Molecular and Cellular Endocrinology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Involvement of glucocorticoid prereceptor metabolism and signaling in rat visceral adipose tissue lipid metabolism after chronic stress combined with high-fructose diet

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ARTICLE INFO

Keywords:
Glucocorticoids
Chronic unpredictable stress
Visceral adipose tissue
Lipid metabolism
Fructose

ABSTRACT

Both fructose overconsumption and increased glucocorticoids secondary to chronic stress may contribute to overall dyslipidemia. In this study we specifically assessed the effects and interactions of dietary fructose and chronic stress on lipid metabolism in the visceral adipose tissue (VAT) of male Wistar rats.

We analyzed the effects of 9-week 20% high fructose diet and 4-week chronic unpredictable stress, separately and in combination, on VAT histology, glucocorticoid prereceptor metabolism, glucocorticoid receptor subcellular redistribution and expression of major metabolic genes. Blood triglycerides and fatty acid composition were also measured to assess hepatic Δ9 desaturase activity.

The results showed that fructose diet increased blood triglycerides and Δ9 desaturase activity. On the other hand, stress led to corticosterone elevation, glucocorticoid receptor activation and decrease in adipocyte size, while phosphoenolpyruvate carboxykinase, adipose tissue triglyceride lipase, FAT/CD36 and sterol regulatory element binding protein-1c (SREBP-1c) were increased, pointing to VAT lipolysis and glyceroneogenesis. The combination of stress and fructose diet was associated with marked stimulation of fatty acid synthase and acetyl-CoA carboxylase mRNA level and with increased 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase protein levels, suggesting a coordinated increase in hexose monophosphate shunt and *de novo* lipogenesis. It however did not influence the level of peroxisome proliferator-activated receptor-gamma, SREBP-1c and carbohydrate responsive element-binding protein.

In conclusion, our results showed that only combination of dietary fructose and stress increase glucocorticoid prereceptor metabolism and stimulates lipogenic enzyme expression suggesting that interaction between stress and fructose may be instrumental in promoting VAT expansion and dysfunction.

1. Introduction

Overconsumption of energy-dense food in combination with everyday stress represents a hallmark of modern lifestyle and coincides with the rising incidence of obesity, metabolic syndrome and type 2 diabetes. It is well documented that the consumption of large amounts of liquid fructose may induce features of metabolic syndrome, including dyslipidemia, insulin resistance and adipose tissue dysfunction (Basciano et al., 2005). Fructose consumption was largely increased over past few decades mainly through utilization of high fructose corn syrup in food industry, which expanded its use as a sweetener in soft drinks and juice beverages (Bray et al., 2004). It is now well documented that fructose is involved in the genesis and progression of the metabolic syndrome mainly through deregulation of signaling

Abreviations: VAT, visceral adipose tissue; GR, glucocorticoid receptor; SREBP-1c, sterol regulatory element binding protein-1c; 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; H6PDH, hexose-6-phosphate dehydrogenase; ChREBP, carbohydrate responsive element-binding protein; FFA, free fatty acid; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; HSL, hormone-sensitive lipase; ATGL, adipose tissue triglyceride lipase; PPARγ, peroxisome proliferator-activated receptor-gamma

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https://doi.org/10.1016/j.mce.2018.04.015
Received 14 February 2018; Received in revised form 4 April 2018; Accepted 29 April 2018

pathways in the liver and adipose tissue, which are tissues with insulin-modulated metabolism (Miller and Adeli, 2008). Apart from the diet, it is also known that chronic stressors cause physiological and neuroendocrine changes that are associated with increased food intake and have been related to visceral obesity and insulin resistance in both animals and humans (Kouvonen et al., 2005). It was previously shown that this type of obesity, characterized by the accumulation of excessive fat around the abdomen, is strongly correlated with cardiovascular and metabolic diseases (Despres et al., 2008).

Glucocorticoids are known as stress and anti-inflammatory hormones, but they also act as potent regulators of adipose tissue energy metabolism (Wang, 2005). It has been suggested that systemic hypercortisolemia or local generation of active glucocorticoids in visceral adipose tissue (VAT), mediated by the enzymes 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and hexose-6-phosphate dehydrogenase (H6PDH), can increase visceral adiposity and have deleterious metabolic effects (Walker, 2006). Despite considerable evidence that connects 11βHSD1 overexpression in adipose tissue and development of visceral obesity (Livingstone et al., 2000; Seckl et al., 2004), data addressing the effects of stress and fructose interaction on adipose tissue prereceptor glucocorticoid metabolism in adult rats are still scarce. Glucocorticoids dually affect lipid storage depending on the adipose tissue depot and nutritional and/or hormonal conditions (Lundgren et al., 2004; Peckett et al., 2011). Although some human and *in vitro* studies showed that glucocorticoids preferably induce lipolysis in subcutaneous adipose tissue (Ryden and Arner, 2017; Stimson et al., 2017), rodent studies revealed that glucocorticoid-induced lipolysis is rather related to VAT (Bjorndal et al., 2011; Chusyd et al., 2016). Furthermore, several lines of evidence support the hypothesis that VAT lipolysis is actually the major contributor for obesity-related insulin resistance through release of free fatty acids (FFAs) (Lebovitz and Banerji, 2005; Nielsen et al., 2004). In addition, when insulin level rise with feeding, glucocorticoids may act synergistically with insulin, increasing the expression of numerous genes involved in lipogenesis and fat deposition (Lee et al., 2014). Glucocorticoids are involved in direct transcriptional regulation of lipolytic enzymes, hormone-sensitive lipase (*Hsl*) and adipose tissue triglyceride lipase (*Atgl*) (Campbell et al., 2009a), through activation of glucocorticoid receptor (GR). In addition, fatty acid translocase (FAT/CD36) was also shown to be involved in glucocorticoid-mediated lipolysis (Lee et al., 2014; Yu et al., 2010), as well as in fatty acid re-esterification (Wan et al., 2013). On the other hand, glucocorticoids can modulate expression of several prolipogenic factors, including genes involved in lipid intake (lipoprotein lipase, *Lpl*), *de novo* lipogenesis (DNL) (acetyl-CoA carboxylase, *Acc* and fatty acid synthase, *Fas*) (Campbell et al., 2011; Wang et al., 2012) and glyceroneogenesis (phosphoenolpyruvate carboxykinase, *Pepck*) (Nechushtan et al., 1987).

Apart from GR, a complex network of transcription factors regulated by insulin is involved in the control of adipose tissue development and lipid homeostasis. Among them, the peroxisome proliferator activated receptor γ (PPARγ) upregulates the transcription of genes involved in fatty acid metabolism and triglyceride (TG) storage, promoting adipogenesis and the fatty acid flux into adipocytes (Rosen and Spiegelman, 2001; Rosen et al., 2000), while the sterol regulatory element binding protein-1c (SREBP-1c) assists adipo/lipogenesis (Kim and Spiegelman, 1996; Tontonoz et al., 1993). Recently, carbohydrate responsive element binding protein (ChREBP) emerged as a pivotal transcriptional inducer of glucose usage and DNL in the adipose tissue, especially in response to a large amount of carbohydrates (Herman et al., 2012).

Although excessive fructose consumption has been suggested to contribute to the pathogenesis of metabolic syndrome, the contributory role of stress in the worsening of dietary fructose lipogenic properties in animal models is still missing. The aim of this work was to characterize metabolic disturbances driven by combination of dietary fructose and chronic stress on adult male Wistar rats and to investigate whether glucocorticoid signaling in VAT is involved in their harmful metabolic effects. To that end, we analyzed the effects of a 9-week 20% fructose-enriched diet and 4-week chronic unpredictable stress, separately and in combination, on energy intake, body mass, VAT mass and histology, GR activity, as well as the expression of glucocorticoid-target genes related to fatty acid release, transport and storage in the VAT of male Wistar rats.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats (2.5 months old), bred in our laboratory, were randomly divided into four experimental groups (n = 8–9 animals per group), housed three per cage and kept in a temperature-controlled room (22 ± 2 °C) with a 12 h light/dark cycle (lights on at 7 a.m.) during 9 weeks. Control group (C) had standard diet (commercial chow and drinking water), while fructose group (F) had access to the same food and 20% (w/v) fructose solution instead of drinking water. The detailed composition of the diet was described previously by Velickovic et al. (2013). Stress group (S) had standard diet like the C group and was subjected to chronic unpredictable stress during the last 4 weeks of the 9-week dietary treatment. The Stress + Fructose group (SF) had the diet regime like the F group and was exposed to stress like the S group. The stress protocol was a modified protocol of Joels et al. (2004) and included the following daily stressors: forced swimming in cold water for 10 min, physical restraint for 30 min, exposure to a cold room (4 °C) for 50 min, wet bedding for 4 h, rocking cages for 1 h and cage tilt (45°) overnight. The time and type of daily stressors were randomly selected at the beginning of the treatment. All experimental groups had *ad libitum* access to food and drinking fluid and daily food and fluid intake were measured during the 9 weeks. Energy intake for control and stressed rats was calculated by summing the calories ingested as food, while for fructose-fed rats it was calculated as sum of calories ingested by both food and fructose solution. All animal procedures were in compliance with the EEC Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Siniša Stanković”, University of Belgrade.

2.2. Determination of plasma TGs, FFAs, insulin and corticosterone

At the end of 9-week dietary treatment, animals were exposed to overnight fasting and sacrificed by rapid decapitation with a guillotine (Harvard Apparatus, Holliston, MA, USA). Trunk blood was rapidly collected into EDTA containing tubes and TG concentrations in the blood were immediately measured by MultiCare strips (Biochemical Systems International, Arezzo, Italia).

For determination of FFAs blood plasma was prepared by low speed centrifugation (1600 g/10 min). The separated plasma was stored at −20 °C for subsequent processing. Plasma FFAs were measured colorimetrically by the Semi-auto Chemistry Analyzer (Rayito, 1904C) using a Randox NEFA kit (Randox Laboratories Ltd, Crumlin, UK).

Plasma insulin concentrations were determined by RIA method, using rat insulin standards (INEP, Belgrade, Serbia). The assay sensitivity was 0.6 mIU/l and intra-assay coefficient of variation (CV) was 5.24%.

Plasma corticosterone concentrations were determined by using Corticosterone EIA kit according to manufacturer's instructions (Immunodiagnostic Systems LTD, The Boldons, UK). Absorbance at 450 nm (reference 650 nm) was measured spectrophotometrically (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, MA, USA). Corticosterone concentrations were determined using 4PL curve fitting method (Prism 5.0, GraphPad Software, Inc., La Jolla, CA, USA) and given as ng/ml. The assay sensitivity was 0.17 ng/ml. Intra-assay and inter-assay CVs were 5.9% and 8.9%, respectively.

2.3. Determination of fatty acid profile of total plasma lipids

Total plasma lipids were extracted by the method of Glaser et al. (2010) as follows: 100 µl of plasma was combined with 1.5 ml methanolic HCl (3 N, containing 2 g/l 2,6-di-tert-butyl-p-cresol) and heated to 85 °C for 45 min. When cooled, 1 ml hexane was added and centrifuged (657 g/10 min). After phase separation, 0.5 ml of the upper hexane phase was evaporated under nitrogen stream and stored at −20 °C until use. Fatty acid composition of total plasma lipids was analyzed according to protocol described by Petrovic et al. (2016). The sample was dissolved in 10 µl of hexane and 1 µl was injected into a gas chromatograph Shimadzu GC 2014 (Shimadzu Co., Tokyo, Japan) equipped with a flame ionization detector and Rtx 2330 fused silica gel capillary column (60 m × 0.25 mm x 0.2 µm) (Restek Co., Bellefonte, PA, USA). The flame ionization detector was set at 260 °C and the injection port at 220 °C, the oven temperature was 140 °C for 5 min and heating rate 3 °C/min from 140 °C to 220 °C. Individual fatty acid methyl esters in the samples were identified by comparing sample peak retention times with authentic standards (Sigma Chemical Co., St Louis, MO, USA) and/or polyunsaturated fatty acid (PUFA)-2 standard mixture (Restek Co., Bellefonte, PA, USA). Fatty acid profiles were expressed as the percentage areas of total fatty acids. The Δ9 desaturase activity was estimated as the product-to-precursor ratio of 18:1/18:0 (Petrovic et al., 2016).

2.4. Isolation and histological analysis of VAT

VAT (pooled depots of retroperitoneal and perirenal white adipose tissue) (Chusyd et al., 2016) was excised immediately after decapitation, washed by 0.9% NaCl, dried, weighed and frozen in liquid nitrogen for subsequent processing. For RNA and protein isolation, tissues from three animals per experimental group were pooled. Additionally, for histological analysis of VAT, samples from individual rats were fixed in 10% neutral formalin for 24 h, dehydrated in an ethanol gradient, cleared in xylene and embedded in paraffin. Tissue blocks were sectioned at 10 µm thickness and stained with hematoxylin and eosin. Morphometric analysis, *i.e.* determination of adipocyte cell diameter and sectional area, was carried out using Adiposoft (automated software for the analysis of white adipose tissue cellularity in histological sections) (Galarraga et al., 2012). Images for analysis were acquired using a workstation comprising a microscope (Olympus, BX-51, Olympus Corp., Tokyo, Japan) equipped with a CCD video camera (PixeLINK, Ottawa, ON, Canada) connected to a PC monitor. The whole system was controlled by the newCAST software package (Visiopharm Integrator System, version 3.2.7.0, Visiopharm, Horsholm, Denmark). Three high-resolution, randomly located images per section were acquired at 10 × magnification. The cell diameter and area were determined in 100 adipocytes per section (three sections per animal and six animals per group).

2.5. Preparation of subcellular fractions

For the preparation of subcellular fractions, frozen adipose tissue was homogenized (w/v = 1:1) in ice-cold 20 mM Tris-HCl (pH 7.2) buffer containing 10% glycerol, 50 mM NaCl, 1 mM EDTA-Na2, 1 mM EGTA-Na2, 2 mM dithiothretiol (DTT), protease inhibitors (20 mM Na_2MoO_4, 0.15 mM spermin, 0.15 mM spermidin, 0.1 mM PMSF, 5 µg/ml antipain, 5 µg/ml leupeptin, 5 µg/ml aprotinin) and phosphatase inhibitors (20 mM β-glycerophosphate, 5 mM $Na_4P_2O_7x10H_2O$, 25 mM NaF). The homogenates were further processed to obtain cytoplasmic and nuclear fractions, as described previously (Bursac et al., 2013). Total protein fraction was isolated from the adipose tissue using TRIzol® Reagent (AmBion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Briefly, tissues were mechanically homogenized by adding TRI Reagent Solution containing TRIzol® Reagent, 1 mM PMSF and 2 mM EDTA and centrifuged (12 000 g for 15 min at 4 °C). The protein fraction (phenol ethanol supernatant) was precipitated with acetone for 10 min at room temperature and centrifuged (12000 g for 10 min at 4 °C). Pellets were dispersed in 0.3 M guanidine hydrochloride in 95% ethanol with 2.5% glycerol by sonication on ice (3 × 5 s, 1 A, 50/60 Hz), centrifuged (8000 g for 5 min at 4 °C) and washed two more times in 0.3 M guanidine hydrochloride in 95% ethanol with 2.5% glycerol followed by low speed centrifugation (8000 g for 5 min at 4 °C). Finally, pellets were dispersed in the lysis buffer (2.5 mM Tris-HCl pH 6.8, 2% w/v sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM DTT) and used as total protein fractions. All samples were stored at −70 °C until use.

2.6. RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated from VAT using TRIzol® Reagent (AmBion, Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions simultaneously with total protein isolation described above. Quantitative and qualitative evaluation of the isolated RNA was performed spectrophotometrically (OD 260/280 > 1.8 was considered satisfactory) and on 2% agarose gel. Prior to cDNA synthesis, DNA contamination was removed by DNAse I treatment (Fermentas, Burlington, ON, Canada). Reverse transcription was performed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The cDNAs were stored at −70 °C until use.

TaqMan® gene expression probe sets (Applied Biosystems Assay-on-Demand Gene Expression Products) were used for all gene expression experiments. All probe sets used, *Acc* (Rn00573474_m1*), *Lipe/Hsl* (Rn00563444_m1*), *Pepck* (Rn01529014_m1*), *Atgl* (PNPLA2, Rn01479969_m1*), *Lpl* (Rn00561482_m1*), *Cd36* (Rn02115479_g1*) and *Fas* (Rn01463550_m1*), were FAM labeled. Quantitative normalization of cDNA in each sample was performed using *Hprt1* (Rn01527840_m1*) as endogenous control. Real-time PCR reaction was performed using QuantStudio™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 µl containing 1 × TaqMan Universal Master Mix with AmpErase UNG, 1 × Assay Mix (Applied Biosystems, Foster City, CA, USA) and cDNA template (20 ng). Thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. No template control was used in each run. All reactions were run in triplicates. Relative quantification of gene expression was examined using comparative $2^{-\Delta\Delta ct}$ method described by Livak and Schmittgen (2001). The results were analyzed by Quant Studio™ Design & Analysis v1.3.1 (Applied Biosystems, Foster City, CA, USA) with a confidence level of 95% ($p \leq 0.05$).

2.7. Western blot analysis

Protein concentration in all fractions was determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference. The samples were boiled in equal volumes of 2× Laemmli's buffer for 5 min and 50 µg of proteins were subjected to electrophoresis on 7.5% SDS-polyacrylamide gel along with molecular mass references (10–170 kDa) (Thermo Fisher Scientific, Waltham, MA, USA). After electrophoresis, proteins were transferred onto PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) using a blot system (Transblot, Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 1 h with 2% non-fat dry milk dissolved in phosphate buffered saline (PBS) at room temperature and incubated with the respective primary and secondary antibodies. GR was detected by sc-8992 antibody (1:250) (Santa Cruz Biotechnology, Dallas, TX, USA), PPARγ by sc-9000 antibody (1:500) (Santa Cruz Biotechnology, Dallas, TX, USA), 11βHSD1 by ab393364 antibody (1:1000) (Abcam, Cambridge, UK), H6PDH by sc-67394 antibody (1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA), SREBP-1c by sc-366 antibody (1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA) and ChREBP by NB400-135 (1:500) (Novus

Biologicals, Littleton, CO, USA). For cytoplasmic fraction and total protein, anti-β-actin AC-15 (1:10000) (Sigma-Aldrich, St. Louis, MO, USA) was used as loading control, while anti-TBP ab63766 (Abcam, Cambridge, UK) antibody was used as loading control for nuclear fraction. Secondary antibodies were horseradish peroxidase (HRP)-linked goat anti-rabbit antibody (IgG H&L) ab6721 (1:20000) (Abcam, Cambridge, UK) or HRP-linked rabbit anti-mouse (IgG H&L) antibody ab97046 (1:20000) (Abcam, Cambridge, UK). Immunoreactive bands were developed by enhanced chemiluminescent substrate and exposed to X-ray films (Kodak, Rochester, NY, USA). Densitometry of protein bands on X-ray film was performed by Image J analysis PC software (NIH, Bethesda, MD, USA). To correct for equal protein load in all samples, membranes were incubated in stripping buffer, reblocked and probed with β-actin antibody.

2.8. Statistical analysis

Physiological and biochemical data are presented as means ± SD, while histological data and data from Western blot analysis and real-time PCR are presented as means ± SEM. To determine the effects of the fructose and stress treatment, as well as their interaction, two-way ANOVA followed by *post hoc* Tukey test was used. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Physiological measurements

To reveal the effects of stress and dietary fructose and their combination on physiological parameters we analyzed energy intake, as well as body and VAT mass. As shown in Table 1, both groups consuming fructose ingested more total kJ than the rats drinking water ($p < 0.001$, F *vs.* C; SF *vs.* C), and also fructose-fed stressed rats consumed more total kJ in comparison to stress alone ($p < 0.001$, SF *vs.* S). Despite increased energy intake in fructose-fed rats, body weight was similar in all groups, regardless of diet regime or stress exposure. On the other hand, despite a normal energy intake, stressed rats showed a significantly reduced VAT mass ($p < 0.01$, S *vs.* C) and VAT/body ratio (Table 1, $p < 0.01$, S *vs.* C) compared to control animals.

3.2. Biochemical measurements of plasma TGs, FFAs, insulin and corticosterone

As shown in Table 2, fructose-fed rats had significantly increased plasma TG compared to the control animals ($p < 0.001$, F vs. C). Furthermore, plasma TG were elevated in stressed rats on fructose diet in comparison to the stressed animals ($p < 0.05$, SF *vs.* S), and decreased in comparison to fructose-fed animals ($p < 0.05$, SF *vs.* F).

An increase of plasma FFAs level was observed in fructose-fed stressed rats in comparison to both control and stressed animals ($p < 0.05$, SF *vs.* C; SF *vs.* S).

Dietary fructose significantly increased plasma insulin levels in both fructose-fed groups compared to the control group (Table 2, $p < 0.05$, F *vs.* C; SF *vs.* C). Furthermore, a significant increase of plasma insulin level in stressed animals on fructose diet in comparison to stressed animals on standard diet was also observed (Table 2, $p < 0.05$, SF *vs.* S).

A significant increase of plasma corticosterone level was detected in stressed animals on standard diet in comparison to the control animals (Table 2, $p < 0.001$, S *vs.* C), while corticosterone level was significantly decreased in fructose-fed stressed rats in comparison to the stressed animals (Table 2, $p < 0.01$, SF *vs.* S).

3.3. Fatty acid alterations and Δ9 desaturase activity

Table 3 shows that dietary sugar induced significant changes in Δ9 desaturase activity, as estimated from the product-to-precursor ratio of 18:1/18:0. A significant decrease of stearic and concomitant increase of oleic acid was observed in all fructose-fed animals in comparison to the control and stressed rats ($p < 0.01$, $p < 0.001$, F *vs.* C; $p < 0.001$, SF *vs.* C and SF *vs.* S). A significant increase of Δ9 desaturase activity was detected in all fructose-fed animals in comparison to the control and stressed ones ($p < 0.001$, F *vs.* C; SF *vs.* C and SF *vs.* S).

3.4. Histological and morphometrical analysis of VAT

As shown in Fig. 1, histological analysis of VAT revealed that both adipocyte cell diameter and area were significantly decreased only in stressed rats as compared to the control animals (Fig. 1a and b, $p < 0.001$, S *vs.* C).

3.5. Glucocorticoid prereceptor metabolism and signaling

To investigate the effects of fructose overconsumption and/or chronic exposure to unpredictable stress on the glucocorticoid prereceptor metabolism, protein levels of 11βHSD1 and H6PDH enzymes were analyzed in the total protein extract of VAT. As shown in Fig. 2a, a significant increase of 11βHSD1 and H6PDH protein levels was observed only in stressed animals on fructose diet in respect to all other experimental groups ($p < 0.01$ for SF *vs.* C and SF *vs.* F; $p < 0.05$, SF *vs.* S).

In order to analyze effects of fructose diet and/or stress on the GR expression and function in the VAT, we followed the redistribution of GR protein from the cytoplasm to the nuclei. The results showed a significant increase of GR protein level in the VAT nuclei of stressed rats in comparison to the control group (Fig. 2b, $p < 0.05$, S *vs.* C).

Table 1
Physiological measurements: energy intake, body and VAT mass in control rats, fructose-fed rats, rats exposed to chronic unpredictable stress, and fructose-fed stressed rats.

	Control	Fructose	Stress	Stress + Fructose	Two-way ANOVA		
					Fructose	Stress	Interaction
Energy intake (kJ/day/animal)	182.0 ± 18.0	262.0 ± 51.9[b]	175.7 ± 23.2	248.7 ± 47.0[b,c]	$p < 0.001$	NS	NS
Body mass (g)	369 ± 18	330 ± 38	338 ± 39	338 ± 43	NS	NS	NS
Mass of VAT (g)	7.67 ± 1.67	7.80 ± 2.40	4.43 ± 1.39[a]	6.11 ± 1.86	NS	$p < 0.001$	NS
VAT/body ratio (× 1000)	20.80 ± 4.53	21.54 ± 5.11	13.00 ± 3.09[a]	18.55 ± 6.55	$p < 0.05$	$p < 0.001$	NS

The data are presented as means ± SD (n = 8–9 animals per group). A value of $p < 0.05$ was considered statistically significant. Significant between-groups differences obtained from two-way ANOVA followed by *post hoc* Tukey test are given as follows:
NS: not significant.
[a] $p < 0.01$.
[b] $p < 0.001$, treated animals *vs.* C.
[c] $p < 0.001$, SF *vs.* S.

Table 2
Biochemical measurements in plasma: TGs, FFAs, insulin and corticosterone in control rats, fructose-fed rats, rats exposed to chronic unpredictable stress, and fructose-fed stressed rats.

	Control	Fructose	Stress	Stress + Fructose	Two-way ANOVA		
					Fructose	Stress	Interaction
TG (mmol/L)	1.13 ± 0.21	1.87 ± 0.40[b]	1.03 ± 0.18	1.48 ± 0.38[c,d]	$p < 0.001$	$p < 0.05$	NS
FFAs (mmol/L)	0.77 ± 0.22	0.90 ± 0.32	0.80 ± 0.17	1.15 ± 0.27[a,d]	$p < 0.01$	NS	NS
Insulin (mIU/L)	10.07 ± 6.41	19.44 ± 8.48[a]	10.62 ± 6.22	20.84 ± 10.74[a,d]	$p < 0.001$	NS	NS
Corticosterone (ng/ml)	135.78 ± 45.26	118.91 ± 39.64	570.16 ± 190.05[b]	205.34 ± 68.45[e]	$p < 0.001$	$p < 0.001$	$p < 0.001$

The data are presented as means ± SD (n = 8–9 animals per group). A value of $p < 0.05$ is considered statistically significant. Significant between group differences obtained from two-way ANOVA followed by *post hoc* Tukey test are given as follows:
NS: not significant.
[a] $p < 0.05$.
[b] $p < 0.001$, treated animals *vs.* C.
[c] $p < 0.05$, SF *vs.* F.
[d] $p < 0.05$.
[e] $p < 0.01$, SF *vs.* S.

Table 3
Fatty acid alterations in total plasma lipids and estimated plasma Δ9 desaturase activities in control rats, fructose-fed rats, rats exposed to chronic unpredictable stress, and fructose-fed stressed rats.

Fatty acid (%)	Control	Fructose	Stress	Fructose + Stress	Two-way ANOVA		
					Fructose	Stress	Interaction
Stearic acid (18:0)	13.94 ± 0.77	11.81 ± 1.53[a]	14.65 ± 0.91	11.17 ± 1.05[b,c]	$p < 0.001$	NS	NS
Oleic acid (18:1n-9)	8.28 ± 0.76	13.60 ± 3.29[b]	7.71 ± 0.68	12.50 ± 1.59[b,c]	$p < 0.001$	NS	NS
Δ9 desaturase (18:1n-9/18:0)	0.60 ± 0.08	1.18 ± 0.40[b]	0.53 ± 0.07	1.14 ± 0.27[b,c]	$p < 0.001$	NS	NS

The data are presented as means ± SD (n = 8 animals per group). A value of $p < 0.05$ is considered statistically significant. FAs concentrations are expressed in % of totally detected FA. Significant between group differences obtained from two-way ANOVA followed by *post hoc* Tukey test are given as follows:
NS: not significant.
[a] $p < 0.01$.
[b] $p < 0.001$, treated animals *vs.* C.
[c] $p < 0.001$, SF *vs.* S.

3.6. Transcriptional regulators involved in VAT lipid metabolism

To determine whether fructose diet and stress affect activation of adipo/lipogenic transcriptional regulators, the protein levels of PPARγ, SREBP-1c and ChREBP were determined. The total protein level of PPARγ was not affected by any of the treatments (Fig. 3, left). Nuclear SREBP-1c protein level was significantly increased in stressed rats on standard diet compared to the controls, and significantly decreased after the combined treatment in comparison to stress alone (Fig. 3, middle, $p < 0.01$, S *vs.* C and SF *vs.* S). However, important regulator of adipocyte lipogenesis, ChREBP was not affected by any of the treatments (Fig. 3, right).

3.7. GR-regulated genes involved in VAT lipid metabolism

The expression of GR target genes involved in lipid metabolism was

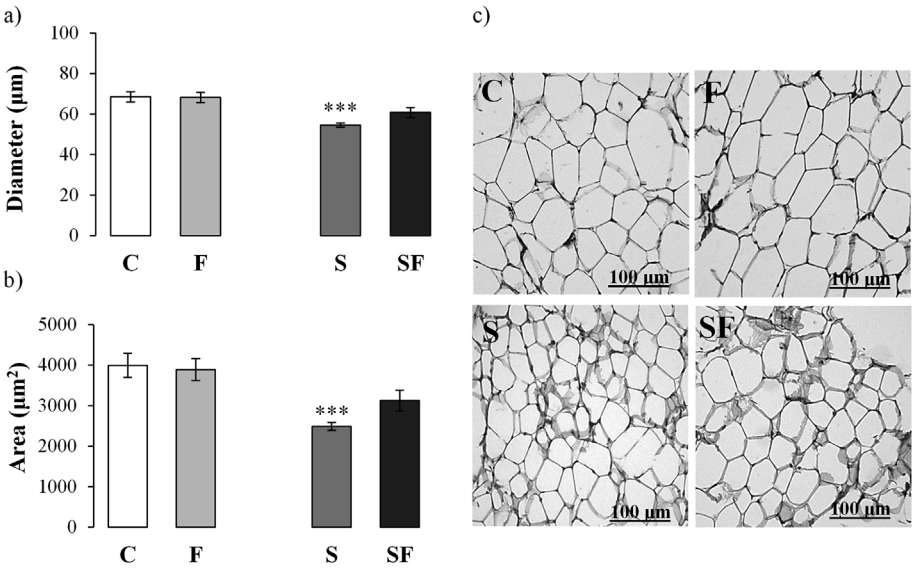


Fig. 1. Histological and morphometric analysis of VAT. Morphometric analysis of adipocyte cell diameter **(a)** and area **(b)** and representative micrographs of hematoxylin-eosin-stained sections of VAT of control (C), fructose-fed (F), stressed (S) and fructose-fed stressed (SF) rats (magnification × 10, bar = 100 µm) **(c)**. Data are presented as mean ± SEM (100 adipocytes per section, three sections per animal and six animals per group). A value of $p < 0.05$ was considered statistically significant. Statistical analysis by two-way ANOVA showed significant effect of stress on the adipocyte cell diameter and area ($p < 0.001$). Significant between-group differences from *post hoc* Tukey test are given as follows:
***$p < 0.001$; treated animals *vs.* C.

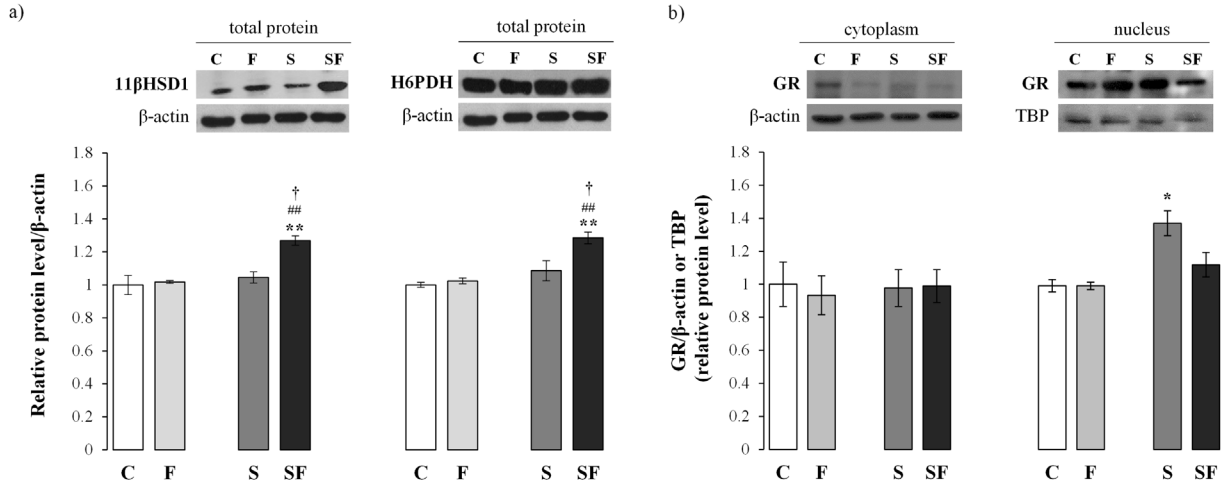


Fig. 2. Prereceptor glucocorticoid metabolism and GR protein level in the VAT. Representative Western blots and relative quantification of 11βHSD1 and H6PDH protein level in the total protein extract **(a)** and GR protein level in cytoplasm and nuclei **(b)** in the VAT of control (C), fructose-fed (F), stressed (S) and fructose-fed stressed (SF) rats. Immunoreactivities of the 11βHSD1, H6PDH and GR are normalized to β-actin or TBP as loading controls and are expressed as mean ± SEM (n = 9 animals per group). A value of $p < 0.05$ was considered statistically significant. Two-way ANOVA showed that 11βHSD1 protein level was significantly affected by fructose ($p < 0.05$), stress ($p < 0.01$), and their interaction ($p < 0.05$). For the H6PDH protein level the effect of fructose ($p < 0.05$), stress ($p < 0.01$) and their interaction ($p < 0.05$) was observed. ANOVA also revealed that stress had the effect on nuclear GR level ($p < 0.01$). Significant between-group differences from *post hoc* Tukey test are given as follows:
$^{*}p < 0.05$; $^{**}p < 0.01$; treated animals *vs.* C
$^{\#\#}p < 0.01$; SF *vs.* F
$^{\dagger}p < 0.05$; SF *vs.* S.

examined by real-time PCR (Fig. 4). As shown in Fig. 4, we found a significant increase of *Atgl*, *Pepck* and *Cd36* mRNA levels in stressed rats in comparison to the control ones ($p < 0.01$ for *Atgl* mRNA; $p < 0.05$ for *Cd36* and *Pepck* mRNA, S *vs.* C), as well as a significant increase of *Atgl*, *Fas* and *Acc* mRNA levels in fructose-fed stressed rats in comparison to the controls ($p < 0.05$ for *Atgl*, *Fas* and *Acc* mRNA, SF *vs.* C). In addition, in stressed animals on fructose diet, we observed a significant increase of mRNA levels for *Fas* and *Acc* in comparison to fructose-fed ($p < 0.05$, SF *vs.* F) and stressed animals ($p < 0.01$ for *Fas* mRNA; $p < 0.05$ for *Acc* mRNA, SF *vs.* S).

4. Discussion

Glucocorticoid hormones mediate the metabolic effects of both dietary carbohydrates and stress, and play an important role in adipogenesis and adipocyte function (Lee et al., 2014). It was previously shown that both fructose consumption (Bray et al., 2004; Dekker et al., 2010) and chronic stress (Dallman et al., 2003) are associated with

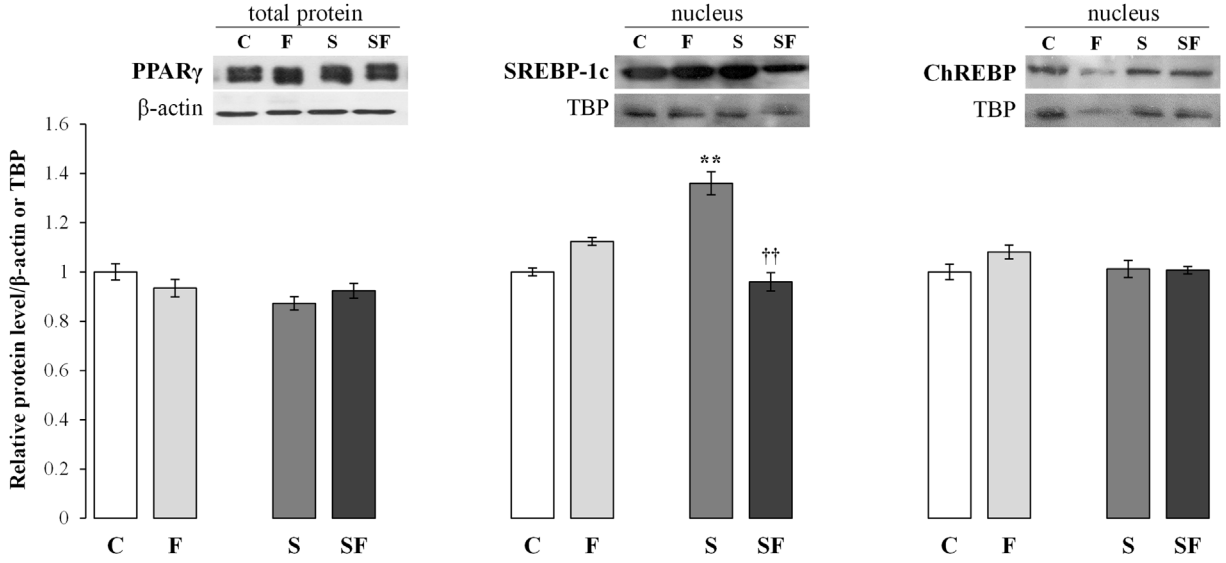
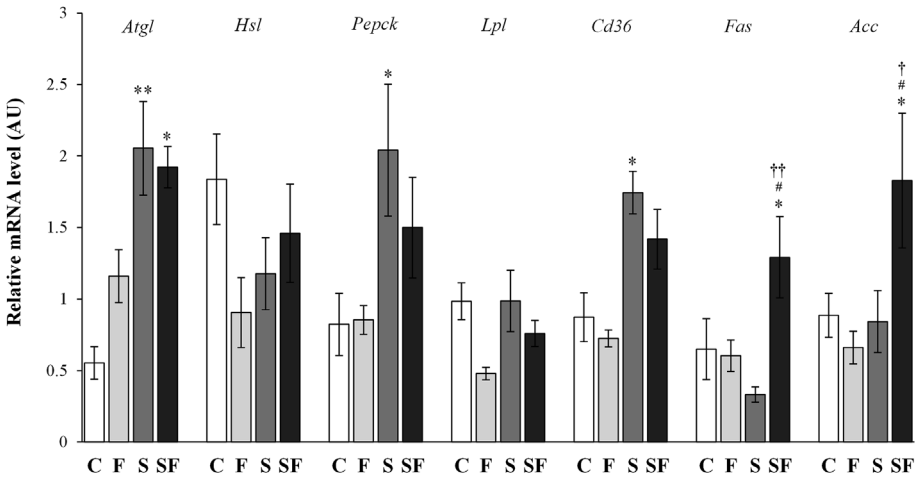


Fig. 3. Transcriptional regulators involved in VAT lipid metabolism. Representative Western blots and relative quantification of PPARγ, nuclear SREBP-1c and nuclear ChREBP in the VAT of control (C), fructose-fed (F), stressed (S) and fructose-fed stressed (SF) rats. Immunoreactivities of the proteins are normalized to β-actin or TBP as loading controls and are expressed as mean ± SEM (n = 9 animals per group). A value of $p < 0.05$ was considered statistically significant. ANOVA showed significant effect of fructose ($p < 0.05$), as well as the interaction between fructose and stress on nuclear SREBP-1c level ($p < 0.05$). Between-group differences from *post hoc* Tukey test are given as follows:
$^{**}p < 0.01$; treated animals *vs.* C
$^{\dagger\dagger}p < 0.01$; SF *vs.* S.



#$p < 0.05$; SF *vs.* F
†$p < 0.05$; ††$p < 0.01$; SF *vs.* S.

Fig. 4. mRNA levels of genes involved in VAT lipid metabolism. The levels of *Atgl*, *Hsl*, *Pepck*, *Lpl*, *Cd36*, *Fas* and *Acc* mRNAs normalized to *Hprt1* mRNA were determined by TaqMan real-time PCR in the VAT from control (C), fructose-fed (F), stressed (S) and fructose-fed stressed (SF) rats. Data are presented as mean ± SEM (n = 9 animals per group) of the triplicate analysis of RNA samples. A value of $p < 0.05$ was considered statistically significant. The effect of fructose was detected by two-way ANOVA for *Fas* ($p < 0.05$) and *Lpl* ($p < 0.01$), while stress affected *Cd36* ($p < 0.001$), *Pepck* ($p < 0.05$) and *Atgl* ($p < 0.001$) mRNA levels. Interaction between fructose and stress affected *Fas* ($p < 0.01$), *Acc* ($p < 0.05$) and *Hsl* ($p < 0.05$) mRNA levels as detected by two-way ANOVA. Significant between-group differences from *post hoc* Tukey test are given as follows:
*$p < 0.05$; **$p < 0.01$; treated animals *vs.* C

obesity and related metabolic disorders, but how these two factors can interact to affect total body energy homeostasis and adipose tissue metabolism is still largely unknown. Thus, the aim of the present study was to investigate whether fructose consumption in combination with chronic stress leads to an aggravation of metabolic function through changes in VAT glucocorticoid signaling. The concentration of fructose solution was chosen to resemble the consumption of fructose-sweetened beverages in human population (Ventura et al., 2011), while chronic unpredictable stress protocol consisted of psychological and physical stressors reflecting their variability in modern human life (Joels et al., 2004). The results showed typical metabolic effects of fructose consumption, such as dyslipidemia, and typical effects of stress, such as elevated plasma corticosterone level and decreased VAT mass in stressed animals. However, only when stress was applied in combination with fructose diet, a disturbed adipose tissue lipid metabolism accompanied by increased plasma FFAs was observed, which was not mediated by insulin-dependent lipogenic transcriptional factors, but rather by enhanced glucocorticoid prereceptor metabolism.

All rats on fructose diet had elevated energy intake, but without change of total body mass (Table 1), which is in accordance with previously published data (Bursac et al., 2013, 2014). It is well documented that fructose diet induces dyslipidemia in humans (Tappy and Le, 2010), while animal studies, including ours, indicated that it can lead to insulin resistance, dyslipidemia and high blood pressure (Bursac et al., 2013, 2014; Jurgens et al., 2005). The results from the present study confirmed hypertriglyceridemia and hyperinsulinemia as metabolic hallmarks of fructose-enriched diet (Table 2). Furthermore, fructose diet affected stearic and oleic fatty acid levels in the similar manner in both fructose-fed groups, regardless of stress. Also, the activity of Δ9 desaturase was increased in fructose-fed rats, probably leading to increased levels of oleic acid (Table 3). It was previously shown that increased activity of Δ9 desaturase is the main factor closely associated with cardiometabolic risk and hypertriglyceridemia (Warensjo et al., 2009). Finally, while fructose diet alters plasma fatty acids in the direction of metabolic derangements, stress does not negatively contribute to the metabolic alteration.

As already mentioned, glucocorticoids can modulate both lipolysis and lipogenesis in the adipose tissue, which may be determined by the insulin level and/or examined adipose tissue depot (Lee et al., 2014; Stimson et al., 2017). In our study fructose feeding alone did not affect plasma corticosterone level (Table 2), which is consistent with other studies showing that metabolic disturbances caused by fructose are not necessarily followed by changes in the plasma glucocorticoid levels (Bursac et al., 2013, 2014; London and Castonguay, 2011). As expected, plasma corticosterone level was significantly elevated in stressed animals on standard diet (Table 2). This was accompanied by a decrease in VAT mass and its relative ratio to total body mass (Table 1), which could result from GR activation (Fig. 2) and a consequent GR-mediated induction of lipolytic pathways through increased expression of *Atgl*, although *Hsl* mRNA level was unchanged (Fig. 4) (Shen et al., 2017). Accordingly, *in vitro* study by Langin (2006) suggested that *Hsl* is the major lipase for catecholamine-stimulated lipolysis, whereas *Atgl* mediates triglyceride hydrolysis regulated by glucocorticoids (Villena et al., 2004). As already mentioned, it was previously shown that glucocorticoids induce lipolysis in rodent VAT (Bjorndal et al., 2011). In line with this, histological analysis of rat VAT from our study revealed the presence of significantly smaller adipocytes in normal-fed stressed animals as a presumable consequence of glucocorticoid-induced lipolysis (Campbell et al., 2009a,b) (Fig. 1). Interestingly, decreased visceral adiposity and elevated *Atgl* mRNA level have not been associated with increased plasma FFAs in these animals (Table 2). The possible explanation could be found in FFA re-esterification in other tissues, possibly liver and skeletal muscle (Reshef et al., 2003), but also in the adipose tissue itself (Franckhauser et al., 2002). Our results point at involvement of adipose tissue, since adipose *Pepck* and *Cd36* mRNA levels were increased in stressed rats (Fig. 4) and recent findings showed that *Cd36* can modulate FFA re-esterification through regulation of *Pepck* expression (Zhou et al., 2015).

However, the main resultant of fructose consumption combined with stress on adipose tissue lipid metabolism were elevated plasma FFAs, which could partially be the result of unchanged *Cd36* expression in the VAT of these animals implying that their *Atgl*-induced release was not supported by the adequate uptake (Table 2 and Fig. 4) (Goldberg et al., 2009). Also, it could be proposed that under the conditions of fructose overload, FFA-dependent tissues such as skeletal muscles could rather be using fructose as fuel, leaving the stress-released FFAs in the circulation. Indeed, Laughlin (2014) have previously showed that skeletal muscle can directly metabolize fructose when both its plasma level and energy demands are high, as in stressed animals on fructose diet. Tracer studies in humans suggested that fructose can decrease fatty acid oxidation in muscle, which could also be a contributory factor for increased FFAs in the circulation (Sun and Empie, 2012). Interestingly, although in combined treatment stressful stimuli were present, it seems that fructose diet was able to cancel stress-induced rise of corticosterone at the end of treatment. Similar results were previously reported by other authors, where stressed rats subjected to hypercaloric cafeteria diet were hyporesponsive to stress due to reduced central reactivity (Macedo et al., 2012), while others postulated that the increased intake of palatable food under stress could be associated with reward-based eating, as a mean to reduce the stress response (Adam and Epel, 2007).

As reviewed by Ulrich-Lai (2016), stress promotes consumption of sugar, since both hedonic and metabolic effects of sucrose contribute to its relief through glucocorticoid action in adipose tissue and through modulated plasticity in brain reward regions. In addition, when high fat diet was combined with chronic stress model utilized to induce glucocorticoids elevation, the results were contradictory to the effects of glucocorticoids injected exogenously. The chronic stress actually improved the fat diet-induced adiposity and glucose intolerance (Balsevich et al., 2014; Bruder-Nascimento et al., 2013), which could be clinically relevant, since it implies that exogenous therapy with glucocorticoids represents a higher risk of further metabolic complications if combined with hypercaloric diet.

However, endogenous glucocorticoids availability and action depend not only on their circulating level but also on intracellular regeneration of their active forms, mediated by the enzymes 11βHSD1 and H6PDH (Bujalska et al., 2005; Rose et al., 2010). Our results showed that only the combination of fructose diet and chronic stress significantly increased both enzymes involved in the prereceptor metabolism (Fig. 2), which was also confirmed by significant interaction of factors found by two-way ANOVA analysis. Interestingly, this increase in 11βHSD1 and H6PDH was associated with an important parameter of dyslipidemia, increased plasma FFA level in fructose-fed stressed animals (Table 2). This result is consistent with the finding that mice overexpressing 11βHSD1 in adipose tissue exhibit a threefold increase in plasma FFA in the portal vein and a specific fat accumulation in the VAT (Masuzaki et al., 2001).

Apart from elevated plasma FFAs in fructose-fed stressed rats, significant hyperinsulinemia was also present, while plasma TG showed a trend of increase ($p = 0.08$, SF *vs.* C). As already mentioned, in the setting of hyperinsulinemia, glucocorticoids can simultaneously increase pathways of both TG synthesis and breakdown (Lee et al., 2014). Indeed, expression of genes involved in both DNL (*Acc* and *Fas*) and TG hydrolysis (*Atgl*) was concomitantly increased in response to combined treatment in the present study (Fig. 4), while the net effect of these changes were normal-sized adipocytes. It seems logical to assume that *Acc* and *Fas* would be elevated in the VAT through insulin-regulated lipogenic transcriptional factors, such as PPARγ, SREBP-1c and ChREBP, especially after high-fructose diet (Herman et al., 2012; Janevski et al., 2012; Muhlhausler and Smith, 2009). However, this was not the case in the present study, since both PPARγ and ChREBP were unchanged in all experimental groups, while SREBP-1c was increased only by stress in normal-fed rats (Fig. 3). These findings imply that the observed changes of concomitantly increased *Fas*, *Acc* and *Atgl* could rather be related to the changes in glucocorticoid prereceptor metabolism in the VAT. In support of this proposal is the study demonstrating association of elevated 11βHSD1 protein level with significantly increased *Fas* expression in the intraperitoneal white adipose tissue of male rats (Veyrat-Durebex et al., 2012).

In conclusion, our results showed that only the combination of dietary fructose and chronic stress, as hallmarks of modern lifestyle, exerts harmful effects on lipid homeostasis, as judged by the increased circulatory FFAs and stimulated lipogenic enzyme expression in the VAT. These effects are most likely conducted through the enhanced glucocorticoid prereceptor metabolism, implying the inevitable involvement of glucocorticoids in the metabolic diseases of modern mankind. The results from this study could help fighting the ongoing obesity epidemic through nonpharmacological approach, such as lifestyle intervention, with the special emphasis on stress management and healthy well-balanced diet combined with physical activity.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant III41009) and SCOPES JRP (Grant No IZ73ZO_152331).

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