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

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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.

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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
Summary

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 17\(\beta\)-estradiol treatment. Increased myocardial hexokinase appears as a potential mechanism to explain increased myocardial glucose uptake by 17\(\beta\)-estradiol. Increased cardiac glucose uptake in response to 17\(\beta\)-estradiol in ovariectomized SHR may provide a novel mechanism to explain the reduction of cardiac hypertrophy in E2 treated SHR. Therefore, 17\(\beta\)-estradiol improves cardiac glucose utilization in ovariectomized SHR which may give rise to possible mechanism for its protective effects against cardiac hypertrophy.
I(a). Zusammfassung

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

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).

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.](image-url)
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.
Introduction

(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 known.
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 \textit{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:**

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

**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 % xylene cyanol
TAE (50x) 242 g Tris base
57.1 ml acetic acid
100ml 0.5M EDTA
Add H$_2$O to 1 liter, adjust pH to 8.5.

For RNA electrophoresis:

MOPS running buffer (10x) 41.86 g MOPS
6.8 g NaOAc.3H$_2$O
3.8 g Na$_4$EDTA
H$_2$O to 1L

RNA electrophoresis 1 g Agarose
88 μml H$_2$O 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 H$_2$O
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$_2$HPO$_4$ 2H$_2$O 1 mM
KH$_2$PO$_4$ 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
Material & Methods

SDS 0.1 %

Transfer Buffer (Western Blotting)

200 ml of 5x Electrophoresis + 200ml of Methanol (absolute) + 800 ml distilled H₂O

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:

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<td>dH₂O</td>
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</tbody>
</table>
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+E2’, 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-(18F)-fluoro-deoxyglucose (18FDG) and Positron Emission Tomography scanner (Oxford Positron Systems, Oxford, UK). 18FDG 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 isoflurane / O₂ 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, ¹⁸FDG (~15MBq) was injected in a bolus. The tissue paper was placed under the rat to collect any urine. Then rats were anaesthetized with isoflurane/O₂ and placed in HIDAC for scanner. After 60 minutes of (¹⁸F) 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 (¹⁸F) 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
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10 minutes. Then the samples were run on 8-10% SDS-PAGE gel using 1X Tris-Glycine running buffer. 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 minutes 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 8 minutes. After centrifugation at 15000 rpm 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 H₂O 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 MgCl₂, 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 performed 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

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<th>Ovx+E2</th>
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<td><img src="image2" alt="Ovx Image" /></td>
<td><img src="image3" alt="Ovx+E2 Image" /></td>
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**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 \[^{18}\text{F}\] 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. 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/100 ml tissue of myocardium in SHR. Myocardial $[^{18}\text{F}]$ 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
Results

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), 17β-estradiol treatment did not alter total content of either GLUT4 or GLUT1 in SHR rat hearts.
Results

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)).
Results

15 (a)

Cytosol

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<td></td>
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<tr>
<td>Glut1</td>
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<td></td>
<td></td>
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<tr>
<td>GAPDH</td>
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15 (b)

Glut 4

Glut4 Levels (Arbitrary Units)

- Sham
- Ovx+placebo
- Ovx+ E2
Results

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. 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).

![Western Blot Images]

**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*. 
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 expressed genes are listed in table 2.
### Results

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

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank™ accession number</th>
<th>Fold Difference</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>Eif4ebp1</strong></td>
<td>NM_053857</td>
<td>+2.29</td>
<td>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</td>
</tr>
<tr>
<td><strong>Frap1</strong></td>
<td>NM_019906</td>
<td>+1.97</td>
<td>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.</td>
</tr>
<tr>
<td><strong>Map2k1</strong></td>
<td>NM_031643</td>
<td>+1.71</td>
<td>A kinase that activates Mapk3 (Erk1) and Mapk1 (Erk2) kinases</td>
</tr>
<tr>
<td><strong>Raf1</strong></td>
<td>NM_012639</td>
<td>+1.68</td>
<td>Acts as a mitogenic protein kinase; mutant forms may play a role in transformation</td>
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<tr>
<td><strong>Fbp1</strong></td>
<td>NM_012558</td>
<td>+1.64</td>
<td>Catalyzes the hydrolysis of fructose 1,6-biphosphate to fructose 6-phosphate and inorganic phosphate in gluconeogenesis</td>
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<tr>
<td><strong>Gpd1</strong></td>
<td>NM_022215</td>
<td>+1.68</td>
<td>Target gene of PPARγ, involved in glycolysis</td>
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<td><strong>Erec1_predicted</strong></td>
<td>XM_214833</td>
<td>+1.78</td>
<td>Target gene in MAPK pathway</td>
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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).

![Figure 9](image_url)

**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, Kano 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 phosphorylation of beta subunit of insulin receptor (Lee and Pilch 1994). This interaction activates tyrosine phosphorylation of many other insulin receptors substrates and interact with phosphatidylinositol 3-kinase (PI3K), in turn activates Akt, a downstream serine/threonine kinase that stimulates uptake of glucose through glucose transporter GLUT4 translocation into 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 cause the defect in glucose metabolism and will cause severe metabolic changes in cardiac muscles. In the 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)
Discussion

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 17\(\beta\)-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


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
References


# Abbreviations

10. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenine Diphosphate</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine Nuclear Transition pore complex</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine Triphosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CVD</td>
<td>Cardio-Vascular Disease</td>
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<td>DCM</td>
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<td>DNA</td>
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<td>E2</td>
<td>$17\beta$-estradiol</td>
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<td>ER</td>
<td>Estrogen Receptor</td>
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<td>FDG</td>
<td>F(18)-Deoxyglucose</td>
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<td>GLUT</td>
<td>Glucose Transporter</td>
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<td>Hexokinese</td>
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<td>Oral Glucose Tolerance Test</td>
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<td>Peroxisome Proliferator Activated Receptor</td>
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<tr>
<td>RNA</td>
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<td>SDS</td>
<td>Sodiumdodecylsulfate</td>
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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