Title: Estradiol-mediated regulation of hepatic iNOS in obese rats: Impact of Src, ERK1/2, AMPKα, and miR-221

Anastasija Panic[1], Julijana Stanimirovic[1], Milan Obradovic[1], Emina Sudar-Milovanovic[1], Milan Perovic[3], Milena Lackovic[4], Nina Petrovic[1,2] and Esma R Isenovic[1]

[1]Vinca Institute of Nuclear Sciences, University of Belgrade, Laboratory of Radiobiology and Molecular Genetics, Mike Petrovica Alasa 12-14, 11000 Belgrade, Serbia

[2] Institute for Oncology and Radiology of Serbia, Pasterova 14, Belgrade 11000, Serbia

[3] Clinic for Gineacology and Obstetrics "Narodni front", Kraljice Natalije 62, 11000 Belgrade, Serbia

[4] Clinical Hospital Centre Zemun, Clinic for internal medicine, University of Belgrade, Vukova 9, 11000 Belgrade, Serbia

Correspondence

Esma R. Isenovic, Ph.D. & Anastasija Panic, MSc, Vinca Institute of nuclear science, 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 & anastasijap16@gmail.com

Abstract

Purpose:

This study aimed to investigate *in vivo* effects of estradiol on the regulation of hepatic inducible nitric oxide synthase (iNOS) expression in the high fat (HF) diet-induced obesity. Also, we aimed to investigate whether activation of the extracellular regulated kinase (ERK1/2), adenosine monophosphate-activated protein kinase (AMPK), Src kinase, and miR-221 is involved in estradiol-mediated regulation of iNOS in the liver of obese male Wistar rats. Male Wistar rats were fed a standard laboratory diet or a HF diet for ten weeks. Half of HF rats were treated with estradiol intraperitoneally (40 μg/kg), while the other half were placebo-treated 24 hours before euthanasia. Results show that estradiol treatment of HF rats decreased hepatic iNOS mRNA ($p < 0.05$) and protein expression ($p < 0.01$), the protein levels of p65 subunit of NFκB ($p < 0.05$) and ERα ($p < 0.05$), ERK1/2 phosphorylation ($p < 0.001$) and ERα/Src kinase association ($p < 0.05$). By contrast, hepatic Src protein level ($p < 0.05$), AMPKα phosphorylation ($p < 0.05$) and miR-221 expression ($p<0.05$) were increased in HF

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rats after estradiol treatment. Our results indicate that estradiol *in vivo* regulates hepatic iNOS expression in obese rats via molecular mechanisms involving ERK1/2, AMPK, Src, and miR-221 signaling.

Key words: estradiol; liver; HF diet; iNOS; miR-221.

1. Introduction

Visceral obesity is increasingly being suggested as an independent risk factor for the development of diabetes, cardiovascular diseases, and metabolic syndrome. These disorders share a common pathological condition, insulin resistance (IR) [1]. The precise triggers of inflammation in obesity are not yet fully understood. A hypothesis posits that nutrient overload in metabolic cells induces intracellular stress which results in the activation of inflammatory cascades and impaired insulin signaling [2], through the activation of the nuclear factor κB (NFκB) pathway [3]. In order to mediate inflammation-involved IR, NFκB regulates expression of inducible nitric oxide synthase (iNOS) gene [4]. A growing body of evidence indicates that iNOS expression and the excessive production of NO are involved in the development of various types of liver diseases [5, 6]. Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver diseases at the moment, and it usually develops in the setting of obesity and IR [7].

Estrogens regulate various aspects of glucose and lipid metabolism in different organs [8]. Estradiol is the most important biologically relevant form of estrogens [8]. Estradiol regulates iNOS protein expression via estrogen receptors (ER) [9] As estradiol binds to its receptors with identical affinity, the metabolic effects of individual ER isoforms, ERα and ERβ, are hard to differentiate and appear to be tissue- and species-specific. There is evidence that ERα mediates protective anti-inflammatory effects in various tissues, including liver [10-12]. Non-receptor intracellular Tyr kinase Src has a central role in estradiol-ER cytoplasmic signaling [13]. The association of ER and Src is followed by activation of different intracellular kinases, including extracellular signal-regulated kinases 1/2 (ERK1/2) [14]. Phosphorylated ERK1/2 further phosphorylates and activates nuclear transcription factors, including NFκB [13]. Adenosine monophosphate-activated protein kinase (AMPK) is a major regulator of energy metabolism and an effective inhibitor of inflammatory responses through iNOS [15] and NFκB [16] inhibition. The activation of ERK1/2 is required for induction of miR-221 [17], which modulates an inflammatory response by controlling the NFκB activity [18]. miR-221 is a member of microRNAs (miRNAs) family whose abnormal expression was observed in obesity [19] and in mouse and human liver diseases [20, 21]. In this study, we examined the effects of *in vivo* treatment with 17β-estradiol of high fat (HF)-induced obese male rats on the regulation of hepatic iNOS expression. Furthermore, we examined

the involvement of NFκB, ERα, Src, ERK1/2, AMPKα, and miR-221 in the estradiol-regulated expression of hepatic iNOS.

2. Experimental procedures

2.1 Animals and experimental treatment

Eight weeks old male Wistar rats (body mass 150 g - 200 g) bred at the Institute of Nuclear Sciences (Vinca, Belgrade) were used in this study. The animals were kept under a 12:12-hour light/dark cycle at 22 ± 2 °C and divided into three experimental groups. The first group (labeled as CONTROL) was a group fed standard laboratory diet (Veterinarski zavod Subotica, Subotica, Serbia), the second group (labeled as HF) was fed standard laboratory diet enriched with 42% fat (HF diet) and the third group (labeled as HF+Estradiol) was a HF diet-fed group treated intraperitoneally with a bolus injection of 40 μg/kg of 17β-estradiol (Sigma E8875, purity ≥98%; Sigma-Aldrich Corporation, St. Louis, MO, USA) dissolved in 1% ethanol in phosphate-buffer solution (PBS), 24 hours before euthanasia. The control group and the HF group were injected at the same time with the same volume of 1% ethanol in PBS. The diet was free of phytoestrogens, and food and water were available to rats *ad libitum*. After ten weeks, all the rats were anesthetized with ether (Lek, Ljubljana, Slovenia) and euthanized. The livers were excised, weighed, snap frozen in liquid nitrogen and stored at −70 °C until further experiments. The experimental treatment was approved by the official Vinca Institute Ethical Committee for Experimental Animals.

2.2 Liver lysate preparation

The livers were homogenized on ice with an Ultra-Turrax homogenizer in a lysis buffer (150 mM NaCl, 20 mM Tris, 2 mM EDTA, 2 mM DTT, 1% Triton X-100, 10% glycerol, pH 7.4) containing protease (Complete Ultra Tablets, Mini, EDTA-free, EASYpack) and phosphatase inhibitor (PhosStop) cocktails (Roche, Mannheim, Germany), and 2 mM sodium orthovanadate. Following a 60-minute incubation of homogenates with constant rotation at 4 °C, the samples were centrifuged for 30 min at 100,000 × g at the same temperature. The supernatants were obtained, and total protein concentration was determined using the Lowry assay [22]. The final lysates were stored at -70°C until further experiments.

2.3 SDS-PAGE and western blotting

Total lysates protein extracts (80 μg/lane) were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [23] and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% bovine albumin and probed with rabbit polyclonal anti-ERα, ERβ, anti- NFκB -p65, goat polyclonal anti- NFκB -p50, mouse monoclonal anti-β-actin (Santa Cruz

Biotechnology, CA, USA), rabbit polyclonal anti-Src, anti-phospho-p44/42 ERK1/2 (Thr^{202}/Tyr^{204}), anti-total-p44/42 ERK1/2, anti-phospho-AMPKα (Thr^{172}), anti-total-AMPKα (Cell Signaling Technology, Beverly, MA), or anti-iNOS (Abcam, Cambridge, UK) antibodies. After washing with TBS-T, the membranes were incubated with the appropriate secondary horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgG antibodies (Santa Cruz Biotechnology, CA, USA) and used for subsequent detection with the electrochemiluminescence (ECL) (GE Healthcare Life Sciences, Buckinghamshire, UK) method. Following phospho-p44/42 ERK1/2 and phospho-AMPK α analysis, the membranes were stripped and reblotted with antibodies detecting the total p44/42 ERK1/2 and total AMPKα content, respectively. To ensure that protein loading was equal in all samples, the blots were re-probed with anti-β-actin antibody and appropriate secondary antibody. Image J 1.45s software (NIH, USA) was used for the quantification of the signals on membranes.

2.4 Co-immunoprecipitation

Liver lysates were normalized for protein content (500 mg of protein) and incubated overnight with 2 mg of anti-ERα antibody. Immunocomplexes were absorbed with protein A/G-sepharose (prepared according to manufacturer's instructions, Santa Cruz Biotechnology, CA, USA) overnight at 4ºC, recovered by centrifugation (2500 × g; 5 min), washed three times with lysis buffer and separated on SDS-PAGE gel. Next, the proteins were transferred from a gel to a PVDF membrane, and membranes were probed with an anti-Src antibody. The signals obtained were detected and quantified using ImageJ software.

2.5 Total RNA isolation and qPCR

Total hepatic RNA (mRNA and miRNA) was extracted using Trizol reagent according to the manufacturer's recommended procedure (Invitrogen Life Technologies, Paisley, GB). Concentration and purity of isolated RNA were determined by measuring the absorbance at 260 nm/280 nm. To ensure that isolated RNA was not degraded, total RNA samples were analyzed by 1.2% agarose gel electrophoresis. The reverse transcription reaction was performed using 1 μg of total RNA and a commercially available RevertAid H minus First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). For reverse transcription of miR-221, we used 10 ηg of total RNA, TaqMan MicroRNA Reverse Transcription Kit, Taqman Universal master mix 2x, No UNG amperase, according to manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The expression levels of hepatic iNOS and β-actin mRNA and miR-221 were measured by the quantitative real-time PCR (qPCR) assay performed on 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in 96-well reaction plates (MicroAmp Optical, ABI Foster City, CA) in 20 μl volume/well containing 10 μl of Brilliant III SYBR qPCR MasterMix with lowROX (Agilent

Technologies, Santa Clara, CA, USA) and 10 µl of appropriate sample diluted in demineralized water and pairs of primers. The level of expression of *Nos2* gene was normalized to β-actin mRNA level detected in the identical sample. All assays were performed in triplicate. Primers for rat Actb (β-actin) and Nos2 (iNOS) genes were designed using Primer Express1 software v2.0 (Applied Biosystems) and purchased from Metabion, Martinsried, Germany. Primers for iNOS (GenBank accession number: NM_012611) were 5′-AGAAGTCCAGCCGCACCAC- 3′ (forward primer) and 5′-TGGTTGCCTGGGAAAATCC-3′ (reverse primer) with PCR product length of 103 bp and primers for rat Actb (GenBank accession number: NM_031144) were 5′-CCCTGGCTCCTAGCACCAT-3′ (forward primer) and 5′-GAGCCACCAATCCACACAGA-3′ (reverse primer) with PCR product length of 76 bp. TaqMan microRNA assays (Thermo Fisher Scientific, Waltham, MA, USA) were used for miR-221 and small nuclear RNA-U6B amplification. miR-221 expression levels were normalized to endogenous control U6B. Cycle threshold values (Ct) were analyzed and relative quantification of mRNA and miRNA expression was performed by the $2^{-\Delta\Delta Ct}$ method [24].

2.7 Statistical analysis

Results are expressed as mean ± SEM (standard error of the mean). Statistical significance of the data was evaluated using a two-tailed student's *t-test*. For correlation analysis, we used non-parametric Spearman's correlational test. For target prediction analysis, we used miRGrate prediction tool [25]. A p-value < 0.05 was considered as statistically significant.

3. Results

Since NO has important roles in the regulation of energy metabolism and insulin sensitivity in the state of obesity [26-29] and since iNOS is an important inflammatory mediator linking metabolic disorders and inflammation [30], in this study we first examined whether *in vivo* estradiol treatment of HF rats had an effect on hepatic iNOS gene and protein expression. Our results show that estradiol treatment of HF rats causes a reduction in hepatic iNOS mRNA ($p < 0.05$) expression compared with HF group (Figure 1A). Additionally, estradiol treatment decreased the level of hepatic iNOS protein in HF rats compared with untreated HF rats ($p < 0.01$) (Figure 1B). Since NFκB is a transcription factor for iNOS gene [4], we further examined protein levels of the hepatic NFκB subunits p50 (NFκB-p50) and p65 (NFκB-p65) in HF rats after estradiol treatment. The results show that estradiol treatment did not cause any significant changes in the level of the hepatic NFκB-p50 subunit (Figure 1C), while estradiol treatment induced a decrease of hepatic NFκB-p65 subunit protein level in HF rats ($p < 0.05$) (Fig. 1D) compared with untreated HF group.

In the next set of experiments, we examined the changes in the hepatic ERα and ERβ protein levels and in the association level of ERα/Src in estradiol-treated HF rats. We additionally examined the effects of estradiol treatment of HF rats on hepatic Src protein level. The results show that estradiol treatment of HF rats decreases the protein level of hepatic ERα ($p < 0.05$) (Figure 2A) while not affect the ERβ protein level (Figure 2B). Treatment with estradiol also decreases ERα/Src association ($p < 0.05$) (Figure 2C), while it increases the Src protein level ($p < 0.05$) (Figure 2D) compared with HF rats.

To examine the involvement of ERK1/2 and AMPKα kinases in the estradiol-mediated regulation of hepatic iNOS in HF rats, we measured total and phospho forms of both kinases using immunoblot analysis only when phosphorylated at Thr^{202}/Tyr^{204} of ERK1/2 and Thr^{172} of AMPKα. The results show that estradiol treatment of HF rats induces a decrease ($p < 0.001$) in phosphorylation of hepatic ERK1/2 at Thr^{202}/Tyr^{204} (Figure 3A) compared with HF rats. By contrast, the phosphorylation of hepatic AMPKα at Thr^{172} increases after estradiol treatment in HF rats ($p < 0.05$) (Figure 3B) compared with untreated HF group.

Because the abnormal expression of miR-221 was observed in the state of obesity [19] and various liver diseases [31], we next examined the effects of estradiol treatment on the level of hepatic miR-221 expression in HF diet-induced obese rats. Results show that treatment with estradiol increased miR-221 expression ($p < 0.05$) in HF rats compared with untreated HF group (Figure 4A). To examine whether the expression of hepatic miR-221 in the HF rats treated with estradiol shows relation to the levels of protein components of Src/ERK1/2/NFκB signaling pathway, we used Spearman's correlation test. The analysis shows a negative correlation between miR-221 and Src in estradiol-treated HF group ($p = 0.026$, $r = -0.974$) (Figure 4B).

4. Discussion

The principal discovery in this study is that estradiol regulates hepatic iNOS expression *in vivo* in the HF diet-induced obesity via molecular mechanisms involving interaction between ERα and Src, and miR-221 signaling. We have previously reported that a HF diet leads to the development of obese phenotype, whole body IR and a significant increase in liver mass and liver/body mass ratio [32, 33]. Additionally, we have also reported that a HF diet caused lipid dysregulation in the circulation [32]. In the liver, increased lipid accumulation leads to altered hepatic lipid metabolism [34] and activation of inflammatory pathways [35]. The expression of iNOS can be induced in the liver by cytokines and other inflammatory agents. Also, dysregulation of iNOS is involved in the pathogenesis of obesity-linked IR [35], and iNOS has both detrimental and protective effects in IR and hepatic insulin signaling [36, 37]. We have previously shown a significant reduction in hepatic iNOS protein level in HF diet-fed male rats [33]. In this study, we show that HF diet induces a significant increase in the

hepatic iNOS mRNA level, confirming that HF diet-induced obesity leads to the activation of certain inflammatory responses and that decreased level of hepatic iNOS protein of HF rats is probably a consequence of the post-translational regulatory mechanisms [33, 38].

Our current results show that treatment with estradiol decreased both hepatic iNOS mRNA and protein levels compared with HF rats. Similarly to our results, Nweze et al. demonstrated that incubation of rat hepatocytes with estradiol inhibited cytokine-stimulated hepatocyte NO production and iNOS mRNA and protein levels [39]. The level of iNOS expression is primarily regulated at the transcriptional level [40], and NO produced after stimulation with cytokines inhibits hepatic iNOS gene and protein expression through a negative feedback mechanism, whereby inhibition of transcription is achieved, at least in part, through a decrease of NFκB DNA-binding activity [41]. Our previously published results show that HF diet induced a significant increase of plasma nitrite/nitrate level [33], while estradiol treatment of HF rats did not change plasma nitrite/nitrate level (data not shown). A high level of nitrite/nitrate in the plasma of HF rats treated with estradiol could be responsible for decreased hepatic iNOS expression, as NO can diffuse rapidly into cells [38] and inhibit iNOS gene and protein expression [41]. Negative feedback regulation of iNOS gene and protein expression limit overproduction of NO during pathophysiological conditions and prevent further liver tissue injury [41].

Hepatocytes contain both of the primary ER mediating the physiological effects of estradiol, ERα and ERβ [42]. Although ERα is the dominant ER in the liver, the phenotype of the ERβ-deficient mice is characterized by increased insulin sensitivity and decreased accumulation of triglycerides in the liver, indicating that ERβ might have a diabetogenic function [43]. However, we have reported no changes in the ERβ protein level in the liver of obese rats compared with control, non-obese rats [44]. In this study, our results related to the effects of estradiol treatment on the protein level of hepatic ERβ show no significant change in HF rats compared with untreated HF rats. In contrary, the protein level of hepatic ERα was decreased in estradiol-treated HF rats compared with untreated HF rats in our study. Several studies have shown that estradiol-mediated induction of iNOS promoter activity is related to ERβ, while inhibition of iNOS expression was achieved via ERα [45, 46]. Also, the primary ER shown to be responsible for estradiol-mediated regulation of iNOS expression in the liver is ERα [12]. Thus, we assume that the effect of estradiol on the changes of hepatic iNOS expression in our study is mediated primarily via ERα. We have previously demonstrated that ERα protein level was decreased in the liver of HF rats [44], and we have assumed that this could be associated with these rats being predetermined to develop IR [47]. Treatment with estrogens induces an ERα-mediated decrease in liver fat storage and diacylglycerol content and, induces insulin action in ovariectomized mice fed a HF diet [48]. However, ER

protein undergoes translational regulation, and this phenomenon has been reported to occur in diabetes [49]. Estradiol induces rapid proteasome-dependent down-regulation of ERα receptors and consequent ERα degradation, linked to ERα-mediated transcriptional responses [50]. Also, long-term exposure to estradiol is needed to up-regulate steady-state ERα and maintain the estrogen target tissues such as liver in an estrogen-responsive condition [51]. In our study, rats were treated with a single injection of estradiol 24 hours before euthanasia, and this could be a reason for the observed decrease in the hepatic ERα protein level. Another mechanism that may contribute to decreased hepatic ERα protein level in our HF rats treated with estradiol could be a translocation of ligand-bound ERα to the nucleus [52]. In order to stimulate cytoplasmic signaling after binding of estradiol, ERα needs to interact with some other molecules since ERα does not exhibit kinase activity. One of such molecules is non-receptor Tyr kinase Src, a key proximal signaling molecule in multiple pathways [13]. Besides their critical role in the progression of cancers [53, 54], Src family kinases are also involved in the inflammation-related pathways that have important role in the activation of inflammatory cells in normal tissues [55]. Activation of Src is a consequence of a cytokine-mediated inflammatory microenvironment, and, inversely, the cytokine production is driven by Src kinases [56, 57]. The treatment of mice with the Src inhibitor PP1 blocks the increase in Src activation and consequent elevation in IL-6 production by Kupffer cells under hypoxic conditions [58], suggesting that Src may be an inhibitory target for liver inflammation. The Src kinases are basally inactive as their catalytic domain is constrained in an inactive state through intramolecular interactions. Binding of Src protein to other phosphorylated Tyr residues induces a release of these constraints and catalytic activation of the protein [59]. ERα has been reported to be Tyr phosphorylated by c-Src or other enzymes, which enables the interaction of the receptor with the SH2-domain of c-Src [59]. In our study, the treatment of HF rats with estradiol increases the level of total hepatic Src protein content, but decreases association of ERα and Src proteins. Our results suggest that estradiol treatment decreases hepatic Src activity dependent on the interaction with ERα in the conditions of obesity and inflammation.

The expression of iNOS is primarily regulated at the transcriptional level. NFκB is a key transcription factor controlling molecular mechanisms related to immediate-early inflammation. The activation of NFκB has been demonstrated in a number of liver diseases [60]. Most members of NFκB family of proteins can homodimerize, as well as form heterodimers with each other. Mammary NFκB heterodimers mainly consisting of p50 and p65 subunits are involved in the activation of inflammatory genes, with iNOS being one of the main NFκB-p65 downstream targets [61]. In resting cells, NFκB proteins are predominantly located in the cytoplasm in the association with the members of inhibitory IκB protein family. Phosphorylation and degradation of IκB expose

nuclear localization signals on the NFκB proteins, leading to their nuclear translocation and binding to specific regulated sequences in the DNA [62]. Our results show that estradiol treatment of HF rats did not cause any significant change in the level of hepatic NFκB-p50 protein, while it induced a significant decrease in the hepatic NFκB-p65 protein level. The results of this study suggest that estradiol treatment of HF rats stimulates the activation and nuclear translocation of hepatic NFκB-p65 protein. Previous studies reported an inhibitory effect of prolonged treatment with estradiol on NFκB activation [63, 64], while the rapid activation of NFκB protein points to a non-genomic response to estradiol [65], which is consistent with acute estradiol treatment applied in our rats. Stice et al [65] reported that activation of NFκB by estradiol is highly regulated and requires coordination of multiple pathways to induce the protective effects of acute *in vivo* estradiol treatment. ERK1/2-dependent signaling mechanism may temporally control the activation of NFκB and NFκB-dependent gene expression. Even though the activation of ERK1/2 is required to induce persistent inflammation-induced activation of NFκB [66], inhibition of ERK1/2 activation has no effect on early transient NFκB activation. Thus, one of the roles of ERK1/2 signaling in iNOS induction is apparently to maintain the persistent activation of NFκB [67]. In our study, we assume that acute estradiol treatment of HF rats induced early NFκB activation and translocation, despite the decreased ERK1/2 activity in the liver. However, decreased hepatic iNOS gene expression in HF rats treated with estradiol indicates that NFκB-p65 translocation into nucleus did not lead to its full transcriptional activation. As NFκB may achieve its function in concert with other transcription factors, such as AP-1 [68], the absence of activation of some other transcription factor could be a possible explanation for decreased iNOS gene expression despite the NFκB-p65 nuclear translocation. In addition, molecular cross-talk between nuclear transcription factors in which the ER mediates inhibition of NFκB activity has been demonstrated [69]. The functional interaction between ER and NFκB-p65 has been demonstrated both *in vitro* and *in vivo* [70] and several groups have identified the potential for a reciprocal transcription inhibition between agonist-bound ER and activated NFκB-p65 [71]. Treatment with estradiol of Sprague-Dawley male rats prevented increases in iNOS protein and mRNA levels through an ER-dependent pathway [72]. An increased estradiol concentration enhanced the translocation of ER into nucleus [73] and hence the association of NFκB-p65/ER was increased causing decreased NFκB transcriptional activation. A direct interaction between ER and NFκB may introduce conformational changes in both proteins that prevent them from binding DNA or result in the formation of inactive complexes on the DNA, which prevent interaction with essential cofactors or basal transcriptional machinery [74]. Since our results show decreased hepatic ERα protein level in the cytoplasm, it is possible that translocated ERα inhibits transcriptional activation of NFκB-p65 in the nucleus.

The association of ER and Src initiates signaling through a number of intracellular pathways, including ERK1/2 [75]. The activation of ERK1/2 cascade might play a key role in the pathogenesis of obesity and its associated metabolic alterations [76]. We have previously shown that the level of ERK1/2 phosphorylation was decreased in the liver of HF male rats [44], which may be associated with the IR developed in our rats [32]. The results of the current study show that estradiol treatment of HF rats led to a further decrease in the Thr^{202}/Tyr^{204} phosphorylation of the hepatic ERK1/2. Even though the treatment with estradiol leads to the activation of ERK and its translocation to the nucleus [77], Lobenhofer et al. failed to detect any activation of ERK by estradiol [78]. Caristi et al. reported that the activation of ERK by estradiol is a poorly reproducible event [79], while Gaben et al. reported that estradiol does not induce rapid MAPK/ERK phosphorylation [80]. Decreased hepatic ERK1/2 phosphorylation in our study is in accordance with the decreased association of ERα and Src proteins after estradiol treatment.

Adenosine monophosphate-activated protein kinase is an intracellular energy sensor that plays a key role in the regulation of glucose and lipid metabolism in muscle and liver. Common features of the metabolic abnormalities observed in metabolic syndrome or diabetes mellitus type 2 include the dysregulation of AMPK [81]. We have previously reported that phosphorylation of AMPKα was decreased in the liver of HF male rats [44], and this could be in the connection to the development of hyperglycemia and IR [32]. In the current study, we show that estradiol treatment of the HF rats leads to the increase in AMPKα phosphorylation and this result is in line with literature data showing that estradiol activates AMPK [82]. Kim et al. reported that estradiol stimulates the activation of AMPK via an ER, probably ERα [82]. Our results showing an increased activity of AMPKα are in accordance with the observed decreased protein level of hepatic iNOS in estradiol-treated HF rats. It has been reported that AMPK-activating agents inhibit iNOS induction and NO production in cells and tissues exposed to inflammatory mediators. AMPK activation also suppressed NO production in cytokine/LPS-treated adipocytes and in adipose tissue of LPS-challenged rats [15]. The inhibitory effects of AMPK activation on NO production were highly correlated with the reduction in iNOS protein content, suggesting that AMPK modulates iNOS protein turnover [83]. Our results also suggest that estradiol-mediated activation of AMPKα could contribute to the reduced hepatic iNOS protein content in HF rats.

miRNAs are short non-coding RNA molecules that base-pair imperfectly with target mRNA molecules and act as negative regulators of gene expression via modulation of the mRNA stability and/or translational efficiency. miRNAs have a variety of roles in modulating immune and inflammatory responses [84]. miR-221 is a member of the miRNA family, which participates in the development of diabetes and tumors, and a common factor in the

pathogenesis of both diseases is IR [19, 20]. The expression of miR-221 is upregulated in rats and humans with fatty liver disease [21]. In the liver of obese mice, miR-221 is highly expressed and the silencing of miR-221 can improve the insulin sensitivity and decrease blood glucose [20]. In the current study, we have also examined the changes of hepatic miR-221 expression after estradiol treatment in HF rats. We have observed that treatment of HF rats with estradiol, induced a significant increase in the level of hepatic miR-221 expression. The conversion rate of estradiol from estrone in peripheral tissues, such as adipose tissue, is higher in obese people than that in lean people and estrogen level is positively correlated with body weight [85]. White adipose tissue in obese men exhibits elevated aromatase activity leading to elevated estrogen levels [86]. It is possible that estradiol treatment of HF rats in our study has induced an increase in hepatic miR-221 level due to high estradiol levels in the system [87], since increased expression of miR-221 has also been observed in estrogen-dependent breast cancer tissues [88]. Furthermore, increased miR-221 level could be a contributing factor leading to a decreased hepatic NFκB-p65 protein level observed in our experiments, since miR-221 might promote degradation of IkBα [18]. Phosphorylation of IkB leads to its ubiquitination and subsequent degradation, which in turn liberates the p65 subunit of NFκB to be translocated into the nucleus [62]. In addition, a prediction analysis of miR-221 targets extracted Src mRNA as one of the potential targets in the liver of estradiol-treated rats, indicating the connection between estradiol, miR-221 and Src/ERK1/2 signaling pathway in the regulation of iNOS expression. However, the results of our study show negative correlation between increased miR-221 and total Src protein levels in estradiol-treated HF rats. Based on our results, we can speculate an existence of some additional estradiol-regulated mechanism that enables maintenance of increased Src protein level despite the elevated miR-221.

In summary, the results of our study indicate that *in vivo* estradiol treatment of HF male rats partially restores the detrimental effects of a HF diet-induced IR on the hepatic metabolism through the reduction of iNOS mRNA and protein expression. The molecular mechanism responsible for decreased iNOS mRNA and protein levels following estradiol treatment includes decreased ERα/Src interaction, decreased ERK1/2 activation and decreased level of NFκB-p65 protein. Estradiol treatment increased AMPKα activation, contributing to the reduction in iNOS protein level. Also, our results also suggest the involvement of miR-221 in the regulation of iNOS expression possibly influencing NFκB-p65 translocation (Figure 5).

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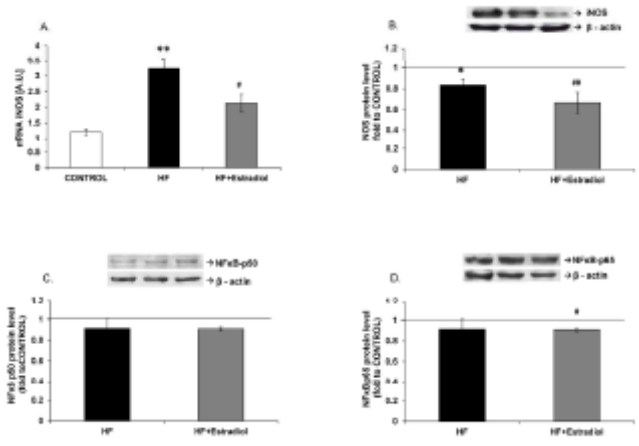

Figure 1. Effects of estradiol on the regulation of hepatic iNOS expression and p50/p65 subunits of NFκB protein. (A) mRNA level of hepatic iNOS expressed as arbitrary units (A. U.). (B) Densitometry data for six separate Western blots (each bar is mean ± SEM). The Y-axis represents iNOS protein level expressed as - fold

change to an appropriate control (arbitrary control set at 1). (C) Densitometry data for six separate Western blots (each bar is mean ± SEM). The Y-axis represents NFκB-p50 protein level expressed as - fold change to an appropriate control (arbitrary control set at 1). (D) Densitometry data for 3-5 separate Western blots (each bar is mean ± SEM). The Y-axis represents NFκB-p65 protein level expressed as - fold change to an appropriate control (arbitrary control set at 1). The X-axis represents treatment. Inserts show representative Western blots. */#p < 0.05, **/##p < 0.01 indicate significant differences. *fold to CONTROL, #fold to HF

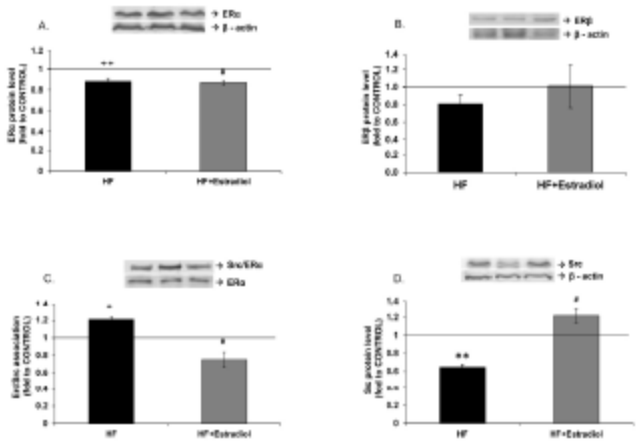

Figure 2. Effects of estradiol on the hepatic ERα protein level, ERα/Src association and Src protein level. (A) Densitometry data for 3-6 separate Western blots (each bar is mean ± SEM). The Y-axis represents ERα protein level expressed as - fold change to an appropriate control (arbitrary control set at 1). (B) Densitometry data for five separate Western blots (each bar is mean ± SEM). The Y-axis represents ERβ protein level expressed as - fold change to an appropriate control (arbitrary control set at 1). (C) Densitometry data for three separate Western blots (each bar is mean ± SEM). The Y-axis represents Src protein level in association with ERα expressed as - fold change to an appropriate control (arbitrary control set at 1). (D) Densitometry data for six separate Western blots (each bar is mean ± SEM). The Y-axis represents Src protein level expressed as - fold change to an appropriate control (arbitrary control set at 1). The X-axis represents treatment. Inserts show representative Western blots. */#p < 0.05, **p < 0.01 indicates a significant difference. *fold to CONTROL, #fold to HF

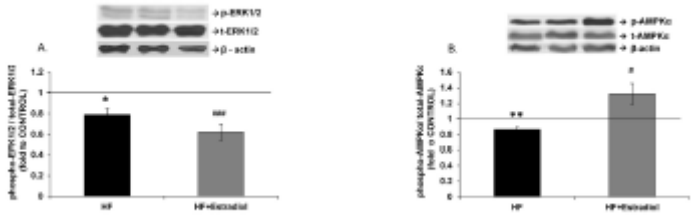

Figure 3. Effects of estradiol on the phosphorylation of hepatic ERK1/2 on Thr202/Tyr 204 and AMPKα on Thr172. (A) Densitometry data for seven separate Western blots (each bar is mean ± SEM). The Y-axis represents the ratio of phosphorylated and total ERK1/2 expressed as - fold change to an appropriate control (arbitrary control set at 1). (B) Densitometry data for seven separate Western blots (each bar is mean ± SEM). The Y-axis represents the ratio of phosphorylated and total AMPKα expressed as - fold change to an appropriate control (arbitrary control set at 1). The X-axis represents treatment. Inserts show representative Western blots. */#p < 0.05, **p < 0.01, ###p < 0.001 indicate significant differences. *fold to CONTROL, #fold to HF

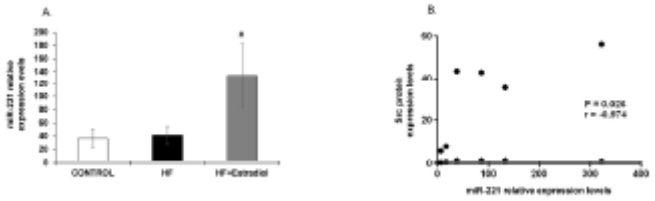

Figure 4. Effects of estradiol on the hepatic miR-221 expression. (A) The level of miR-221 gene expression. The Y-axis represents miR-221 level expressed in relative units (each bar is mean ± SEM), and the X-axis represents treatment. #p<0.05 indicates a significant difference. (B) Correlation analysis between miR-221 expression level and Src protein level. The analysis shows a negative correlation between miR-221 and Src in estradiol-treated HF group. The Y-axis represents Src protein expression levels, and the X-axis represents miR-221 level expressed in relative units. R → -1 indicates a negative correlation; p < 0.05 indicates the statistical significance of the R-value.

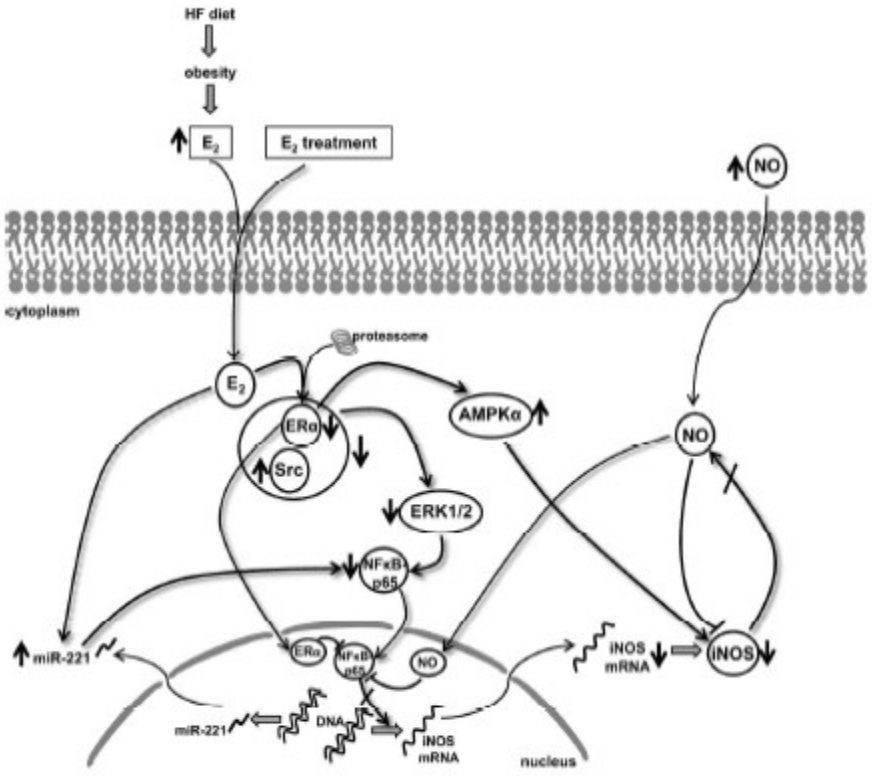

Figure 5. A proposed mechanism of iNOS expression regulation in the liver of estradiol-treated obese rats. Estradiol passes through the cell membrane into the cell and bounds to the specific ERα receptors. Estradiol-bound hepatic ERα receptors translocate into the nucleus, contributing to decreased cytoplasmic ERα protein level. Increased level of estradiol may induce proteasome-dependent down-regulation and consequent degradation of ERα protein. Association of ERα receptor and Src kinase, needed to enable cytoplasmic signaling downstream of estradiol-ERα binding, decreases in the liver after estradiol treatment, suggesting decreased hepatic Src activity. Accordingly, treatment with estradiol also results in decreased hepatic ERK1/2 activation. However, decreased activation of ERK1/2 has no effect on NFκB-p65-activation, and hepatic NFκB-p65 protein translocates into the nucleus after estradiol treatment. Decreased hepatic iNOS gene expression indicates that translocated NFκB-p65 protein did not achieve its full transcriptional activation in the nucleus, possibly due to the molecular cross-talk between ERα and NFκB-p65 and NO-mediated inhibition of iNOS gene transcription (treatment of HF rats with estradiol increases plasma nitrite/nitrate level). The reduction of iNOS protein level may also be a consequence of the increased hepatic AMPKα phosphorylation and its activation after estradiol treatment. The elevated level of estradiol in estradiol-treated HF rats may be a reason for the increased hepatic miR-221 level. miR-221 promotes degradation of IkBα, which in turn liberates the p65

subunit of NFκB to be translocated into the nucleus. **HF** - high fat; **iNOS** - inducible nitric oxide synthase; **ERα** - estrogen receptor alpha; **ERK1/2** - extracellular signal-regulated kinases 1/2; **NFκB** - nuclear factor kappa B; **AMPKα** - adenosine monophosphate-activated protein kinase; **IκBα** - inhibitor of kappa B alpha; - decreased level of RNA/protein; - increased level of RNA/protein; - proposed mechanism.