Fructose-rich diet induces gender-specific changes in expression of the renin–angiotensin system in rat heart and upregulates the ACE/AT1R axis in the male rat aorta

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Abstract

Introduction: The cardiovascular renin–angiotensin system (RAS) could be affected by gender and dietary regime. We hypothesized that male rats will be more susceptible to activation of RAS in the heart and aorta, as a response to a fructose-rich diet (FRD).

Materials and methods: Both male and female Wistar rats were given a 10% (w/v) fructose solution for 9 weeks. We measured the biochemical parameters, blood pressure (BP) and heart rate. We used Western blot and real-time polymerase chain reaction (PCR) to quantify protein and gene expression.

Results: In the male rats, the FRD elevated BP and expression of cardiac angiotensin-converting enzyme (ACE), while the expression of angiotensin-converting enzyme 2 (ACE2) and angiotensin II Type 2 receptor (AT_2R) were significantly decreased. In female rats, there were no changes in cardiac RAS expression due to FRD. Furthermore, the ACE/AT_1R axis was overexpressed in the FRD male rats' aortae, while only AT_1R was upregulated in the FRD female rats' aortae. ACE2 expression remained unchanged in the aortae of both genders receiving the FRD.

Conclusions: The FRD induced gender-specific changes in the expression of the RAS in the heart and aortae of male rats. Further investigations are required in order to get a comprehensive understanding of the underlying mechanisms of gender-specific fructose-induced cardiovascular pathologies.

Introduction

Increased intake of fructose has been shown to induce insulin resistance, Type 2 diabetes and the development of metabolic syndrome (MetS).[1,2] These states are associated with increased cardiovascular risk.[3–5] Blockade of the renin–angiotensin system (RAS) represents a potential therapeutic strategy against MetS.[6–9]

The main effector molecule of RAS, angiotensin II (Ang II), is synthesized by the angiotensin-converting enzyme (ACE).[10] The main mediators of Ang II action are angiotensin II Type I receptor (AT_1R) and angiotensin II Type II receptor (AT_2R). AT_1R activation in vascular tissue leads to cardiac myocyte hypertrophy, cardiac fibrosis and accumulation of inflammatory cells.[11,12] Also, AT_1R mediates the effect of a fructose-rich diet

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(FRD) on blood pressure (BP) elevation. On the other hand, AT_2R mainly counteracts AT_1R action.[13] Another substantial enzyme of the RAS cascade, ACE2, degrades Ang II into Ang-(1-7),[14] which has an ameliorating effects on insulin resistance, hypertriglyceridemia, fatty liver, obesity, adiposities, and myogenic and adipogenic differentiation.[15] In addition, ACE2 overexpression has a protective effect against Ang II-induced cardiac hypertrophy and fibrosis.[16]

It was previously shown that a high consumption of fructose (60% fructose by weight) increases the AT_1R messenger ribonucleic acid (mRNA) level in the rat heart and aorta, as well as the ACE mRNA level in the aorta, suggesting that vascular RAS mediates vascular dysfunction in fructose-fed male rats.[17] A recent study shows higher Ang II at the local level in the heart of male rats on a 10% FRD,[18] but it remains unknown in what way the 10% FRD affects the cardiac expression of four main components of RAS: ACE, ACE2, AT_1R and AT_2R.

Cardiovascular RAS expression could be affected by gender and sex hormones in a number of ways.[19,20] Still, most studies were performed either in those of male or of female gender. It has been suggested that female rats are protected against fructose-induced changes in metabolism and blood pressure.[21] Recently, we showed that estradiol opposed the fructose diet's (10% w/v) effects on cardiac RAS.[22] According to the data presented, we hypothesized that male rats will be more susceptible to activation of cardiovascular RAS and changes in BP than female rats, after consumption of a FRD. Thus, we investigated if there is a gender-specific effect of FRD (10% w/v) on BP and the expression of four RAS molecules in cardiovascular tissue: two main enzymes, ACE and counter-regulatory ACE2; and two main RAS receptors, AT_1R and AT_2R, which are supposed to mediate Ang II actions in an opposing manner.

Materials and methods

Animal model

All experiments with animals conformed to Directive 2010/63/EU of the European Parliament and were approved by the Vinca Institute's official Ethical Committee for Experimental Animals (protocol 01/11). Experiments were conducted on 36 animals grown in the Vinca Institute of Nuclear Sciences: 18 male and 18 female Wistar rats, aged 21 days. The animals were maintained under a 12-h light and 12-h dark cycle (lights on at 7 a.m.) at 22ºC and constant humidity. The animals were not kept in individual boxes, in order to avoid social isolation stress. Two control groups were comprised of 18 animals (9 female rats and 9 male rats), and the other 18 (9 female rats and 9 male rats) comprised the two experimental groups on FRD. The body weight of the rats in the two experimental groups wasn't different at the beginning of the diet. Fructose was purchased from API-PEK (Becej, Serbia).

Table 1. Chemical composition of standard laboratory chow.

Protein, not less than (%)	20
Moisture, not more than (%)	13
Ash, not more than (%)	10
Cellulose, not more than (%)	8
Ca, not less than (%)	1
Na (%)	0.15–0.25
P, not less than (%)	0.5
Lys, not less than (%)	0.9
Met + cystine, not less than (%)	0.75
Vitamin A, not less than (mg/kg)	3440
Vitamin D3, not less than (mg/kg)	0.04
Vitamin E, not less than (mg/kg)	25
Vitamin B12, not less than (mg/kg)	0.02
Zn, not less than (mg/kg)	100
Fe, not less than (mg/kg)	100
Mn, not less than (mg/kg)	30
Cu, not less than (mg/kg)	20
I, not less than (mg/kg)	0.5
Se, not less than (mg/kg)	0.1
Antioxidant, not less than (mg/kg)	100

Ca: calcium; Cu: copper; Fe: iron; I: iodine; kg: kilogram; Lys: lysine; Met: methionine; mg: milligram; Mn: manganese; Na: sodium; P: phosphor; Se: selenium; Zn: zinc.

The control animals had free access to tap water and standard laboratory chow (Table 1). The animals on the FRD were fed with the same standard laboratory food, but instead of tap water, they drank a 10% (w/v) fructose solution. The high fructose diet regime lasted for 9 weeks, and then the rats were sacrificed by decapitation.

Measurement of food and liquid intake, body and heart mass

At the beginning of the diet, the body mass of the rats in the two experimental groups was not significantly different (Table 2). Body mass was recorded during the treatment, as well as food and liquid intake. The total caloric intake in the fructose-fed rats was calculated as the sum of calories ingested as food and the 10% fructose solution. Immediately after the killing of the animals, the hearts and aortae were excised, washed in saline, dried and weighted. After weighing, the whole hearts were immediately cut in pieces on ice, mixed and aliquoted for further RNA and protein isolation (aliquots of the tissue were immediately snap-frozen in liquid nitrogen), in order to avoid the use of different parts of the heart for RNA and protein isolation.

Measurement of blood glucose, insulin and triglyceride

Before the collection of blood samples, the animals were fasted overnight. This was done in order to avoid changes in

Table 2. Food, liquid, caloric intake and biochemical parameters.

	ND female rat	FRD female rat	ND male rat	FRD male rat
Food intake (g/day/animal)	17.41 ± 2.99	12.59 ± 0.67[b]	20.57 ± 2.63	14.20 ± 2.01[b]
Liquid intake (g/day/animal)	33.02 ± 6.58	56.19 ± 20.41	49.33 ± 5.39	74.10 ± 11.36
Calorie intake (kJ/day/animal)	191.51 ± 32.89	236.06 ± 30.21	226.23 ± 28.9	283.65 ± 24.51
Insulin (mIU/l)	6.60 ± 2.5	8.87 ± 3.97	12.68 ± 6.33	7.51 ± 4.94
Glucose (mmol/l)	5.38 ± 0.65	4.58 ± 0.84	5.20 ± 0.42	4.84 ± 0.62
HOMA index	1.62 ± 0.69	1.86 ± 0.92	2.94 ± 1.49	1.72 ± 1.29
Triglyceride (mmol/l)	1.39 ± 0.29	1.87 ± 0.38[a]	1.49 ± 0.36	1.9 ± 0.35

Results are presented as means with standard deviations. Each experimental group consisted of 9 animals. The statistical significance between the animal groups was determined by Kruskal-Wallis test followed by Dunn's multiple comparison test.
[a]$p < 0.05$ versus ND female rat.
[b]$p < 0.01$ versus ND.
FRD: fructose-rich diet; HOMA: homeostasis model assessment index; kJ: kilo-Joule; l: liter; mmol: millimol; ND: normal diet not supplemented with fructose.

glucose level. We used an Accutrend glucometer (Roche Diagnostics GmbH, Mannheim, Germany) and Multicare analyzer (Biochemical Systems International, Arezzo, Italy) to determine blood glucose and triglyceride concentration, respectively. In order to obtain plasma samples, after decapitation we collected blood samples in EDTA-pretreated tubes and centrifuged at 1000 x g for 10 minutes. The plasma insulin level was determined by radioimmunoassay (RIA), while the homeostasis model assessment index (HOMA) was calculated from the fasted plasma insulin and glucose concentration, using previously published formulas.[23]

Blood pressure and heart rate

A previously described indirect tail-cuff method with external preheating was used for measurements of BP and heart rate in conscious, pre-conditioned rats.[24] This indirect method is recommended for detecting substantial differences in systolic BP between groups.[25]

Preparation of tissue lysate

Tissue specimens were homogenized on ice with an Ultra-turrax homogenizer in a buffer (pH 7.4) containing 10 mM-Tris, 150 mM-NaCl, 1 mM-EGTA, 1% Triton X-100, protease inhibitors (2 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The homogenates were centrifuged at 600 x g for 20 min at 4°C. The obtained supernatants were ultracentrifuged for 60 min at 100,000 x g. The BCA method was used to determine protein concentration.[26] We used supernatants as a cardiac cell lysate for Western blot analysis, after boiling in Laemmli sample buffer.

Western blot analysis

Cardiac and aortic lysate proteins (100 μg) were run through a 10% polyacrylamide gel and this was followed by electronic transfer to Polyvinylidene difluoride (PVDF) membranes at 4°C. The membranes were blocked with 5% bovine serum albumin (BSA) for 2 h. We then incubated them with primary antibodies (ACE (sc-12187, Santa Cruz Biotechnology, dilution 1:250), ACE2 (sc-17720, Santa Cruz Biotechnology, dilution 1:250), AT_1R (sc-1173, Santa Cruz Biotechnology, dilution 1:200 and PA5-2-812, Thermo Scientific, dilution 1:500) and AT_2R (ab19134, Abcam, dilution 1:2500)) on the membranes at 4°C, overnight. After extensive washing, the membranes were incubated with secondary anti-rabbit or anti-goat horseradish peroxidase-conjugated antibody (dilution 1:10,000) for 2 h at room temperature. After the last washing step, signals were detected using ECL reagents. The blots were stripped and re-probed with an anti-β-actin antibody (Santa Cruz Biotechnology), which served as a loading control for protein input. The films were scanned and analyzed using ImageJ software (National Institute of Health (NIH), USA).

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

Hearts were obtained with minimal manipulation of the sample, and these were snap-frozen in liquid nitrogen and stored at −70°C until the isolation of RNA. The extraction of total RNA from heart tissue was done using the TRI Reagent (Ambion, Inc.) according to the manufacturer's instructions. The RNA was quantified by reading the optical density (OD) at 260 and 280 nm with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed on 2μg of RNA that was treated with DNAse I (Fermentas, Lithuania), using a First Strand cDNA Synthesis kit with oligo-dT18 primers, according to the manufacturer's instructions (Fermentas, Lithuania). Mock reactions lacking reverse transcriptase were performed during the cDNA synthesis step, in order to exclude genomic contamination. Real-time PCR was performed in duplicate in an ABI Real-time 7500 system (ABI, Foster City, CA). Detection of AT_1R and AT_2R gene expression was done by amplification in a total volume of 25μl, using the pre-developed TaqMan® Gene Expression

Table 3. Body mass, absolute and relative mass of the heart, blood pressure and heart rate.

	ND female rat	FRD female rat	ND male rat	FRD male rat
Body mass (g)	263.2 ± 30.26	279.5 ± 20.52	338.9 ± 40.45[c]	311.3 ± 37.58
Heart mass (g)	0.83 ± 0.13	0.83 ± 0.08	1.05 ± 0.14[b]	0.94 ± 0.16
Heart to body index (×10⁻³)	3.17 ± 0.16	3.04 ± 0.21	3.11 ± 0.19	3.00 ± 0.26
BP (mm Hg)	121.43 ± 7.01	124.14 ± 12.4	109 ± 4.33	120.6 ± 7.93[a]
Heart rate (beats/min)	433.71 ± 47.21	437.14 ± 24.84	479.33 ± 43.63	475.2 ± 36.6

Results are presented as means with SD for a total of nine animals per each experimental group. The statistical significance between animal groups was determined by Kruskal-Wallis test, followed by Dunn's multiple comparison test.
[a]$p < 0.05$ versus ND male rat.
[b]$p < 0.01$ versus ND female rat.
[c]$p < 0.001$ versus ND female rat.
BP: blood pressure; FRD: fructose-rich (supplemented) diet; mm Hg: millimeters of mercury in pressure; ND: normal diet without fructose added.

Assays Rn02758772_s1 and Rn00560677_s1 (ABI, Foster City, CA). Detection of internal reference 18s rRNA was done by the pre-developed TaqMan® Gene Expression Assays ID Hs99999901_s1 (ABI, Foster City, CA). The reaction mixture was initially denatured at 95°C for 10 min, which was followed by 40 cycles of a denaturation step at 95°C for 15 s, and an annealing step at 60°C for 60 s. The 18s rRNA was used as a reference gene and the $2^{-\Delta Ct}$ method was used to process the data and compare the differences in relative gene expression between groups.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software). The data were presented as a mean ± SD. To determine the statistical significance between groups, we used either the Mann Whitney U test or the Kruskal-Wallis test, followed by Dunn's multiple comparison test when the data were not distributed normally. For normally distributed data, we used one-way analysis of variance (ANOVA), followed with the Tukey post-hoc test. To determine the effects of gender and diet, as well as their interaction, we used a two-way ANOVA. In all tests, the differences with two-tailed alpha-probability and $p < 0.05$ were considered significant.

Results

Food, liquid and caloric intake

As presented in Table 2, both male and female fructose-fed rats had significantly decreased intake of solid food ($p < 0.01$ normal diet (ND) versus FRD), while their liquid and total caloric intake were unchanged ($p > 0.05$ ND female rats versus FRD female rats; $p > 0.05$ ND male rats versus FRD male rats).

Biochemical parameters

The FRD did not affect blood glucose levels, the plasma insulin level nor the HOMA index in both male and female rats ($p > 0.05$ ND versus FRD). As seen in Table 2, the triglyceride level increased in female rats after 9 weeks consumption of the 10% fructose solution ($p < 0.05$ ND versus FRD).

Body mass and the mass of the heart

Both body and heart mass were significantly different between the female and male rats that were fed the normal diet ($p < 0.001$ and $p < 0.01$ for ND female rats versus ND male rats, respectively). The FRD didn't cause alteration in body mass, absolute mass of the whole heart, nor the heart-to-body ratio (Table 3).

Systolic blood pressure and heart rate

As presented in Table 3, systolic BP was significantly increased in the FRD male rats, compared to the ND male rats ($p < 0.05$, ND versus FRD). In female rats, there were no significant changes in systolic BP ($p > 0.05$, ND versus FRD). The fructose diet regime did not influence heart rate in either the male or female rats ($p > 0.05$, ND versus FRD) (Table 3).

Effects of the FRD on the expression of ACE and ACE2 in the heart of male and female rats

The present study showed that the FRD significantly increased ACE (Figure 1(a)) and decreased ACE2 (Figure 1(b)) protein expression in the heart of male rats ($p < 0.01$, ND versus FRD); while in the female rats, both proteins remained unchanged ($p > 0.05$, ND versus FRD). The results of the two-way ANOVA revealed that only the interaction of gender and fructose diet showed a statistically significant effect on ACE protein expression ($p < 0.05$), while gender and diet alone did not impact ACE protein expression. The two-way ANOVA showed that both gender and diet impact ACE2 protein expression ($p < 0.01$ and $p < 0.05$, respectively). The ACE protein was significantly more abundant in the heart of FRD male rats, compared to FRD female rats ($p < 0.01$, FRD male rats versus
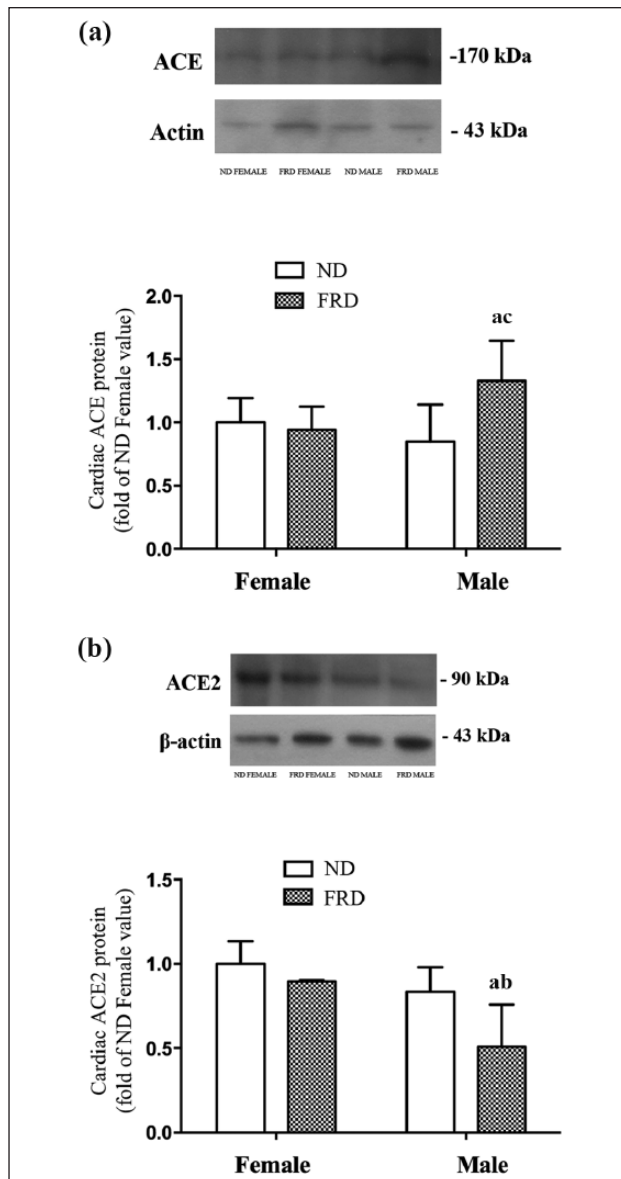
Figure 1. Alteration of cardiac ACE and ACE2 protein expression in male and female rats, due to a FRD. Data were normalized using β-actin expression. The statistical significance of the differences in mean values between the four animal groups was assessed by one-way ANOVA, followed with a post-hoc Tukey test. The FRD significantly increased ACE expression only in male rats. ACE protein expression was significantly higher in the fructose-fed male rats than in the fructose-fed female rats (Figure 1(a)). The FRD significantly decreased ACE2 expression in male rats, compared to both male rats on a normal diet and fructose-fed female rats (Figure 1(b)). The Western blot of heart tissue shows the results for three independent experiments, with a total of nine rats per each experimental group. Results are expressed as a fold of the appropriate control value (ND female rats). Values are means, with the SDs represented by vertical bars. Representative Western blots are shown.
[a]$p < 0.01$ versus ND male rats.
[b]$p < 0.05$ versus FRD female rats.
[c]$p < 0.01$ versus FRD female rats.
ACE: angiotensin-converting enzyme; ANOVA: analysis of variance; FRD: fructose-rich diet; ND: normal diet.

FRD female rats), while ACE2 protein expression was significantly lower ($p < 0.05$, FRD male rats versus FRD female rats).

We did not detect significant differences in cardiac ACE and ACE2 protein expression between the ND male and ND female rats.

Effects of the FRD on the expression of AT_1R and AT_2R in the heart of male and female rats

We did not detect a gender-specific effect for FRD on AT_1R protein expression in heart tissue. Still, both the AT_1R protein and mRNA had significantly higher expression in the hearts of FRD male rats, compared to FRD female rats ($p < 0.05$, FRD male rats versus FRD female rats) (Figure 2(a) and 2(b), respectively). The two-way ANOVA revealed a significant effect of gender on AT_1R protein expression ($p < 0.05$), and a significant effect of gender ($p < 0.05$) and interaction of gender and the fructose diet ($p < 0.05$) on the AT_1R mRNA expression.

For the AT_2R, we detected a male gender-specific significant decrease in AT_2R protein expression in the heart, originated as a consequence of a FRD ($p < 0.01$; ND versus FRD) (Figure 3(a)). A similar decrease was noticed when comparing FRD male rats with FRD female rats ($p < 0.05$, FRD male rats versus FRD female rats). The two-way ANOVA detected a significant effect of both gender ($p < 0.001$) and diet ($p < 0.05$) on AT_2R protein expression. The AT_2R mRNA was expressed in the heart of ND male rats, but it was not even detectable in the heart of the FRD male rats. In both the ND- and FRD-fed female rats the AT_2R mRNA was expressed, but it was not changed by the diet regime (Figure 3(b)).

We did not detect a significant difference in the heart AT_1R and AT_2R, either as protein or mRNA expression, between the ND male and ND female rats.

Effect of FRD on the expression of ACE, ACE2 and AT_1R protein in the aortas of male and female rats

As we found a gender-specific effect for FRD on the expression of RAS molecules in the heart, with significant changes in males, as well as an increase in BP only in the FRD males, we performed an experiment to investigate these molecules in the vasculature of male and female rats. The FRD significantly increased both ACE ($p < 0.001$; ND versus FRD) and AT_1R ($p < 0.001$; ND versus FRD) protein expression in the aortic tissue of male rats, while ACE2 protein expression was unaffected by the diet regime (Figure 4(a), 4(b) and 4(c), respectively). In female rats, the ACE and ACE2 levels remained unchanged in the FRD-fed rats, compared to the ND rats (Figure 4(d) and 4(e), respectively), while the AT_1R level increased (Figure 4(f)).
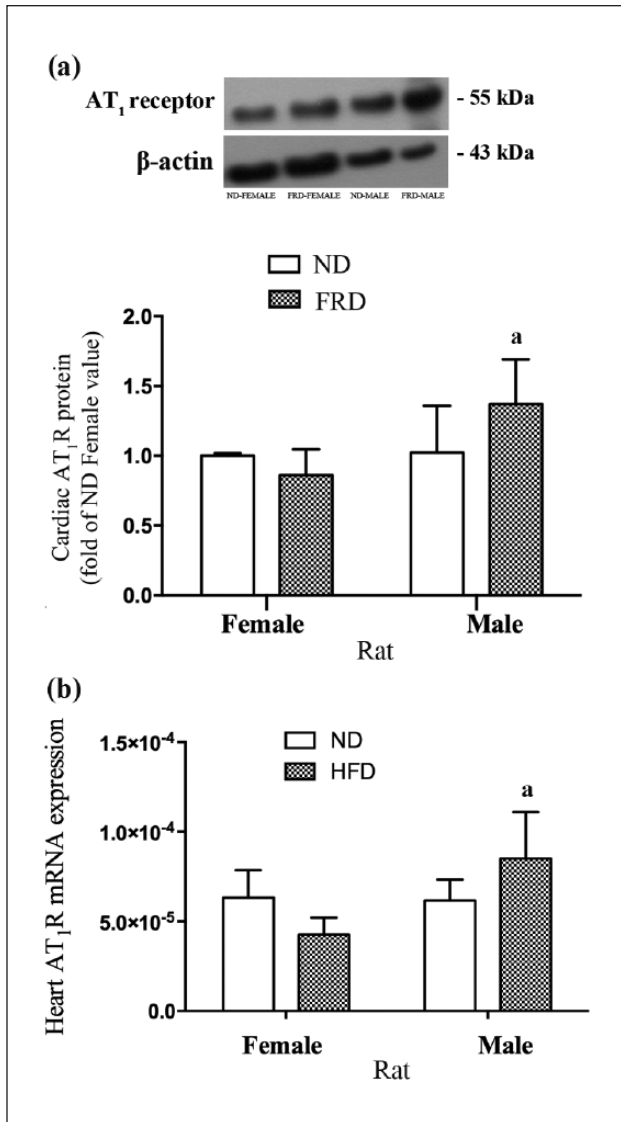
Figure 2. (a) Alteration of cardiac AT_1R protein expression in male and female rats due to a FRD. Data were normalized using β-actin expression. The statistical significance of the differences in mean values between the four animal groups was assessed by one-way ANOVA, followed with a post-hoc Tukey test. FRD significantly increased AT_1R protein expression in fructose-fed male rats, as compared to fructose-fed female rats. The Western blot of heart tissue shows the results for three independent experiments, with a total of nine rats per each group. Results were expressed as a fold of the appropriate control value (ND females). Values are means, with the SDs represented by vertical bars. Representative Western blots are also shown.
(b) Alterations of cardiac AT_1R mRNA expression in male and female rats due to a FRD, as measured by qRT-PCR. FRD significantly upregulated AT_1R mRNA expression in fructose-fed male rats, as compared to fructose-fed female rats. Results were expressed as the mean with SD.
[a]$p < 0.05$ versus FRD female rats.
ANOVA: analysis of variance; AT_1R: angiotensin II Type 1 receptor; FRD: fructose-rich diet; mRNA: messenger ribonucleic acid; ND: normal diet; RT-PCR: reverse transcriptase polymerase chain reaction.
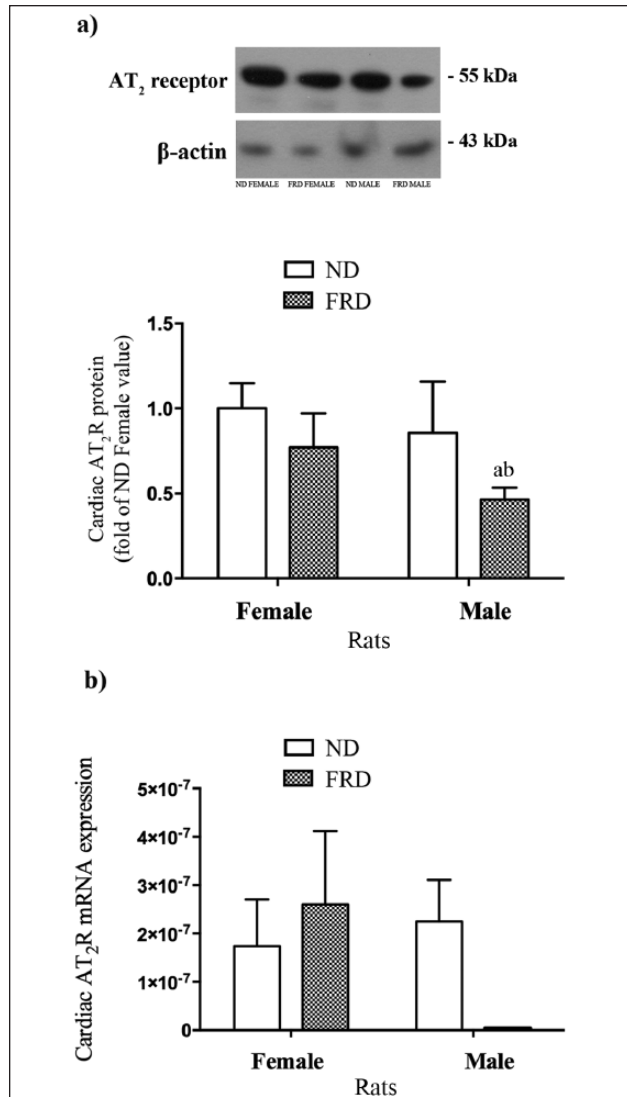
Figure 3. (a) Alteration of cardiac AT_2R protein expression in male and female rats due to a FRD. Data were normalized using β-actin expression. The statistical significance of the differences in mean values between the four animal groups was assessed by one-way ANOVA, followed with a post-hoc Tukey test. The FRD significantly decreased AT_2R protein expression in fructose-fed male rats, compared to both male rats on a ND and fructose-fed female rats. Western blot of heart tissue shows the results for three independent experiments, with a total of nine rats per group. Results are expressed as a fold of the appropriate control value (ND female rats). Values are means, with the SDs represented by vertical bars. Representative Western blots are also shown. (b) Alterations of cardiac AT_2R mRNA expression in male and female rats due to FRD, measured by qRT-PCR. There were no statistically significant differences among the groups compared. AT_2R mRNA was not detected in the FRD male group. Results are expressed as mean with SDs.
[a]$p < 0.05$ versus FRD female rats.
[b]$p < 0.01$ versus ND male rats.
ANOVA: analysis of variance; AT_2R: angiotensin II Type 2 receptor; FRD: fructose-rich diet; mRNA: messenger ribonucleic acid; ND: normal diet; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction.
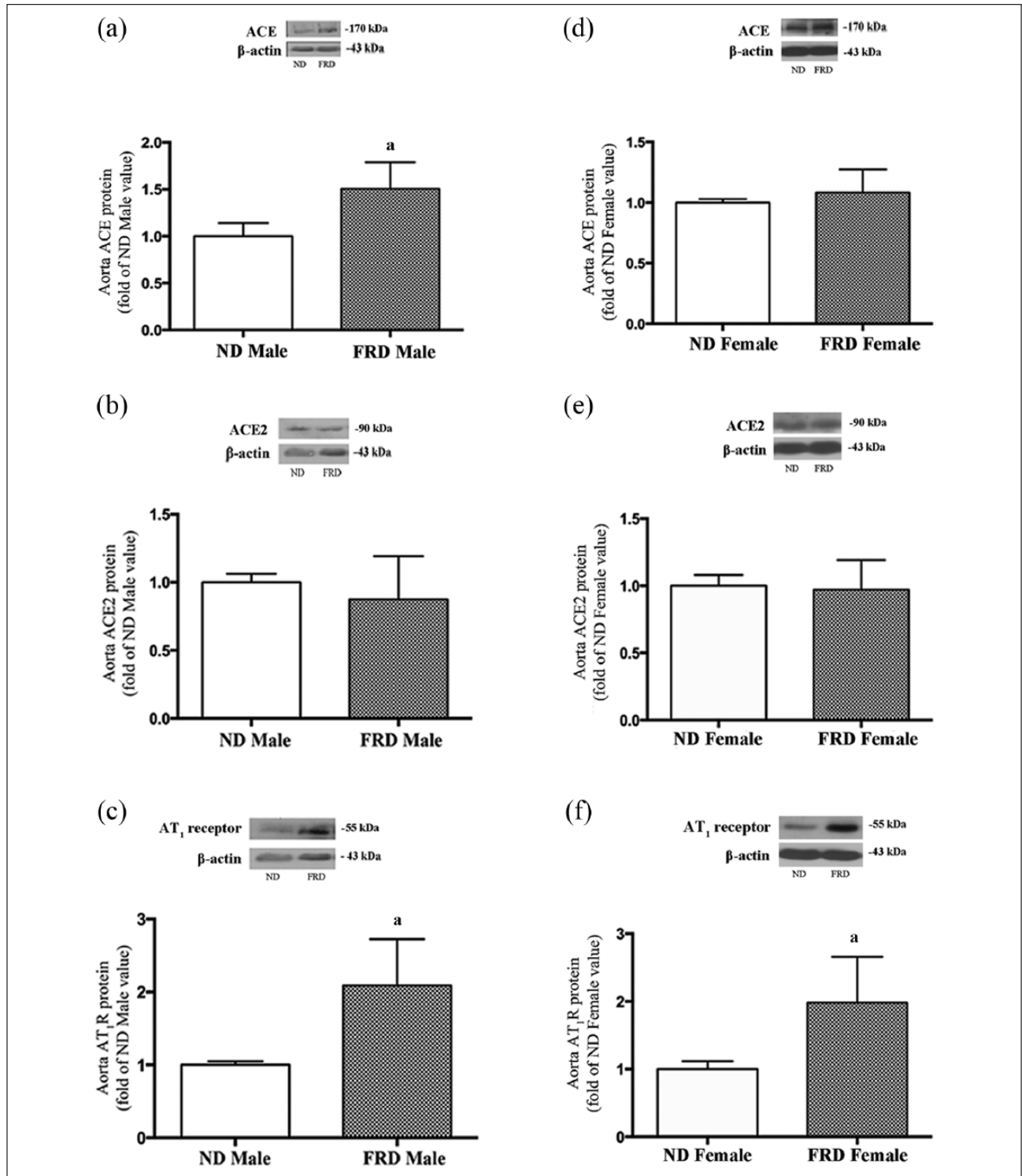
Figure 4. Effect of FRD on ACE, ACE2 and AT$_1$R protein expression in aortae of male and female rats. Data were normalized using β-actin expression. The statistical significance of the differences in mean values between the two animal groups was assessed by Mann Whitney U test. The FRD significantly increased (a) ACE and (c) AT$_1$R protein expression in male rats, while (b) ACE2 protein expression was unaffected by this diet regime. FRD significantly increased (f) AT$_1$R protein expression in female rats, while (d) ACE and (e) ACE2 protein expression remained unchanged. Western blot of aortic tissue is shown (results for three independent experiments, with a total of nine rats per group). The results are expressed as a fold of appropriate control values (ND male rats and ND female rats). Values are shown as means, with the SD represented by vertical bars. Representative Western blots are also shown.
[a]$p < 0.001$ versus ND male rats
ACE: angiotensin-converting enzyme; AT$_1$R: angiotensin II Type 1 receptor; FRD: fructose-rich diet; ND: normal diet.

Discussion

The main finding of the present study is that the cardiac RAS upregulation induced by FRD shows a gender-specific pattern. The significant changes of RAS molecules expression in the heart were detected only in the fructose-fed male rats and was accompanied by fructose-induced upregulation of the ACE/AT$_1$R axis in male rats' aortas. Furthermore, the changes in the RAS molecules' expression in the cardiovascular tissue of fructose-fed male rats coincided with elevated systolic BP.

It is know that there are gender differences in susceptibility to disease and treatment; still, there is the gender imbalance in research, especially in animal models.[27] Only a few studies investigated the influence of FRD according to gender.[28,29] In this study, we did not observe changes in blood glucose level, insulin level, or the HOMA index after consumption of a FRD; however, we recently reported FRD-impaired glucose tolerance in male rats exposed to FRD for 9 weeks between weaning and adulthood, in terms of having an increased AUC value under the glucose curve and a prolonged glucose removal time in the glucose tolerance test.[29] In addition, we observed an increased level of phospho Ser[307] IRS-1, an insulin resistance marker, in the heart of male rats that drank 10% fructose for 9 weeks.[30]

Previously, it was demonstrated that a diverse protein diet differently affected RAS and BP in male and female rats.[31] Nevertheless, this is the first study investigating the gender-specific effect of a FRD on the expression of RAS molecules in the heart and aortae. It is known that overactivation of the ACE/Ang II/AT$_1$R pathway is involved in cardiovascular pathologies such as hypertension, hypercholesterolemia, diabetes and heart failure. Previously, a single study had shown that a HFD (60% fructose by weight) triggered an increase of the ACE and AT$_1$R mRNA expression in aortae and similarly in the heart, but that study was performed exclusively in male rats.[17]

In this study, we investigated both genders and four main components of the cardiac RAS. A significant effect of the interaction between gender and a fructose diet on the cardiac AT$_1$R mRNA expression was observed in our study. It is known that AT$_1$R activation causes hypertrophy of cardiac myocytes and promotes cardiac fibrosis.[11] The mass of the heart or heart-to-body ratio in the fructose-fed animals were unchanged, compared to adequate controls. This is in a line with our previous finding of unchanged matrix metalloproteinase 9 (MMP-9) expression, a marker of cardiac remodeling, between the rats that were fed a FRD and standard laboratory chow; however, we detected higher MMP-9 mRNA expression in the FRD-consuming males, compared to the FRD females.[32] Previous studies have implied that either a prolonged fructose diet regime or the increased percentage of consumed fructose finally do result in cardiac hypertrophy.[33,34] Although we didn't

detect the effect of a FRD on AT$_1$R protein expression in heart tissue, our finding of ACE protein upregulation in the heart of male rats on a FRD (10% fructose by weight) is in agreement with the previous finding.[17]

We also detected a significant effect of both gender and diet on AT$_2$R and ACE2 protein expression in the heart. It was suggested that in female rats the vasodepressor arm (ACE2/AT$_2$R) could be upregulated, compared to male rats.[20] The protein expression of ACE2 and AT$_2$R in the heart were significantly lower in the FRD male rats, compared with the FRD female rats. Moreover, AT$_2$R mRNA was not even detected in the FRD male rats. We can propose that a FRD attenuates the *de novo* synthesis of AT$_2$R mRNA, which can further lead to a decrease in protein expression. Previously, male and female rats treated with Ang II had significantly less AT$_2$R mRNA expression in the left ventricle than the controls.[20] Ang II could downregulate AT$_2$R expression by decreasing the stability of its mRNA.[35,36] Considering the fact that 10% FRD increased plasma Ang II in male rats,[18] we can speculate that a FRD, through increasing the Ang II level in the heart of fructose-fed male rats, attenuates AT$_2$R mRNA expression. This could be the reason why we did not detect AT$_2$R gene transcription in the fructose-fed male rats.

Here we detected early gender-specific changes in cardiac RAS expression on the molecular level, even on a 10% fructose diet and even before morphological changes of cardiac tissue appeared, which are of great importance, because they reflect changes on the subclinical level.

We detected a significant increase of systolic BP only in the FRD-consuming male rats. It seemed that the female rats were protected against fructose-induced changes in BP, which is in agreement with previous findings.[21,22] It was shown that fructose has a direct, deleterious effect on aortic vascular contractility.[37] An increase of the plasma Ang II level in male rats was described after 10% FRD.[18] The aorta hyporeactivity to Ang II was shown in spontaneously hypertensive females, in comparison to males.[19] Furthermore, in the isolated aortae of male rats, the contraction induced by Ang II was more potent in rats fed a 60% fructose diet, in comparison with controls. The AT$_1$R mRNA was overexpressed in the same animals.[38] There are no results about the expression of RAS in the aortae of FRD fed female rats. In the current study, AT$_1$R protein expression was enhanced in the aortae of FRD-fed animals of both genders, but ACE expression was upregulated only in aortae of the male rats. Although ACE2 protein expression in aortic tissue was unaffected by FRD, the ACE/ACE2 ratio in males increased. The ACE/ACE2 disbalance could lead to higher levels of Ang II. It is known that activation of AT$_1$R stimulates various tyrosine kinases that besides stimulating cell growth and proliferation, also lead to vasoconstriction and a subsequent increase of BP.[39,40] Because FRD elevates AT$_1$R in the aortae of both genders of rats, but the ACE level and BP

only in male rats, we propose that simultaneous upregulation of ACE and AT_1R in the aorta contributes to the elevated BP detected in FRD male rats.

A progressive induction of hypertension and hyperuricemia was noticed in regard to higher fructose intake (Controls < 10% FRD < 60% FRD).[41] Hyperuricemia, which could lead to an increase of BP, renal arteriolar damage and glomerular hemodynamic changes, was associated with the activation of RAS.[42] Still, feeding rats with a 10% fructose solution did not produced the effect on glomerular hemodynamic of change, while feeding rats with a 60% fructose solution led to differences in the previously mentioned pathologies. The 10% fructose solution did not demonstrate renal cortical vasoconstriction in rats, whereas 60% fructose did.[41,43] Although fructose in drinking water induces an increment in water consumption,[41] which was not statistically significant in the current study, it was previously hypothesized that the volume expansion in rats fed with a 10% fructose solution may have a renal vasodilatory effect.[41]

The current study has certain limitations that should be discussed. There is a possibility that female rats might generally ingest less fructose than males; thus, one of the study limitations was the lack of tracking of how much fructose was ingested by each animal. Also, keeping each animal alone in the cage would allow more precise measurement of fructose intake; but on the other hand, it could influence the social isolation stress, which alters the hormonal status of rats and consequently could influence results. Nevertheless, we did not detect a significant difference in liquid intake in female rats, in comparison to male rats. Also, we have not monitored the BP evolution during the 9-week fructose diet. All the parameters that were analyzed in the study (BP, biochemical parameters, mass of the body and heart and the expression of RAS molecules) were measured at the end of the experiment; however, in previous studies, the level of systolic BP increased gradually throughout the entire experimental period in the 10% w/v fructose-fed rats, reaching or maintaining the significant differences with respect to the control group at the end of the protocol (12 and 6 weeks, respectively).[44,18] Thus, we assume that our current design is comparable with previous studies. Although the cardiac hypertrophy and remodeling was usually evaluated based on relative cardiac weight,[44] such as in the current study, we might consider a limitation of our study to be the lack of measurement of cardiomyocyte diameter or the thickness of the left ventricle wall. Further studies should also determine the average diameter and the wall thickness of the aortae.

Conclusions

Male rats are more prone to changes of cardiac RAS molecules expression after consumption of a 10% FRD. Upregulated expression of ACE/AT_1R axis that was detected in male rats' aortas were accompanied by the increase in BP, in the FRD male rats. Further investigations are required, in order to get a comprehensive understanding of the underlying mechanisms and to investigate the capacity of RAS as a reasonable target for prevention of gender-specific fructose-induced cardiovascular changes and consequent pathologies.

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