Estradiol enhances effects of fructose rich diet on cardiac fatty acid transporter CD36 and triglycerides accumulation

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ABSTRACT

Fructose rich diet increases hepatic triglycerides production and has deleterious cardiac effects. Estrogens are involved in regulation of lipid metabolism as well, but their effects are cardio beneficial. In order to study effects of fructose rich diet on the main heart fatty acid transporter CD36 and the role of estrogens, we subjected ovariectomized female rats to the standard diet or fructose rich diet, with or without estradiol (E2) replacement. The following parameters were analyzed: feeding behavior, visceral adipose tissue mass, plasma lipids, cardiac CD36 expression, localization and insulin regulation, as well as the profile of cardiac lipids. Results show that fructose rich diet significantly increased plasma triglycerides and decreased plasma free fatty acid (FFA) concentration, while E2 additionally emphasized FFA decrease. The fructose diet increased cardiac plasma membrane content of CD36 in the basal and insulin-stimulated states, and decreased its low density microsomes content. The E2 in fructose-fed rats raised the total cardiac protein content of CD36, its presence in plasma membranes and low density microsomes, and cardiac deposition of triglycerides, as well. Although E2 counteracts fructose in some aspects of lipid metabolism, and separately they have opposite cardiac effects, in combination with fructose rich diet, E2 additionally enhances CD36 presence in plasma membranes of cardiac cells and triglycerides accumulation, which paradoxically might promote deleterious effects of fructose diet on cardiac lipid metabolism. Taken together, the results presented in this work are of high importance for clinical administration of estrogens in females with a history of type 2 diabetes.

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1. Introduction

Under normal conditions heart uses fatty acids (60–90%) and glucose (10–40%) as key energy substrates (Kodde et al., 2007). Utilization of fatty acids and glucose depends mostly on their plasma concentration, but is also regulated by different agents. Insulin resistance is accompanied by altered cardiac usage of energetic substrates, shifting cardiac metabolic balance toward increased fatty acid utilization (Coort et al., 2007).

Enhanced intake of fructose initiates development of metabolic syndrome phenotype in humans and experimental animals (Tran et al., 2009). Insulin resistance is a hallmark of metabolic syndrome and enhanced intake of fructose causes decrease in insulin sensitivity in the heart, as we recently reported (Zakula et al., 2011). It is also well known that insulin regulates cardiac free fatty acid (FFA) transport (Luiken et al., 2004). On the other hand, specific fructose metabolism in the liver results in increased production of triglycerides which leads to further disturbances in lipid metabolism and development of obesity (Dekker et al., 2010). One of the numerous consequences of fructose rich diet is altered plasma concentration of heart energy substrates.

Estrogen hormones are also involved in regulation of lipid metabolism (Jensen et al., 1994) and selection of energy substrate (Herrero et al., 2005). Moreover, estrogen effects on insulin sensitivity are, at least partly, mediated by changes in lipid metabolism (Gao et al., 2006). Physiological concentrations of estrogens are cardio protective (Patten and Karas, 2006) and beneficial for insulin action (Gonzalez et al., 2002). Furthermore, estrogens affect specific cardiac insulin action (Koricancic et al., 2009). These hormones have also been assigned as protective against damaging effects of fructose rich diet (Galipeau et al., 2002).

Having in mind that both agents, fructose and estradiol, affect lipid metabolism as well as the insulin sensitivity, with strong direct and indirect effects on heart function, in this work, we have investigated potential protective role of E2 in anticipated fructose...
induced deterioration of lipid metabolism and alterations of main cardiac FFA transporter, CD36, and accumulation of cardiac triglycerides. For that purpose, ovariectomized female rats were subjected to fructose rich diet and to estradiol (E2) replacement and feeding behavior, visceral adipose tissue mass, plasma lipids, cardiac CD36 expression, localization and insulin regulation, as well as the profile of cardiac lipids were determined. This issue is of great importance for human medicine, concerning wide usage of estrogens in population of women with insulin resistance and type 2 diabetes.

2. Materials and methods

2.1. Chemicals

Fructose was purchased from API-PEK (Becej, Serbia). Polyclonal anti-CD36 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Reagents for the bicinchoninic acid (BCA) assay were purchased from Pierce (Rockford, IL, USA). Secondary anti-rabbit antibody, insulin, 17β-estradiol, and matrix for matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS), 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Animals

The research was approved by the official Vinca Institute's Ethical Committee for Experimental Animals. Female 21-day-old Wistar rats were separated from their mothers and divided into two main groups according to the diet regime. Control animals had free access to tap water and standard laboratory chow (normal diet). Animals held on fructose enriched diet were also fed standard food, but water was replaced with a 10% (w/v) fructose solution in tap water. Diet regime lasted 9 weeks.

Two weeks before sacrifice, all animals were bilaterally ovariectomized (OVX) under ketamine (40 mg/kg, i.p.)—xylazine (5 mg/kg, i.p.) anesthesia, to remove endogenous estrogens and prevent their effects (Vasudevan et al., 2005). Half of the fructose-fed rats were subjected to E2 replacement (40 μg/kg, s.c., every second day) the next day after ovariectomy, which continued until the day before sacrifice. Non-estrogen treated animals were injected with vehicle to avoid the effects of injection stress. In order to study fructose and E2 effects on insulin regulation of the analyzed molecule, experimental groups were divided into two subgroups, one of each was treated with insulin (12 IU/kg of b.w., 40 min before sacrifice, i.p.) and the other one with vehicle.

The dose and way of E2 administration were adopted from literature as a replacement protocol, to achieve the concentration near the physiological level (Sales et al., 2010). The dose and timing of insulin treatment were also estimated on the basis of literature data (Carvalheira et al., 2003), and optimized to observe the peak of induction of CD36 translocation to plasma membranes, as we recently reported (Tepavcevic et al., 2011). The suggestion of the authors of the original protocol (Luiken et al., 2002) was used to make decision which fractions to refer to as plasma membranes and low density microsomes.

2.3. Determination of plasma triglycerides and free fatty acid concentration

For measurement of plasma FFA and triglycerides levels the animals were fasted and fructose solution was replaced with water overnight, before collection of blood samples. The triglycerides level was measured using a Multicare analyzer (Biochemical Systems International, Arezzo, Italy) and the plasma FFA level was determined using a modified colorimetric method (Duncombe, 1964).

2.4. Preparation of cardiac plasma membranes and low density microsomes

Immediately after sacrifice, hearts were excised from the body and their mass was measured. Tissue was minced in small pieces and incubated for 30 min in a cold high-salt solution (2 mol/l NaCl, 20 mmol/l HEPES pH 7.4, and 5 mmol/l Na3HPO4). Thereafter, the plasma membranes and low density microsomes were isolated by sequential centrifugations, as we described earlier in detail (Tepavcevic et al., 2011). The suggestion of the authors of the original protocol (Luiken et al., 2002) was used to make decision which fractions to refer to as plasma membranes and low density microsomes.

2.5. Heart lysate preparation

After sacrifice, heart tissue was homogenized on ice with an Ultra-turrax homogenizer in a buffer (pH 7.4) containing 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and protease inhibitors (Dupont et al., 1998). The homogenates were centrifuged at 600 × g for 20 min at 4 °C, and the obtained supernatants were ultracentrifuged for 60 min at 100,000 × g. After determination of protein concentration by the BCA method (Smith et al., 1985) supernatants were prepared as cardiac cell lysate samples for Western blot analysis.

2.6. SDS-PAGE and Western blotting

Cardiac plasma membranes, low density microsomes, or lysate proteins (50 μg/lane) were separated on 10% SDS polyacrylamide gels and transferred to PVDF membranes (Laemmli, 1970). The membranes were blocked with 5% nonfat dry milk and blotted with an antibody against CD36. After extensive washing, membranes were incubated with secondary HRP-conjugated antibody and used for detection with ECL reagents. To ensure that protein loading was equal in all lysate samples, blots were stripped and reprobed with the actin antibody. Films were scanned and analyzed using ImageJ software (NIH, USA).

2.7. RNA isolation and quantitative real-time reverse transcriptase-PCR (qRT-PCR)

Total RNA from the heart tissue was isolated with the TRI Reagent according to the manufacturer (Ambion, Inc.). The structural integrity of RNA was confirmed by formaldehyde-agarose gel electrophoresis. One microgram of RNA was treated with DNase I and reverse transcription was performed using First Strand cDNA Synthesis kit, with oligo-dT18 primers, according to manufacturer’s instructions, (Fermentas, Lithuania). Mock reactions lacking reverse transcriptase were performed during the cDNA synthesis to additionally exclude genomic contamination. Real-time PCR was performed in duplicate in an ABI Real-time 7500 system (ABI, Foster City, CA). Detection of CD36 gene expression was done by the pre developed TaqMan® Gene Expression Assay ID Rn02115479_g1 (ABI, Foster City, CA). Detection of internal reference 18 s rRNA were done by the pre-developed TaqMan® Gene Expression Assays ID Hs99999901_s1 (ABI, Foster City, CA). Differences in mRNA expression between the groups were tested by the REST 09 software.

2.8. Extraction of cardiac lipids and MALDI-TOF MS

Lipids from heart muscle were extracted according to the modified Folch procedure (Folch et al., 1957). Briefly, 100 mg of heart tissue was homogenized in 2.5 ml of chloroform-methanol (1:5:1) mixture, upon which the water containing 0.45 M HCl was added, and homogenate vigorously mixed. The extraction mixture was centrifuged to separate individual layers, and chloroform (lower) layer was taken for further analysis of lipid composition.

Heart muscle was screened for lipid composition by MALDI-TOF MS using a Bruker Autoflex device with a nitrogen laser emitting at 337 nm. The chloroform solution of lipids (0.5 μl) extracted from the heart was applied onto the gold-coated MALDI target plate and immediately dried under a warm stream of air. After this, the same volume of 0.5 M solution of DHB in methanol was applied and also dried. All MALDI-TOF mass spectra were acquired under delayed extraction conditions and in the reflector mode. Each spectrum represents the average of 100 laser shots.

2.9. Statistical analysis

Values are expressed as the means ± S.D. (if not otherwise stated) of at least three independent experiments performed with a total of 9 animals per group. The significance of differences between two groups was estimated by the one-way ANOVA test. A value of $P < 0.05$ was considered statistically significant. In addition, differences in mRNA expression between groups were tested by a pairwise randomization and boot strapping technique using the relative expression software tool REST 09 (Corbett Life Science, http://rest.gene-quantification.info) (Pfaffl et al., 2002).

3. Results

3.1. Food, liquid and calorie intake

It is well known that a sweet fructose solution has a palatable effect. Increased intake of fructose leads to appetite deregulation due to absence of its acute effect on plasma glucose and insulin concentration. In accordance with these data, food and liquid intake were decreased ($P < 0.05$, vs. OVX-normal diet) and increased ($P < 0.001$, vs. OVX-normal diet), respectively in ovariectomized fructose-fed rats. The calorie intake was also increased ($P < 0.05$, vs. OVX-normal diet) (Table 1). Estradiol treatment additionally decreased food intake ($P < 0.001$ vs. OVX-normal diet; $P < 0.05$ vs. OVX-fructose rich diet), while liquid intake was partially reverted towards the control value ($P < 0.05$ vs. OVX-normal diet; $P < 0.05$ vs. OVX-fructose rich diet). Calorie intake was decreased in animals that received estrogen replacement in comparison with the non-estrogen treated fructose-fed rats ($P < 0.05$) (Table 1).

3.3. Cardiac plasma membranes and low density microsomes content of fatty acid transporter CD36

The CD36 is the main cardiac FFA transporter, which migrates between intracellular pool and plasma membranes, where it performs its primary function (Luiken et al., 2004). As presented in Fig. 1, the fructose rich diet increased the plasma membranes content of CD36 under basal and insulin-stimulated conditions ($P < 0.05$, vs. OVX-normal diet; $P < 0.05$, vs. OVX-normal diet+insulin), at the expense of the decrease in the low density microsomes content of transporter ($P < 0.01$, vs. OVX-normal diet; $P < 0.01$, vs. OVX-normal diet+insulin). It indicates transporter translocation from intracellular pool to plasma membranes as a mechanism of fructose effect.

The E2 and fructose diet act in the same direction regarding the CD36 plasma membranes level (Fig. 1a). The E2 additionally

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<th>OVX-normal diet</th>
<th>OVX-fructose rich diet</th>
<th>OVX-fructose rich diet + E2</th>
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<tbody>
<tr>
<td>Food intake (g/day)</td>
<td>19.23 ± 1.85</td>
<td>16.31 ± 3.37$^a$</td>
<td>14.46 ± 1.62$^{abc}$</td>
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<tr>
<td>Liquid intake (ml/day)</td>
<td>28.51 ± 4.54</td>
<td>48.75 ± 14.81$^b$</td>
<td>37.93 ± 11.49$^{abc}$</td>
</tr>
<tr>
<td>Caloric intake (kJ/day)</td>
<td>211.57 ± 20.31</td>
<td>263.26 ± 58.29$^c$</td>
<td>224.31 ± 32.62$^c$</td>
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Abbreviations: ovariectomy (OVX); estradiol treatment (E2).

Results are presented as mean ± S.D. from 9 animals per group.

$^a$ $P < 0.05$, vs. OVX-normal diet.

$^b$ $P < 0.001$, vs. OVX-normal diet.

$^c$ $P < 0.05$, vs. OVX-fructose rich diet.
increased plasma membranes CD36 in the fructose-fed rats (P < 0.01, vs. OVX-normal diet; P < 0.05, vs. OVX-fructose rich diet). A stimulating effect of E2 was observed in insulin-treated group as well (P < 0.001, vs. OVX-normal diet + insulin; P < 0.05, vs. OVX-fructose rich diet + insulin). In contrast to the effect of fructose rich diet, an increase of plasma membranes CD36 in the heart of E2-treated fructose-fed rats was accompanied by a significant increase in low density microsomes CD36 with (P < 0.05, vs. OVX-normal diet + insulin; P < 0.01, vs. OVX-fructose rich diet + insulin) or without (P < 0.05, vs. OVX-normal diet; P < 0.01, vs. OVX-fructose rich diet) insulin stimulation (Fig. 1b). This indicates an increase of the total cardiac expression of CD36 in animals subjected to E2 replacement.

3.4. Cardiac CD36 protein and mRNA expression

Changes in the membrane distribution of CD36 in the cardiac cells of fructose-fed rats were not accompanied by changes in the total CD36 protein expression (Fig. 2a). In contrast, the increase in plasma membranes and low density microsomes CD36 induced by E2 in the fructose-fed rats coincided with the increase of the CD36 content in the cardiac cell lystate, as compared to the fructose-fed rats (P < 0.05).

Analysis of the cardiac CD36 mRNA expression (Fig. 2b) indicated that the effects of fructose diet are in agreement with the results obtained for the cardiac CD36 protein expression (Fig. 2a), in terms of absence of the diet regime effect (OVX-fructose rich diet vs. OVX-normal diet, expression mean factor 1.773, S.E. range 0.724–3.438, 95% CI 0.648–5.718, P = 0.497). At the level of CD36 mRNA expression we did not find a significant effect of E2 replacement in the fructose-fed rats (OVX-fructose rich diet + E2 vs. OVX-fructose rich diet, expression mean factor 1.089, S.E. range 0.651–1.647, 95% CI 0.525–1.794, P = 0.804), but the increase of the CD36 mRNA level in fructose-fed rats subjected to E2 replacement with respect to control ovariectomized rats was close to significant (OVX-fructose rich diet + E2 vs. OVX-normal diet, expression mean factor 1.931, S.E. range 1.013–3.980, 95%CI 0.860–4.209, P = 0.09).

3.5. Composition of cardiac lipids

The increase of the plasma membranes level of CD36 induced by the fructose rich diet and E2 treatment (Fig. 1a) indicates increased entry of fatty acids into the heart, and changes in the cardiac lipid metabolism and deposition. Cardiac lipids were screened for their composition by MALDI-TOF MS. Representative spectra of heart lipids are shown in Fig. 3. The mass region of the main cellular phospholipids and triglycerides is shown in the spectra, and signals arising from phosphatidylincholines and triglycerides are indicated by their m/z ratios. They are also identified in the figure. In summary, no major differences could be observed between the samples obtained from lipids isolated from the OVX animals and those on the fructose diet (Fig. 3a and b, respectively). Signals in the region of triglycerides were not detected, most probably due to their lower concentrations and peak suppression by the presence of phosphatidylincholines (Petricovc et al., 2001). On the other hand, a significant increase in the signal intensity of triglycerides in the OVX animals on fructose diet treated with E2 (Fig. 3c) was detected, implying an increase in the content of these lipids in the heart. The following triglycerides were detectable: triglycerides (50:2), triglycerides (50:1), triglycerides (52:3), triglycerides (52:2), and triglycerides (54:3). The summarized compositions of triglycerides are given, where the first number indicates the total number of C atoms in the fatty acid residues, and the second indicates the total number of double bonds, since the exact position of fatty acid residue in a glycerol backbone and the position of a double bond on the fatty acid residue is not possible to determine by MALDI-TOF MS. In all cases, only Na-adducts of the triglycerides could be detected in the MALDI-TOF mass spectra, which agrees with their mass spectrometric behavior (Schiller et al., 2002).

4. Discussion

Altered myocardial substrate metabolism is one of the pathophysiological mechanisms which may contribute to the development of diabetic cardiomyopathy. Reduced myocardial glucose and lactate utilization with increased consumption of fatty acids is often associated with myocardial triglycerides accumulation in the insulin-resistant heart (Boudina and Abel, 2007). Fatty acids may impair insulin signaling and may activate several signaling pathways involved in cardiac oxidative metabolism. Increased uptake of plasma FFA probably contributes to increased fatty acid utilization, which is related to the reduced glucose oxidation that leads to the development of cardiac lipotoxicity and diabetic cardiomyopathy (Boudina and Abel, 2007).

Although there is a general consensus that fructose rich diet increases liquid intake at the expense of food intake and enhances the plasma triglycerides level, published data concerning the effects of this diet on plasma FFA are controversial. The decrease in the plasma FFA level observed in our study in ovariectomized fructose-fed rats agrees with the study which reported that FFA also tended to be lower in high fat/high fructose/streptozotocin-
Menard et al., 2010). Menard suggested that the higher insulin concentration in the HFHFS group may have contributed to the relatively low plasma FFA levels and myocardial fatty acid uptake in HFHFS rats by suppression of adipose tissue lipolysis (Menard et al., 2010). On the other hand, our results show that E2 decreased food, liquid, and calorie intake in fructose-fed rats. In addition, the FFA level is also decreased in the E2-treated rats, which is in accordance with previous findings (Jensen et al., 1994). Although the observed E2-induced decrease of the plasma triglycerides concentration in fructose-fed rats was not significant, it was unexpected. It has previously been suggested that a potential increase in the plasma triglycerides level could be an important issue in estrogen replacement therapy in women (Erberich et al., 2002; Stevenson, 2009).

In cardiomyocytes the most important fatty acid transporters are fatty acid translocase CD36, and two members of the family of 6 fatty acid transport proteins, FATP 1 and 6 (Luiken et al., 2004). In this study redistribution of CD36 from the low density microsomes fraction to plasma membranes of cardiac cells was observed in ovariectomized fructose-fed rats. This indicates a cardiac metabolic shift towards increased fatty acid utilization, which is characteristics of insulin resistance (Coort et al., 2007).

![Fig. 1. Effects of estradiol replacement treatment on the level of the CD36 free fatty acid transporter in cardiac plasma membranes (a) and the low density microsomes fraction (b) of ovariectomized female rats on fructose rich diet. Plasma membrane and low density microsomes content of the CD36 transporter were analyzed by Western blot. Results of three independent experiments with 9 animals per group are expressed as a fold of the appropriate control value (ovariectomized rats on normal diet or their insulin-treated counterparts) and presented as mean ± S.D. Representative Western blots are also shown. Ovariectomy (OVX); normal diet (ND); insulin treatment (INS); fructose rich diet (FRD); estradiol treatment (E2); plasma membranes (PM); low density microsomes (LDM). *p < 0.05, **p < 0.01, vs. OVX-normal diet; p < 0.05, **p < 0.01, vs. OVX-fructose rich diet; $p < 0.05, $$$p < 0.001, vs. OVX-normal diet + insulin; &p < 0.05, &&p < 0.01, vs. OVX-fructose rich diet + insulin.](image-url)
Impaired insulin signaling and action in the rat heart has previously been observed with fructose rich diet (Zakula et al., 2011). In addition, the CD36 mRNA expression shows the absence of significant change in the fructose-fed rats, as well as total protein expression does. In contrast, in a recent study the HFHFS treatment leads to an increase in the cardiac CD36 mRNA expression both under fasting and fed conditions (Menard et al., 2010). However, these results are not quite comparable to the present ones, since the nutrition model was different and the protein expression level of fatty acid transporter was not assessed in that study.

Although there are results that indicate the role of E2 in the regulation of CD36 expression in other tissues (Cheung et al., 2007), data concerning E2 effects on the cardiac CD36 are exceptionally rare. It was only shown that E2 administration following a trauma-hemorrhage increases the cardiac CD36 protein level (Hsieh et al., 2006), which could be connected with E2-induced increase of CD36 protein expression in the fructose-fed rats.

**Fig. 2.** Effect of estradiol on the CD36 protein (a) and mRNA (b) expression in the cardiac cells of ovariectomized female rats on fructose rich diet. Total CD36 protein content was determined by Western blot in lysate of cardiac cells. Analysis of cardiac CD36 mRNA expression was performed by quantitative RT PCR. Results of three independent experiments with 9 animals per group are expressed as a fold of the appropriate control value (ovariectomized rats on normal diet) and presented as mean ± S.D. and mean ± S.E., for Western blot and RT PCR, respectively. Representative Western blots are also shown. Ovariectomy (OVX); normal diet (ND); fructose rich diet (FRD); estradiol treatment (E2). *P < 0.05, vs. OVX-fructose rich diet.
rats observed in the present study. Regarding the fact that in the present study just a trend of the CD36 mRNA increase has been observed, it is possible that the effect of E2 on the CD36 gene, which resulted in an increase of the CD36 protein level in cardiac cell compartments of E2-treated fructose-fed rats, is more complex. It is well documented that E2 activates signaling pathways which regulate the protein metabolism (Koh et al., 2008; Morelli et al., 2003). Thus, genomic effects of E2 on the specific gene could be combined with general effects of this hormone on the stimulation of protein synthesis and the suppression of protein degradation (Kamanga-Sollo et al., 2010). In addition, an increase of the plasma membranes content of CD36 could be, at least partially, attributed to the translocation of CD36 from intracellular pools via activation of Akt signaling by E2 (Tepavcevic et al., 2011). We recently reported that single injection of E2 stimulates a rapid increase of CD36 in the plasma membranes fraction of cardiac cells of ovariectomized female rats (Tepavcevic et al., 2011) and this result is in agreement with the results on the effects of E2 replacement in the present study.

Based on the present results we speculated about the changes in cardiac lipid metabolism provoked by the fructose rich diet combined with estradiol replacement, which need to be confirmed by additional studies. The stimulation of the cardiac CD36 transporter by the fructose diet and E2 treatments might direct myocardial fatty acids to different metabolic pathways. The E2 probably primarily directs fatty acids to beta oxidation, producing energy for the cardiac functions (Maher et al., 2010; Tarnopolsky, 2011) and this result is in agreement with these findings and indicates enhanced FFA pool in the hearts of E2-treated fructose-fed rats, which indicates an increase in the amount of fatty acids amount in the heart muscle. The results obtained by MALDI-TOF MS analysis show that fatty acids at least make a triglycerides pool in the hearts of E2-treated fructose-fed rats, which indicates a stimulation of a triglycerides synthesis pathway by E2. Paradoxically, E2 might promote detrimental effects of fructose with fatal consequences for the heart. Therefore, this topic is of particular significance for clinical administration of estrogens to women with insulin resistance and type 2 diabetes. As a perspective subject, it would be very useful to analyze in detail the cardiac lipid metabolism of fructose-fed rats and the role of E2 in the regulation of these processes.

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