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ORIGINAL CONTRIBUTION

Impairment of cardiac insulin signaling in fructose-fed ovariectomized female Wistar rats

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Abstract
Background Fructose consumption produces deleterious metabolic effects in animal models. The sites of fructose-induced insulin resistance are documented to be the liver, skeletal muscle, and adipose tissue, but effects of fructose-rich diet on cardiac insulin signaling and action were not investigated.
Purpose and methods In order to study the potential fructose effects on development of cardiac insulin resistance, we analyzed biochemical parameters relevant for insulin action and phosphorylation of insulin signaling molecules, plasma membrane glucose transporter type 4 (GLUT4) content, and phosphorylation of endothelial nitric oxide synthase (eNOS), in ovariectomized female rats on fructose-enriched diet, in basal and insulin-stimulated conditions.
Results Fructose-fed rats (FFR) had increased content of visceral adipose tissue, but not body weight. Food intake was decreased, while fluid and caloric intake were increased in FFR. Additionally, fructose diet increased plasma insulin, blood triglycerides level, and HOMA index. Stimulation of protein kinase B (Akt) signaling pathway by insulin was reduced in rats on fructose-enriched diet, but effect of fructose on extracellular signal-regulated kinase (Erk 1/2) phosphorylation was not observed. Furthermore, insulin-induced GLUT4 presence in plasma membranes of cardiac cells was decreased by fructose diet, as well as insulin stimulation of eNOS phosphorylation at Ser^{1177}.
Conclusion In summary, these results strongly support our hypothesis that fructose diet-induced changes of plasma lipid profile and insulin sensitivity are accompanied with decrease in cardiac insulin action in ovariectomized female rats.

Keywords Fructose · Heart · Insulin resistance · Glucose transporter type 4 · Nitric oxide synthase type III

Introduction

Fructose, a naturally found sugar in many fruits, is now commonly used as an industrial sweetener and is excessively consumed in Western diets. An increase in fructose consumption over the past decades has been linked with a rise in obesity and metabolic disorders and recognized as causative in the development of prediabetes and metabolic syndrome. These effects are not seen in glucose-fed animals suggesting that the mechanism is not mediated by excessive caloric intake [1–3].

Fructose produces deleterious metabolic effects in animal models. Rats fed with a high-fructose diet provide a useful animal model of insulin resistance [4]. The sites of fructose-induced insulin resistance are recognized to be the liver, skeletal muscle, and adipose tissue. The fructose-fed rats (FFR) also develop a cluster of abnormalities, which include hypertension, hypertriglyceridemia, and glucose intolerance in addition to hyperinsulinemia [2]. This raises

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concern regarding the effects of fructose and its risk in humans. In humans, fructose consumed in moderate to high quantities in the diet increases plasma triglycerides and alters hepatic glucose homeostasis, but does not appear to cause muscle insulin resistance or high blood pressure in the short term. Further human studies are required to define the effects of fructose in humans [5].

The mechanisms underlying fructose-induced metabolic disturbances are not completely clear. A high flux of fructose to the liver perturbs glucose metabolism and glucose uptake pathways and leads to a significantly enhanced rate of lipogenesis and triacylglycerol synthesis. Fructose is more lipogenic than glucose. The consumption of high levels of fructose functions as an unregulated source for triglyceride (TG) production [6–9].

Studies using fructose-fed rats have demonstrated that the degree of insulin resistance developed in males is greater than that in females [10]. The results demonstrate that females do not develop hypertension or hyperinsulinemia upon fructose feeding except after ovariectomy, suggesting that female sex hormones provide protection against the effects of a fructose diet [11].

Cardiac muscle is a target of insulin [12, 13]. Binding of insulin to its receptor activates signaling pathways leading to stimulation of different downstream serine/threonine kinases, protein kinase B (Akt), and extracellular signal-regulated kinase (Erk) 1/2 [14]. Activation of these pathways consequently induces glucose uptake via translocation of glucose transporter GLUT4 to the plasma membrane [15] and cardiac nitric oxide (NO) production via phosphorylation of endothelial nitric oxide synthase (eNOS) [16]. Impairment of cardiac glucose uptake and NO synthesis have been described in animal models of diabetes, obesity, and hypertension. Abnormalities in insulin signaling account for insulin resistance. Insulin resistance is an important risk factor for the development of hypertension, atherosclerotic heart disease, left ventricular hypertrophy and dysfunction, and heart failure [17].

On the basis of published data that fructose-enriched diet induces insulin resistance in main insulin target tissues and in view of importance of cardiac insulin action, we hypothesized that heart could also be targeted by fructose diet-induced disturbances in insulin signaling and insulin-regulated cardiac processes, implicating disregulation of important cardiac function. In order to test this hypothesis and investigate exact mechanism of potential cardiac insulin resistance induced by high fructose diet, we subjected ovariectomized female Wistar rats to model of 9-week-diet containing 10% fructose in drinking water and analyzed parameters relevant for insulin action in the circulation and at cardiac tissue level.

Materials and methods

Material

Fructose was purchased from API-PEK (Becej, Serbia). The RIA insulin kit was a product of INEP (Zemun, Serbia). Estradiol (E2) Enzyme Immunoassay test kit BC-1111 was a product of BioCheck, Inc., Foster City, CA, USA. Polyclonal anti-phospho-Akt (Ser^{473}, Thr^{308}) (pAkt), anti-GLUT4, anti-phospho eNOS (Ser^{1177}) (peNOS), and secondary horse radish peroxidase (HRP)- and alkaline phosphatase (ALP)-conjugated anti-rabbit antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal anti-Akt, anti-Erk 1/2, and anti-phospho Erk 1/2 (Thr^{202}/Tyr^{204}) (pErk 1/2) were products of Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal anti-eNOS antibody was a product of Affinity Bioreagents (Rockford, IL, USA). Reagents for the bicinchoninic acid (BCA) assay were purchased from Pierce (Rockford, IL, USA). Polyclonal rabbit anti-actin antibody and insulin were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA).

Animals

Twenty-one-day-old female Wistar rats were separated from their mothers and divided into two main groups according to diet regime. All animals were kept under standard temperature and dark/light conditions. Control animals had free access to tap water and standard laboratory chow. Animals on fructose-enriched diet were fed by the same food, but water was replaced with 10% fructose solution in tap water (w/v). Body weight of rats in two experimental groups was not significantly different at the beginning of diet. Food and fluid intake and body weight were recorded during the treatment. Animals were exposed to these diets during 9 weeks. Two weeks before killing, all animals were bilaterally ovariectomized, under ketamine anesthesia (40 mg/kg, i.p.) combined with xylazine (5 mg/kg, i.p.), to remove endogenous estrogen that could prevent development of insulin resistance. According to published data, E2 is highly protective against damaging effects of fructose-enriched diet [10]. In order to underline fructose effects on analyzed molecules in the context of insulin signaling, because they are also involved in other signaling pathways, both main experimental groups, standard and fructose diet group, were additionally divided into two subgroups, one of which was treated with insulin:

1. C group—rats on standard diet treated with vehicle (40 min before killing, i.p.)
2. FFR group—fructose-fed rats treated with vehicle (40 min before killing, i.p.)

3. C + INS group—rats on standard diet treated with insulin (12 IU/kg of b.w., 40 min before killing, i.p.)
4. FFR + INS group—fructose-fed rats treated with insulin (12 IU/kg of b.w., 40 min before killing, i.p.)

Rats were exposed to overnight fasting before biochemical measurements. Immediately after killing, heart was carefully excised from the body, dropped in chilled saline and blotted with tissue paper to remove as much blood as possible and weighted. Visceral adipose tissue was also completely removed and its weight was measured. Animal experiments were conducted according to standards approved by the official Vinca Institute's Ethical Committee for Experimental Animals.

Biochemistry

Blood glucose and TG levels were measured by multiCare analyzer (Biochemical Systems International S.r.l., Arezzo, Italy). Plasma was separated from blood samples using EDTA as an anticoagulant. Plasma insulin concentration was determined using RIA kit and rat insulin standards, with assay sensitivity of 0.6 mIU/L and an intra-assay coefficient of variation of 5.24%. The plasma E2 level was determined by E2 Enzyme Immunoassay test kit BC-1111, according to the protocol provided by manufacturer. The plasma free fatty acids (FFA) level was measured using a modified method of Duncombe [18]. Homeostasis model assessment (HOMA) index, as an indicator of insulin resistance, was calculated from fasted plasma insulin and glucose concentration using the formula described by Matthews et al. [19]: insulin (mU/L) × [glucose (mmol/L)/22.5].

Lysate preparation

Isolated rat hearts were homogenized on ice with an Ultra-turrax homogenizer in buffer (pH 7.4) containing 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitors (2 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin), and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate) [20]. Homogenates were centrifuged at 600*g* for 20 min at 4 °C, after which supernatants were ultracentrifuged for 60 min at 100,000*g*. Protein concentration was determined by the BCA method. Supernatants were used for Western blot analysis of protein content.

Plasma membrane preparation

Plasma membranes (PM) were prepared according to Luiken et al. [17]. In brief, hearts were diced and incubated for 30 min in a high-salt solution (2 mol/L NaCl, 20 mmol/L HEPES pH 7.4, and 5 mmol/L NaN_3) at 4 °C. Thereafter, the suspension was centrifuged for 5 min at 1,000*g*, and the pellet was homogenized in TES buffer (pH 7.4, 20 mmol/L Tris, 250 mmol/L sucrose, and 1 mmol/L EDTA) using an Ultra-turrax homogenizer. The resulting homogenate was centrifuged for 5 min at 1,000*g*, after which the pellet was rehomogenized in TES buffer with 10 strokes using glass-Teflon homogenizer and then recombined with the 1,000*g* 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 was resuspended in 300 μL of TES buffer and saved. According to the suggestion of Luiken et al. [21], we decided to refer this pellet as PM-fraction.

SDS–PAGE and Western blotting

Lysate proteins (50 μg/lane) were separated by 7.5% or 10% SDS polyacrylamide gels [22] and transferred to polyvinylidene fluoride membranes. The membranes were blocked 1.5 h with 5% bovine albumin and probed with anti-Akt, anti-pAkt (Ser^{473}, Thr^{308}), anti-Erk, anti-pErk 1/2 (Thr^{202}/Tyr^{204}), GLUT4, eNOS, or peNOS (Ser^{1177}) antibodies. After washing with Tris-buffered saline Tween-20, membranes were incubated with the appropriate secondary HRP- or ALP-conjugated anti-rabbit or anti-goat antibody and used for subsequent chemiluminescent or ALP detection. After analysis of phosphorylated molecules, membranes were stripped and reblotted with antibody for detecting total content of molecule. In order to be sure that protein loading was equal in all samples, blots were probed with rabbit anti-actin monoclonal antibody. Signals on membranes or films were quantified using ImageJ software (NIH, USA).

Statistics

Fructose effect in basal state (in the absence of exogenous insulin) and insulin effect were estimated against control (FFR vs. C and C + INS vs. C, respectively), and fructose effects in insulin-stimulated state were estimated against control rats treated with insulin (FFR + INS vs. C + INS). Values are given as means ± SD of three independent experiments with at least seven animals per group. The SPSS program for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. The results were evaluated by parametric statistics, and the significance of differences between two groups was estimated by the Student's t-test. Values of $p < 0.05$ were considered significant (compared with appropriate control).

Table 1 Physical characteristics of the rats in different groups at the end of the experiment

C control group of rats on standard diet, *FFR* fructose-fed rats; Values are given as means ± SD of three independent experiments with seven animals per group

** $p < 0.01$; *** $p < 0.001$ versus C

	C	FFR
Body weight (g)	276.45 ± 25.46	285.40 ± 25.19
Heart weight (g)	0.80 ± 0.08	0.83 ± 0.09
Weight of visceral adipose tissue (g)	6.22 ± 0.83	8.77 ± 2.23**
Relative heart weight (mg/g body weight)	2.90 ± 0.12	2.89 ± 0.15
Relative adipose tissue weight (g/100 g body weight)	2.20 ± 0.33	3.08 ± 0.76**
Fluid intake (mL/day/rat)	29.45 ± 4.82	45.61 ± 4.44***
Food intake (g/day/rat)	19.98 ± 1.14	16.22 ± 1.93**
Caloric intake (kJ/day/rat)	219.80 ± 12.51	256.88 ± 22.65**

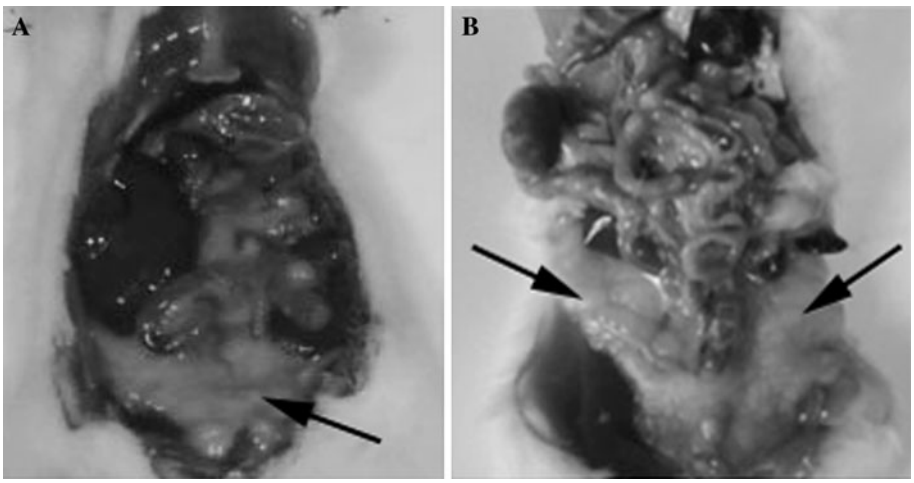

Fig. 1 Effects of fructose-rich diet on visceral adipose tissue of rats. Visceral adipose tissue of ovariectomized female rats on standard (**a**) and fructose-rich diet (**b**). *Arrows* indicate visceral adipose tissue

Results

Fructose diet did not change body weight, despite increase in absolute ($p < 0.01$) and relative (related to body weight) ($p < 0.01$) content of visceral adipose tissue (Table 1; Fig. 1). Absolute and relative weight (heart-to-body weight ratio) of FFR heart was at the control level. Rats exposed to fructose-rich diet had increased intake of fluid ($p < 0.001$) and total caloric intake ($p < 0.01$), but significantly decreased intake of food ($p < 0.01$) (Table 1). E2 plasma level was low due to ovariectomy and did not differ significantly after fructose diet and/or insulin injection (Table 2). Blood glucose level did not change in FFR compared to control, in difference to plasma insulin level that was significantly increased in FFR group ($p < 0.001$) (Table 2). Due to hyperinsulinemia and slight nonsignificant increase in glucose level, HOMA index was significantly higher in FFR ($p < 0.001$), indicating decrease in insulin sensitivity (Table 2). Lipid status of rats was partially influenced by fructose diet. There was no change in FFA concentration in plasma, but blood TG level was increased after 9 weeks of fructose diet ($p < 0.05$) (Table 2).

In order to stimulate insulin signaling pathway in the heart, one subgroup of FFR received insulin intraperitoneally just before the experiment. Comparing FFR underwent insulin stimulation to FFR received no insulin, we observed that fructose diet did not influence insulin regulation of plasma FFA and TG, but significantly decreased insulin capacity to lower glucose level ($p < 0.001$) (Table 2). This observation is in line with decrease in insulin sensitivity observed at basal level (without insulin stimulation). It was not expected to observe any changes in body, heart, and adipose tissue weight, as well as fluid, food, and caloric intake 40 min after insulin injection, so we did not analyze these parameters. HOMA index calculation in insulin-stimulated state is not applicable.

Effects of fructose-enriched diet on insulin signaling and insulin action in the heart were analyzed in basal (without insulin administration) and insulin-stimulated state. In accordance with published data, insulin stimulated Akt signaling pathway by phosphorylation of serine residue at

Table 2 Biochemical characteristics of the rats in different groups at the end of the experiment

	C	FFR	C + INS	FFR + INS
Estradiol (pg/mL)	1.66 ± 0.61	1.68 ± 0.69	4.25 ± 3.04	1.31 ± 0.34
Glucose (mmol/L)	5.90 ± 0.27	6.53 ± 1.38	2.06 ± 0.29***	3.27 ± 0.47###
Insulin (mIU/L)	23.89 ± 9.76	51.69 ± 15.84***	376.93 ± 43.26***	344.32 ± 24.68
FFA (mmol/L)	0.66 ± 0.16	0.62 ± 0.18	0.42 ± 0.11**	0.40 ± 0.12
TG (mmol/L)	1.38 ± 0.25	1.96 ± 0.66*	1.25 ± 0.07	1.15 ± 0.35
HOMA index	5.71 ± 2.67	14.73 ± 6.17***	N/A	N/A

C rats on standard diet, *FFR* fructose-fed rats; *INS* insulin treatment (12 IU, i.p.) 40 min before the experiment. *N/A* not applicable. Values are given as means ± SD of three independent experiments with seven animals per group. *FFA* free fatty acids, *TG* triglycerides, *HOMA* homeostasis model assessment

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus C; ### $p < 0.001$ versus C + INS

position 473 ($p < 0.05$, C + INS vs. C) (Fig. 2a). We did not observe any diet- or insulin treatment-dependent changes in total Akt content. Fructose diet significantly decreased basal level of Akt phosphorylation at Ser^{473} expressed as a phospho Ser^{473} Akt/total Akt ratio ($p < 0.01$, FFR vs. C). In addition, FFR had decreased level of insulin-stimulated phosphorylation of Ser^{473} ($p < 0.05$, FFR + INS vs. C + INS). Insulin stimulation of Akt signaling pathway was also due to strong phosphorylation of Thr residue at position 308 in Akt molecule ($p < 0.01$, C + INS vs. C). Fructose diet impairs insulin-stimulated phosphorylation (phospho Thr^{308} Akt/total Akt ratio, $p < 0.05$, FFR + INS vs. C + INS) but not the basal one (Fig. 2b).

We also analyzed the other insulin-regulated cardiac signaling pathway of Erk 1/2 kinases, regularly connected with mitogenic action of insulin, but we did not observe any significant change in phosphorylation of these kinases at Thr^{202}/Tyr^{204} position, expressed as a phospho Erk/total Erk ratio, neither by insulin treatment (40 min) nor by fructose diet, compared to appropriate control (Fig. 3). Total cardiac Erk 1/2 content was not modified by diet or insulin treatment, as well.

Insulin regulates glucose transport in the heart by promoting GLUT4 movement from intracellular pools to the PM. We analyzed changes in total cell lysate content of GLUT4 transporters and their content in PM of cardiac cells. Insulin injection 40 min before experiment did not influence total level of GLUT4. We also did not observe any change in total GLUT4 content in FFR in basal or insulin-stimulated state (Fig. 4a). However, insulin raised GLUT4 content in PM (1.27-fold, $p < 0.05$, C + INS vs. C), and fructose diet reduced insulin effect (35% $p < 0.05$, FFR + INS vs. C + INS) (Fig. 4b). Furthermore, fructose diet did not change basal level of PM GLUT4. When PM content of GLUT4 was normalized to GLUT4 total content, recorded in lysate of cardiac cells, all observed changes were slightly emphasized (1.5-fold increase C + INS vs. C, 47% decrease FFR + INS vs. C + INS), but level of statistical significance did not change.

Endothelial NOS is one of the enzymes generating NO in the heart, and its activity is regulated by phosphorylation at Ser^{1177}. We did not observe changes in total eNOS content in the heart of rats dependent on diet or insulin treatment. Basal level of this phosphorylation (expressed as a phospho eNOS/total eNOS ratio) was not influenced by fructose diet, but insulin-stimulated phosphorylation of eNOS ($p < 0.05$, C + INS vs. C) was significantly reduced in FFR ($p < 0.05$ FFR + INS vs. C + INS) (Fig. 5).

Discussion

Fructose-enriched diet is a well-accepted diabetogenic protocol in experimental animals [6, 23, 24]. Hyperinsulinemia and hypertriglyceridemia are accompanied by insulin resistance in main insulin target tissues [1–3]. In addition, insulin was identified as an important regulator of cardiac functions, and insulin resistance in the heart was described in other models of diabetogenic diet [25]. The aim of our study was to confirm the presumption that cardiac insulin resistance is a feature of FFR model.

Nine-week-long 10% fructose (in drinking water) diet increased fluid and caloric intake and weight of visceral adipose tissue and decreased food intake of ovariectomized female rats (Table 1). Body weight and whole heart weight did not change in FFR (Table 1). Rats subjected to fructose diet were hyperinsulinemic and hypertriglyceridemic compared to control (Table 2). Increased level of insulin and TG is expected and in accordance with putative mechanism of fructose-induced insulin resistance [6]. Similar pattern of changes in biochemical characteristics due to increased intake of fructose was observed by other authors [24]. In difference to Xi et al. [24], we observed changes in caloric intake and did not observe changes in

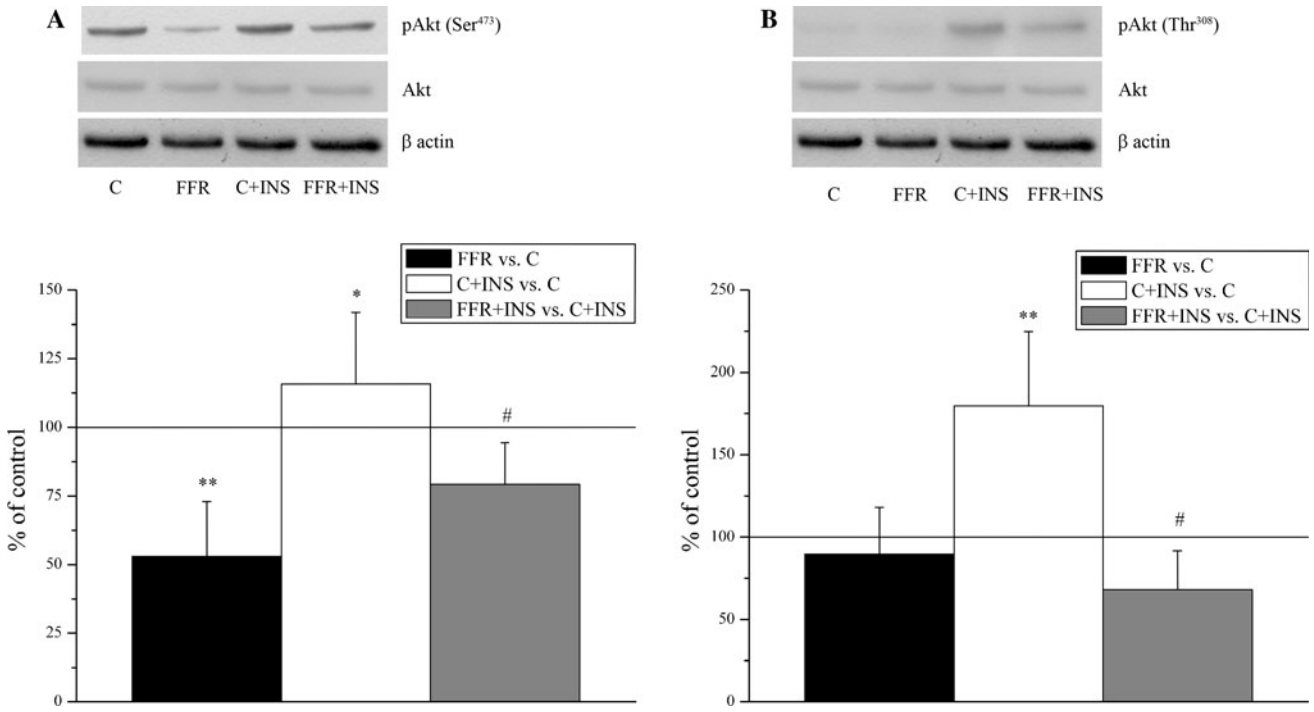

Fig. 2 The influence of fructose-enriched diet on Akt phosphorylation at Ser^{473} and Thr^{308} in the heart. Equal amounts of proteins were resolved on 10% SDS–PAGE and analyzed by immunoblotting with anti-phospho-Akt (Ser^{473}), anti-phospho-Akt (Thr^{308}) or anti-Akt antibodies, as described in "Materials and methods". Representative immunoblots and the quantification of basal and insulin-stimulated Akt phosphorylation on Ser^{473} (**a**) and Thr^{308} (**b**) are given, respectively. Three independent experiments with a total of 7 rats per group were quantified to yield the ratio between phospho-Akt and total Akt. Values are expressed as % of the appropriate control. *C* control rats on standard diet, *FFR* fructose-fed rats, *C + INS* control insulin-treated rats, *FFR + INS* insulin-treated fructose-fed animals. $*p < 0.05$ vs. C; $**p < 0.01$ vs. C; $\#p < 0.05$ vs. C + INS

FFA concentration in FFR (Tables 1, 2), which can be a consequence of sex dependence of fructose effects [10].

In the study of Qin et al., fructose diet did not change Akt mRNA expression in the myocardium [26]. In skeletal muscle removed immediately after the clamp procedure, high-fructose feeding did not alter protein levels of Akt protein kinase. However, insulin-stimulated phosphorylation of Akt was reduced [23]. Our results confirm the effects of high-fructose feeding observed in myocardium and skeletal muscle. Insulin-induced stimulation of Akt at both position Ser^{473} and Thr^{308} was decreased in lysate of whole FFR heart (Fig. 2a, b), indicating diminished metabolic action of insulin. Insulin stimulation of cardiac Thr^{308} phosphorylation was stronger, but only basal level of Ser^{473} phosphorylation was reduced in FFR, indicating different role of phosphorylation at these positions for cardiac insulin action. Furthermore, we did not observe expected mitogenic insulin-induced increase in Erk 1/2 phosphorylation and consequent effect of fructose diet (Fig. 3). This result is in line with results of Hyakukoku et al. showing no significant differences in the degree of phosphorylation of Erk-1/2 in the thoracic aorta or in the soleus muscle between FFR and controls [27]. To specify, results designate that fructose influences metabolic (Akt) but not mitogenic (Erk 1/2) insulin pathway in the rat heart. The absence of change in mitogenic pathway in FFR can be connected with the absence of change in absolute and relative heart weight (Table 1).

Changes in Akt signaling pathway in the heart of FFR point toward the reduction in GLUT4 and eNOS, downstream molecules regulated by this pathway. In course with this presumption, we observed a decrease in PM GLUT4 content in the heart of FFR in insulin-stimulated state (Fig. 4b). Qin et al. [26] showed a decrease in GLUT4 mRNA level in the myocardium of FFR. However, in our study, total cardiac GLUT4 protein level (measured in the lysate of cardiac cells) was unchanged by fructose diet (Fig. 4a). Li et al. [23] observed the same effects in skeletal muscle. High-fructose feeding did not alter the protein levels of GLUT4. Moreover, insulin-stimulated GLUT4 translocation to the plasma membrane was reduced [23]. Reduction of GLUT4 protein in FFR plasma membranes of skeletal muscle was also observed in the study of Shih et al. [28]. Observed changes in PM content of GLUT4, in light of role of cardiac glucose transport, point out to modification of balance in transport and consumption of glucose and FFA, main energetic substrates of heart [15, 29].

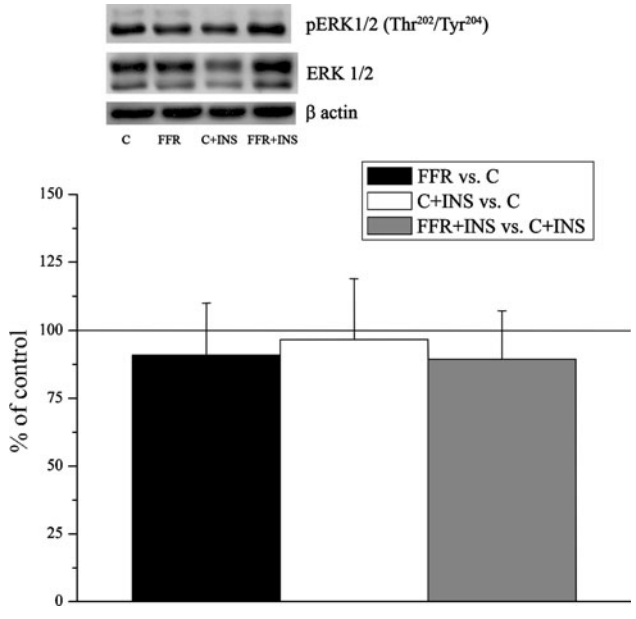

Fig. 3 The influence of fructose-enriched diet on the phosphorylation of extracellular signal-regulated kinase (pErk 1/2) in the heart. Data are presented as the phospho-Erk 1/2 per total Erk 1/2 ratio and expressed as % of the appropriate control. Values are given as means ± SD of three independent experiments with a total of seven animals per group. Representative immunoblots are shown at the *top* of the figure. The abbreviations are the same as in Fig. 2

Published results concerning fructose diet effects on cardiac eNOS do not agree about observed changes. Fulton et al. observed that the relative expression of eNOS and phosphorylation of Ser 1176 eNOS in the heart was not significantly different between control and FFR [30]. However, FFR showed decreased eNOS activity in both aortic endothelium and cardiac tissue, in the study of Miatello et al. [4, 31]. In agreement with Miatello study are results of Nyby et al. showing that expression of eNOS protein and mRNA was reduced in the FFR aortas and hearts [32]. Eventually, we observed no changes in total content of cardiac eNOS protein, but insulin-induced eNOS phosphorylation of Ser^{1177} was reduced in rats after 9 weeks of fructose diet (Fig. 5). Regarding the role of eNOS in the heart, observed changes in eNOS phosphorylation indicate changes in enzyme activity, cardiac NO production, and regulation of cardiac function. Namely, it is well established that agonist-stimulated release of NO from eNOS in the coronary endothelium exerts paracrine effects on cardiomyocytes, predominantly affecting the timing of relaxation as well as myocardial oxygen consumption. Myocardial eNOS, on the other hand, may be involved in mediating the inotropic response to sustained stretch [33].

In summary, fructose-enriched diet increases weight of visceral adipose tissue, plasma insulin and blood triglycerides concentration and reduces insulin stimulation of Akt signaling pathway and activation of downstream insulin targets, GLUT4, and eNOS. These results indicate changes in plasma lipid profile and insulin sensitivity accompanied with cardiac insulin resistance**,** as a consequence of high fructose consumption.

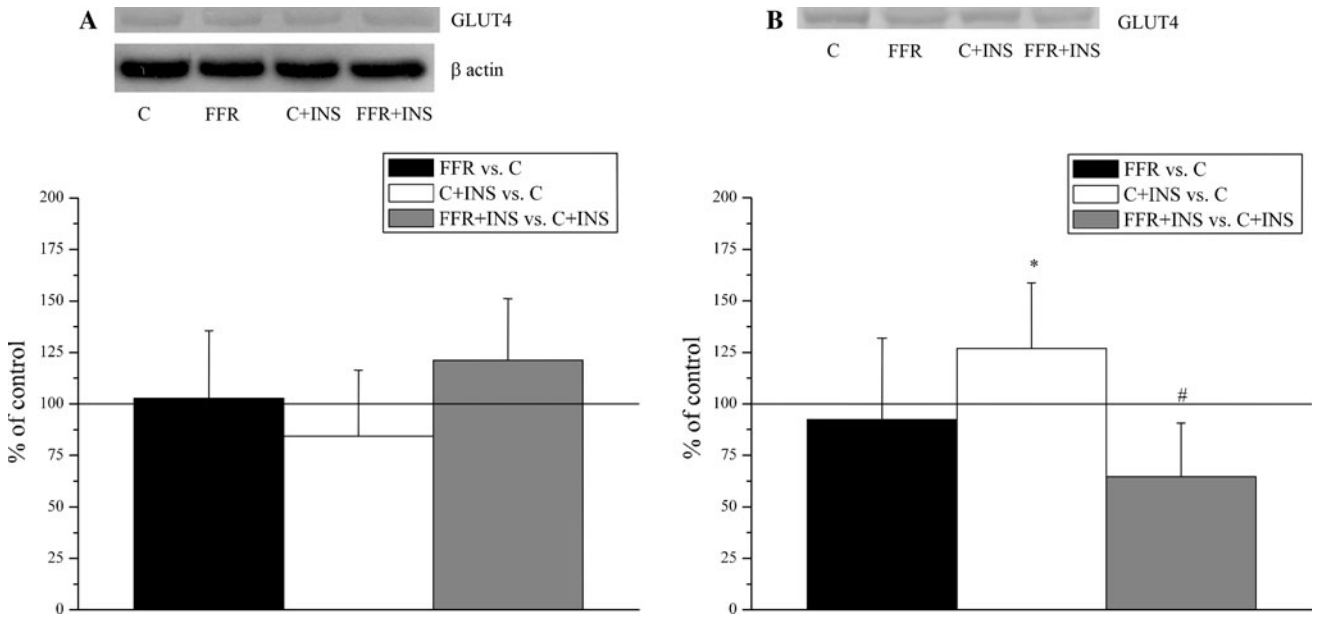

Fig. 4 Effect of fructose-enriched diet on the glucose transporter GLUT4 protein content in total cell lysate (**a**) and in plasma membrane (**b**) of cardiac cells in basal or insulin-stimulated state. Equal amounts of proteins were resolved on 10% SDS–PAGE and blotted with respective GLUT4 antibodies. *Upper panels* show representative blots, and quantified ratio is shown in the *lower panels*. The abbreviations are the same as in Fig. 2. Values are given as means ± SD of three independent experiments with a total of seven animals per group. $^{*}p < 0.05$ versus C; $^{\#}p < 0.05$ versus C + INS

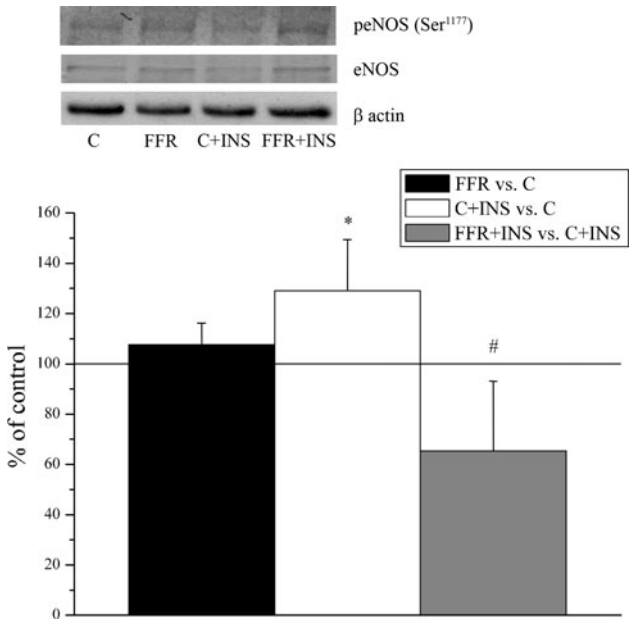

Fig. 5 Effects of fructose-enriched diet on eNOS signaling in rat heart. *Upper panels* show representative Western blots for phosphorylation of eNOS at Ser^{1177} (peNOS) and total eNOS from rats on standard and fructose-rich diet in the absence or presence of insulin. Quantified ratio of peNOS/eNOS is shown in the lower panel. The abbreviations are the same as in Fig. 2. Values are given as means ± SD of three independent experiments with a total of seven animals per group. $^{*}p < 0.05$ vs. C; $^{\#}p < 0.05$ vs. C + INS. *eNOS* endothelial nitric oxide synthase, *peNOS* phospho-eNOS

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