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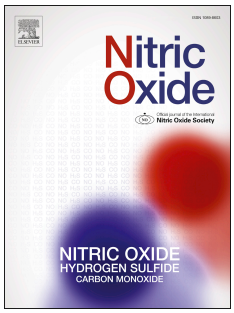

A high fat diet induces sex-specific differences in hepatic lipid metabolism and nitrite/nitrate in rats

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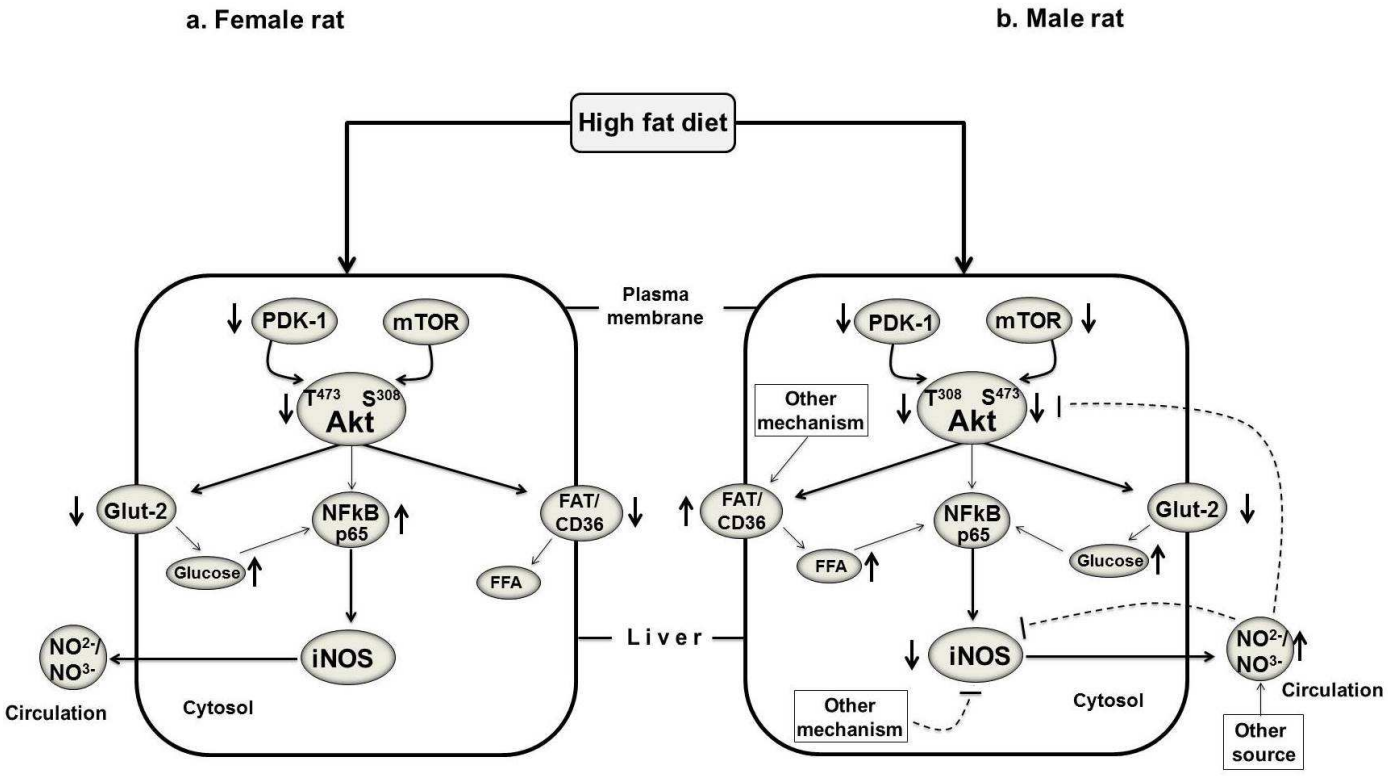
Graphical abstract



The proposed mechanism by which a HF diet induces altered hepatic lipid metabolism and affects regulation of hepatic iNOS expression and nitrite/nitrate production in a sex-specific manner in rats. Altered hepatic lipid metabolism caused by a HF diet influence iNOS/NO pathway and thus promotes IR by attenuating insulin action. This is likely a consequence of decreased Akt activation by PDK-1 and mTOR, which further allows regulation of glucose and FFA membrane transporters. Differences in HF-fed female and male rats in IR manifestation, insulin signalling disturbance, iNOS induction and nitrite/nitrate production is likely due to protective effects of estradiol which mitigate effects of a HF diet on insulin action. Among other mechanisms, large amounts of nitrite/nitrate produced in other tissues may easily enter the liver and inhibit iNOS protein expression, but also disturb insulin action. Akt, protein kinase B; FAT/CD36, fatty acid translocase/cluster of differentiation 36; FFA, free fatty acids; Glut-2, glucose transporter 2; IR, insulin resistance; p65, p65 subunit of nuclear factor κB; iNOS, inducible nitric oxide (NO) synthase; mTOR, mammalian target of rapamycin; NO^{2-}/NO^{3-}, nitrite/nitrate; PDK-1, phosphoinositide-dependent kinase-1.

A high fat diet induces sex-specific differences in hepatic lipid metabolism and nitrite/nitrate in rats

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ABSTRACT

Men and women differ substantially in regard to the severity of insulin resistance (IR) but the underlying mechanism(s) of how this occurs is poorly characterized. We investigated whether a high fat (HF) diet resulted in sex-specific differences in nitrite/nitrate production and lipid metabolism and whether these variances may contribute to altered obesity-induced IR.

Male and female Wistar rats were fed a standard laboratory diet or a HF diet for 10 weeks. The level of plasma nitrite/nitrate, as well as free fatty acid (FFA), in both plasma and liver lysates were assessed. The levels of inducible nitric oxide (NO) synthase (iNOS), p65 subunit of NFκB, total and phosphorylated forms of Akt, mTOR and PDK-1 in lysates, and the levels of glucose transporter 2 (Glut-2) and fatty acid translocase/cluster of differentiation 36 (FAT/CD36) in plasma membrane fractions of liver were assessed.

HF-fed male rats exhibited a significant increase in plasma nitrite/nitrate, and hepatic FFA and FAT/CD36 levels compared with controls. They also displayed a relative decrease in iNOS and Glut-2 levels in the liver. Phosphorylation of Akt (at Ser^{473} and Thr^{308}), mTOR and PDK-1 was also reduced. HF-fed female rats exhibited increased levels of NFκB-p65 in liver compared with controls, while levels of Glut-2, FAT/CD36 and Akt phosphorylation at Thr^{308} and PDK-1 were decreased.

Our results reveal that altered lipid and glucose metabolism in obesity, lead to altered iNOS expression and nitrite/nitrate production. It is likely that this mechanism contributes to sex-specific differences in the development of IR.

Keywords: high fat diet; sex differences; insulin resistance; liver metabolism; nitrite/nitrate

List of abbreviations:

Akt, protein kinase B; FAT/CD36, fatty acid translocase/cluster of differentiation 36; FFA, free fatty acids; Glut-2, glucose transporter 2; HF, high fat; HOMA, homeostasis model assessment index; HOMA-IR, HOMA-index of insulin resistance; HOMA-β, HOMA-index of β-cell function; IkB, inhibitor of NFkB; IkK, IκB Kinase; iNOS, inducible NOS; IR, insulin resistance; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin; NFκB, nuclear factor κB; NO, nitric oxide; PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; TC, total cholesterol

1. INTRODUCTION

Obesity is highly associated with insulin resistance (IR). In obese individuals the disturbance of glucose and lipid metabolism in the liver is a critical component of the pathogenesis of IR and is linked with inflammatory responses [1]. Inducible nitric oxide (NO) synthase (iNOS) has been proposed as an important inflammatory mediator, and may be the key link between metabolic disorders and inflammation [2, 3]. Besides its vital role in immunity, iNOS is known to be expressed in various metabolic disorders including diabetes [2, 4]. Inhibition of iNOS results in increased liver damage during ischemia reperfusion and sepsis [5, 6] highlighting a beneficial role of NO in the liver. However, increased expression of iNOS causes overproduction of NO which may lead to cell damage in the liver [3] suggesting that the balance of NO is crucial in determining its protective and cytotoxic effects.

The liver is an essential organ for energy homeostasis and takes an active part in synthesis, storage, and redistribution of free fatty acids (FFA) and glucose [7]. Disturbance in glucose and FFA metabolism is a common feature of IR [8]. The development of IR is suggested to be sex-specific due to different hormonal status between the sexes [9]. Excessive lipid accumulation can induce iNOS expression and NO production in metabolic tissues of both dietary and genetic models of obesity [2, 4, 7]. Experimental data suggest that altered hepatic lipid metabolism interferes with activation of inflammatory signaling pathways by pro-inflammatory cytokines, leading to overexpression of iNOS and consequent IR in the liver [3, 7, 10].

The risk of obesity related metabolic disorders and diabetes is well established in men, but less so for age matched pre-menopausal women [11]. Estradiol has a pivotal role in the protection against IR, since it regulates carbohydrate and lipid metabolism [12, 13]. A

potential protective mechanism proposed for estradiol involves its ability to regulate iNOS activity/expression [14, 15]. Previous data showed that significant sex differences in the synthesis of NO occur in multiple organs including the liver, and this is a direct result of both an increased expression of the iNOS protein [16] and an increase in the total level of NO [17].

The iNOS gene promoter is a binding site for several transcription factors, including NFκB [18, 19]. Inactive NFκB protein is localized in the cytosol where it complexes with the inhibitor of NFκB (IκB) [20]. Induction of iNOS is mediated through the stimulation of NFκB by different stimuli, including protein kinase B (Akt) [21]. Complete activation of Akt requires phosphorylation at two sites, whereby phosphoinositide-dependent protein kinase 1 (PDK-1) is responsible for phosphorylation of Akt at Thr^{308} and the mammalian target of rapamycin (mTOR) at Ser^{473}. However, increases in inflammatory mediators including iNOS may be linked with impaired insulin signaling in hepatocytes which is characterized by reduced expression of the insulin receptor substrate (IRS) and Akt [7, 22]. In obesity, hepatic lipid accumulation promotes production of numerous inflammatory cytokines, which are able to activate NFκB and further upregulate iNOS activity/expression [23, 24].

Previous studies showed differences related to tissues in which iNOS inhibition or deficiency exerts its insulin-sensitizing effects [4, 25]. However, most observations revealed that obesity and disturbed liver lipids metabolism lead to increased iNOS activity/expression causing hepatic insulin signaling disruption and promoting IR [2, 7, 26, 27]. Men and women differ substantially with regard to the severity of IR but the underlying mechanism(s) of how this sexual dimorphism occurs is poorly characterized. Thus, we propose that altered hepatic lipid metabolism in obesity leads to modification in iNOS expression and nitrite/nitrate

production, resulting in different hepatic pathology including IR, in female and male rats fed a HF diet. We also hypothesised that increased nitrite/nitrate production induced by a HF diet in male rats attenuates insulin action, leading to decreased Akt activation by PDK-1 and mTOR, which further allows regulation of glucose and FFA membrane transporters. This is likely a consequence of changes in hepatic iNOS through molecular mechanisms involving transcriptional regulator NFκB which also may be activated by high levels of glucose and FFA [28, 29]. In female rats disturbance in insulin action and iNOS induction caused by a HF diet may be attenuated by the protective effects of estradiol. Here we explore the effects of a HF diet on lipid and glucose metabolism, and regulation of plasma nitrite/nitrate levels and iNOS protein expression in livers of female and male rats. We also assess if changes in the level of iNOS are related to changes in the protein level of the p65 subunit of transcriptional regulator NFκB and the protein level and phosphorylation status of PDK-1, mTOR and Akt kinases.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Ether was obtained from Lek (Ljubljana, Slovenia). Protease inhibitor (Complete, Ultra Mini, EDTA-free) and phosphatase inhibitor (PhosStop) cocktails were obtained from Roche (Mannheim, Germany). Luminol and p-coumaric acid were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). The RIA insulin kit was a product of INEP (Zemun, Serbia). GLUC-PAP commercial kit was a product of Randox (Crumlin, UK). Commercially available kit for Beckman Coulter Olympus AU400 Analyzer was used for determination of total cholesterol (TC) (Beckman enzymatic reagent kit). The Nitrate/Nitrite Colorimetric Assay Kit was purchased from Cayman Chemical (Michigan, USA). Antibodies directed toward Akt, phospho-Akt (Ser^{473}) and phospho-Akt (Thr^{308}) were obtained from Abcam

(Cambridge, UK). Polyclonal anti-phospho-PDK-1 (Ser^{241}), anti-total PDK-1, anti-phospho-mTOR (Ser^{2448}) and anti-total mTOR, antibodies were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal anti-iNOS/NOS type-II antibody, anti-NFκB-p65 antibody, anti-Glut2 antibody, anti-FAT/CD36 antibody, monoclonal anti-actin antibody and secondary anti-mouse and anti-rabbit IgG horseradish peroxidase-linked antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Animals

Adult female and male Wistar rats (150-200 g, 8 weeks old) bred at the Institute of Nuclear Sciences (Vinca, Belgrade) were divided into 4 experimental groups: control female (female), female rats fed a high fat diet (HF female), control male rats (male), and male rats fed a high fat diet (HF male). Rats were kept under a 12:12 hour light-dark cycle at 22 ± 2°C with rat chow and water *ad libitum*. Control groups were fed for 10 weeks with a standard laboratory diet for rats composed of 20% proteins, 8% cellulose, 13% moisture, 1% calcium, 0.90% lysine, 0.75% methionine, 0.75% cysteine, 0.5% phosphorus, 0.15 – 0.25% sodium, vitamin mixture (A 10,000 IU/kg, D_3 1,600 IU/kg, E 25 mg/kg, B_{12} 0.02 mg/kg), mixture of minerals (in milligrams per kilogram: 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 (Veterinarski zavod Subotica, Subotica, Serbia). HF groups were fed a standard laboratory diet for rats enriched with 42% fat.

Animals were anesthetized with ether and decapitated. All experimental protocols were approved by Vinca Institute's Ethical Committee for Experimental Animals. Blood samples were collected and livers were excised, weighed, snap frozen in liquid nitrogen and stored at -70°C. Half of the collected blood was transferred to EDTA-containing vacutainer tubes,

incubated on ice for 60 min and subsequently centrifuged for 15 min at 2,000 × g. Obtained supernatants (plasma) were stored at -20°C prior to analysis. Serums were isolated by incubation of blood at room temperature for 30 min without anticoagulants, followed by 15 min centrifugation at 1,800 × g. Serums were stored at -20°C until further biochemical analysis.

2.3. Liver lysate preparation

Following decapitation, the liver was removed and then homogenized on ice with an Ultra-Turrax homogenizer in buffer (pH 7.4) containing (in mM) 150 NaCl, 20 Tris, 2 EDTA, 2 DTT, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail (Complete Ultra Tablets, Mini, EDTA-free, EASYpack), phosphatase inhibitor cocktail (PhosSTOP), and 2 sodium orthovanadate. Homogenates were then incubated at 4°C for one hour and then centrifuged for 30 min at 100,000 × g at 4°C. Protein concentration was determined by the Lowry method. Supernatants were used for determination of the glucose, TC and FFA levels and Western blot analysis.

2.4. Measurement of serum insulin, glucose and total cholesterol concentration (TC), and liver lysate glucose and TC

The serum insulin level was determined by the radioimmunoassay (RIA) method using a commercially available RIA kit and insulin standards. The glucose level was measured using standardized GLUC-PAP method (Randox, Crumlin, UK) in Rx Daytona automatic biochemical analyzer (Randox, UK) according to the manufacturer's manual and expressed as mmol/l. Serum concentrations of both glucose and insulin were used to calculate the indexes of IR (HOMA-IR) and insulin secretion (HOMA-β), by using the following formulas:

HOMA-IR= fasting glucose (mM) × fasting insulin (μU/ml) / 22.5

HOMA-β= 20 × fasting insulin (μU/ml) / (fasting glucose (mM) - 3.5).

The concentration of TC was measured using a Beckman Coulter Olympus AU400 Analyzer using a commercially available kit according to the manufacturer's manual. TC was determined by standardized enzymatic colour test (enzymatic assay) using cholesterol oxidase-peroxidase enzyme.

2.5. Measurement of plasma and liver lysate FFA and plasma nitrite/nitrate levels

The concentration of FFA was determined using a modified version of the Duncombe's method [30]. The principle of the method is based on the fact that extracted FFA in chloroform, in the presence of an appropriate reagent (aqueous solution of $Cu(NO_3)_2$ x $3H_2O$ with triethanolamine (TEA), pH 7.8), forms salts of copper, which in contact with diethyldithiocarbamate (DDC) builds a yellow complex compound with a maximum absorbance at 436 nm. The concentration of nitrite and nitrate, were determined by using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical 780001 IN).

2.6. Liver plasma membrane protein extraction

Plasma membranes were prepared according to Luiken et al. [31]. In brief, rat livers (200 mg pieces of liver) were cut 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. Thereafter, the suspension was centrifuged for 5 min at 1,000 × g. The pellet was homogenized on ice with an Ultra-turrax homogenizer in a TES buffer (20 mM Tris, 250 mM sucrose and 1 mM EDTA, pH 7.4) with protease inhibitor cocktail with additional 2 mM sodium orthovanadate. The resulting homogenate was centrifuged for 5 min at 1,000 g and the pellet was rehomogenized in a TES-buffer and then recombined with the supernatant. Subsequently, the homogenate was centrifuged for 10 min at 100 × g. The supernatant was centrifuged for 10 min at 5,000 × g.

The final pellet (referred to as “plasma membrane fraction”) was resuspended in a TES buffer and stored at -80 °C. Protein concentration was determined by the Lowry method [32].

2.7. SDS-PAGE and Western blotting

The lysate proteins or plasma membrane protein extracts (100 μg/lane) were separated by SDS-PAGE and transferred to PVDF membranes [33]. Membranes were blocked with a 5% bovine serum albumin and total cell lysate fractions were probed with phospho-Akt (Ser^{473}), phospho-Akt (Thr^{308}), total-Akt, phospho-PDK-1 (Ser^{241}), total-PDK-1, phospho-mTOR (Ser^{2448}), total mTOR, iNOS, NFκB-p65 or actin antibodies. Membrane fractions were probed with anti-glucose transporter 2 (Glut-2) and anti-fatty acid translocase - cluster of differentiation 36 (FAT/CD36) antibodies. After washing, the membranes were incubated with the appropriate secondary HRP-conjugated antibody and used for subsequent detection with the ECL reagent. After p-Akt, p-mTOR and p-PDK-1 analysis, the membranes were stripped and reblotted with an antibody detecting the total Akt, total mTOR and total PDK-1 content, respectively. In order to be sure that protein loading was equal in all samples, the blots were reprobed with the mouse anti-β-actin monoclonal antibody. The signals were quantified using ImageJ software (NIH, USA).

2.8. Statistical Analysis

Values are expressed as means ± SEM. Statistical analysis of data was evaluated using a two-tailed student's *t* test. A p-value <0.05 was considered significant.

3. RESULTS

3.1. Effects of a high fat diet on body mass, plasma and serum metabolic parameters

We investigated the effects of a high fat (HF) diet on body mass, insulin secretion and glucose and lipid metabolism in female rats. The results related to body mass, serum insulin, glucose and TC, plasma FFA and HOMA-β and HOMA-IR indexes, are presented in Table 1. A HF diet significantly increased the body mass of HF-fed female rats ($p<0.05$) compared with their respective controls. HF-fed females had a significant decrease in serum glucose concentration ($p<0.05$) compared with control, while changes of other parameters of insulin resistance were not observed. Quantitative measurements revealed that HF diet did not induced significant changes in the concentration of serum TC or plasma FFA in female rats compared to their controls. Similarly, HOMA-β and HOMA-IR indexes were not significantly different between HF-fed and control rats.

3.2. A high fat diet leads to sex-specific differences in liver mass and metabolism

In order to determine whether a HF diet influences liver size and function in male and female rats, we measured liver mass, and concentration of liver glucose, TC and FFA in control and HF-fed groups. The resultant values are shown in Table 2. Liver mass was significantly higher only in HF-fed males ($p<0.01$) but not HF-fed females. In liver lysates the glucose concentration was significantly increased in HF male ($p<0.01$) and HF female ($p<0.01$) groups compared with their controls. The FFA concentration in liver lysates was significantly higher ($p<0.05$) in HF males compared with controls, whilst there was no significant change in the FFA concentration between HF-fed females and control rats. The TC concentration in liver lysates was significantly increased in both HF male ($p<0.05$) and HF female ($p<0.001$) rats compared to controls.

3.3. A high fat diet leads to sex-specific biochemical differences

To further explore the effects of a HF diet on male and female rats, we next examined biochemical markers of insulin resistance. First we assessed the level of Glut-2 (Fig. 1A) and FAT/CD36 (Fig. 1B) in the plasma membrane fraction in liver. The obtained results show that the level of Glut-2 protein expression is significantly decreased in HF-fed male ($p<0.01$) and HF female ($p<0.01$) rats compared with controls. Measurement of plasma membrane FAT/CD36 protein levels reveal a significant elevation in HF-fed male rats ($p<0.05$), while in HF-fed female rats the level of FAT/CD36 was significantly decreased ($p<0.01$), compared with their respective controls.

We next determined the effects of a HF diet on plasma nitrite/nitrate concentration and iNOS protein expression in liver lysates. Our results show that plasma nitrite/nitrate concentrations were significantly increased ($p<0.01$) in HF-fed male rats, while in HF-fed female rats the plasma nitrite/nitrate concentration was not significantly altered relative to controls (Fig. 2A). Moreover, our results show significant reduction in iNOS protein levels in HF-fed male rats ($p<0.001$), whilst the level of iNOS did not change in HF-fed female rats compared with controls (Fig. 2B). Consistent with a reduction in iNOS protein levels, no significant change was observed in the level of the p65 subunit of NFκB in HF-fed male liver lysates relative to controls (Fig. 2C). A significant elevation in the level of the p65 subunit of NFκB was observed in liver lysates of HF-fed females ($p<0.05$).

We also investigated the effects of a HF diet on the total and phosphorylated forms of mTOR, PDK-1 kinases in male and female rats (Fig. 3A). A HF diet in liver lysates led to a significant decrease ($p<0.01$) in phosphorylation of Akt at Thr^{308} in male and female rats (Fig. 3C), while only in male rats there was a significant reduction ($p<0.01$) in phosphorylation of Akt at Ser^{473} compared with their control (Fig. 3B). A HF diet induced a significant decrease

in phosphorylation of mTOR at Ser^{2448} and PDK-1 at Ser^{241} in HF-fed male rats compared with their control, while in female rats only phosphorylation of PDK-1 at Ser^{241} was significantly decreased (Fig. 3D and E).

4. DISCUSSION

In the present study we demonstrate that a HF diet leads to significant sex-specific differences in rats, in regard to iNOS expression, nitrite/nitrate production and manifestation of IR. We previously demonstrated that a HF diet in the same male rats led to the development of an obese phenotype and IR characterized by significant increase in body mass, serum insulin and glucose and HOMA-IR and HOMA-β indexes [34]. We also show in the same study that a HF diet caused lipid dysregulation in the circulation of male rats, with significant increases in observed serum TC and plasma FFA levels [34]. Obesity is a cause of several diseases, including liver steatosis and non-alcoholic fatty liver disease [35]. Due to the excess of lipids in obesity, the capacity of adipose tissue is impaired as the primary fat depot, causing other tissues, including the liver, to accumulate fat [36]. Fat accumulation in the liver leads to structural and functional changes and eventually causes hypertrophy [36]. Measuring liver mass in our study, we show that a HF diet leads to a significant increase in liver mass and liver/body mass ratio in HF male rats with no increase in HF female rats. This increase of liver mass in HF male rats is in agreement with the reported literature [36, 37].

Previous studies suggest that increased dietary fats cause whole-body and regional (liver) IR [38], and also that the severity of IR is sex-specific [39, 40]. A HF diet led to the manifestations of whole body IR in male rats [34], which is again consistent with results reported in other studies [41, 42]. However, a HF diet did not induce IR in female rats in our study. Although a HF diet significantly increased the serum glucose level and HOMA-IR in

female rats [43], previous data has shown that a HF diet does not necessary lead to the development of IR in female rats [39, 40]. Whilst investigating the effect of a HF diet on the development of IR and its pathology in the liver, we examined the glucose level and plasma membrane Glut-2 expression in liver lysate. In liver lysates we show that the glucose levels are significantly higher, while Glut-2 levels are significantly decreased from both HF-fed groups compared with their controls. In two different studies [37, 44], it was reported that a HF diet decreased the level of Glut-2 expression in the liver of male mice. These results indicate that glucose metabolism is disturbed in both HF-fed groups or it could be a response to a postprandial elevation in the level of glucose in the liver. Considering that a HF diet caused the development of whole body IR in HF-fed male but not in HF-fed female rats, the increased level of glucose and decreased level of Glut-2 in liver lysates of both HF-fed groups may be explained by the presence of large amounts of lipids. For example, the presence of excessive amounts of FFA in the liver may inhibit insulin-stimulated glucose uptake [45].

Previous studies have revealed sex-specific differences in the levels of circulating lipids in premenopausal women, who exhibited a lower concentration of TC compared to age-matched men [46]. In a study performed by De Marinis *et al.* the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase was lower in female rats compared to male rats, indicating sex-specific differences in the neo-synthesis of cholesterol [47]. Our results highlight differences between serum TC levels in HF-fed female and male rats, which could be explained by the cholesterol-reducing effects of estrogen [48]. However, maintaining excessive intrahepatic lipid accumulation caused by obesity could be the reason behind our observation of elevated hepatic TC levels in HF-fed female rats.

The link between FFA and IR in obesity is significant. Excessive FFA uptake in obesity leads to storage of triglycerides, providing a substrate for lipid peroxidation and increasing steatosis [45, 49]. Elevated FFA levels reduce the extraction of insulin by the liver and contribute to the development of IR [49]. Furthermore, FFA are cytotoxic and induce hepatocyte lipoapoptosis [50]. One mechanism by which FFA are taken up by the liver is through the membrane transporter, FAT/CD36. In obesity, the increased translocation of FAT/CD36 protein to the plasma membrane in hepatocytes is commonly associated with IR and type 2 diabetes [51]. Our results indicate that a HF-fed diet induced a significant increase in plasma and liver lysate FFA levels as well as FAT/CD36 protein, but only in male rats compared to controls. Koonen *et al.* previously demonstrated that diet-induced obesity in male mice led to a significant elevation of FAT/CD36 protein levels in the liver, and these elevations correlate with increased hepatic lipid accumulation [51]. In the same study, these authors provide evidence that increased hepatic FAT/CD36 protein expression in response to obesity contributes to aberrant liver FFA uptake and may play a role in the pathogenesis of IR [51]. The unchanged level of FFA in HF-fed female rats may be explained by the increased lipolytic activity of adipose tissue, and therefore decreased lipogenesis which is mediated by estradiol [48]. Estradiol is known to influence FAT/CD36 translocation to the plasma membrane [52], and estrogen deficiency caused by ovariectomy in rodents leads to decreased lipid oxidation [53].

Increased lipid accumulation in the liver leads to altered hepatic lipid metabolism and consequently to lipotoxicity [23]. This leads to activation of inflammatory pathways and overexpression of iNOS resulting in increased production of NO [3, 7, 10]. Dysregulation of iNOS is known to be involved in the pathogenesis of obesity-linked IR [7, 54]. In our experiments only HF-fed male rats exhibited significantly elevated nitrite/nitrate levels in

plasma. It has been reported that NO derived from iNOS causes hepatic IR by nitrosylating tyrosine residues within the insulin receptor and Akt impairing insulin signalling and Akt activity [2, 7]. Previous studies however, have reported varying levels of hepatic iNOS protein expression in different animal models of obesity-induced IR [4, 25]. Most studies report that obesity and disturbed liver lipids metabolism lead to increased hepatic iNOS expression and that iNOS plays an important role in the pathogenesis of hepatic IR [2, 7, 26, 27]. In contrast, Perreault and Marette observed that in mice fed a HF diet iNOS expression was increased in skeletal muscle and adipose tissue but not in the liver, and that iNOS disruption restored HF diet–induced defects in insulin signaling in skeletal muscle but not in the liver [4]. Also it was reported that obesity induced by HF feeding does not affect the protein levels of endothelial NOS and neuronal NOS isoforms [4]. Thus, increased plasma nitrite/nitrate level, which suggests an increased NO level, in HF-fed male rats in our study may be due to an increased iNOS activity/expression in other tissues, such as skeletal muscle. NO is a highly lipophilic molecule that can diffuse rapidly into cells, thus it is possible that increased NO produced in other tissues may suppress iNOS expression in the liver of male HF–fed rats. NO may coordinate with the heme of the iNOS enzyme and thereby inhibit its activity [55]. In addition, results reported by Taylor *et al.* indicate that NO inhibits iNOS gene transcription, and that the effect is mediated in part by inhibiting NFκB activity [56]. In our study, iNOS protein expression in HF-fed female rats was comparable to control animals. We suggest that in HF-fed female rats, estradiol plays a key role in regulating the expression of iNOS which helps control inflammatory responses and the development of IR [57]. Consistent with this, Yeh *et al.* previously showed that treatment of septic mice with 2-methoxyestradiol significantly reduced the levels of interleukines and tumor necrosis factor-α, in addition to NO levels in serum and peritoneal fluid [14].

To gain insight into the molecular mechanism of iNOS regulation and its insulin-sensitizing effects in sex-specific manner, we examined the role of NFκB and Akt activation by PDK-1 and mTOR. Previous data revealed that obesity/lipid-induced increase of hepatic iNOS expression and NO production decreases phosphorylation and expression of insulin receptor, IRS and Akt [2, 7], but the effects of PDK-1 and mTOR on regulation of Akt activation and further influence on glucose and FFA membrane transporters in the same condition are missing from literature.

The serine/threonine kinase Akt is a downstream target of insulin action and is involved in the regulation of glucose and FFA memebrane transporters. Thus, disregulation of Akt plays an important role in hepatic lipid accumulation in obesity and IR induced by a HF diet [58]. Once activated, mTOR phosphorylates S6 kinase, which in turn leads to serine phosphorylation and degradation of IRS1/2 [59, 60]. Therefore, hyperactivation of mTOR/S6 kinase cascade exerts negative effects on the downstream mediators of insulin/PI3K pathway, such as Akt, and in that way contributes to the development of IR and consequently type 2 diabetes [61]. However, Sarbassov *et al.* have shown that a decrease in mTOR expression leads to inhibition of Akt activity [62]. In addition, chronic inhibition of mTOR was found to increase peripheral IR and to downregulate both basal and insulin induced Akt phosphorylation [61]. Moreover, Mora et al. showed that mice that lack PDK-1 in the liver display glucose intolerance and liver failure [63], while insulin fails to activate Akt in PDK-1 deficient adipocytes and cardiomyocytes [64, 65]. In our study the level of Akt phosphorylation was significantly decreased at both Ser^{473} and Thr^{308} in HF-fed male rats, while in HF-fed female rats only phosphorylation at Thr^{308} was reduced. Moreover, the phosphorylation of mTOR was significantly decreased only in HF-fed male rats, while the phosphorylation of PDK-1 was decreased in both HF-fed groups compared with controls.

These changes are consistent with previous studies which show that a HF diet leads to the development of IR characterized by disturbed PDK-1, mTOR and Akt activation [66, 67].

The molecular regulation of iNOS expression is complex and occurs at the transcriptional, translational and post-translational level [5]. The promoter of the *NOS2* gene (which encodes iNOS) is a binding site for several transcription factors, including NFκB [18, 19]. Increased hepatic lipid accumulation, consequently leads to the upregulation of inflammatory cytokines [23], which activate NFκB and upregulate hepatic iNOS expression [24]. Our results show that in liver lysates of HF-fed male rats, the expression of the p65 subunit of NFκB was not significantly changed but was significantly increased in liver lysates of HF-fed female rats. In a study performed by Hattori *et al.* processes necessary for the activation of NFκB (IκB degradation, nuclear translocation, and increased NFκB DNA binding) were suppressed by inhibitors of Akt [68]. Therefore, there is evidence to indicate that Akt is upstream of NFκB activation in the signalling cascade, whereby activation of Akt upregulates iNOS promoter activity, leading to transcription and translation of iNOS. Given that IκB kinase is a substrate of Akt, the absence of Akt activation in HF-fed male rats in our study, might inhibit translocation of NFκB to the nucleus and further decrease expression of iNOS. The increased expression of the p65 subunit of NFκB and the decreased Akt phosphorylation in the liver of HF-fed female rats indicates a protective role of estradiol in pathological conditions such as obesity and IR. Fig 4. combines our current findings into a predictive model of how a HF diet leads to modification in iNOS expression and nitrite/nitrate production, resulting in different manifestation of IR, in female and male rats.

Based on our published work where we examined the *in vivo* effects of estradiol administration in ovarectomized rats [52, 69] and male rats [34, 70, 71], we emphasized the protective role of estradiol as a main reason for less detrimental effects of obesity in female, unlike obese/IR male rats. Furthermore, ovariectomy in rats leads to an increase in body

mass, intra-abdominal fat, fasting glucose and insulin levels, and IR, while estrogen treatment reverses all of these effects [72].

The results presented in this study suggest that a HF diet induces a disturbance of circulatory and liver lipids in male rats but not female rats, which is likely a result of increased FAT/CD36 expression. In addition, we reveal that male rats fed a HF diet are more predisposed to develop IR. Consistent with this, biochemical markers including reduced hepatic iNOS and Glut2 expression, increased levels of plasma nitrite/nitrate and liver glucose, and decreased PDK-1, mTOR and Akt phosphorylation in liver lysates were observed in HF-fed male rats. Sex-specific differences in rats fed a HF diet may be explained by the protective role of estradiol in female rats. This work provides a mechanistic explanation as to why females are more protected against IR than males.

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CONFLICT OF INTEREST

None declared.

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Table and figure legends:

Table 1. Whole body mass, plasma and serum metabolic parameters of control and HF-fed female rats. The data shown represent the mean ± SEM (*n*=5-6).

Table 2. Liver mass and metabolic profile of control and HF-fed female and male rats. The data shown represent the mean ± SEM (*n*=4-6).

Fig. 1. Levels of liver plasma membrane Glut-2 and FAT/CD36 proteins in control and HF-fed female and male rats (A) The results for the level of Glut-2 protein represent the mean ± SEM (*n*=4-5); insert shows representative Western blots for Glut-2 protein. (B) Western blot analysis showing relative FAT/CD36 protein levels. Insert shows representative Western blots for FAT/CD36 protein. Data represents the mean ± SEM (*n*=4). Significant differences in HF compared with the corresponding control (*p<0.05; **p<0.01) are indicated. In each case β-actin levels were measured as a loading control.

Fig. 2. The levels of plasma nitrite/nitrate, iNOS and NFκBp65 proteins in the liver lysates of control and HF-fed female and male rats (A) Plasma nitrite/nitrate concentrations (*n*=6). (B) Western blot analysis showing relative iNOS protein levels (*n*=6). (C) Western blot analysis showing relative NFκBp65 protein levels (*n*=5-6). In (B and C) inserts show representative blots. For (A-C) data represents the mean ± SEM. Significant differences in HF-fed rats compared with the corresponding controls (*p<0.05; **p<0.01 ***p<0.001) are indicated.

Fig. 3. Phosphorylated liver Akt (Ser^{473} and Thr^{308}), mTOR Ser^{2448} and PDK-1 Ser^{241} proteins in control and HF-fed female and male rats (A) Representative Western blots for

total and phosphorylated Akt (Ser^{473} and Thr^{308}), mTOR (Ser^{2448}) and PDK-1 (Ser^{241}) (B) Relative levels of Akt phosphorylation (Ser^{473}; *n*=3-4). (C) Relative levels of Akt phosphorylation (Thr^{308}; *n*=4). (D) Relative levels of mTOR phosphorylation (Ser^{2448}; *n*=5-6) (E) Relative levels of PDK-1 phosphorylation (Ser^{241}; *n*=4). For (B-D) data represents the mean ± SEM. Significant differences in HF-fed rats compared with the corresponding controls (*p<0.05; **p<0.01) are indicated.

Fig. 4. The proposed mechanism by which a HF diet induces altered hepatic lipid metabolism and affects regulation of hepatic iNOS expression and nitrite/nitrate production in a sex-specific manner in rats. Altered hepatic lipid metabolism caused by a HF diet influence iNOS/NO pathway and thus promotes IR by attenuating insulin action. This is likely a consequence of decreased Akt activation by PDK-1 and mTOR, which further allows regulation of glucose and FFA membrane transporters. Differences in HF-fed female and male rats in IR manifestation, insulin signalling disturbance, iNOS induction and nitrite/nitrate production is likely due to protective effects of estradiol which mitigate effects of a HF diet on insulin action. Among other mechanisms, large amounts of nitrite/nitrate produced in other tissues may easily enter the liver and inhibit iNOS protein expression, but also disturb insulin action. Akt, protein kinase B; FAT/CD36, fatty acid translocase/cluster of differentiation 36; FFA, free fatty acids; Glut-2, glucose transporter 2; IR, insulin resistance; p65, p65 subunit of nuclear factor κB; iNOS, inducible nitric oxide (NO) synthase; mTOR, mammalian target of rapamycin; NO^{2-}/NO^{3-}, nitrite/nitrate; PDK-1, phosphoinositide-dependent kinase-1.

Table 1.

	Experimental groups		
Parameter	**Female**	**HF Female**	**Sig. diff**
Initial body mass [g]	202 ± 8	190 ± 8	p=0.34
Final body mass [g]	260 ± 12	275 ± 12	p=0.40
Body mass difference [g]	58 ± 6	85 ± 8	p<0.05
Serum insulin [mlU/l]	79 ± 19	85 ± 8	p=0.76
Serum glucose [mM]	11.6 ± 0.5	10.2 ± 0.4	p<0.05
HOMA-β	209 ± 48	281 ± 22	p=0.21
HOMA-IR	34 ± 10	36 ± 4	p=0.80
Serum TC [mM]	2.07 ± 0.11	1.77±0.09	p=0.76
Plasma FFA [mM]	0.70 ± 0.08	0.81 ± 0.11	p=0.45

Table 2.

	Experimental groups					
Parameter	**Female**	**HF Female**	**Sig. diff.**	**Male**	**HF Male**	**Sig. diff.**
Liver mass [g]	10.6 ± 0.1	10.3 ± 0.09	p=0.73	11.4 ± 0.2	14 ± 0.2	p<0.01
Liver/body mass ratio	0.0405 ± 0.0007	0.0407 ±0.0013	p=0.92	0.0321 ± 0.0010	0.0352 ± 0.0001	p<0.05
Liver glucose [mM/mg tissue]	12.66 ± 0.8	21.12 ± 1.5	p<0.01	10.86 ± 0.3	19.74 ± 1.3	p<0.01
Liver TC [mM/mg tissue]	1.60 ± 0.10	2.58 ± 0.13	p<0.001	1.54 ± 0.09	1.96 ± 0.14	p<0.05
Liver FFA [mM/mg tissue]	0.43 ± 0.03	0.40 ± 0.03	p=0.58	0.29 ± 0.02	0.38 ± 0.03	p<0.05

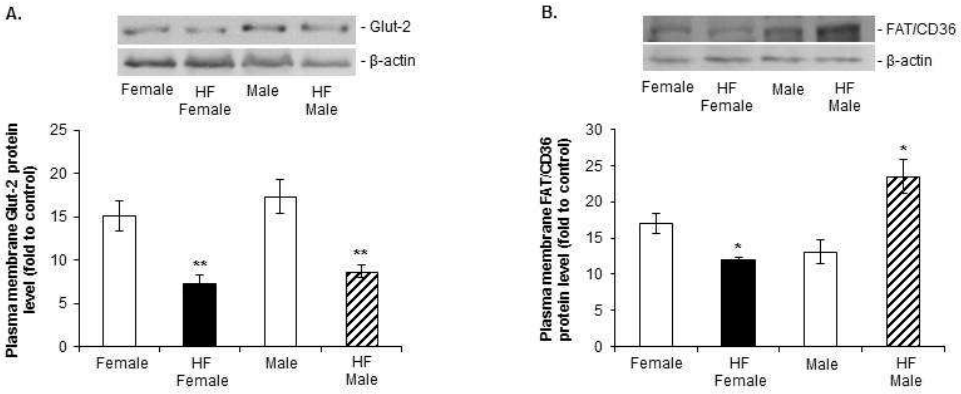

Fig. 1

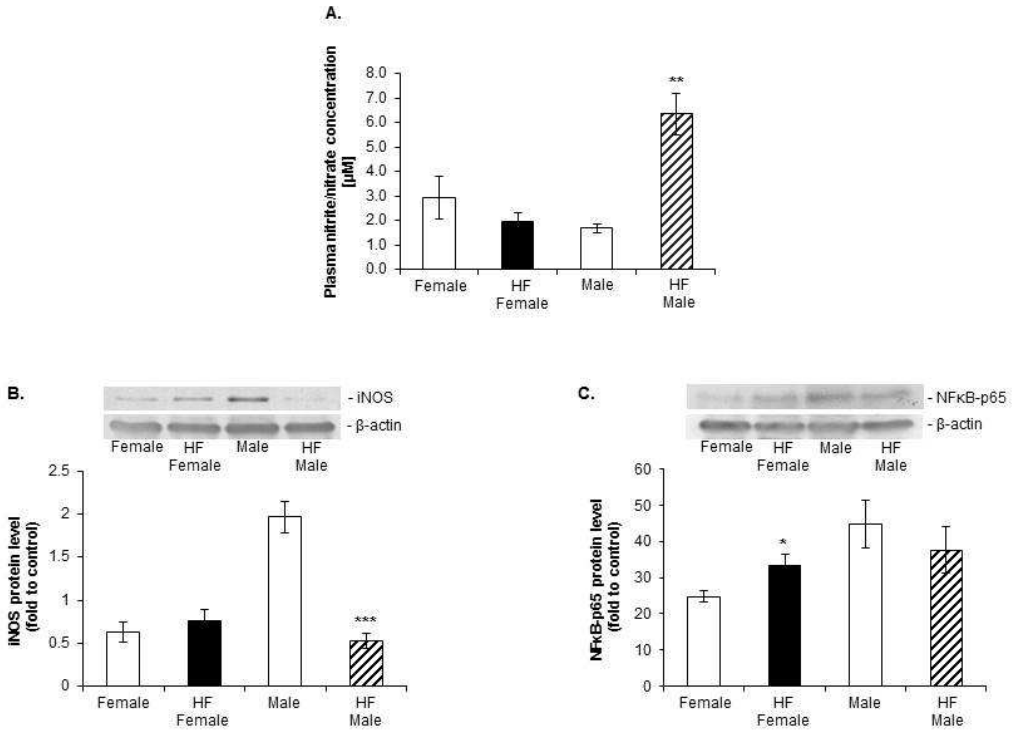

Fig. 2

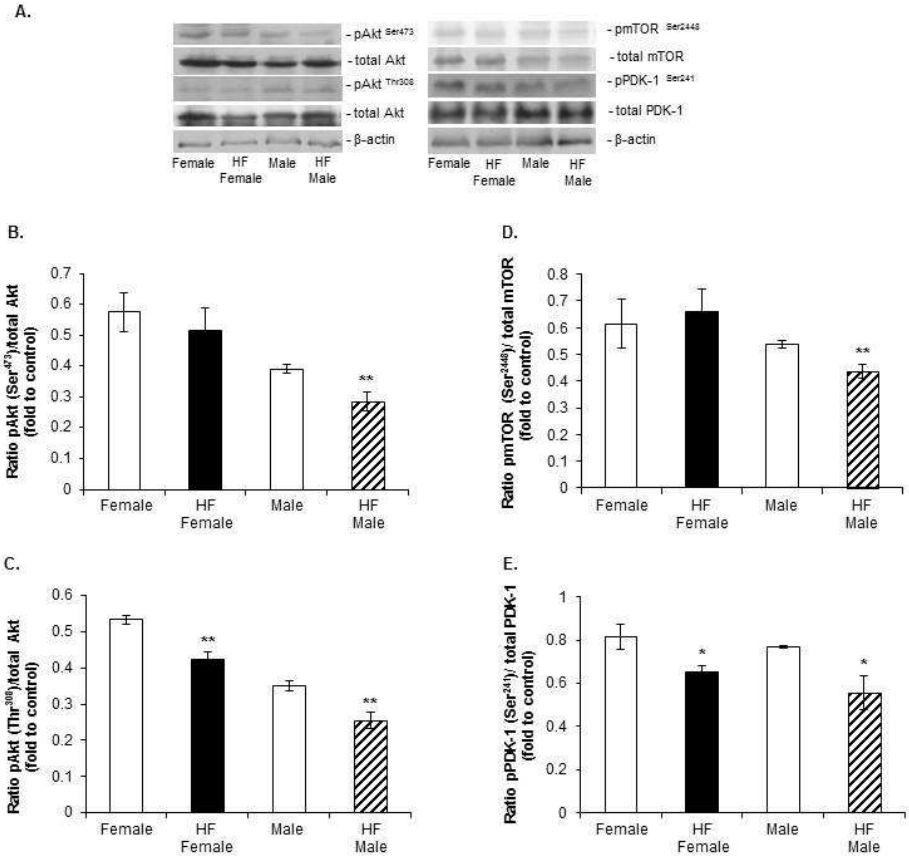

Fig. 3

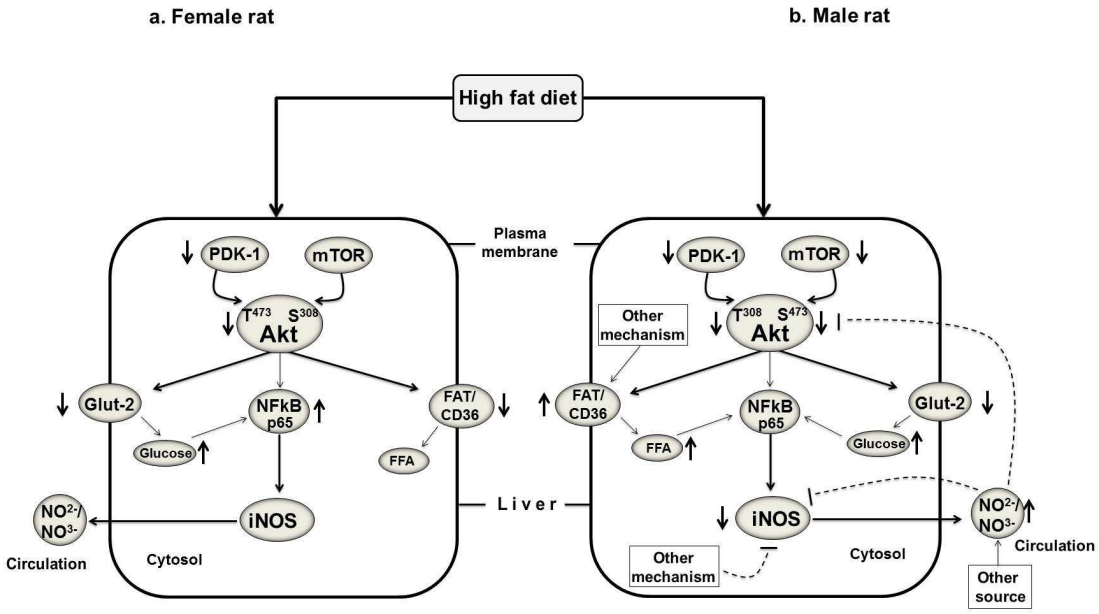

Fig. 4

Highlights:

- Men and women differ substantially in regard to the severity of IR.
- HF diet differently modifies nitrite/nitrate production between the sexes.
- Changes in nitrite/nitrate level are related to changes of Akt, mTOR, PDK-1 activation.
- This work provides a mechanistic explanation why females are more protected from IR than males.