Influence of a high-fat diet on cardiac iNOS in female rats

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Abstract

Overexpression of inducible nitric oxide synthase (iNOS) is a key link between high-fat (HF) diet induced obesity and cardiovascular (CV) disease. Several studies have reported that oestradiol has cardioprotective effects that may be mediated through reduction of iNOS activity/expression. In the present study, female Wistar rats were fed a standard diet or a HF diet (balanced diet for laboratory rats enriched with 42% fat) for 10 weeks. Gene and protein expression of iNOS were measured in heart tissue. HF diet-fed rats exhibited a significant increase in cardiac iNOS mRNA by 695% ($p<0.05$), iNOS protein level by 248% ($p<0.01$), without changes in nitrate/nitrite levels. Expression of CD36 protein in plasma membranes was increased by 37% ($p<0.05$), while the concentration of free fatty acids (FFA) was reduced by 25% ($p<0.01$) in HF diet-fed rats. Expression of the p50 subunit of nuclear factor-κB (NFκB-p50) in heart lysate was increased by 77% ($p<0.01$) in HF diet-fed rats. Expression and phosphorylation of protein kinase B (Akt) and extracellular signal-regulated kinases 1/2 (ERK1/2) in control and HF diet-fed rats were also examined. Expression of Akt and ERK1/2 were unchanged between the groups. There was a significant increase in the ratio of phospho-Akt/total Akt but not for phospho-ERK1/2/total ERK1/2/ in HF-fed rats. Estrogen receptor-α levels (by 50%; $p<0.05$) and serum oestradiol concentrations (by 35%; $p<0.05$) were examined and shown to be significantly reduced in HF diet-fed rats. Our results revealed that a HF diet led to increased iNOS expression, most likely via a mechanism involving Akt and NFκB-p50 proteins. Decreased levels of oestradiol and ERα protein in the HF-fed group, in combination with increased iNOS levels are consistent with the hypothesis that oestradiol has a cardioprotective effect through its ability to regulate iNOS expression.

Key words: cardioprotection, cardiovascular disease, oestradiol, inducible nitric oxide synthase, obesity

Abbreviations: Akt, protein kinase B; CD36, cluster of differentiation 36; CHD, coronary heart disease; CV, cardiovascular; CVD, cardiovascular disease; DMT2, diabetes mellitus type 2; ERα, oestrogen receptor-α; ERs, oestrogen receptors; ERK1/2, extracellular signal-regulated kinases 1/2; FFA, free fatty acids; HF, high fat; HOMA-IR, HOMA-index of insulin resistance; HOMA-β, HOMA-index of β-cell function; IκB, inhibitor of NFκB; iNOS, inducible nitric oxide synthase; IR, insulin resistance; NFκB-p50, the p50 subunit of nuclear

factor-κB; TES, N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TG, triglycerides VSMCs, vascular smooth muscle cells.

1. Introduction

Obesity is reaching global epidemic proportions, is associated with inflammatory responses and a number of comorbidities including insulin resistance (IR), type 2 diabetes mellitus (T2DM) and cardiovascular (CV) diseases (CVD) [1-3]. A high fat (HF) diet is believed to cause fat accumulation and consequentially metabolic disorders and is generally used to create valid animal models of obesity and IR [4,5]. The risk of obesity-related comorbidities is well established in men, but less so for age matched pre-menopausal women [6]. There are a number of experimental and clinical studies showing sex differences in the susceptibility to various diseases like IR, T2DM and metabolic syndrome [7-11]. Moreover, CVD develops earlier and are more frequently in men compared with premenopausal women [12]. It is generally accepted that the reason for these differences is oestradiol, the primary female sex steroid. Oestradiol improves insulin sensitivity, impedes hyperglycaemia and IR and also displays cardioprotective effects [13-15]. There is a clear association between obesity and CVD [1,4,5], but the exact molecular mechanism of this pathophysiology remains to be elucidated, especially in premenopausal women.

Inducible nitric oxide synthase (iNOS) is an important inflammatory mediator with a vital role in immunity [16]. However, overexpression of iNOS is hypothesized to be involved in the development of IR and CVD, and may be the key link between obesity related metabolic disorders and inflammation [17,18]. In dietary and genetic models of obesity, excessive accumulation of lipids can induce iNOS expression and nitric oxide (NO) production in metabolic tissues [17,19,20]. Increased cardiac iNOS expression was observed in spontaneously hypertensive rats and rabbits fed a HF-diet, as well as in obese patients [3]. Overexpression of cardiac iNOS in mice was reported to cause ventricular hypertrophy, which is an independent risk factor for coronary heart disease, sudden death, heart failure and stroke [21,22]. Moreover, numerous studies reported iNOS-induced myocardial dysfunction which was manifested either as a decrease in baseline myocardial contractile function [23,24], or as a reduction in β-adrenergic inotropic responsiveness [25]. In addition, induction of iNOS is positively associated with the expression of CD36, also known as a fatty acid translocase. This can lead to enhanced free fatty acid (FFA) uptake and triglyceride (TG) accumulation, which can eventually cause oxidative/nitrosative stress and cardiac dysfunction, especially in obese and diabetic individuals [26-28].

The cardioprotective effects of oestradiol are proposed to be associated with its ability to regulate iNOS activity/expression [29,30]. Regulation of iNOS expression occurs primarily at the transcriptional level and involves nuclear factor-κB (NFκB). When inactive, NFκB

protein is located in cytosol and bound to the inhibitor of NFκB (IκB). Phosphorylation of IκB results in its degradation and subsequent NFκB translocation to the nucleus where it initiates iNOS gene expression [31]. Induction of iNOS is mediated by various stimuli, including protein kinase B (Akt) as well as extracellular signal-regulated kinases 1 and 2 (ERK 1/2) signalling pathways, both of which are altered in obesity and IR [32]. In vascular smooth muscle cells (VSMCs) [33] and macrophages [34] oestradiol blocks the synthesis of iNOS induced by inflammatory stimuli. This occurs through two types of specific oestrogen receptors (ERs), oestrogen receptor α (ERα) and oestrogen receptor β, both of which are expressed in cardiomyocytes [35,36]. There is also evidence indicating that ERs may interact with other transcription factors, including NFκB [37]. In addition, oestrogen ERα has been shown to decrease iNOS level in VSMCs [38].

In male rats fed a HF-diet the expression of iNOS in the heart is up-regulated [17,19,20], and *in vitro* studies show that regulation of iNOS expression is altered by oestradiol [33]. Additionally, we have recently shown sex-specific differences in hepatic iNOS regulation in HF-diet fed rats [39]. There is limited knowledge as to the effects of a HF-diet on cardiac iNOS in female rats and the mechanisms by which obesity reverses the cardioprotective effects of oestradiol are poorly understood. Here we investigate the effects of a HF-diet on the expression of cardiac iNOS in female rats and explore the signalling pathways by which this is regulated.

2. Material and Methods

2.1. Materials

Ether was purchased from Lek (Ljubljana, Slovenia). Luminol and p-coumaric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protease (Complete, Ultra Mini, EDTA-free) and phosphatase inhibitor cocktails (PhosStop) were purchased from Roche (Mannheim, Germany). Trizol reagent was purchased from Invitrogen Life Technologies (Paisley, GB). The rabbit polyclonal antibodies (anti-phospho-Akt (Ser^{473}), anti-Akt and anti-iNOS) were obtained from Abcam (Cambridge, UK). The rabbit polyclonal (anti-phospho ERK1/2 (Thr^{202}/Tyr^{204}) and anti-ERK1/2) antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). The rabbit anti-NFκB, anti-ERα and CD36 polyclonal antibodies, mouse anti-actin monoclonal antibody and the secondary anti-mouse and anti-rabbit antibodies conjugated to alkaline phosphatase or to horseradish peroxidase and the 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium chloride, were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The primers for rat β-actin (for gene Actb) were

obtained from Sigma-Aldrich (St. Louis, MO, USA) and primers for iNOS (*Nos2* gene) were obtained from Metabion (Martinsried, Germany).

2.2. Animals

In this study we used adult female Wistar rats (150-200 g, 8-weeks old) bred at the Institute of Nuclear Sciences (Vinca, Belgrade). The animals were divided into 2 groups: control (Control) and HF diet fed rats (Obese). The rats were kept under a 12:12 h light: dark cycle at 22±2°C. For the next 10 weeks control groups were fed *ad libitum* with standard laboratory chow composed of 20% proteins, 8% cellulose, 13% moisture, 1% calcium, 0.90% lysine, 0.75% methionine+cystine, 0.5% phosphorus, 0.15-0.25% sodium, vitamin mixture (A 10000 IU/kg, D_3 1600 IU/kg, E 25 mg/kg, B_{12} 0.02 mg/kg), mixture of minerals (in mg/kg: zinc 100, iron 100, manganese 30, copper 20, iodine 0.5, selenium 0.1), antioxidants 100 mg/kg, and digestible/metabolizable energy 11 MJ/kg (prepared by D. D. Veterinarski zavod Subotica, Subotica, Serbia). Obese rats were fed the standard laboratory diet enriched with 42% fat. After 10 weeks all animals were euthanized under deep ether anesthesia. Hearts were excised, weighed, snap-frozen in liquid nitrogen and stored at -80°C. We have previously reported the body mass, levels of insulin, glucose, the HOMA-index of insulin resistance (HOMA-IR) and HOMA-index of β-cell function (HOMA-β) and plasma nitrate/nitrite levels in control and obese female rats [39], and shown that our obese female rats did not develop IR (assessed by unchanged levels of HOMA-IR and HOMA β). Experimental protocols were approved by the Vinca Institute Ethical Committee for Experimental Animals.

2.3. Heart lysate preparation

Approximately 200 mg of rat heart tissue was homogenized on ice with an Ultra-turrax homogenizer in lysis buffer (pH 7.4) containing: 10 mM Tris, 150 mM sodium chloride, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM sodium orthovanadate, phosphatase and protease inhibitor cocktails. Following incubation of homogenates for 1 h at 4°C, the samples were centrifuged at 4°C at 100,000 × g for 20 min. The supernatants were obtained and concentration of proteins was determined by the Lowry method [40]. The final lysate was stored at -80°C until further experiments.

2.4. Heart plasma membrane protein extraction

Membrane proteins were isolated according to Luiken *et al.* [41]. Briefly, rat hearts (200 mg) were dissected on ice and incubated for 30 min in a high-salt solution (20 mM HEPES, 2 M NaCl, and 5 mM sodium azide, pH 7.4) at 4°C. Afterwards samples were centrifuged for 5 min at 1000×*g*, and the pellet was homogenized on ice with an Ultra-Turrax homogenizer in

N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)-buffer (pH 7.4) containing: 20 mM Tris, 250 mM sucrose, and 1 mM EDTA, 2 mM sodium orthovanadate, phosphatase and protease inhibitor cocktails. The resulting homogenate was centrifuged for 5 min at 1000×*g*. The pellet was then re-homogenized in a TES-buffer and recombined the supernatant obtained in previous centrifugation. This was followed by another centrifugation of the homogenate for 10 min at 100×*g*, and the obtained supernatant was additionally centrifuged for 10 min at 5000×*g*. The final pellet (referred to as the "plasma membrane fraction") was resuspended in TES buffer and stored at -80°C for further analysis. Protein concentrations were determined by the Lowry method.

2.5. SDS-PAGE and Western blotting

Equal amounts of total lysates protein extracts (80 μg/lane) were separated by 10% or 12% SDS-polyacrylamide gel electrophoresis PAGE [42] and transferred to polyvinylidene difluoride membranes as previously described [43,44]. The membranes were afterwards blocked with 5% bovine serum albumin and probed with antibodies directed against phospho-ERK1/2 (Thr^{202}/Tyr^{204}), total ERK1/2, phospho-Akt (Ser^{473}), total Akt, iNOS, NFκB-p50 and ERα. After washing, membranes were incubated with the appropriate secondary antibodies and used for subsequent detection with either BCIP/NBT or with the electrochemiluminescence method. Following analysis of phospho-ERK1/2 and phospho-Akt, the membranes were stripped and re-blotted with antibody detecting non-phosphorylated forms of these proteins. In order to insure that protein loading was equal in all samples, all blots were probed with anti-actin antibody and appropriate secondary antibody. Signals on membranes were quantified using ImageJ 1.45s software (National Institutes of Health, USA, http://rsb.info.nih.gov).

2.6. RNA isolation

Total RNA from rat heart tissue was extracted by Trizol reagent according to the manufacturer's instructions. Briefly, 100 mg of heart tissue was homogenized in 1 ml of Trizol, and after 5 min of incubation at room temperature, samples were centrifuged at 12000 × g for 15 min at 4°C. Afterwards 200 μl of chloroform was added and samples were first incubated at room temperature for 15 min, and then centrifuged at 12000 × g for 15 min at 4°C and aqueous phase was collected. RNA was precipitated with isopropyl alcohol and washed with 75% ethanol. The RNA pellet was dried and re-dissolved in diethylpyrocarbonate-water. Concentration and purity of isolated RNA were determined by measuring the absorbance at 260 nm/280 nm. In order to ensure that RNA samples were not degraded, 2.5 μg of total RNA was analysed by 1.2% agarose gel electrophoresis.

2.7. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

The reverse transcription reaction was performed using 1 µg of isolated heart RNA and M-MuLV reverse transcriptase with random hexamers according to the manufacturer's instructions. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed on 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in 96-well reaction plates (MicroAmp Optical, Applied Biosystems) in 20 µl volume/well, containing 10 µl of reaction mix (ABM) and 10 µl of appropriate sample diluted in demineralized water and pairs of primers. The wells were sealed with optical adhesive film, and the plate was centrifuged for few seconds at high speed and thereafter placed into the thermocycler. The cycling conditions were 95°C for 4 min (1 cycle) followed by 95°C for 15 s and 61°C for 1 min (40 cycles). All assays were performed in duplicate and the level of expression of iNOS was normalized to β-Actin mRNA levels. 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′-CCCTGGCTCCTAGCACCAT-3′ (forward primer), 5′-GAGCCACCAATCCACACAGA-3′ (reverse primer) with PCR product length of 76 bp. iNOS, and β-Actin primers were designed using Primer Express1 software v2.0 (Applied Biosystems).

2.8. Measurement of heart lysate nitrate/nitrite concentration

The levels of NO in heart lysates were measured by Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. In this assay, nitrate/nitrite is measured indirectly by determining the total concentration of nitrate/nitrite, in a two-step process. In the first step nitrate is converted to nitrite by utilizing nitrate reductase, while the second step includes addition of Griess reagents and conversion of nitrite into a deep purple azo compound. The absorbance at 540 nm was measured and the nitrate concentrations were calculated from a sodium nitrate standard curve.

2.9. Measurement of free fatty acid (FFA) concentration in heart lysates

The level of FFAs in heart lysates was determined by a modified version of Duncombe's colorimetric method [45]. 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), which was followed by the addition of 1125 µl of chloroform, intensive shaking for 20 min and centrifugation for 10 min at 3000×g. After removing the top blue-green layer, in the lower chloroform phase containing the extracted FFA 45 µl of 0.2% diethyldithiocarbamate was added. The samples were then incubated for 20 min at room temperature and the absorbance

was measured at 436 nm using a Lambda 35UV/VIS spectrometer (Perkin Elmer, Waltham, Massachusetts, USA). The FFA concentration was calculated from a palmitate standard curve and expressed as mmol FFA per µg tissue.

2.10. Measurement of serum oestradiol concentration

The estradiol concentration in rat sera was measured by a standardized immunoassay system (Immulite 1000 Estradiol; Siemens, Surrey, UK) using an Immulite autoanalyzer (Diagnostic Products Corp., Los Angeles, CA, USA) according to the manufacturer's protocol.

2.11. Statistical Analysis

Statistical analyses of data were evaluated with Student's *t* test. A two-tailed $p<0.05$ was considered significant.

3. Results

Gene and protein expression of cardiac iNOS in obese female rats

Overexpression of iNOS is hypothesized to be a key link between obesity and CVD. We therefore examined the effects of a HF diet on cardiac iNOS mRNA and protein expression in female rats using western blot and qRT-PCR analysis, respectively. Cardiac iNOS protein levels were found to increase ~2.5-fold in the obese female rats relative to controls (by 248%, $p<0.01$; Figure 1A and B). Similarly, the iNOS mRNA levels in the heart were found to increase by ~7-fold in the HF-fed (obese) group compared with control (695%, $p<0.05$; Figure 1C). However, there were no significant differences in the level of nitrate/nitrite in heart lysates between obese and control female rats (Figure 1D).

Effects of HF diet feeding on cardiac CD36 expression and FFA levels in female rats

Since iNOS has been shown to regulate the expression of CD36 [27,28], we measured the level of CD36 protein in membrane fraction of heart lysates as well as the level of FFAs. The level of CD36 protein in plasma membranes was increased in the obese female rats relative to controls (by 37%, $p<0.05$; Figure 2B). Furthermore, our results revealed that FFA levels were significantly decreased (by 25%, $p<0.01$; Figure 2C).

Factors that regulate cardiac iNOS in control and obese female rats

Considering that the iNOS gene promoter contains a binding site for the transcription factor, NFκB [46,47], we next examined whether a HF diet affects the protein level of p50 subunit of NfκB protein (NFκB-p50) in females rat hearts by western blotting. Our results revealed that the level of NFκB-p50 protein was significantly increased (77%; $p<0.01$; Figure 3) in the hearts of obese female rats compared with controls.

To gain further insight into the effects of a HF diet on iNOS regulation in female rat hearts, we explored the phosphorylation state of Akt and ERK1/2 kinases. These kinases have previously been implicated as upstream regulators of NFκB, and consequently iNOS. Western blotting was carried out to determine the relative levels of phospho-Akt (Ser^{473}) and total Akt in heart lysates from obese and control female rats (Figure 4A). The results show that there was an increase in the level of phospho-Akt in the rats fed a HF diet relative to controls but the change was not found to be statistically significant (Figure 4B). There was no change in the level of total Akt between the groups (Figure 4C). However, in obese female rats the ratio of phospho-Akt/total Akt was significantly increased by 95% ($p<0.05$) compared to control (Figure 4D). The relative levels of phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and total ERK1/2 in heart lysates taken from obese and control female rats were also examined (Figure 5A). There were no significant changes in phospho-ERK1/2 (Figure 5B), total ERK1/2 (Figure 5C) or the ratio of phospho-ERK1/2/total ERK1/2 (Figure 5D).

Oestradiol is known to reduce iNOS activity/expression through interaction between its receptor ERα and NFκB [37,38]. Therefore, we next explored whether a HF diet causes changes in cardiac ERα protein expression and serum oestradiol concentration. The level of ERα protein was assessed using western blotting (Figure 6A) and was found to decrease by 50% ($p<0.05$) in the obese group (Figure 6B). The concentration of serum oestradiol was measured using a standardized immunoassay and found to be significantly reduced (by 36%; $p<0.05$) in HF-fed female rats relative to controls (Figure 6C).

4. Discussion

We previously reported that a HF diet causes the development of an obese phenotype in both male and female rats [39,48,49]. In addition, our previous results show that the level total cholesterol was increased in the liver obese female, suggesting excessive intrahepatic lipid accumulation caused by obesity [39]. However, only in male rats did a HF diet induce obesity leading to hyperlipidaemia, hyperglycaemia and IR [39,48]. This was attributed (at least in part) to a sex-specific reduction of iNOS expression in rat liver regulated through the action of oestradiol. In addition, oestradiol is known to exert cardioprotective effects in premenopausal females but it is unclear whether its actions are compromised in obesity. Here we demonstrate for the first time that a HF diet causes up-regulation of cardiac iNOS expression in female rats by a mechanism involving Akt and NFκB in combination with a decreased serum oestradiol concentration and reduced cardiac ERα expression. We also

reveal that increased iNOS expression is associated with increased expression of the fatty acid translocase, CD36 and a reduction in the level of FFA in cardiac tissue.

Overexpression of iNOS is known to cause cardiac fibrosis, apoptosis of cardiomyocytes and cardiac hypertrophy, whilst iNOS deficiency prevents congestive heart failure [50]. In this study we found that cardiac iNOS gene and protein expression was increased in obese females hearts compared with controls. Surprisingly, the increase in iNOS mRNA and protein levels were not associated with increased nitrate/nitrite levels. A possible explanation is that much of the NO generated as a consequence of increased iNOS expression reacts with other molecules. Several studies have shown that CD36 is an important downstream target of iNOS [27,28,51]. It has been shown that incubation of cardiomyocytes with the saturated fatty acid, palmitate leads to increase in intracellular TG stores, iNOS expression and translocation of CD36, which can be inhibited by the specific iNOS inhibitor, 1400W [27]. Similarly, in our study a HF diet augmented CD36 expression at the plasma membrane in obese female rat hearts, potentially due to increased iNOS levels. In addition, the results revealed decreased cardiac FFA levels in these rats. This may be a consequence of the increased levels of CD36 at the plasma membrane, since it has been shown that overexpression of CD36 directs FFA to a TG pool [52]. This is in agreement with observations suggesting that obesity and IR in the heart are characterized by decreased glucose metabolism and that energy production is shifted towards oxidation of fatty acids [53,54].

The regulation of iNOS expression is multifactorial and dependent on several transcription factors, including NFκB [31]. Increased accumulation of lipids in heart leads to the activation of proinflammatory pathways, which is followed by the activation of NFκB and up-regulation of cardiac iNOS expression [3]. In our study NFκB p50 was significantly increased in heart lysates of obese female rats. This finding is in agreement with others showing that a HF diet has a proinflammatory effect, increasing NFκB activity in several tissues [55-58]. Interestingly, Mariappan *et al.* reported increased NFκB expression in hearts of obese db/db mice and that blockade of NFκB in these mice protects the heart from oxidative stress and improves cardiac function [52].

Since NFκB is located in the cytosol in an inactive form, bound to I-κB, phosphorylation of I-κB by I-κB kinase (IκK) is necessary for proteolytic digestion, dimerization and translocation of NFκB subunits to the nucleus where it binds to DNA and initiates transcription. Several lines of evidence indicate that Akt is an upstream activator of NFκB, whereby phosphorylated Akt (or ERK1/2) activate I-κK, and consequently leads to transcription and translation of iNOS [46,59]. Hattori *et al.* reported that the activation of NFκB is suppressed by inhibitors

of Akt [60]. Our results show increased Akt phosphorylation at Ser^{473} in the hearts of obese female rats, which further support the hypothesis that Akt regulates NFκB activity and thereby iNOS expression. Moreover, the role of ERK1/2 in iNOS activation has been demonstrated and increased ERK1/2 phosphorylation usually upregulates both NFκB activity and iNOS protein expression [46,49,61-63]. However, in the present study there were no differences in ERK1/2 phosphorylation between obese and control female rats. A possible explanation is that *in vivo* activation of ERK1/2 depends on various stimuli and there is periodic activation and deactivation of ERK1/2 kinase [64]. In addition, ERK1/2 may be deactivated by dual-specificity phosphatase 6 (DUSP6), since it has been shown that DUSP6 is transcriptionally induced during stress, such as obesity, where it specifically dephosphorylates ERK1/2 [65].

Obesity impacts all functions of the body, including reproduction, and may cause ovulatory disorders and infertility [66]. Balasubramanian *et al.* reported that a 6-week HF diet, where 45% of calories came from fat, reduced the concentration of oestradiol in female rats [67]. Similarly, in our study the level of oestradiol was significantly reduced in obese compared with control rats. Even though the reason for this reduction is not clear, it may be related to leptin, an adipokine secreted from adipose tissue [68]. Increased level of leptin, which is generally associated with obesity, was shown to impair reproductive function and cause infertility [69]. Furthermore, leptin can interfere with oestradiol synthesis through the inhibition of pregnenolone synthesis, the precursor for oestradiol synthesis [70]. On the other hand, Guo *et al.* found that the expression of aromatase, a key enzyme regulating oestradiol biosynthesis, was significantly decreased in adipose tissue of HF diet-fed female mice [71]. It is well documented that healthy premenopausal women are naturally protected from CVD, partially via ERα signalling in the vasculature [72]. However, these beneficial effects of ERα signalling are blunted in obesity and IR conditions [73]. In addition, several lines of evidence point out that HF diet alter the expression of ER [71,74,75]. Our results show that the level of ERα expression was significantly reduced in obese female rat hearts. Likewise, Gorres *et al.* reported that 6 weeks HF diet with 60% of calories coming from fat in female rats induces reduction of ERα protein expression in adipose tissue [74].

In conclusion, our results indicate that a HF diet decreases serum oestradiol levels, as well as cardiac ERα signalling. These findings strongly suggest that the oestradiol production and action are disrupted by a HF diet, which may be the reason why cardioprotective effects of oestradiol are blunted in obesity and IR. Furthermore, due to the lack of beneficial action of oestradiol, HF dietary effects on female rat hearts are similar to those observed in male rats

and they include the up-regulation of cardiac iNOS expression by a mechanism involving Akt and NFκB [17,19,20,49], and this may be the first step in the development of CVD.

Given that the incidence and prevalence of CVD is much lower in premenopausal women, while this sex difference gradually disappears after menopause and CV risk becomes even higher in older women, numerous studies have been conducted over the past few decades in order to assess the influence of hormone replacement therapy (HRT) on the risk of CVD in postmenopausal women [76-84]. One of the largest observational studies, the Nurses' Health Study (NHS), suggested that use of HRT significantly reduces the risk of coronary heart disease (CHD) [77]. Furthermore, randomized clinical trials, such as the Postmenopausal Estrogen/Progestin Intervention (PEPI) and the Kronos Early Estrogen Prevention Study (KEEPS) showed that HRT in postmenopausal women improves the levels of low- and high-density lipoprotein cholesterol and reduces the levels of fibrinogen and C-reactive protein [76,85], while the preliminary data from the Early versus Late Intervention Trial with Estradiol (ELITE) indicate a reduction in progression of vascular disease in the HRT groups, but only if HRT was initiated within the first 6 years of the menopause [81,86]. However, other clinical trials, such as the Women's Health Initiative (WHI) reported that HRT increases the rate of nonfatal myocardial infarction, stroke and venous thromboembolism [82]. Moreover, this trial was stopped early because the risk of invasive breast cancer was increased by 26% in group receiving HRT [82]. In addition, the Heart and Estrogen/Progestin Replacement Study I and II (HERS I and II) investigated the effects of HRT in individuals with established CHD and lower rates of CHD events were seen in the final years of HERS I (years 3-5) among women who were receiving HRT, but these differences did not persist during additional years (HERS II) and it was eventually shown that HRT for 6.8 years does not reduce the risk of CV events in women with CHD [79,87]. Even though the reason for these discrepancies remains unclear, potential factors that may contribute to the adverse outcome have been proposed and they include the type and dosage of HRT given, pre-existing CVD or the time when HRT was initiated [88-90].

Cardioprotective effects of oestrogen are based on activation of eNOS through an ERα-dependent mechanism, rapid release of NO from endothelial cells and consequent vasorelaxation [91]. On the other hand, it has been shown that ERα signalling reduces iNOS gene expression, thereby preventing cardiac tissue damage [37,38]. Hwang et al. reported that ERα knockout mice significantly increase iNOS protein expression in the hippocampus and cortex [92]. Furthermore, ERα-selective agonist 4,4',4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) significantly reduces cytokine induced iNOS protein expression in aortic

smooth muscle cells [93]. However, in conditions that are affecting the level of endogenous oestrogen, such as obesity, T2DM or advancing atherosclerosis, there is loss of ERα expression in the vasculature, which is similar to the results from our study, and there is consequentially loss vasculoprotection in response to exogenous oestrogen [91]. Therefore, there may be a window of opportunity where HRT could be beneficial to the progression of atherosclerosis, and that is early in menopause, while ERα signalling is still preserved. Overall, the use of HRT has become one of the most controversial topics related to women health, and increasing our understanding of the effects of oestrogen on cardiovascular system both in physiological and pathophysiological, such as obesity and T2DM, in humans may help to develop new strategies for the treatment of CVD.

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Conflict of interest

None declared.

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FIGURE LEGENDS

Figure 1 Effects of a HF diet on iNOS gene expression and iNOS protein in rat heart (A) Representative western blots for iNOS and β-actin proteins in control and obese female rats. (B) iNOS protein levels expressed as arbitrary units (A.U.) in control and obese female rats. (C) mRNA levels of cardiac iNOS in control and HF diet-fed (obese) female rats expressed as a percentage of β-actin mRNA expression. (D) Nitrate/nitrite concentrations in control and HF diet-fed (obese) female rats. All values represent mean ± SEM (n=5-8; * $p<0.05$; ** $p<0.01$; N.S.-not significant). **HF**, high fat; **iNOS**, inducible nitric oxide synthase.

Figure 2 Effects of HF diet on CD36 plasma membrane protein expression and FFA concentration in rat heart (A) Representative western blot for CD36 in control and HF diet-fed (obese) female rats. (B) CD36 protein levels in plasma membranes expressed as a percentage of the value obtained for the control. (C) FFA concentrations in control and HF diet-fed (obese) female rats. Values represent mean ± SEM (n=5; * $p<0.05$; ** $p<0.01$). **HF**, high fat; **CD36**, cluster of differentiation 36; **FFA**, free fatty acids.

Figure 3 Effects of HF diet on NFκB protein expression in rat heart (A) Representative western blots for NFκB-p50 and β-actin proteins in control and HF diet-fed (obese) female rats. (B) NFκB-p50 protein levels in control and obese female rats expressed in arbitrary units (A.U.). Values represent mean ± SEM (n=5; ** $p<0.01$). **HF**, high fat; **NFκB-p50,** the p50 subunit of nuclear factor-κB.

Figure 4 Effects of HF diet on Akt expression and phosphorylation at Ser^{473} in lysates of rat heart (A) Representative western blots of Akt phosphorylated at Ser^{473}, total Akt and β-actin proteins in control and HF diet-fed (obese) female rats. (B) Phosphorylation of Akt at Ser^{473} in control and obese female rats. (C) Expression of total Akt protein in control and obese female rats. (D) Ratio of phospho-Akt/total Akt in control and obese female rats. (B-D) are expressed in arbitrary units (A.U.) and represent mean ± SEM (n=4; * $p<0.05$, N.S.-not significant). **Akt**, protein kinase B; **HF**, high fat.

Figure 5 Effects of HF diet on ERK1/2 expression and phosphorylation in lysates of rat heart (A) Representative western blots of phospho-ERK1/2, total ERK1/2 and β-actin proteins in control and HF diet-fed (obese) female rats. (B) Phosphorylation of ERK1/2 at

Tyr^{202}/Tyr^{204} in control and obese female rats. (C) Expression of total ERK1/2 in control and obese female rats. (D) Ratio of phospho-ERK1/2/total ERK1/2 in control and obese female rats. (B-D) are expressed in arbitrary units (A.U.) and represent mean ± SEM (n=4; * $p<0.05$, N.S.-not significant). **HF**, high fat; **ERK1/2**, extracellular signal-regulated kinases ½.

Figure 6 Effects of HF diet on ERα protein expression in rat heart and the concentration of serum oestradiol (A) Representative western blots of ERα and β-actin proteins in control and HF diet-fed (obese) female rats. (B) Expression of ERα in control and obese female rats expressed as arbitrary units (A.U.). (C) Serum oestradiol concentration expressed in pg/ml in control and obese female rats. (B and C) values represent mean ± SEM (n=5; * $p<0.05$). **HF**, high fat; **ERα**, oestrogen receptor-α**.**

Figure 1.

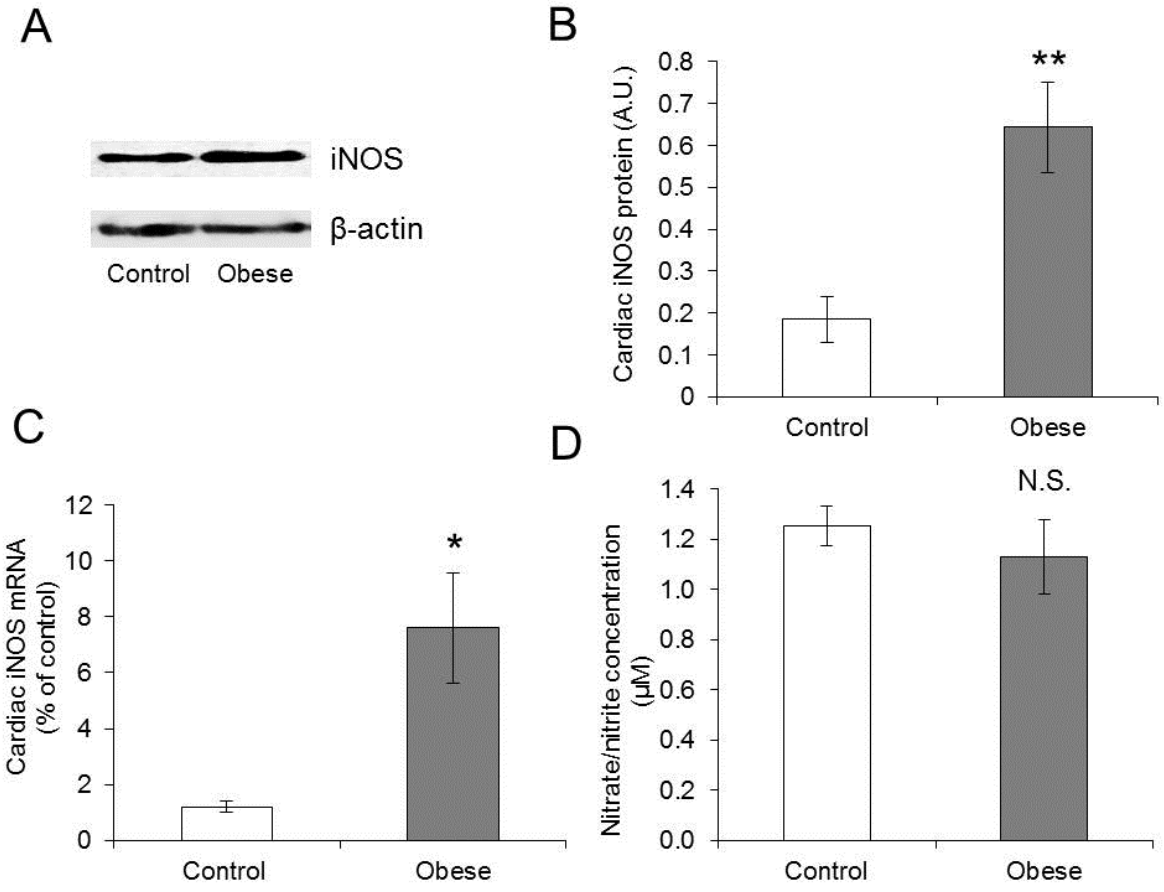

A
iNOS
β-actin
Control
Obese
B
Cardiac iNOS protein (A.U.)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
Control
Obese
C
Cardiac iNOS mRNA (% of control)
12
10
8
6
4
2
0
*
Control
Obese
D
Nitrate/nitrite concentration (μM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N.S.
Control
Obese

Figure 2.

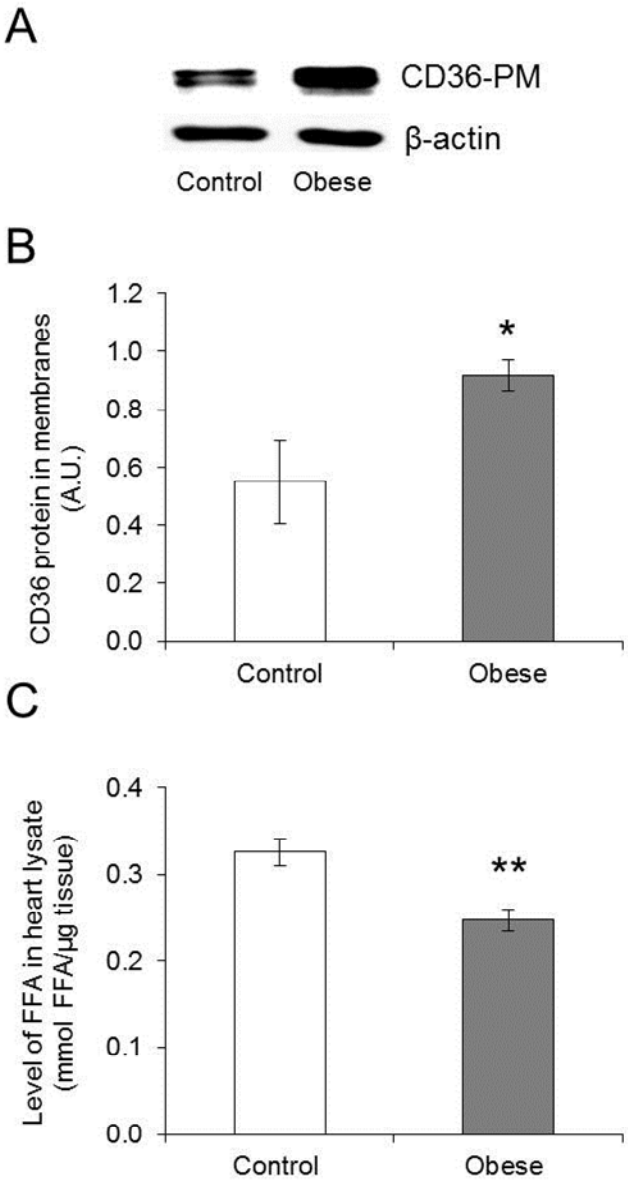

A
CD36-PM
β-actin
Control
Obese
B
CD36 protein in membranes (A.U.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Obese
C
Level of FFA in heart lysate (mmol FFA/µg tissue)
0.4
0.3
0.2
0.1
0.0
**
Control
Obese

Figure 3.

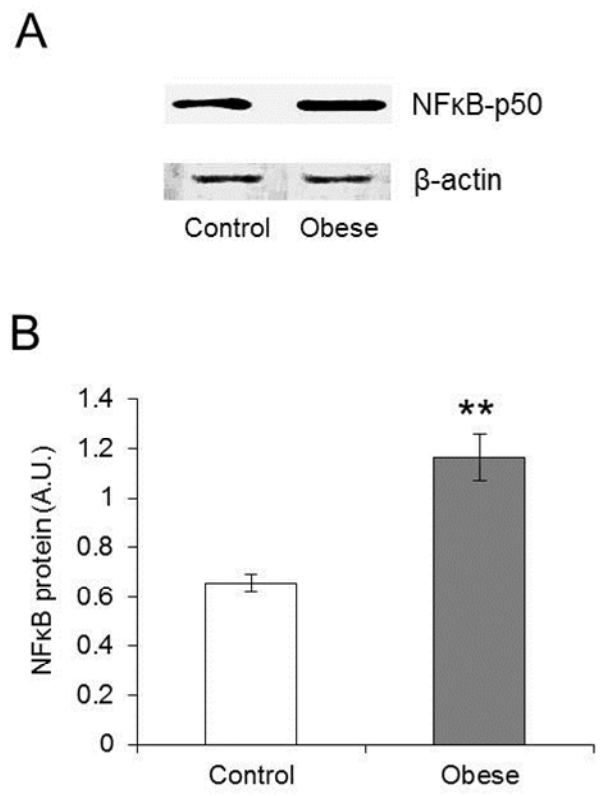

A
NFκB-p50
β-actin
Control
Obese
B
NFκB protein (A.U.)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Control
Obese

Figure 4.

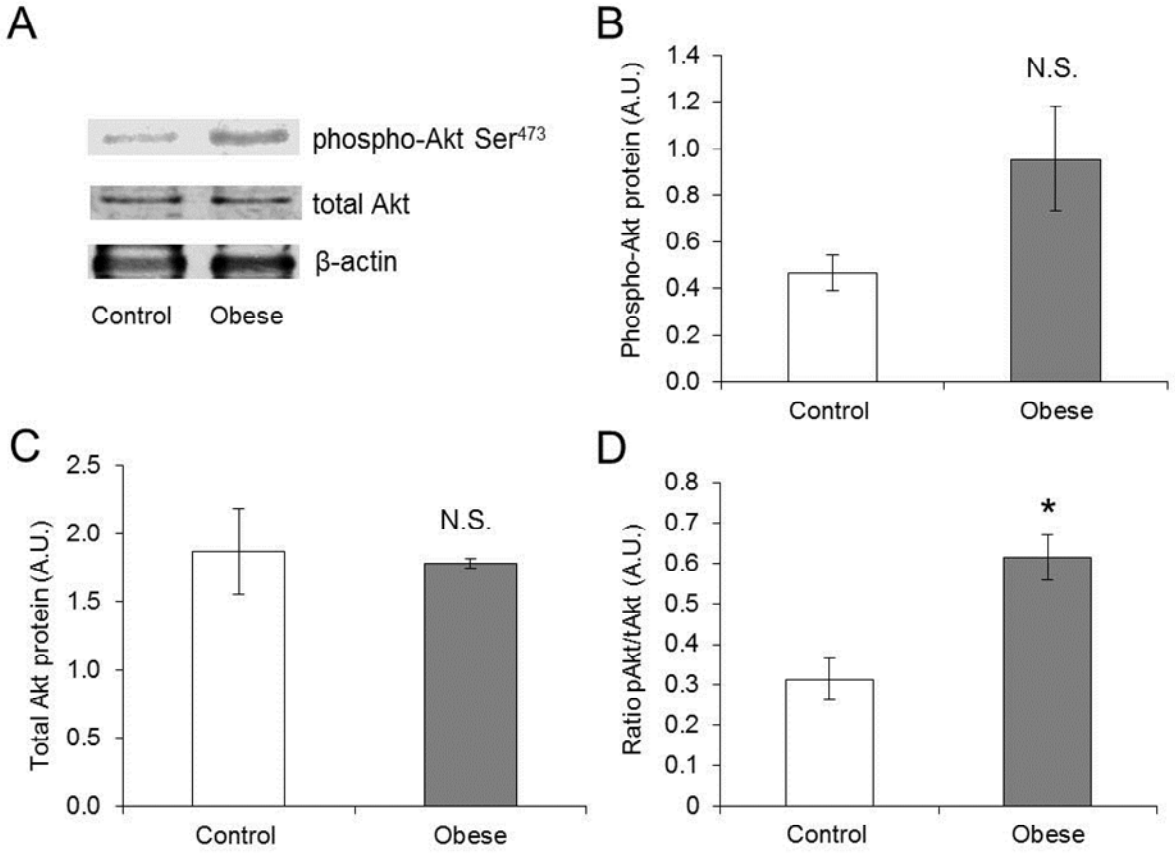

A
phospho-Akt Ser473
total Akt
β-actin
Control
Obese
B
Phospho-Akt protein (A.U.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N.S.
Control
Obese
C
Total Akt protein (A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
N.S.
Control
Obese
D
Ratio pAkt/tAkt (A.U.)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
Obese

Figure 5.

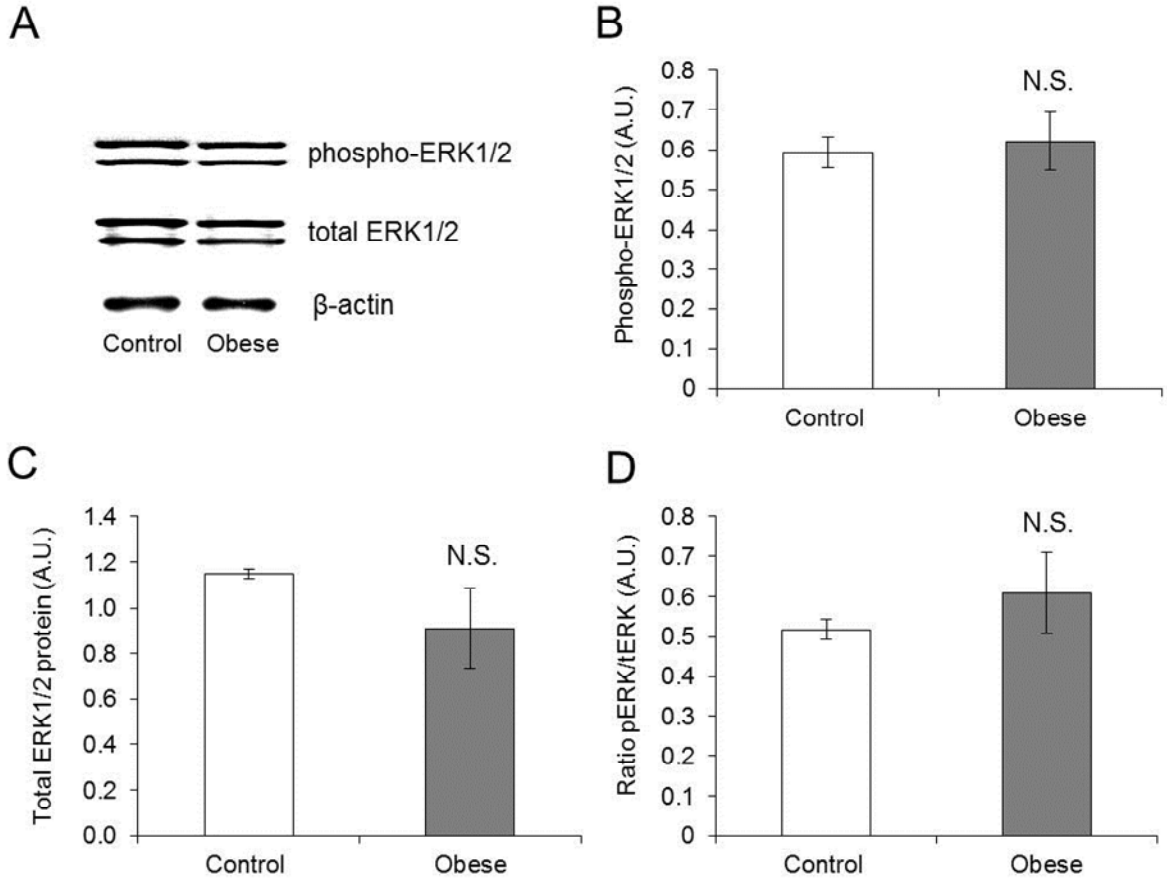
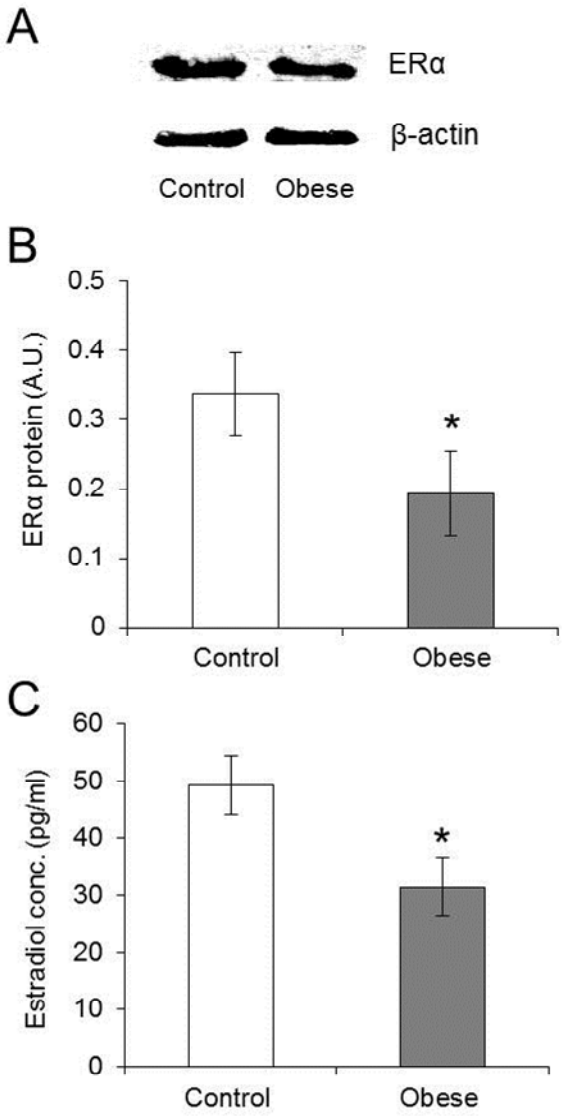

A
phospho-ERK1/2
total ERK1/2
β-actin
Control
Obese
B
Phospho-ERK1/2 (A.U.)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N.S.
Control
Obese
C
Total ERK1/2 protein (A.U.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N.S.
Control
Obese
D
Ratio pERK/tERK (A.U.)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N.S.
Control
Obese

Figure 6.


A
ERα
β-actin
Control
Obese
B
ERα protein (A.U.)
0.5
0.4
0.3
0.2
0.1
0
*
Control
Obese
C
Estradiol conc. (pg/ml)
60
50
40
30
20
10
0
*
Control
Obese