hariharan R, Bray M, Ganim R, Doenst T, Goodwin GW, Taegtmeyer H (1995) Fundamental limitations of [18F]2-deoxy-2-fluoro-D-glucose for assessing myocardial glucose uptake. *Circulation* **91**, 2435-44.

Hayat SA, Patel B, Khattar RS, Malik RA (2004) Diabetic cardiomyopathy: mechanisms, diagnosis and treatment. *Clin Sci (Lond)* **107**, 539-57.

Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC, Bender JR (2000) Membrane Estrogen Receptor Engagement Activates Endothelial Nitric Oxide Synthase via the PI3-Kinase-Akt Pathway in Human Endothelial Cells. In. pp. 677-682. (Am Heart Assoc)

Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS (2000) Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A* **97**, 12729-34.

Hoerter JA, Opie LH (1978) Perinatal changes in glycolytic function in response to hypoxia in the incubated or perfused rat heart. *Biol Neonate* **33**, 144-61.

Holman GD, Cushman SW (1994) Subcellular localization and trafficking of the GLUT4 glucose transporter isoform in insulin-responsive cells. *Bioessays* **16**, 753-9.

Holman GD, Kozka IJ, Clark AE, Flower CJ, Saltis J, Habberfield AD, Simpson IA, Cushman SW (1990) Cell surface labeling of glucose transporter isoform GLUT4 by

bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J Biol Chem* **265**, 18172-9.

Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E (1998) Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *Jama* **280**, 605-13.

James DE, Strube M, Mueckler M (1989) Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* **338**, 83-7.

Jazbutyte V, Arias-Loza PA*, et al.* (2008) Ligand-dependent activation of ER{beta} lowers blood pressure and attenuates cardiac hypertrophy in ovariectomized spontaneously hypertensive rats. *Cardiovasc Res* **77**, 774-81.

Jones ME, Thorburn AW*, et al.* (2001) Aromatase-deficient (ArKO) mice accumulate excess adipose tissue. *J Steroid Biochem Mol Biol* **79**, 3-9.

Joost HG, Weber TM, Cushman SW, Simpson IA (1987) Activity and phosphorylation state of glucose transporters in plasma membranes from insulin-, isoproterenol-, and phorbol ester-treated rat adipose cells. *J Biol Chem* **262**, 11261-7.

Kadokami T, McTiernan CF, Kubota T, Frye CS, Feldman AM (2000) Sex-related survival differences in murine cardiomyopathy are associated with differences in TNF-receptor expression. *Journal of Clinical Investigation* **106**, 589.

Kagaya Y, Kanno Y, Takeyama D, Ishide N, Maruyama Y, Takahashi T, Ido T, Takishima T (1990) Effects of long-term pressure overload on regional myocardial

glucose and free fatty acid uptake in rats. A quantitative autoradiographic study. *Circulation* **81**, 1353-61.

Kahn BB (1992) Facilitative glucose transporters: regulatory mechanisms and dysregulation in diabetes. *J Clin Invest* **89**, 1367-74.

Kahn BB, Charron MJ, Lodish HF, Cushman SW, Flier JS (1989) Differential regulation of two glucose transporters in adipose cells from diabetic and insulin-treated diabetic rats. *J Clin Invest* **84**, 404-11.

Kannel WB, McGee DL (1979) Diabetes and cardiovascular disease. The Framingham study. *Jama* **241**, 2035-8.

Kemppainen J, Tsuchida H*, et al.* (2003) Insulin signalling and resistance in patients with chronic heart failure. *J Physiol* **550**, 305-15.

King LM, Opie LH (1998) Glucose delivery is a major determinant of glucose utilisation in the ischemic myocardium with a residual coronary flow. *Cardiovasc Res* **39**, 381-92.

Klein-Hitpass L, Schorpp M, Wagner U, Ryffel GU (1986) An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. *Cell* **46**, 1053-61.

Klein R (1995) Hyperglycemia and microvascular and macrovascular disease in diabetes. *Diabetes Care* **18**, 258-68.

Kraegen EW, Sowden JA, Halstead MB, Clark PW, Rodnick KJ, Chisholm DJ, James DE (1993) Glucose transporters and in vivo glucose uptake in skeletal and cardiac

muscle: fasting, insulin stimulation and immunoisolation studies of GLUT1 and GLUT4. *Biochemical Journal* **295**, 287.

Krasinski K, Spyridopoulos I, Asahara T, van der Zee R, Isner JM, Losordo DW (1997) Estradiol Accelerates Functional Endothelial Recovery After Arterial Injury. *Circulation* **95**, 1768-1772.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863-70.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* **93**, 5925-30.

LaCroix AZ (2005) Estrogen with and without progestin: benefits and risks of short-term use. *The American Journal of Medicine* **118**, 79-87.

Landers JP, Spelsberg TC (1992) New concepts in steroid hormone action: transcription factors, proto-oncogenes, and the cascade model for steroid regulation of gene expression. *Crit Rev Eukaryot Gene Expr* **2**, 19-63.

Laybutt DR, Thompson AL, Cooney GJ, Kraegen EW (1997) Selective chronic regulation of GLUT1 and GLUT4 content by insulin, glucose, and lipid in rat cardiac muscle in vivo. *American Journal of Physiology- Heart and Circulatory Physiology* **273**, 1309-1316.

Lee J, Pilch PF (1994) The insulin receptor: structure, function, and signaling. *Am J Physiol* **266**, C319-34.

Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP (1990) Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* **322**, 1561-6.

Louet JF, LeMay C, Mauvais-Jarvis F (2004) Antidiabetic actions of estrogen: insight from human and genetic mouse models. *Curr Atheroscler Rep* **6**, 180-5.

Manchester J, Kong X, Nerbonne J, Lowry OH, Lawrence JC, Jr. (1994) Glucose transport and phosphorylation in single cardiac myocytes: rate-limiting steps in glucose metabolism. *Am J Physiol* **266**, E326-33.

Mendelsohn ME (2002) Protective effects of estrogen on the cardiovascular system. *Am J Cardiol* **89**, 12E-17E; discussion 17E-18E.

Mendelsohn ME, Karas RH (2007) HRT and the young at heart. *N Engl J Med* **356**, 2639-41.

Mermelstein PG, Becker JB, Surmeier DJ (1996) Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. *J Neurosci* **16**, 595-604.

Miya Y, Sumino H, *et al.* (2002) Effects of hormone replacement therapy on left ventricular hypertrophy and growth-promoting factors in hypertensive postmenopausal women. *Hypertens Res* **25**, 153-9.

Moore RL, Korzick DH (1995) Cellular adaptations of the myocardium to chronic exercise. *Prog Cardiovasc Dis* **37**, 371-96.

Mueckler M (1990) Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes. *Diabetes* **39**, 6-11.

Musatov S, Chen W, Pfaff DW, Mobbs CV, Yang XJ, Clegg DJ, Kaplitt MG, Ogawa S (2007) Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. *Proc Natl Acad Sci U S A* **104**, 2501-6.

Nilsson S, Gustafsson JA (2002) Estrogen receptor action. *Crit Rev Eukaryot Gene Expr* **12**, 237-57.

Nilsson S, Makela S*, et al.* (2001) Mechanisms of estrogen action. *Physiol Rev* **81**, 1535-65.

Opie LH (1968) Metabolism of the heart in health and disease. I. *Am Heart J* **76**, 685-98.

Palfreyman RW, Clark AE, Denton RM, Holman GD, Kozka IJ (1992) Kinetic resolution of the separate GLUT1 and GLUT4 glucose transport activities in 3T3-L1 cells. *Biochem J* **284 (Pt 1)**, 275-82.

Panidis IP, Kotler MN, Ren JF, Mintz GS, Ross J, Kalman P (1984) Development and regression of left ventricular hypertrophy. *J Am Coll Cardiol* **3**, 1309-20.

Paolisso G, De Riu S, Marrazzo G, Verza M, Varricchio M, D'Onofrio F (1991) Insulin resistance and hyperinsulinemia in patients with chronic congestive heart failure. *Metabolism* **40**, 972-7.

Peale FV, Jr., Ludwig LB, Zain S, Hilf R, Bambara RA (1988) Properties of a high-affinity DNA binding site for estrogen receptor. *Proc Natl Acad Sci U S A* **85**, 1038-42.

Pedram A, Razandi M, Aitkenhead M, Levin ER (2005) Estrogen Inhibits Cardiomyocyte Hypertrophy in Vitro: ANTAGONISM OF CALCINEURIN-RELATED HYPERTROPHY THROUGH INDUCTION OF MCIP1. *Journal of Biological Chemistry* **280**, 26339.

Pedram A, Razandi M, Levin ER (2006) Nature of functional estrogen receptors at the plasma membrane. *Mol Endocrinol* **20**, 1996-2009.

Pelzer T, Jazbutyte V, *et al.* (2005) The estrogen receptor-alpha agonist 16alpha-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats. *Cardiovasc Res* **67**, 604-12.

Pelzer T, Loza PAA, *et al.* (2005) Increased Mortality and Aggravation of Heart Failure in Estrogen Receptor-ß Knockout Mice After Myocardial Infarction. In. pp. 1492-1498. (Am Heart Assoc)

Pessin JE, Bell GI (1992) Mammalian facilitative glucose transporter family: structure and molecular regulation. *Annu Rev Physiol* **54**, 911-30.

Petersen KF, Shulman GI (2002) Pathogenesis of skeletal muscle insulin resistance in type 2 diabetes mellitus. *Am J Cardiol* **90**, 11G-18G.

Phelps ME, Schelbert HR, Mazziotta JC (1983) Positron computed tomography for studies of myocardial and cerebral function. *Ann Intern Med* **98**, 339-59.

Pratt WB, Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* **18**, 306-60.

Ratib O, Phelps ME, Huang SC, Henze E, Selin CE, Schelbert HR (1982) Positron tomography with deoxyglucose for estimating local myocardial glucose metabolism. *J Nucl Med* **23**, 577-86.

Razeghi P, Young ME, Alcorn JL, Moravec CS, Frazier OH, Taegtmeyer H (2001) Metabolic gene expression in fetal and failing human heart. *Circulation* **104**, 2923-31.

Reaven GM (1991) Insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypertension. Parallels between human disease and rodent models. *Diabetes Care* **14**, 195.

Richey PA, Brown SP (1998a) Pathological versus physiological left ventricular hypertrophy: a review. *J Sports Sci* **16**, 129-41.

Richey PA, Brown SP (1998b) Pathological versus physiological left ventricular hypertrophy: A review. *Journal of Sports Sciences* **16**, 129-141.

Ritchie RH, Delbridge LM (2006) Cardiac hypertrophy, substrate utilization and metabolic remodelling: cause or effect? *Clin Exp Pharmacol Physiol* **33**, 159-66.

Rossouw JE, Anderson GL*, et al.* (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Jama* **288**, 321-33.

Russell RR, 3rd, Li J, *et al.* (2004) AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J Clin Invest* **114**, 495-503.

Saglam K, Polat Z, Yilmaz MI, Gulec M, Akinci SB (2002) Effects of postmenopausal hormone replacement therapy on insulin resistance. *Endocrine* **18**, 211-4.

Sak K, Everaus H (2004) Nongenomic effects of 17beta-estradiol--diversity of membrane binding sites. *J Steroid Biochem Mol Biol* **88**, 323-35.

Santalucia T, Boheler KR, *et al.* (1999) Factors involved in GLUT-1 glucose transporter gene transcription in cardiac muscle. *J Biol Chem* **274**, 17626-34.

Santalucia T, Camps M, Castello A, Munoz P, Nuel A, Testar X, Palacin M, Zorzano A (1992) Developmental regulation of GLUT-1 (erythroid/Hep G2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle, and brown adipose tissue. *Endocrinology* **130**, 837-46.

Sharma S, Guthrie PH, Chan SS, Haq S, Taegtmeyer H (2007) Glucose phosphorylation is required for insulin-dependent mTOR signalling in the heart. *Cardiovasc Res* **76**, 71-80.

Shipp JC, Opie LH, Challoner D (1961) Fatty acid and glucose metabolism in the perfused heart. *Nature* **189**, 2.

Sinha MK, Raineri-Maldonado C, Buchanan C, Pories WJ, Carter-Su C, Pilch PF, Caro JF (1991) Adipose tissue glucose transporters in NIDDM. Decreased levels of muscle/fat isoform. *Diabetes* **40**, 472-7.

Skavdahl M, Steenbergen C, Clark J, Myers P, Demianenko T, Mao L, Rockman HA, Korach KS, Murphy E (2005) Estrogen receptor-ß mediates male-female differences in the development of pressure overload hypertrophy. *American Journal of Physiology- Heart and Circulatory Physiology* **288**, 469-476.

Slot JW, Geuze HJ, Gigengack S, James DE, Lienhard GE (1991) Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat. *Proc Natl Acad Sci U S A* **88**, 7815-9.

Smoak IW, Branch S (2000) Glut-1 expression and its response to hypoglycemia in the embryonic mouse heart. *Anatomy and Embryology* **201**, 327-333.

Stamler J, Vaccaro O, Neaton JD, Wentworth D (1993) Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care* **16**, 434-44.

Stevenson JC, Crook D, Godsland IF, Collins P, Whitehead MI (1994) Hormone replacement therapy and the cardiovascular system. Nonlipid effects. *Drugs* **47**, 35-41.

Sun D, Nguyen N, DeGrado TR, Schwaiger M, Brosius FC, 3rd (1994) Ischemia induces translocation of the insulin-responsive glucose transporter GLUT4 to the plasma membrane of cardiac myocytes. *Circulation* **89**, 793-8.

Swislocki A, Burgie ES, Rodnick KJ (2002) Effects of ovariectomy on indices of insulin resistance, hypertension, and cardiac energy metabolism in middle-aged spontaneously hypertensive rats (SHR). *Horm Metab Res* **34**, 516-22.

Taegtmeyer H (2000a) Genetics of Energetics: Transcriptional Responses in Cardiac Metabolism. *Annals of Biomedical Engineering* **28**, 871-876.

Taegtmeyer H (2000b) Metabolism--the lost child of cardiology. *J Am Coll Cardiol* **36**, 1386-8.

Taegtmeyer H, Overturf ML (1988) Effects of moderate hypertension on cardiac function and metabolism in the rabbit. *Hypertension* **11**, 416-26.

Takano H, Zou Y, Akazawa H, Toko H, Mizukami M, Hasegawa H, Asakawa M, Nagai T, Komuro I (2002) Inhibitory molecules in signal transduction pathways of cardiac hypertrophy. *Hypertens Res* **25**, 491-8.

Thompson LP, Pinkas G, Weiner CP (2000) Chronic 17ß-Estradiol Replacement Increases Nitric Oxide-Mediated Vasodilation of Guinea Pig Coronary Microcirculation. In. pp. 445-451. (Am Heart Assoc)

Tian R, Musi N, D'Agostino J, Hirshman MF, Goodyear LJ (2001) Increased Adenosine Monophosphate-Activated Protein Kinase Activity in Rat Hearts With Pressure-Overload Hypertrophy. In. pp. 1664-1669. (Am Heart Assoc)

Till M, Kolter T, Eckel J (1997) Molecular mechanisms of contraction-induced translocation of GLUT4 in isolated cardiomyocytes. *Am J Cardiol* **80**, 85A-89A.

van der Vusse GJ, Glatz JF, Stam HC, Reneman RS (1992) Fatty acid homeostasis in the normoxic and ischemic heart. *Physiol Rev* **72**, 881-940.

van Eickels M, Grohe C, Cleutjens JPM, Janssen BJ, Wellens HJJ, Doevendans PA (2001) 17ß-Estradiol Attenuates the Development of Pressure-Overload Hypertrophy. In. pp. 1419-1423. (Am Heart Assoc)

Wang W, Lopaschuk GD (2007) Metabolic therapy for the treatment of ischemic heart disease: reality and expectations. *Expert Rev Cardiovasc Ther* **5**, 1123-34.

Warner M, Gustafsson JA (2006) Nongenomic effects of estrogen: why all the uncertainty? *Steroids* **71**, 91-5.

Wisneski JA, Gertz EW, Neese RA, Mayr M (1987) Myocardial metabolism of free fatty acids. Studies with 14C-labeled substrates in humans. *Journal of Clinical Investigation* **79**, 359.

Yonekura Y, Brill AB*, et al.* (1985) Regional myocardial substrate uptake in hypertensive rats: a quantitative autoradiographic measurement. *Science* **227**, 1494-6.

Young LH, Renfu Y, Russell R, Hu X, Caplan M, Ren J, Shulman GI, Sinusas AJ (1997) Low-flow ischemia leads to translocation of canine heart GLUT-4 and GLUT-1 glucose transporters to the sarcolemma in vivo. *Circulation* **95**, 415-22.

Young LH, Russell RR, 3rd, Yin R, Caplan MJ, Ren J, Bergeron R, Shulman GI, Sinusas AJ (1999) Regulation of myocardial glucose uptake and transport during ischemia and energetic stress. *Am J Cardiol* **83**, 25H-30H.

Young ME, Laws FA, Goodwin GW, Taegtmeyer H (2001) Reactivation of peroxisome proliferator-activated receptor alpha is associated with contractile dysfunction in hypertrophied rat heart. *J Biol Chem* **276**, 44390-5.

Zaninetti D, Greco-Perotto R, Assimacopoulos-Jeannet F, Jeanrenaud B (1988) Effects of insulin on glucose transport and glucose transporters in rat heart. *Biochem J* **250**, 277-83.

Zhang J, Merkle H, Hendrich K, Garwood M, From AH, Ugurbil K, Bache RJ (1993) Bioenergetic abnormalities associated with severe left ventricular hypertrophy. *J Clin Invest* **92**, 993-1003.

Zorzano A, Sevilla L, *et al.* (1997) Regulation of glucose transport, and glucose transporters expression and trafficking in the heart: studies in cardiac myocytes. *Am J Cardiol* **80**, 65A-76A.

10. Abbreviations

ADP	Adenine Diphosphate
ANT	Adenine Nuclear Transition pore complex
ATP	Adenine Triphosphate
cDNA	Complementary DNA
CVD	Cardio-Vascular Disease
CHF	Congestive Heart Failure
DCM	Dilated CardioMyopathy
DNA	DeoxyriboNucleic Acid
E2	17β-estradiol
ER	Estrogen Receptor
ER-α	Estrogen Receptor alpha
ER-β	Estrogen Receptor beta
ERT	Estrogen Replacement Therapy
FDG	F(18)-Deoxyglucose
GLUT	Glucose Transporter
GAPDH	Glycerine aldehyde phosphate dehydrogenase
HK	Hexokinase
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
LVH	Left Ventricle Hypertrophy
mRNA	messenger RiboNucleic Acid
OGTT	Oral Glucose Tolerance Test
PAGE	Polyacrylamide gelelektrophoresis
PET	Positron Emission Tomography
PI3K	PhosphoInositide 3‘-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator Activated Receptor
RNA	Ribonucleic acid
SDS	Sodiumdodecylsulfate

12. ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation in allen Teilen selbst angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich habe die Dissertation weder in gleicher noch in ähnlicher Form in anderen Prüfungsverfahren vorgelegt.

Ich erkläre weiterhin, dass ich bislang noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg,
January 2009