Accepted Manuscript

17β-Estradiol protects against the effects of a high fat diet on cardiac glucose, lipid and nitric oxide metabolism in rats

Sonja Zafirovic, Milan Obradovic, Emina Sudar-Milovanovic, Aleksandra Jovanovic, Julijana Stanimirovic, Alan J. Stewart, Samantha J. Pitt, Esma R. Isenovic

PII: S0303-7207(17)30077-1

DOI: 10.1016/j.mce.2017.02.001

Reference: MCE 9828

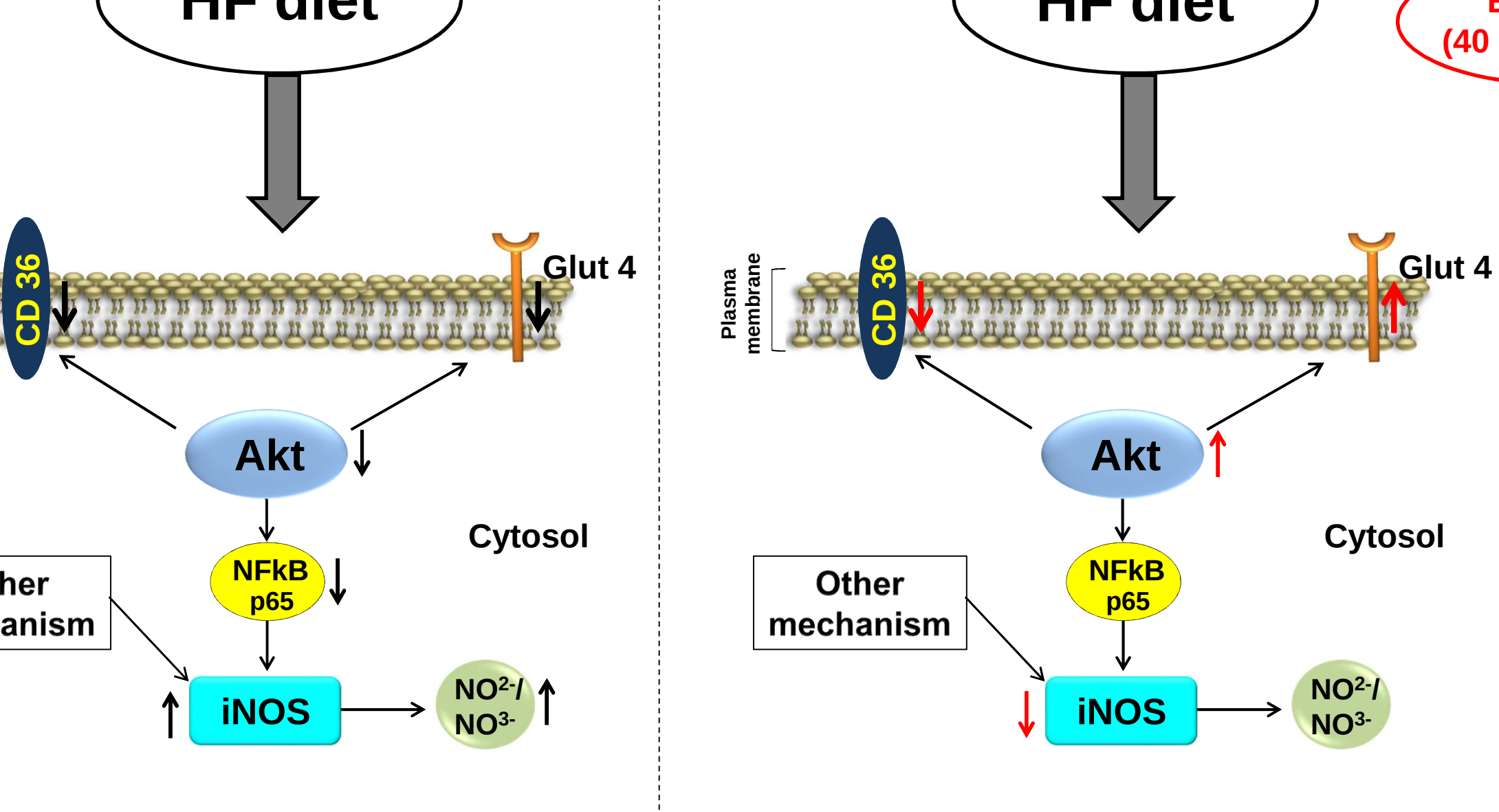
To appear in: *Molecular and Cellular Endocrinology*

Received Date: 1 November 2016

Revised Date: 17 January 2017

Accepted Date: 1 February 2017

Please cite this article as: Zafirovic, S., Obradovic, M., Sudar-Milovanovic, E., Jovanovic, A., Stanimirovic, J., Stewart, A.J., Pitt, S.J., Isenovic, E.R., 17β-Estradiol protects against the effects of a high fat diet on cardiac glucose, lipid and nitric oxide metabolism in rats, *Molecular and Cellular Endocrinology* (2017), doi: 10.1016/j.mce.2017.02.001.


HF diet
CD 36
Glut 4
Akt
Cytosol
NFkB
p65
her
anism
iNOS
NO2-/
NO3-
HF diet
Plasma
membrane
CD 36
Glut 4
Akt
Cytosol
Other
mechanism
NFkB
p65
iNOS
NO2-/
NO3-

17ß-Estradiol protects against the effects of a high fat diet on cardiac glucose, lipid and nitric oxide metabolism in rats

Sonja Zafirovic[a*], Milan Obradovic[a*], Emina Sudar-Milovanovic[a], Aleksandra Jovanovic[a], Julijana Stanimirovic[a], Alan J. Stewart[b], Samantha J. Pitt[b], Esma R. Isenovic[a]

[a]Institute of Nuclear Sciences Vinca, University of Belgrade, Laboratory of Radiobiology and Molecular Genetics, Mike Petrovica Alasa 12-14, 11000 Belgrade, Serbia.
[b]School of Medicine, University of St Andrews, North Haugh, St Andrews, KY16 9TF, United Kingdom.

Sonja Zafirovic, E-mail: sonjazafirovic@hotmail.com
Milan Obradovic, E-mail: obradovicmilan@hotmail.com
Emina Sudar-Milovanavic, E-mail: emma_crash@yahoo.com
Aleksandra Jovanovic, E-mail: jovsale@gmail.com
Julijana Stanimirovic, E-mail: julijana1008@gmail.com
Alan J. Stewart, E-mail: ajs21@st-andrews.ac.uk
Samantha J. Pitt, E-mail: sjp24@st-andrews.ac.uk
Esma R. Isenovic, E-mail: isenovic@yahoo.com

* Equally contributed as a first author.

Corresponding author:
Prof. Dr Esma R. Isenovic
Institute Vinca, University of Belgrade
Laboratory of Radiobiology and Molecular Genetics
P.O.Box 522, 11000 Belgrade, Serbia
Tel/ Fax: +38111-3408147
E-mail: isenovic@yahoo.com

Abstract

The aim of this study was to investigate the *in vivo* effects of estradiol (E_2) on myocardial metabolism and inducible nitric oxide synthase (iNOS) expression/activity in obese rats. Male Wistar rats were fed with a normal or a high fat (HF) diet (42% fat) for 10 weeks. Half of the HF fed rats were treated with a single dose of E_2 while the other half were placebo-treated. 24h after treatment animals were sacrificed. E_2 reduced cardiac free fatty acid (FFA) ($p<0.05$), L-arginine ($p<0.01$), iNOS mRNA ($p<0.01$), and protein ($p<0.05$) levels and translocation of the FFA transporter (CD36) ($p<0.01$) to the plasma membrane (PM) in HF fed rats. In contrast, Akt phosphorylation at Thr^{308} ($p<0.05$) and translocation of the glucose transporter GLUT4 ($p<0.05$) to the PM increased after E_2 tretment in HF rats. Our results indicate that E_2 acts via PI3K/Akt signaling pathway to partially protect myocardial metabolism by attenuating the detrimental effects of increased iNOS expression/activity in HF fed rats.

Key words: estradiol, obesity, myocardial metabolism, cardiovascular diseases, inducible nitric oxide synthase

List of abbreviations:

Akt, protein kinase B; **AS160**, Akt substrate of 160 kDa; **CD36**, fatty acid transporters; **E_2**, estradiol; **FFA**, free fatty acids; **GLUT**, glucose transporters; **HF**, high fat; **IR**, insulin resistance; **IRS1**, insulin receptor substrate-1; **L-arg**, L-arginine; **LDM**, low density microsomes; **NO**, nitric oxide; **PM**, plasma membrane; **NOS**, NO synthase; **iNOS**, inducible NOS; **NFkB**, nuclear factor kappa-B; **OVX**, ovariectomized; **PI3K**, phosphatidylinositol 3-kinase.

1. INTRODUCTION

One of the leading causes of morbidity and mortality in the developed world is obesity-associated cardiovascular disease (Lavie et al. 2014). Substantial evidence implies a direct causation between obesity and the increased risk of endothelial dysfunction, hypertension, coronary artery disease, heart failure and stroke (Lavie et al. 2009, Lavie et al. 2014). A high fat (HF) diet induces body fat accumulation, insulin resistance (IR) and metabolic disorders in animals (Buettner et al. 2007, Obradovic et al. 2015b). In our study, we used male rats fed a HF diet as a model of obesity-associated IR. In this model the HF diet induces whole body IR/hyperinsulinemia, endothelial dysfunction and hypertension (Barnard et al. 1998, Panchal et al. 2011, Sudar et al. 2015). Furthermore, we have previously demonstrated using the same male model rats, that a HF diet applied for 10 weeks induces obesity accompanied with abnormalities such as cardiac hypertrophy, increased serum insulin concentration, whole body IR, hyperlipidaemia, hyperleptinemia and inflammation (Obradovic et al. 2015a, Obradovic et al. 2015b).

Glucose and free fatty acids (FFAs) are the main energy sources in the heart. In cardiac muscle, the energy required for contractile function is mainly attained through FFA oxidation (Rider et al. 2013). In cardiomyocytes transport of glucose and FFAs is mediated via glucose transporters (GLUT) and fatty acid transporters (CD36), which migrate between intracellular pools and plasma membranes (PM). Estradiol (E_2) at physiological concentrations exerts many beneficial effects on the heart, including improved vascular function and reduced atherosclerosis (Patten, Karas 2006, Romic et al. 2013). These E_2-mediated effects include alterations in the activity and

expression of many enzymes, including the regulation of nitric oxide synthase (NOS) (Babiker et al. 2002). E_2 has a direct effect on the heart by influencing lipid metabolism and insulin sensitivity (Koricanac et al. 2012). Furthermore, in the same rat model as used in this study we have previously reported that *in vivo* the administration of E_2 as a bolus injection reduced cardiac hypertrophy, decrease serum total cholesterol and high sensitivity C-reactive protein, and realigning of insulin signalling cascade (Obradovic et al. 2015a, Obradovic et al. 2015b).

Nitric oxide (NO) plays an important role in cardiovascular function by regulating relaxation of blood vessels and also influencing cardiomyocyte contractility (Moncada, Higgs 1995, Kypreos et al. 2014). Endogenously, NO is released as a product during conversion of the amino acid L-arginine (L-arg) to L-citrulline in a reaction mediated by NOS (Stanimirovic et al. 2015, Sudar-Milovanovic et al. 2015). The primary source of NO generation in the vascular system is endothelial NOS (Rhodes et al. 1995, Kypreos et al. 2014). Overproduction of NO as a result of inducible NOS (iNOS) activation leads to reduced myocardial contractility and also has detrimental effects to the heart (Ikeda, Shimada 1997). E_2 exerts many of its effects in cardiomyocytes through activation of the insulin receptor substrate 1 (IRS1), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) signalling cascades (Kypreos et al. 2014, Obradovic et al. 2014). E_2 exerts beneficial effects on the cardiovascular system by reducing nuclear factor kappa-B (NFkB), IkB and iNOS expression during ischemia (Karpuzoglu, Ahmed 2006). Under normal conditions, E_2 also influences energy usage by regulating GLUT and CD36 in heart (Gorres et al. 2011, Tepavcevic et al. 2011).

In the current study, we show that the PI3K/Akt signalling pathway is altered in obesity associated with IR induced by HF feeding in male rats. This likely causes a disruption in the translocation of the cardiac transporters GLUT1 and GLUT4 and CD36 to the PM resulting in impaired glucose and FFA metabolism, and altered expression/activity of iNOS. We also show that the *in vivo* administration of E_2 to obese rats protects against the detrimental effects of obesity, preventing the disruption of myocardial glucose and FFA metabolism and reducing the impact on iNOS expression/activity. The physiological effects of E_2 are very complex and to date our knowledge of these in the context of obesity-related cardiac functioning has been limited. The results from this study provide important insights into the *in vivo* cardioprotective effects of E_2 in obesity, and represent a solid basis for future basic and preclinical studies.

2. MATERIAL AND METHODS

2.1. Chemicals

Ether was purchased from Lek (Ljubljana, Slovenia). Luminol, p-coumaric acid and 17ß-Estradiol (E_2; E8875) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Protease inhibitor (Complete, Ultra Mini, EDTA-free) and phosphatase inhibitor cocktails (PhosStop) were obtained from Roche (Mannheim, Germany). The Nitrate/Nitrite Colorimetric Assay Kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti phospho-Akt (Thr^{308}), and anti total Akt antibodies were obtained from Abcam (Cambridge, UK), while the ERα, ERβ, anti-iNOS, anti-NFκB-p65, anti-GLUT1, anti-GLUT4, anti-FAT/CD36 polyclonal antibodies, and the mouse monoclonal anti-actin antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary anti-rabbit and anti-mouse IgG

horseradish peroxidase-linked antibodies as well as BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium chloride) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). RevertAid H minus First Strand cDNA Synthesis Kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Primers for rat iNOS and β actin were obtained from Metabion (Martinsried, Germany). Brilliant III SYBR QPCR MasterMix with lowROX was purchased from Agilent Technologies (Santa Clara, CA, USA).

2.2. Animals and experimental treatment

This study was performed on eight weeks old, adult male Wistar rats (150g-200g) bred at the Institute of Nuclear Sciences (Vinca, Belgrade). We chose this experimental rat model based on our previous results, and literature data (Barnard et al. 1998, Obradovic et al. 2015b). All metabolic parameters for these rats in response to the treatments administered in this study are detailed in our previous study (Obradovic et al. 2015a). The rats were kept at a 12:12 h, light/dark cycle at 22±2°C and divided into two groups: one group (labelled as CONT) was fed for 10 weeks with a balanced diet for laboratory rats (prepared by Veterinarski zavod Subotica, Subotica, Serbia); and a second group (labeled as HF) was fed with a HF diet, a balanced diet for laboratory rats enriched with 42% fat. The diet was free of phytoestrogens. Food and water were available to rats *ad libitum*. At the end of the 10^{th} week, half of the rats from the HF fed group were treated intraperitoneally with 40 μg/kg of E_2 dissolved in 1% ethanol in saline 24 h before decapitation and labeled as HF+E_2, while the other half of rats from the HF fed group, were at the same time injected with the same volume of 1% ethanol in saline. Animals were sacrificed under anesthesia. The hearts from each animal were weighed after excision, snap frozen in liquid

nitrogen and stored at -80°C until further experiments. Experimental protocols were approved by the official Vinca Institute's Ethical Committee for Experimental Animals.

2.3. Heart lysate preparation

Heart tissue from each animal was measured (200 mg) and homogenized on ice using an Ultra-Turrax homogenizer in buffer with protease and phosphatase inhibitor cocktails (10 mM Tris, 150 mM NaCl, 1 mM EDTA; 10% glycerol, 1% Triton X-100, pH 7.4). Homogenates were incubated (1 h, constant rotation at 4°C) and then ultracentrifuged for 20 min, at 4°C at 100,000 × g. The Lowry assay (Lowry et al. 1951) was used for measurement of total protein concentration in obtained supernatants. Isolated lysates were stored at -80°C and used for further experiments.

2.4. Isolation of plasma membrane (PM) and low-density microsomal (LDM) membrane proteins from heart tissue

Plasma membrane **(**PM) proteins were isolated from 200 mg of heart tissue from each animal, according to Luiken *et al.* (Luiken et al. 2002). Briefly, heart pieces were incubated for 30 min at 4°C, in a high-salt solution (20 mM HEPES, 2 M NaCl, and 5 mM sodium azide, pH 7.4) and centrifuged for 5 min at 1,000 × g. The pellets were homogenized on ice in TES-buffer (20 mM Tris, 250 mM sucrose and 1 mM EDTA, pH 7.4) with protease and phosphatase inhibitor cocktail, using Ultra-turrax homogenizer. Homogenates were then centrifuged (5 min at 1,000 × g at 4°C) and the pellets were rehomogenized in a TES-buffer and then recombined with the supernatant followed by centrifugation (10 min at 100×g at 4°C). Thereafter the supernatants were centrifuged (10 min at 5,000 × g at 4°C) and the final pellets (considered as "PM fraction") were resuspended in TES buffer and stored at -80°C for further experiments. Protein

concentrations were determined by the Lowry assay (Lowry et al. 1951). Low density microsomes (LDM) were isolated from the remaining supernatants by centrifugation (20 min at 20,000 × g at 4°C). The obtained supernatants were additionally centrifuged for 30 min (at 48,000 × g at 4°C) and the resulting supernatants were once more centrifuged for 80 min at 200,000 × g at 4°C. The final pellets were then resuspended in TES buffer and considered as "LDM".

2.5. Measurement of lysate FFA concentration

The lysate FFA concentrations were determined using a modified version of Duncombe's colorimetric method (Duncombe 1964). Lysate samples (45 µl) were mixed with 225 µl of reagent (aqueous solution of $Cu(NO_3)_2 \cdot 3H_2O$ with triethanolamine (TEA) pH 7.8). Into the resulting mixture 1,125 µl of chloroform was added and incubated with intensive shaking for 20 min and centrifuged for 10 min at 3,000 × g. After removing the top blue-green layer, in the lower chloroform phase with extracted FFA, 45 µl 0.2% diethyldithiocarbamate (DDC) has been added. After strong shaking, samples were incubated for additional 20 min at room temperature and the absorbance was measured at 436 nm on a Lambda 35UV/VIS spectrophotometer (Perkin Elmer). The FFA concentration was calculated from a palmitate standard curve and expressed in mM. Assays were performed with at least four biological replicates corresponding to each treatment.

2.6. SDS-polyacrylamide electrophoresis and Western blotting

Total heart protein lysates, PM and LDM (50 µg/lane) were separated on SDS-polyacrylamide gel (Laemmli 1970) and transferred to nitrocellulose or polyvinylidene difluoride (PVDF)

membranes. After protein transfer membranes were blocked with 5% bovine serum albumin and probed with primary antibodies directed against ERα, ERβ, iNOS, NFkBp65, non-phosphorylated Akt, phospho-Akt (Thr^{308}) GLUT1, GLUT4 and CD36. After washing with TBST, membranes were incubated with the appropriate secondary anti-rabbit or anti-mouse antibodies (dilution 1:2,000) and used for detection with electrochemiluminescence method. As a control for equal protein loading, all blots were probed with mouse anti-actin monoclonal antibody and appropriate secondary antibody. Image J 1.45s software (National Institutes of Health, USA) was used for quantification of signals on membranes. In all cases blots were performed on samples prepared from at least three (and in some cases five) rats per treatment group.

2.7. Measurement of nitrite/nitate levels in heart lysates

Nitric oxide (NO) levels in heart lysates were measured indirectly, by determining the concentration of nitrate and nitrite as final stable products of NO, using a commercially available Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical 780001 IN), according to the manufacturer's protocols. In brief, nitrates were reduced to nitrites by nitrate reductase, and nitrites were determined by the method of Griess reagent. Measurements of absorbance at 540 nm were performed in an automated microplate reader (Perkin Elmer, Wallac 1420 Victor). $NaNO_3$ standard curve was used for calculation of the nitrate concentration and results are expressed as μM. Assays were performed with at least four biological replicates corresponding to each treatment.

2.8. Measurement of L-Arg in heart tissue

To measure L-Arg we used spectrophotometric method described by Kowalczuk et al. (Kowalczuk et al. 2007). L-Arg was deaminated and converted by ninhydrin into aldehyde. As a result of interaction of ninhydrin with ammonia and hydrindantin, a colored compound was created, with maximum absorbance at 404 nm. Briefly, heart tissue homogenates with equal protein content were diluted with 25mM phosphate buffer (pH 9.0), with 2% ninhydrin reagent and heated on 80°C for 15 minutes. After cooling, the L-Arg–ninhydrin derivative was determined in a Wallac 1420 Victor microplate reader at 404 nm and L-Arg concentration was calculated from a standard curve and expressed in µg/ml. Assays were performed with at least six biological replicates corresponding to each treatment.

2.9. Quantitative real-time PCR (qPCR)

Total RNA was extracted from heart tissue using Trizol reagent (Invitrogen Life Technologies, Paisley, GB) according to the procedure recommended by the manufacturer. RNA concentration and purity were determined using BioSpec-nano-Spectrophotometer (Shimadzu, USA). Degradation of RNA samples was controlled by using 1.2% agarose electrophoresis. Reverse transcription of 1 µg of heart RNA, was carried out using a commercially available RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The qPCR assay was performed using the 7500 Real-Time PCR System (Applied Biosystems) in 96-well reaction plates (MicroAmp Optical, ABI Foster City, CA) in 20 µl volume/well, containing 10 µl of reaction mix (Brilliant III) and 10 µl of appropriate sample diluted in demineralized water and pairs of primers. The iNOS, and Actb primers were designed using Primer Express1 software v2.0 (Applied Biosystems) and purchased from Metabion (Martinsried, Germany). To selectively amplify cDNA synthesized from mRNA but

not genomic DNA, the primers are designed such that one primer from a pair spans an exon junction. Primers for iNOS (GenBank accession number: NM_012611) were 5′-AGAAGTCCAGCCGCACCAC- 3′ (forward primer) 5′-TGGTTGCCTGGGAAAATCC-3′ (reverse primer) with PCR product length of 103 bp and primers for rat β-Actin (GenBank accession number: NM_031144) were 5′-CCCTGGCTCCTAGCAC CAT-3′ (forward primer), 5′-GAGCCACCAATCCA CACAGA-3′ (reverse primer) with PCR product length of 76 bp. The thermal cycle conditions for iNOS were 95°C for 4 min followed by 40 cycles that were run for 15 s at 95° C and for 1 min at 61°C. The level of expression of analyzed iNOS gene was standardized against that of the Actb gene detected in the identical sample. All assays were performed in triplicate with at least three biological replicates per group. Cycle threshold values (Ct) were analyzed and relative quantification of mRNA expression was performed by the $2^{-\Delta\Delta Ct}$ method (Livak, Schmittgen 2001).

2.10. Statistical Analysis

Values gained from experiments are presented as mean ± SEM. Student's *t*-test was used to evaluate statistical significance. The SPSS program for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. A value of $p<0.05$ was considered as statistically significant.

3. RESULTS

3.1. Effect of estradiol on FFA concentratons in heart lysates from HF fed rats

We have previously shown that the plasma (Obradovic et al. 2015a) and liver lysate FFA concentrations (Stanimirovic et al. 2016) are significantly higher in HF fed IR male rats compared with controls, and that *in vivo* E_2 treatment has no effect on HF fed male rats plasma

FFA concentration (Obradovic et al. 2015a). Here we further examined the effect of a HF diet in combination with E_2 treatment on the FFA concentration in heart lysates (**Figure 1**). The results show that the FFA concentration is significantly reduced by 25% ($p<0.05$) in heart tissue lysates of E_2 treated HF fed rats compared with HF untreated rats (CONT= 0.24±0.03; HF=0.31±0.02; HF+E_2=0.23±0.02 mM/mg).

3.2. Effect of estradiol on expression of cardiac glucose and fatty acid transporters in HF fed rats

Glucose and fatty acid transporters play an important role in myocardial metabolism. We next assessed the expression of the fatty acid transporter, CD36 (**Figure 2A**) and glucose transporter proteins, GLUT1 (**Figure 2C**) and GLUT4 (**Figure 2E**) in heart lysates as well as PM and LDM fractions. The level of CD36 protein was unchanged in both heart tissue lysates and the LDM fraction. *In vivo* treatment with E_2 however significantly decreased ($p<0.01$) the level of CD36 by 29% in PM fractions of HF fed animals compared with HF fed untreated rats (CONT=100%; HF=73±6%; HF+E_2=52±6%). Treatment with E_2 did not induce changes in GLUT1 protein expression in cardiac lysates, PM or LDM fractions of HF fed rats compared with HF fed untreated rats. In contrast, the level of GLUT4 protein was observed to be 27% higher in the PM fraction ($p<0.05$) (CONT=100%; HF=90±4%; HF+E_2=114±2%), while it was unchanged in the tissue lysate and LDM fraction of E_2 treated HF fed rats compared with untreated HF fed rats.

3.3. Effect of estradiol on cardiac NO metabolism in HF fed rats

Since NO has important roles in the regulation of energy metabolism, insulin sensitivity and vasodilatation in obese patients, we further explored the effects of E_2 treatment on NO

metabolism in the heart of HF fed rats. We first measured the concentration of nitrite/nitate and L-Arg in heart tissue lysates. In vivo treatment of HF fed animals with E2 decreased nitrite/nitate concentration ($p=0.10$) but with no statistical significance (Figure 3A). E2 treatment caused a significant decrease ($p<0.01$; **Figure 3B**) in the cardiac L-Arg concentration in HF fed rats compared with HF untreated rats (CONT=65.43±2.77 μg/ml; HF=58.93±0.88 μg/ml; HF+E_2=54.12±0.64 μg/ml). Obesity and accumulation of lipids could increase expression of iNOS, so we further examined whether a HF diet and *in vivo* E_2 treatment of HF fed rats had an effect on iNOS gene expression and protein levels. The level of cardiac iNOS mRNA after E_2 treatment (**Figure 4A**), was decreased by 31% ($p<0.01$) compared with HF fed untreated animals (CONT=0.94±0.05 A.U.; HF=0.99±0.02 A.U.; HF+E_2=0.68±0.06 A.U.). Similarly, when HF fed rats were treated with E_2, cardiac iNOS protein (**Figures 4D**) was significantly decreased by 41% ($p<0.05$) compared with HF fed rats (CONT=100%; HF=136±14%; HF+E_2=80±6%). Since NFkB is a transcription factor for iNOS gene, we further examined the effects of a HF diet and *in vivo* E_2 treatment on the level of the cardiac NFkB-p65 subunit. The results show that NFkB-p65 is increased ($p=0.08$) but was not found to be statistically significant (**Figure 4E**) in E_2 treated HF fed rats compared with HF fed untreated rats.

3.4. Effect of estradiol on cardiac Akt-phosphorylation in HF fed rats

We have previously reported using the same rats (Obradovic et al. 2015a) that E_2 given *in vivo* stimulates phosphorylation of Akt at Ser^{473} in HF fed rats (by 28%, $p<0.05$). Since full activation of Akt requires both Ser^{473} and Thr^{308} to be phosphorylated, we next examined the effects of E_2 on Akt-Thr^{308} phosphorylation. The results show that *in vivo* E_2 treatment induced a significant increase by 40% ($p<0.05$) in the density ratio between the phospho and total forms of Akt at

Thr^{308} compared with untreated HF fed rats (**Figure 5C**) (CONT=100%; HF=91±1%; HF+E_2=127±15%).

3.5. Effect of estradiol on cardiac ERα and ERβ in HF fed rats

Since effects of E_2 are mediated by direct binding to the ER, we have examined the protein levels of the cardiac ERα and ERβ in HF fed rats after E_2 treatment. The obtained results indicate that injected E_2 *in vivo* does not induce any significant changes in the protein levels of ERα and ERβ (**Figure 6**).

4. DISCUSSION

In this study we examined the protective effects of estradiol on lipid and NO metabolism in the heart of rats fed a HF diet. In this study we observed that subjecting rats to a 42% fat (HF) diet for 10 weeks increased cardiac FFA concentration, which can be reduced by i*n vivo* treatment with E_2. It has previously been shown that obesity, accompained with IR shifts myocardial metabolism towards usage of FFAs affecting contractile function (Coort et al. 2007). On a molecular level, increased concentration of FFA also diminishes cardiac glucose metabolism (Coort et al. 2004, Rider et al. 2013). E_2 exerts an anti-atherogenic effect influencing lipolysis and lipogenesis thus reducing the concentration of lipids in both the blood (Shi et al. 2013) and tissues (Hewitt et al. 2004, Jelenik, Roden 2013). Moreover, E_2 might decrease synthesis and increase FFA oxidation during hyperinsulinemia (Musatov et al. 2007, Gorres et al. 2011).

Uptake of FFA into cardiac cells occurs through several mechanisms, including a protein-mediated mechanism (Coort et al. 2004, Rider et al. 2013). CD36 is a major contributor to

protein-mediated FFA movement into cardiomyocytes (Coort et al. 2004, Rider et al. 2013). Lukien et al. reported that cardiomyocytes of obese rats have more CD36 located to the PM compared with lean controls (Luiken et al. 2001). E_2 positively regulates the expression and the translocation of FFA and glucose transporter proteins in rats (Coort et al. 2004, Tepavcevic et al. 2011, Koricanac et al. 2012). We and others have previously shown that a single injection of E_2 to ovariectomized female rats (OVX) increases the expression of CD36 at the PM of cardiac cells in both rats fed normal and fructose-fed diets (Tepavcevic et al. 2011) (Koricanac et al. 2012). We now show that administration of E_2 to rats fed a HF diet significantly reduced the expression of CD36 at the PM. Previous data has shown that leptin treatment reduces the expression of the FFA transporter protein CD36, and also reduces the level of CD36 located at the PM (Steinberg et al. 2002). Our previous data revealed an increase in the level of serum leptin in obese, leptin-resistant male rats (Obradovic et al. 2015b) suggesting that the reduced expression of CD36 may be a result of increased levels of serum leptin. Although current data indicate that E_2 influences central leptin sensitivity by enhancing the expression of the leptin receptor (Clegg et al. 2006), our leptin-resistant rats were treated with a single dose of E_2 which probably was not able to reduce long-term increased level of leptin and thus enables CD36 to be translocated to PM.

In obesity and IR, insulin signalling is disrupted, this may lead to a decreased presence of GLUT1 and/or GLUT4 (Huisamen et al. 2001) in the PM fraction. In our study, the expression of both GLUT1 and GLUT4 transporter proteins was decreased in the heart PM fractions of HF fed animals. This is highly relevant when we consider that GLUT1 and GLUT4 are the most abundant glucose transporters in the heart. Also, other members of the glucose transporter family are detected in the heart, including GLUT3, GLUT8, GLUT10, GLUT11, and GLUT12 (Shao,

D., Tian 2015). GLUT1 is responsible for basal glucose metabolism and is mainly located in PM under basal conditions (Slot et al. 1991, Montessuit, Lerch 2013). GLUT4 is a major cardiac glucose transporter mostly present in the intracellular vesicles. In obese rats with developed IR, the utilization of metabolic substrates is disturbed (Coort et al. 2004) and this is likely the result of altered GLUT translocation and activity mediated by altered insulin signalling. Indeed, previous data have shown that glucose transport is reduced by decreased translocation of GLUT4 to the PM, but not by reduction of total protein expression (Luiken et al. 2002) in obese animals. Results obtained on Zucker obese diabetic rats show significantly lower levels of GLUT4 on the sarcolemmal membrane (Huisamen et al. 2001). Imporantly, our data reveal that E_2 treatment partially restores translocation of GLUT4 (but not GLUT1) to the PM which is likely to have a positive influence on myocardial metabolism. Previous studies have shown that in OVX mice fed with HF diet E_2 treatment improved insulin sensitivity and glucose transport (Riant et al. 2009, Zhu et al. 2013). Furthermore, ERα knockout mice have decreased GLUT4 mRNA level in skeletal muscle linking the E_2 with the regulation of glucose transporters (Barros et al. 2006). The observation that E_2 influences GLUT4 translocation but not GLUT1 translocation in the heart of HF fed rats is interesting. It is likely translocation of these transporters occurs via different mechanisms.

In the heart production of NO, catalysed by NOS, depends on the availability of the substrate L-Arg (Andrew, Mayer 1999). L-Arg deficiency has been strongly implicated in obesity and cardiovascular diseases. Cooke et al. (Cooke et al. 1992) reported that local tissue-arginine deprivation is known to occur at inflammatory sites. Study on rats with increased level of NO derived from iNOS also show reduced level of L-Arg (Zunic et al. 2009). These investigations

are in accordance with our results where a diminished cardiac tissue concentration of L-Arg is present in HF fed rats, while the concentration of nitrite/nitate was increased. This is probably due to increased levels of iNOS as a result of obesity.

The results from our study demonstrate that obesity increases cardiac iNOS gene and protein expression in HF fed rats compared to controls, which coincides with increased levels of FFA and NO production. These results are consistent with our previous findings (Sudar et al. 2015) as well as data gained by others for iNOS expression/activity in states of obesity (de Luca, Olefsky 2008). Noronha et al. (2005) showed that iNOS expression is significantly increased in aortas of obese mice. E_2 also displays an anti-inflammatory role in the hearts of obese rats by decreasing the level of iNOS gene and protein expression, and NO. Activation of iNOS as a result of OVX or inflammation is inhibited by E_2 treatment (Maggi et al. 2003). In our study E_2 treatment diminished iNOS expression which is in accordance with the results of other studies (Maggi et al. 2003, Nweze et al. 2012), likely as a result of decreased level of lipids measured after E_2 treatment. In accordance with the observed decrease in iNOS expression (and the level of L-Arg), a decrease was observed in cardiac nitrate/nitrite concentration following E_2 treatment however, this was not statistically significant. It is possible that activation of another form of NOS, not assessed in the study could, compensate for reduced NO production by iNOS.

Since iNOS expression is regulated primarily by control of gene transcription, a possible explanation for increased iNOS expression in HF fed rats may be activation of the transcription factor NFkB p65 subunit, which has a binding site on the iNOS gene promotor. Phosphorylation of the inhibitor protein NFkB (IkB) is necessary for NFkB to become active, and to translocate to

the nucleus. Phosphorylation is mediated by IkB kinase (IKK) which is under the direct regulation of Akt (Patten, Karas 2006). E_2-induced stabilization of IκBα may be due to inhibition of IKK activity and/or inhibition of IκB degradation. Also, E_2 has ability to enhance synthesis of IκBα and to inhibit NFκB p65 binding to promoters of inflammatory genes such as iNOS (Patten, Karas 2006).

In our study a decrease in pAkt(Thr^{308})/Akt ratio in HF-fed rats is accompanied by a decreased level of NFkB p65, while iNOS expression and activity are increased. The PI3K/Akt pathway plays an important role in the regulation of glucose metabolism in the heart. It controls translocation of GLUT4 from the cytoplasm to the PM and facilitates further uptake together with GLUT1 (Huang, Czech 2007). Akt activation leads to phosphorylation and inhibition of the Akt substrate of 160 kDa (AS160) which enables GLUT4 to translocate to the PM (Miinea et al. 2005). Phosphorylation of Akt at both Ser^{473} and Thr^{308} is necessary for this process (Shao, J. et al. 2000). Beneficial effects of E_2 on PI3K/Akt signalling pathway and thus glucose transporters have been previously reported (Patten et al. 2004, Tepavcevic et al. 2011, Obradovic et al. 2015a, Obradovic et al. 2015b). This is consistent with our observation that following treatement with E_2 to HF fed rats there is an increase in the ratio of pAkt(Thr^{308})/Akt accompanying increased translocation of GLUT4 to the PM. We have previously shown that treatment of HF fed rats with estradiol increases phosphorylation of Akt at Ser^{473} (Obradovic et al. 2015a). In current study no differences in ERα or ERβ expression were observed (either as a result of a HF diet or E_2 treatment) suggesting that the E_2-mediated effects identified in our study were not the result of variations in ER levels.

Beside its effect on glucose metabolism, the PI3K/Akt pathway also has proposed roles in regulating FFA metabolism and the expression and activity of iNOS acting via NFkB which is a downstream target of Akt, where in states of obesity and IR, PI3K/Akt signaling is disrupted (Patten, Karas 2006). We have previsouly shown that E_2 treatment affects insulin signalling in HF fed rats by increasing the expression of both subunits: p85 and p110 of PI3K, and also increases association of p85 with IRS1 (Obradovic et al. 2015b). Despite observing a decreased level of iNOS in obese rats after E_2 treatment, the ratio of pAkt/Akt increased but the level of NFkB was not significantly elevated. This suggests that another alternative signalling pathway is likely involved in overexpression of iNOS. In order to regulate the inflammatory cascade, E_2 interacts with other transcription factors, besides NFkB, such as AP-1 and STAT. Paech et al. (1997) reported that E_2 shows opposite influences on NFkB and AP-1 transcription activity, depending on whether E_2 binds to the ERα or ERβ receptor. This indicates that iNOS expression could potentially be regulated via AP-1, and not NFkB as we hypothesized.

In summary, our results indicate that *in vivo* E_2 treatment of HF fed male rats partially restores the detrimental effects of a HF diet on myocardial metabolism, whilst influencing the Akt/GLUT signalling pathway. Our findings strongly suggest that E_2, acts via iNOS, and also regulates translocation of the CD36 fatty acid transporter resulting in the accumulation of lipids in heart. In clinical trials estrogen has been shown to be a safe and effective therapy in treatment of prostate cancer, bone density loss and aggression in men with dementia or traumatic brain injury (Kulkarni et al. 2013). Increasing our understanding of the molecular mechanisms determining the cardiac action of E_2 in males could be a good starting point for future clinical trials on men with cardiovascular complications.

Declaration of conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgement

This work is supported by the grant No.173033 (to E.R.I.) from the Ministry of Education, Science and Technological Development, Republic of Serbia.

REFERENCES

Andrew, P.J., Mayer, B., 1999. Enzymatic function of nitric oxide synthases. Cardiovasc Res. 43, 521-531.

Babiker, F.A., De Windt, L.J., van Eickels, M., Grohe, C., Meyer, R., Doevendans, P.A., 2002. Estrogenic hormone action in the heart: regulatory network and function. Cardiovasc Res. 53, 709-719.

Barnard, R.J., Roberts, C.K., Varon, S.M., Berger, J.J., 1998. Diet-induced insulin resistance precedes other aspects of the metabolic syndrome. J Appl Physiol (1985). 84, 1311-1315.

Barros, R.P., Machado, U.F., Warner, M., Gustafsson, J.A., 2006. Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. Proc Natl Acad Sci U S A. 103, 1605-1608.

Buettner, R., Scholmerich, J., Bollheimer, L.C., 2007. High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity (Silver Spring). 15, 798-808.

Cooke, J.P., Singer, A.H., Tsao, P., Zera, P., Rowan, R.A., Billingham, M.E., 1992. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. J Clin Invest. 90, 1168-1172.

Coort, S.L., Bonen, A., van der Vusse, G.J., Glatz, J.F., Luiken, J.J., 2007. Cardiac substrate uptake and metabolism in obesity and type-2 diabetes: role of sarcolemmal substrate transporters. Mol Cell Biochem. 299, 5-18.

Coort, S.L., Hasselbaink, D.M., Koonen, D.P., Willems, J., Coumans, W.A., Chabowski, A., van der Vusse, G.J., Bonen, A., Glatz, J.F., Luiken, J.J., 2004. Enhanced sarcolemmal FAT/CD36 content and triacylglycerol storage in cardiac myocytes from obese zucker rats. Diabetes. 53, 1655-1663.

de Luca, C., Olefsky, J.M., 2008. Inflammation and insulin resistance. FEBS Lett. 582, 97-105.

Duncombe, W.G., 1964. The Colorimetric Micro-Determination of Non-Esterified Fatty Acids in Plasma. Clin Chim Acta. 9, 122-125.

Gorres, B.K., Bomhoff, G.L., Morris, J.K., Geiger, P.C., 2011. In vivo stimulation of oestrogen receptor alpha increases insulin-stimulated skeletal muscle glucose uptake. J Physiol. 589, 2041-2054.

Hewitt, K.N., Pratis, K., Jones, M.E., Simpson, E.R., 2004. Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout mouse. Endocrinology. 145, 1842-1848.

Huang, S., Czech, M.P., 2007. The GLUT4 glucose transporter. Cell Metab. 5, 237-252.

Huisamen, B., van Zyl, M., Keyser, A., Lochner, A., 2001. The effects of insulin and beta-adrenergic stimulation on glucose transport, glut 4 and PKB activation in the myocardium of lean and obese non-insulin dependent diabetes mellitus rats. Mol Cell Biochem. 223, 15-25.

Ikeda, U., Shimada, K., 1997. Nitric oxide and cardiac failure. Clin Cardiol. 20, 837-841.

Jelenik, T., Roden, M., 2013. How estrogens prevent from lipid-induced insulin resistance. Endocrinology. 154, 989-992.

Karpuzoglu, E., Ahmed, S.A., 2006. Estrogen regulation of nitric oxide and inducible nitric oxide synthase (iNOS) in immune cells: implications for immunity, autoimmune diseases, and apoptosis. Nitric Oxide. 15, 177-186.

Koricanac, G., Tepavcevic, S., Romic, S., Zivkovic, M., Stojiljkovic, M., Milosavljevic, T., Stankovic, A., Petkovic, M., Kamceva, T., Zakula, Z., 2012. Estradiol enhances effects of fructose rich diet on cardiac fatty acid transporter CD36 and triglycerides accumulation. Eur J Pharmacol. 694, 127-134.

Kowalczuk, D., Pietraś, R., Baran Annales, J., 2007. Spectrophotometric analysis of cefepime and L-arginine the pharmaceutical preparation. Universitatis Mariae Curie-Sklodowska, Lublin, 83-87.

Kulkarni, J., Gavrilidis, E., Worsley, R., Van Rheenen, T., Hayes, E., 2013. The role of estrogen in the treatment of men with schizophrenia. Int J Endocrinol Metab. 11, 129-136.

Kypreos, K.E., Zafirovic, S., Petropoulou, P.I., Bjelogrlic, P., Resanovic, I., Traish, A., Isenovic, E.R., 2014. Regulation of endothelial nitric oxide synthase and high-density lipoprotein quality by estradiol in cardiovascular pathology. J Cardiovasc Pharmacol Ther. 19, 256-268.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680-685.

Lavie, C.J., McAuley, P.A., Church, T.S., Milani, R.V., Blair, S.N., 2014. Obesity and cardiovascular diseases: implications regarding fitness, fatness, and severity in the obesity paradox. J Am Coll Cardiol. 63, 1345-1354.

Lavie, C.J., Milani, R.V., Ventura, H.O., 2009. Obesity and cardiovascular disease: risk factor, paradox, and impact of weight loss. J Am Coll Cardiol. 53, 1925-1932.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25, 402-408.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193, 265-275.

Luiken, J.J., Arumugam, Y., Dyck, D.J., Bell, R.C., Pelsers, M.M., Turcotte, L.P., Tandon, N.N., Glatz, J.F., Bonen, A., 2001. Increased rates of fatty acid uptake and plasmalemmal fatty acid transporters in obese Zucker rats. J Biol Chem. 276, 40567-40573.

Luiken, J.J., Koonen, D.P., Willems, J., Zorzano, A., Becker, C., Fischer, Y., Tandon, N.N., Van Der Vusse, G.J., Bonen, A., Glatz, J.F., 2002. Insulin stimulates long-chain fatty acid utilization by rat cardiac myocytes through cellular redistribution of FAT/CD36. Diabetes. 51, 3113-3119.

Maggi, A., Cignarella, A., Brusadelli, A., Bolego, C., Pinna, C., Puglisi, L., 2003. Diabetes undermines estrogen control of inducible nitric oxide synthase function in rat aortic smooth muscle cells through overexpression of estrogen receptor-beta. Circulation. 108, 211-217.

Miinea, C.P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peranen, J., Lane, W.S., Lienhard, G.E., 2005. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem J. 391, 87-93.

Moncada, S., Higgs, E.A., 1995. Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J. 9, 1319-1330.

Montessuit, C., Lerch, R., 2013. Regulation and dysregulation of glucose transport in cardiomyocytes. Biochim Biophys Acta. 1833, 848-856.

Musatov, S., Chen, W., Pfaff, D.W., Mobbs, C.V., Yang, X.J., Clegg, D.J., Kaplitt, M.G., 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, 2501-2506.

Nweze, I.C., Smith, J.W., Zhang, B., Klinge, C.M., Lakshmanan, J., Harbrecht, B.G., 2012. 17beta-Estradiol attenuates cytokine-induced nitric oxide production in rat hepatocyte. J Trauma Acute Care Surg. 73, 408-412.

Obradovic, M., Stewart, A.J., Pitt, S.J., Labudovic-Borovic, M., Sudar, E., Petrovic, V., Zafirovic, S., Maravic-Stojkovic, V., Vasic, V., Isenovic, E.R., 2014. In vivo effects of 17beta-estradiol on cardiac Na(+)/K(+)-ATPase expression and activity in rat heart. Mol Cell Endocrinol. 388, 58-68.

Obradovic, M., Sudar, E., Zafirovic, S., Stanimirovic, J., Labudovic-Borovic, M., Isenovic, E.R., 2015a. Estradiol in vivo induces changes in cardiomyocytes size in obese rats. Angiology. 66, 25-35.

Obradovic, M., Zafirovic, S., Jovanovic, A., Milovanovic, E.S., Mousa, S.A., Labudovic-Borovic, M., Isenovic, E.R., 2015b. Effects of 17beta-estradiol on cardiac Na(+)/K(+)-ATPase in high fat diet fed rats. Mol Cell Endocrinol. 416, 46-56.

Panchal, S.K., Poudyal, H., Iyer, A., Nazer, R., Alam, A., Diwan, V., Kauter, K., Sernia, C., Campbell, F., Ward, L., Gobe, G., Fenning, A., Brown, L., 2011. High-carbohydrate high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. J Cardiovasc Pharmacol. 57, 51-64.

Patten, R.D., Karas, R.H., 2006. Estrogen replacement and cardiomyocyte protection. Trends Cardiovasc Med. 16, 69-75.

Patten, R.D., Pourati, I., Aronovitz, M.J., Baur, J., Celestin, F., Chen, X., Michael, A., Haq, S., Nuedling, S., Grohe, C., Force, T., Mendelsohn, M.E., Karas, R.H., 2004. 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling. Circ Res. 95, 692-699.

Rhodes, P., Leone, A.M., Francis, P.L., Struthers, A.D., Moncada, S., Rhodes, P.M., 1995. The L-arginine:nitric oxide pathway is the major source of plasma nitrite in fasted humans. Biochem Biophys Res Commun. 209, 590-596.

Riant, E., Waget, A., Cogo, H., Arnal, J.F., Burcelin, R., Gourdy, P., 2009. Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. Endocrinology. 150, 2109-2117.

Rider, O.J., Cox, P., Tyler, D., Clarke, K., Neubauer, S., 2013. Myocardial substrate metabolism in obesity. Int J Obes (Lond). 37, 972-979.

Romic, S., Tepavcevic, S., Zakula, Z., Milosavljevic, T., Stojiljkovic, M., Zivkovic, M., Popovic, M., Stankovic, A., Koricanac, G., 2013. Does oestradiol attenuate the damaging effects of a fructose-rich diet on cardiac Akt/endothelial nitric oxide synthase signalling? Br J Nutr. 109, 1940-1948.

Shao, D., Tian, R., 2015. Glucose Transporters in Cardiac Metabolism and Hypertrophy. Compr Physiol. 6, 331-351.

Shao, J., Yamashita, H., Qiao, L., Friedman, J.E., 2000. Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Leprdb/db mice. J Endocrinol. 167, 107-115.

Shi, H., Kumar, S.P., Liu, X., 2013. G protein-coupled estrogen receptor in energy homeostasis and obesity pathogenesis. Prog Mol Biol Transl Sci. 114, 193-250.

Slot, J.W., Geuze, H.J., Gigengack, S., James, D.E., Lienhard, G.E., 1991. Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat. Proc Natl Acad Sci U S A. 88, 7815-7819.

Stanimirovic, J., Obradovic, M., Jovanovic, A., Sudar-Milovanovic, E., Zafirovic, S., Pitt, S.J., Stewart, A.J., Isenovic, E.R., 2016. A high fat diet induces sex-specific differences in hepatic lipid metabolism and nitrite/nitrate in rats. Nitric Oxide. 54, 51-59.

Stanimirovic, J., Obradovic, M., Zafirovic, S., Resanovic, I., Bogdanovic, N., Gluvic, Z., Mousa, S.A., Isenovic, E.R., 2015. Effects of altered hepatic lipid metabolism on regulation of hepatic iNOS. Clinical Lipidology. 10, 167-175.

Steinberg, G.R., Dyck, D.J., Calles-Escandon, J., Tandon, N.N., Luiken, J.J., Glatz, J.F., Bonen, A., 2002. Chronic leptin administration decreases fatty acid uptake and fatty acid transporters in rat skeletal muscle. J Biol Chem. 277, 8854-8860.

Sudar-Milovanovic, E., Obradovic, M., Jovanovic, A., Zaric, B., Zafirovic, S., Panic, A., Radak, D., Isenovic, E.R., 2015. Benefits of L-Arginine on Cardiovascular System. Mini Rev Med Chem. 16, 94-103.

Sudar, E., Jovanovic, A., Marjanovic, M., Vucicevic, L., Janjetovic, K., Isenovic, E., 2015. Effects of Intracerebroventricularly (ICV) Injected Ghrelin on Cardiac Inducible Nitric Oxide Synthase Activity/Expression in Obese Rats. Exp Clin Endocrinol Diabetes 123, 1-8.

Tepavcevic, S., Koricanac, G., Zakula, Z., Milosavljevic, T., Stojiljkovic, M., Isenovic, E.R., 2011. Interaction between insulin and estradiol in regulation of cardiac glucose and free fatty acid transporters. Horm Metab Res. 43, 524-530.

Zhu, L., Brown, W.C., Cai, Q., Krust, A., Chambon, P., McGuinness, O.P., Stafford, J.M., 2013. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. Diabetes. 62, 424-434.

Zunic, G., Supic, G., Magic, Z., Draskovic, B., Vasiljevska, M., 2009. Increased nitric oxide formation followed by increased arginase activity induces relative lack of arginine at the wound site and alters whole nutritional status in rats almost within the early healing period. Nitric Oxide. 20, 253-258.

Figure legends:

Figure 1. Effects of E_2 on FFA concentration in control and HF fed rats. Results are expressed as a mean±SEM (*p<0.05; #p<0.05; * indicates significance between CONT and HF or HF+E_2; # indicates significance between HF and HF+E_2).

Figure 2. Effects of E_2 on cardiac glucose and lipid metabolism in HF fed rats. (A) Distribution of fatty acids transporter CD36 in lysate, plasma membranes (PM) and low density microsomes (LDM). (B) Representative western blots of CD36 protein distribution in rat heart lysate, PM and LDM. (C) GLUT1 protein distribution in rat heart lysate, PM and LDM. (D) Representative western blots of GLUT1 protein distribution in HF rat heart lysate, PM and LDM. (E) GLUT4 protein distribution in rat heart lysate, PM and LDM. (F) Representative western blots of GLUT4 protein distribution in rat heart lysate, PM and LDM. Results are expressed as a percentage of the value obtained for control and represent the mean±SEM (*p<0.05; **p<0.01; ***p<0.001; #p<0.05; * indicates significance between CONT and HF or HF+E_2; # indicates significance between HF and HF+E_2).

Figure 3. Effects of E_2 on (A) nitrite/nitrate and (B) L-arginine concentrations in heart lysates of control and HF fed rats. Results are presented as the mean±SEM (*p<0.05; **p<0.01; ## p<0.01; * indicates significance between CONT and HF or HF+E_2; # indicates significance between HF and HF+E_2).

Figure 4. Regulation of cardiac inducible nitric oxide synthase (iNOS) by E_2 in control and HF fed rats. (A) iNOS gene expression in HF fed rats. (B and C) Representative Western blots for

iNOS and NFkB-p65 protein expression, respectively. (D) iNOS and (E) NFkBp65 protein levels in HF fed rats. Results are expressed as a percentage of the value obtained for control and represent the mean ±SEM (*p<0.05; #p<0.05; ##p<0.01; * indicates significance between CONT and HF or HF+E_2; # indicates significance between HF and HF+E_2).

Figure 5. Effects of E_2 on Akt phosphorylation at Thr^{308} in control and HF fed rats. (A) Total Akt protein expression. (B), Akt phosphorylation level at Thr^{308}. (C) ratio of pAkt (Thr^{308}) and total Akt (pAkt/tAkt) in heart of HF rat. (D) Representative western blots of pAkt (Thr^{308}) and total Akt protein forms. Results are expressed as a percentage of the value obtained for control and represent the mean±SEM (***p<0.001; #p<0.05; * indicates significance between CONT and HF or HF+E_2; # indicates significance between HF and HF+E_2).

Figure 6. Effects of E_2 on cardiac ERα and ERβ protein expression in HF fed rats. (A) ERα and (C) ERβ protein levels in HF fed rats. (B and D) Representative Western blots for ERα and ERβ protein expression, respectively. Results are expressed as a percentage of the value obtained for control and represent the mean ± SEM.

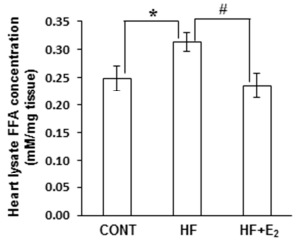


Figure 1.

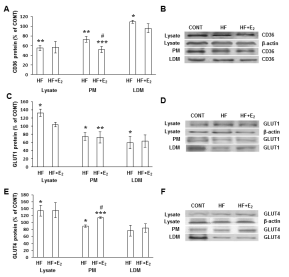


Figure 2.

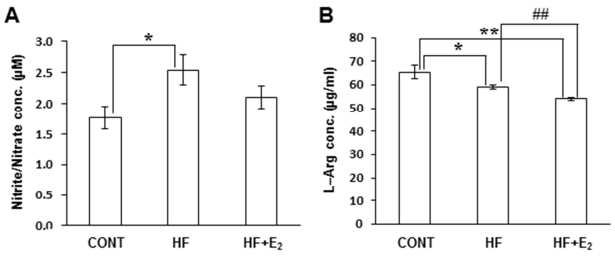


Figure 3.

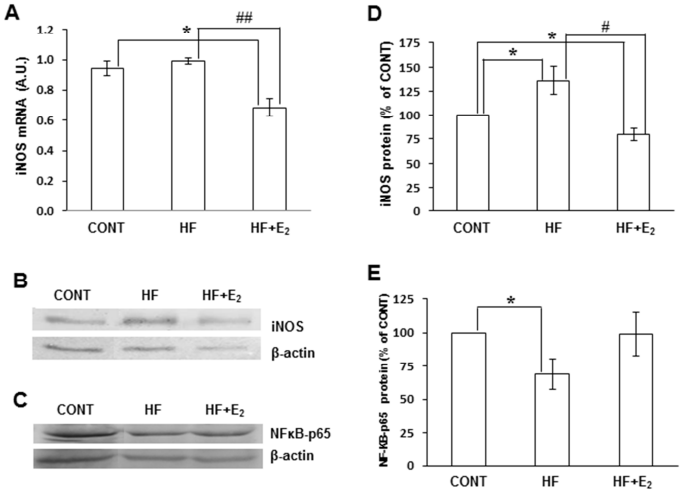


Figure 4.

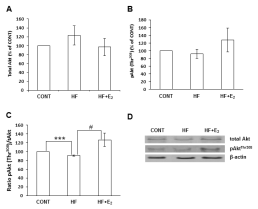


Figure 6.

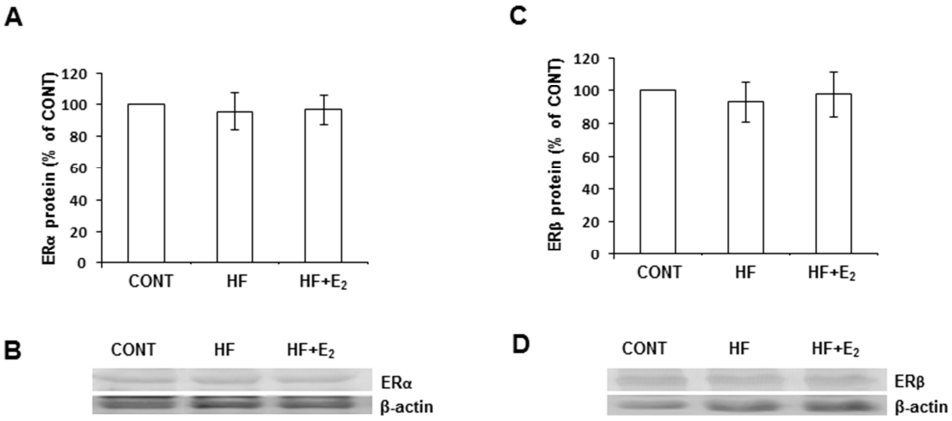


Figure 6.

Highlights:

- Estradiol treatment influences myocardial glucose and free fatty acid metabolism in rats fed a high fat (HF) diet.
- *In vivo* estradiol treatment impacts on cardiac iNOS expression/activity in HF fed rats.
- Estradiol acts via Akt to attenuate the detrimental effects of cardiac iNOS in HF fed rats.