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

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ABSTRACT

The aim of this study was to investigate the *in vivo* effects of 17β-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. 24 h 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 treatment in HF rats. Our results indicate that E_2 acts via the PI3K/Akt signalling pathway to partially protect myocardial metabolism by attenuating the detrimental effects of increased iNOS expression/activity in HF fed rats.

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, 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, 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). 17β-estradiol (E_2) at physiological concentrations exerts many beneficial effects on the heart, including improved vascular function and reduced atherosclerosis (Patten and Karas, 2006; Romic et al., 2013). These E_2-mediated effects include alterations in the activity and expression of many enzymes, including

List of abbreviations: Akt, protein kinase B; AS160, Akt substrate of 160 kDa; CD36, fatty acid transporters; E2, 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.

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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, 2015b).

Nitric oxide (NO) plays an important role in cardiovascular function by regulating relaxation of blood vessels and also influencing cardiomyocyte contractility (Moncada and 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 and 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 and 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 17β-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 (150 g-200 g) 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 (labeled 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 10th 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., (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 1000 × 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 1000 × 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 5000 × 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