Int J Physiol Pathophysiol Pharmacol 2015;7(1):61-75
www.ijppp.org /ISSN:1944-8171/IJPPP0007546

Original Article

Modulation of lipid metabolism in glycyrrhizic acid-treated rats fed on a high-calorie diet and exposed to short or long-term stress

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Received March 3, 2015; Accepted March 18, 2015; Epub March 20, 2015; Published March 31, 2015

Abstract: Stress and high-calorie diets increase the risk of developing metabolic syndrome. Glycyrrhizic acid (GA) has been shown to improve dyslipidaemia in rats fed on a high-calorie diet. This study aimed to examine the effects of GA on lipid metabolism in rats exposed to short- or long-term stress and on a high-calorie diet. The parameters examined included serum lipid profiles, serum free fatty acids and fatty acid profiles in tissues, and expression of peroxisome proliferator-activated receptors (PPAR), lipoprotein lipase (LPL), elongases and desaturases. Within the 14- or 28-day exposure groups, neither stress nor GA affected the lipid profile and serum free fatty acids. Stress did not affect PPAR-α expression in both the 14- and 28-day exposure groups. However, GA-treated rats from the former group had increased PPAR-α expression only in the kidney while all other tissues from the latter group were unaffected. Stress increased PPAR-γ expression in the heart of the 28-day exposure group but its expression was unaffected in all tissues of the 14-day exposure group. GA elevated PPAR-γ expression in the kidney and the skeletal muscles. Neither stress nor GA affected LPL expressions in all tissues from the 14-day exposure group but its expressions were elevated in the QF of the stressed rats and heart of the GA-treated rats of the 28-day exposure group. As for the elongases and desaturases in the liver, stress down-regulated ELOVL5 in the long-term exposure group while up-regulated ELOVL6 in the short-term exposure group while hepatic desaturases were unaffected by stress. Neither elongase nor desaturase expressions in the liver were affected by GA. This research is the first report of GA on lipid metabolism under stress and high-calorie diet conditions and the results gives evidence for the role of GA in ameliorating MetS via site-specific regulation of lipid metabolism gene expressions and modification of fatty acids.

Keywords: Glycyrrhizic acid, high-calorie diet, stress, peroxisome proliferator-activated receptors, lipoprotein lipase, elongases, desaturases

Introduction

Stress is a daily event faced by many people in modern days and has been shown to exert a strong influence on lipid metabolism [1]. The stress response system is made up of the hypothalamic- pituitary-adrenal axis (HPA axis) and the sympathetic nervous system (SNS). Activation of this system leads to the production of the stress hormones catecholamines and glucocorticoids (GCs), which in excess have been associated with the development of dyslipidaemia (characterized by elevated triacylglycerols (TAG), free fatty acids (FFA), low-density lipoprotein (LDL) and reduced high-density lipoprotein (HDL) levels) [2, 3]. Acute increases in blood lipids allows survivorship and adaptation to the stressor but chronic stress causes prolonged changes in lipid metabolism and contributes to the development of various chronic diseases such as cardiovascular disease and type 2 diabetes [4].

Fatty acids are an essential component of the biological system. They form a major part of the membrane, act as an energy source and as signalling molecules in lipid metabolism and inflammation [5]. However, when in excess, free fatty acids (FFA) compete with glucose as the substrate for fuel generation and leads to the

development of insulin resistance (IR) [6]. Research has been shown that a high-fat diet impairs insulin sensitivity and has detrimental effects on lipid metabolism [7, 8].

Peroxisome proliferator activated receptors (PPARs) is a group of ligand-dependent transcription factors that play a pivotal role in regulating lipid and glucose metabolism [9]. There are three isoforms of PPAR i.e. PPAR-α, PPAR-γ and PPAR-δ/β [9, 10]. PPAR-α is predominantly expressed in the liver and skeletal muscles where it controls lipid oxidation; PPAR-γ is mainly found in the adipose tissue to modulate adipogenesis and lipid homeostasis while PPAR-δ/β is ubiquitously expressed and is involved in a wide range of body functions e.g. immunity and reproduction [9].

Lipoprotein lipase (LPL) is an enzyme that hydrolyzes circulating lipid-carrying molecules i.e. triacylglycerol-rich lipoproteins to release free fatty acids (FFAs) [11]. These FFAs are then transported to the target tissues e.g muscles and adipose tissues, either for fatty acid oxidation or re-esterification for storage purposes. Reduced LPL level is associated with the development of dyslipidemia due to elevated circulatory TAG levels which has been found in patients with type 2 diabetes and diabetic animal models [11].

The chemical and physical properties of fatty acids (FA) are determined by their chain length and degree of saturation. The presence of elongases and desaturases systems allows elongation and desaturation to accommodate the multi-functional role of fatty acids in different parts of the body. Recent findings demonstrate the essential role of elongases and desaturases in the development of T2DM and the MetS [12, 13]. Furthermore, patients with these diseases showed similar fatty acid patterns which include increased proportions of palmitic (16:0) and palmitoleic acid, low levels of linoleic acid and a high proportion of dihomo-gamma linolenic acid (DHLA, 20:3 n-6) [5].

In mammals, the rate of FFA elongation is determined by the elongase enzymes known as Elongation Of Very-Long-Chain fatty acids (ELOVLs) [14]. There are seven types of elongases, however, ELOVL5 & 6 are commonly found in the liver. ELOVL6 targets at saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) while ELOVL5 prefers polyunsaturated fatty acids (PUFA) [15]. Fatty acid desaturases are a group of enzymes that catalyzes the incorporation of a single double bond into a specific position within fatty acid molecules [16]. Mammalian cells exhibit Δ9, Δ6 and Δ5 desaturase activities in which the Δnumber indicates the carbon position where the double bond is introduced counting from the carboxy-end [17]. Δ9 desaturase (D9D) belongs to the stearoyl-CoA desaturase (SCD) group that prefers SFA while Δ5 and Δ6 desaturase belong to the fatty acid desaturase (FAD) group that targets PUFA [17].

Glycyrrhizic acid (GA) is an active compound found in the root extract of the licorice plant, *Glycyrrhiza glabra* [18]. GA has been shown to improve glucose and lipid metabolism under different physiological conditions [7, 8, 19-25] via several pathways. Its primary action being the non-selective inhibitory effects on 11β-hydroxysteroid dehydrogenase, an enzyme that catalyzes the inter-conversion of active and inactive GCs [18]. Furthermore, oral administration of GA was shown to improve IR via modulation of lipid metabolism parameters such as PPAR-γ and LPL expressions [7, 8, 26]. Therefore, the present study was designed to investigate the effects of short- or long-term stress and high-calorie diet (HCD) and whether GA is able to improve lipid metabolism via modulation of PPAR, LPL, elongases and desaturases together with modification of fatty acid content in different tissues under these conditions.

Materials and methods

Animals and experimental design

The practice of use and handling of animals in this study had been approved by the Monash University School of Biomedical Sciences Animal Ethics Committee (AEC Approval Number: MARP/2012/043) according to the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and Monash University Animal Welfare Committee Guidelines and Policies (Prevention of Cruelty to Animals Act 1986). Forty-eight male Sprague Dawley rats (*Rattus norvegicus*) with initial weights between 160 to 200 g and ages approximately 9-11 weeks old were obtained from the animal breeding facility of Monash

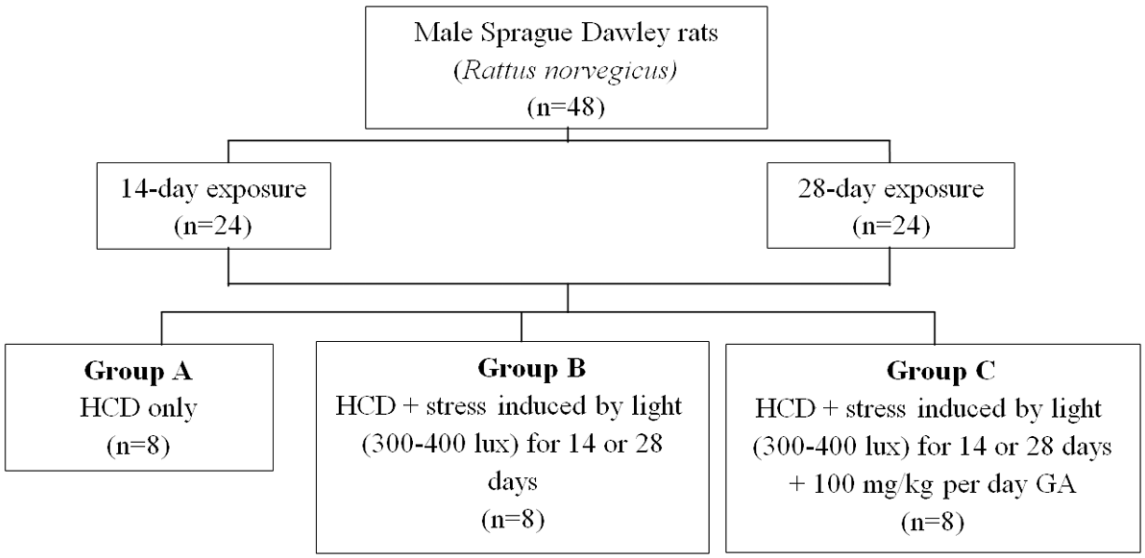

Figure 1. Segregation of animals into different treatment groups (HCD- high-calorie diet; GA- glycyrrhizic acid).

University Malaysia. The rats were housed individually in plastic cages of approximately 35 × 25 × 20 cm which contained shredded paper for bedding.

The rats were randomly assigned equally into two major experimental groups: the 14-day exposure Group and the 28-day exposure Group. Each of these groups were further subdivided equally into three groups: a control group without a stressor, in which rats were fed on a HCD (Group A), the HCD + constant light exposure group (Group B) and the HCD + constant light exposure + GA group (Group C) (**Figure 1**). The HCD fed to all groups was composed of 30% ghee, 30% cane sugar and 40% standard rat chow (Gold Coin, Malaysia). The stressor, continuous light intensity for 14 days or 28 days, was of an intensity between 300 and 400 lux. GA meanwhile was administered orally via water at a concentration of 100 mg per kg of average body weight per day. Food and water were given ad libitum throughout the experiment.

Blood and tissue collection

Prior to sample collection, the rats were fasted for approximately 12 hours. Ketamine (75 mg/kg body weight) and xylazine (10 mg/kg body weight) were administered to each rat via intraperitoneal injection. Blood was drawn from the apex of the cardiac ventricle using 5 mL syringes and 22 G needles for each rat. One ml of blood from each rat was added to blood collection tubes (BD Vacutainer Blood Collection Tubes). The remaining blood was collected in sterile tubes and allowed to clot at room temperature (25°C). The blood was then centrifuged at 1500 × *g* for 15 minutes. The supernatant (serum) was aliquoted into microcentrifuge tubes and stored at -70°C until required for analysis. Liver, kidney, heart, pancreas, abdominal muscles (AM), quadriceps femoris (QF), subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were harvested from each rat. These tissues were put into microcentrifuge tubes and were immediately flash frozen with liquid nitrogen. They were kept at -70°C until required for RNA extraction.

Analysis of biochemical lipid parameters

Serum FFA, TAG and total cholesterol (TC) concentrations were determined using Randox FA115 Non-esterified Fatty Acids kit (Randox, UK), Randox TR1697 Triglycerides kit (Randox, UK) and Randox CH200 Cholesterol (Randox, UK) respectively. High-density lipoprotein (HDL)-cholesterol in serum sample was initially separated from low-density lipoprotein (LDL)-cholesterol and very-low-density lipoprotein (VLDL)-cholesterol fractions by adding Randox CH203 HDL Precipitant (Randox, UK). HDL-cholesterol concentration was measured using TC assay while LDL-cholesterol concentration

Table 1. The probe, forward and reverse primers for LPL, PPAR-α and PPAR-γ specific for *Rattus norvegicus*

Primer/probe	Primer sequence (5'→ 3')
PPAR-α forward primer	TGTGGAGATCGGCCTGGCCTT
PPAR-α reverse primer	CCGGATGGTTGCTCTGCAGGT
PPAR-α probe	(6-FAM) TGCAGGAGGGGATTGTGCACGTGCTCA (BHQ1)
PPAR-γ forward primer	CCCTGGCAAAGCATTTGTAT
PPAR-γ reverse primer	GGTGATTTGTCTGTTGTCTTTC
PPAR-γ probe	(6-FAM) TCCTTCCCGCTGACCA (BHQ1)
BAC forward primer	GTATGGGTCAGAAGGACTCC
BAC reverse primer	GTTCAATGGGGTACTTCAGG
BAC probe	(TET) CCTCTCTTGCTCTGGGC (BHQ1)
LPL forward primer	CAGCAAGGCATACAGGTG
LPL reverse primer	CGAGTCTTCAGGTACATCTTAC
LPL LNA probe	(6-FAM) TTCTCTTGGCTCTGACC (BHQ1)
D5D forward primer	TGGAGAGCAACTGGTTTGTG
D5D reverse primer	GTTGAAGGCTGACTGGTGAA
D5D probe	(6-FAM) TCTCCACCCAGCTACAGGCAACCT (BHQ1)
D6D forward primer	TGTCCACAAGTTTGTCATTGG
D6D reverse primer	ACACGTGCAGGCTCTTTATG
D6D probe	(6-FAM) TGCCTCCGCCAACTGGTGGAACC (BHQ1)
D9D forward primer	ACATACTGCAAGAGATCTC
D9D reverse primer	TGGTGAGGATTCTTTTCA
D9D probe	(6-FAM) CTCTCCTCCATTCTGCTGTCC (BHQ1)
ELOVL5 forward primer	TACCACCATGCCACTATGCT
ELOVL5 reverse primer	GACGTGGATGAAGCTGTTGA
ELOVL5 probe	(6-FAM) TACCACCAGAGGACACGAATAACC (BHQ1)
ELOVL6 forward primer	CTCACTCATGTACCTCAG
ELOVL6 reverse primer	GTCGCTTTCTTCACTTTG
ELOVL6 probe	(6-FAM) ACCTTCTGCTCTTCTGCCATT (BHQ1)

was determined using the Friedewald formula [27].

Real-time reverse transcription polymerase chain reaction (qRT-PCR) for LPL, PPARs, elongases and desaturases

The Qiagen RNeasy Mini Kit (Qiagen, USA) was used to extract total RNA from the liver, kidney, heart, AM and QF whereas the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, USA) was used to extract RNA from SAT and VAT. RNA purity was measured by determining the ratio of absorbance at 260 nm and 280 nm in which a ratio of 1.9 to 2.1 is considered pure. The extracted RNA was treated with the addition of DNase 1, RNase-free (Fermentas, Lithuania) followed by the synthesis of cDNA which was performed using Qiagen Omniscript Reverse Transcriptase Kit (Qiagen, USA). The expression levels of LPL, PPAR-α and PPAR-γ between control and treated rats were performed using the comparative CT (ΔΔCT) method which involves normalising CT values of both controls and samples to an appropriate housekeeping gene: the BAC gene. Sequence of primers and probes specific for *Rattus norvegicus* used to determine LPL, PPAR-α, PPAR-γ elongase and desaturase expression levels were listed in **Table 1**.

Determination of fatty acids content

Fatty acids extraction: Tissues (liver, SAT, VAT and pancreas) were weighed, minced into small pieces and placed into 15 mL Falon tubes containing methanol and chloroform (2:1 ratio). The tissues were homogenized using Heidolph DIAX 900 rotor stator homogenizer. The homogenates were then placed in an orbital shaker and shaken for 20 minutes at 200 rpm. This was followed by centrifugation at 14,000 × *g* for 20 minutes at 4°C. After the addition of dH_2O (1/5 total volume of homogenates), the homogenates were centrifuged at 2000 rpm for 20 minutes at 4°C followed by evaporation using flowing nitrogen gas. For saponification of the lipid extract, alkaline methanol (0.4 M NaOH in 80% methanol) was added to the dried tissue content and the mixture was incubated at 65°C in a water bath for an hour. Then, an equal volume of 0.4M HCl was added into the mixture followed by the addition of acidified hexane (containing 2% glacial acetic acid). The upper (organic) layer of the centrifuged samples were separated and added into a new Falcon tube, while the lower (aqueous) layer and sediments were discarded. The hexane in the organic layer was evaporated using flowing nitrogen gas and the dried content was stored at -80°C.

Table 2. Fasting serum lipid parameters of Groups A, B and C for 14- and 28-day exposure

Lipid profile (mmol/L)		14-day exposure	28-day exposure
TAG	A	0.42 ± 0.08	1.36 ± 0.07
	B	0.33 ± 0.05	0.74 ± 0.10**
	C	0.37 ± 0.04	0.56 ± 0.15
TC	A	1.83 ± 0.07	1.21 ± 0.10
	B	1.67 ± 0.14	1.42 ± 0.10
	C	1.58 ± 0.03	1.43 ± 0.10
HDL	A	1.16 ± 0.08	1.20 ± 0.08
	B	1.14 ± 0.03	1.36 ± 0.13
	C	1.06 ± 0.04	1.37 ± 0.09
LDL	A	0.53 ± 0.07	Too low to be detected
	B	0.40 ± 0.13	Too low to be detected
	C	0.28 ± 0.04	Too low to be detected
FFA	A	0.54 ± 0.06	0.44 ± 0.07
	B	0.47 ± 0.05	0.71 ± 0.12
	C	0.57 ± 0.07	0.53 ± 0.06

**indicates $p<0.01$ for comparison between Group A and B.

Quantification of fatty acids using the high-performance liquid chromatography (HPLC)

HPLC grade methanol was added into each Falcon tube containing the dried fatty acids content. A series of standards for different fatty acids were used to make a standard curve prior to quantification of the fatty acids. An Agilent C18 column with a dimension of 4.6 mm (diameter) × 250 mm (length), 5 μm was used for the separation of the sample fatty acids. A gradient of 70-100% acetonitrile-methanol-hexane (9:8:2 containing 0.2% glacial acetic acid) throughout 60 minutes with constant flow rate of 1.00 mL/min at 55°C. Spectrophotometric absorbance was measured at 208 nm using an Agilent Diode Array Detector. The concentration of fatty acids formed was calculated using a prepared standard curve.

Statistical analysis

Data for lipid parameters were analysed using the Statistical Package for the Social Sciences (SPSS) Version 20.0. Data distribution was analysed using Shapiro-Wilk test. Data with parametric distribution were analysed using one-way analysis of variance (ANOVA) while data with non-parametric distribution were analysed using Kruskal-Wallis test. For PPAR-α and -γ expression, statistical analysis was performed using the Relative Expression Software Tool (REST©) MCS Beta 2006. Results for all analyses were considered statistically significant when the p value was equal to or less than 0.05 ($p \leq 0.05$).

Results

Serum free fatty acids and lipid profile

Serum FFA and lipid profile (TAG, TC, HDL- and LDL-cholesterol) are the key indicators for diabetes and CVD [28, 29]. It is important to examine the above parameters as the development of these diseases is closely associated with disrupted lipid metabolism. For the 14-day and 28-day exposure groups, there was no significant difference in serum free fatty acid concentrations and all lipid parameters across all groups ($p>0.05$) except for TAG of the latter group where Group B had significantly higher TAG level than Group A ($p<0.01$) (**Table 2**). In general, neither stress nor GA affected the serum FFA concentrations and lipid profile parameters in rats subjected to short- or long-term light exposure except TAG of the rats subjected to long-term stress.

Gene expressions for PPAR, LPL, elongase and desaturase

PPAR and LPL are the key genes for lipid metabolism [30] while the elongases and desaturases determine the chemical properties of fatty acids and hence their metabolic fate [14, 16]. Recent studies have demonstrated the importance of these genes in the development of MetS [12, 13, 31].

PPAR-α expression was too low to be detected in both the SAT and VAT of the 14- and 28-day exposure groups (**Figure 2A**, **2B**). In comparing Group A (calibrator) and Group B (target), stress did not affect PPAR-α expression in both the 14- and 28-day exposure groups ($p>0.05$) (**Figure 2A**). By using Group C as the target and Group B as the calibrator, for the 14-day exposure groups, PPAR-α was significantly up-regulated in the kidney ($p<0.05$) (**Figure 2B**). However, GA treatment did not affect PPAR-α in the other tissues and all the tissues of the 28-day exposure group ($p>0.05$). To summarize, stress did not affect PPAR-α expressions in both short and long-term exposure groups while GA increased renal PPAR-α expressions

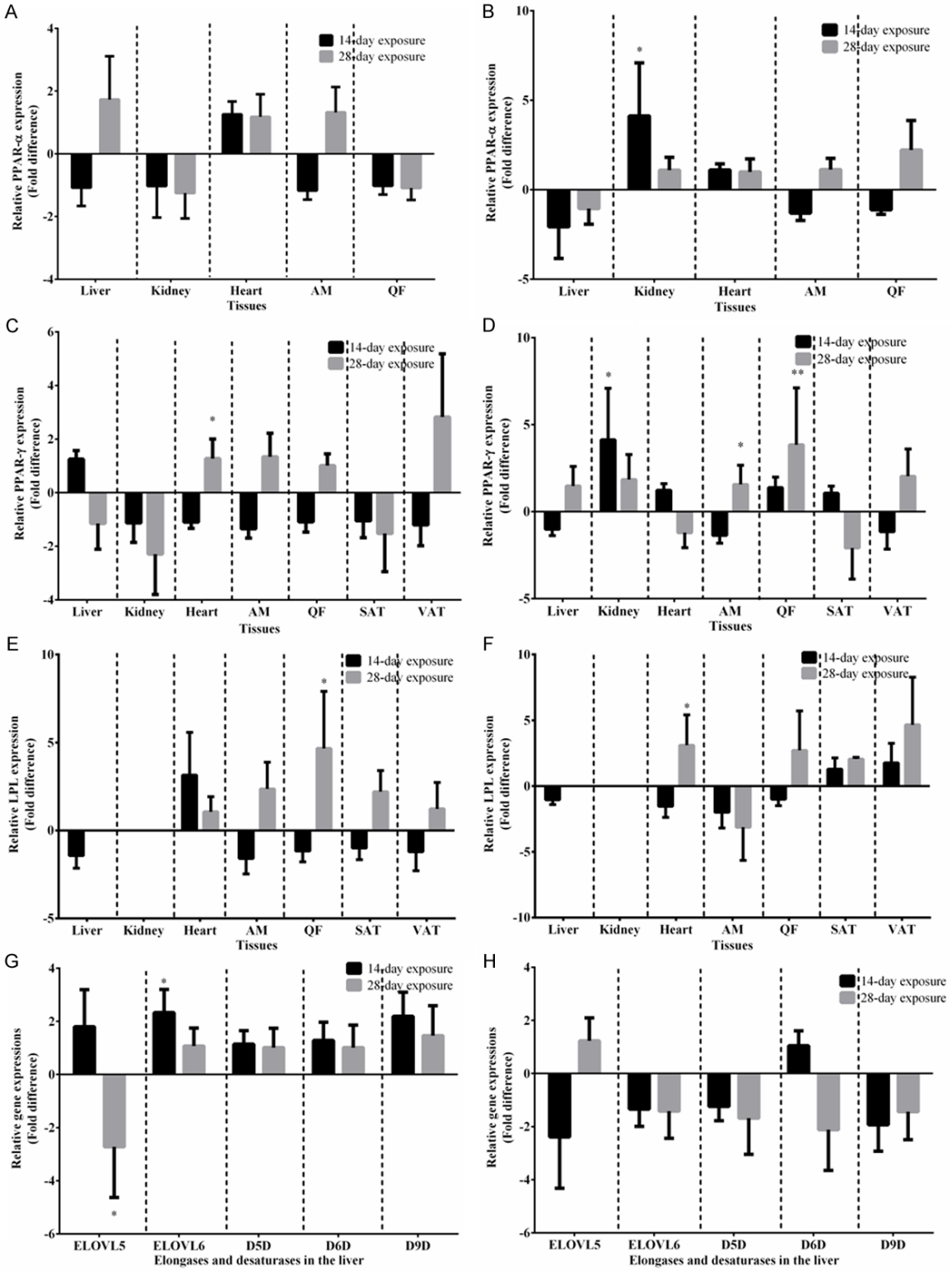

Figure 2. Fold difference of PPAR, LPL, elongase and desaturase expressions with BAC as the reference, Group B as the target and Group A (A, C, E & G) and Group C as the target and Group B as calibrator (B, D, F & H) for both the 14- and 28-day exposure groups in different tissues. [AM, abdominal muscles; QF, quadriceps femoris; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue]. *&**indicates $p<0.05$ and $p<0.01$ respectively.

in the kidneys of rats exposed to short-term stress (14-day exposure).

For PPAR-γ, in comparing Group A (calibrator) and B (target), for the 14-day exposure group,

Table 3. Fatty acid profiles in the liver of Groups A, B and C for 14-day exposure and 28-day exposure

Amount of fatty acid (ng/mL/mg tissue)		14-day exposure	28-day exposure
SFA			
Palmitic acid	A	32.1 ± 2.39	34.30 ± 4.11
	B	11.47 ± 1.18**	23.70 ± 1.91
	C	10.71 ± 1.62	27.50 ± 3.58
Stearic acid	A	40.13 ± 1.82	37.40 ± 3.74
	B	15.29 ± 0.53**	25.30 ± 2.03
	C	34.75 ± 1.41##	21.90 ± 1.52
MUFA			
Oleic acid	A	19.36 ± 0.78	17.90 ± 0.51
	B	12.86 ± 1.17**	17.00 ± 1.02
	C	13.53 ± 0.18	17.90 ± 1.37
PUFA			
Linolenic acid	A	0.12 ± 0.01	0.14 ± 0.00
	B	0.07 ± 0.01*	0.09 ± 0.01
	C	0.07 ± 0.01	0.10 ± 0.02
DHA	A	2.82 ± 0.13	2.68 ± 0.06
	B	2.36 ± 0.26	2.94 ± 0.08
	C	3.18 ± 0.09#	2.70 ± 0.16
AA	A	60.43 ± 0.81	50.60 ± 2.15
	B	51.81 ± 3.07	54.20 ± 2.35
	C	66.34 ± 1.59##	63.40 ± 3.83
Linoleic acid	A	5.02 ± 0.12	3.63 ± 0.08
	B	3.92 ± 0.26**	3.40 ± 0.04*
	C	4.62 ± 0.09#	3.17 ± 0.13

*&** indicates p<0.05 and p<0.01 respectively for comparison between Group A and B; #&## indicates p<0.05 and p<0.01 respectively for comparison between Group B and C.

stress did not affect PPAR-γ expression (p>0.05) while stress significantly increased its expression only in the heart of the 28-day exposure group (p<0.05) (**Figure 2C**). **Figure 2D** depicts the PPAR-γ expressions by comparing Group C (target) to Group B (calibrator) for both 14- and 28-day exposure groups. GA-treated rats (Group C) of the 14-day exposure group demonstrated significant up-regulation in the kidney (p<0.05) while significant up-regulation was observed in both the AM and QF of the 28-day exposure group (p<0.01). However, no difference was observed for the other tissues (p>0.05). Stress increased PPAR-γ expression only in the heart of the long-term stressed (28-day exposure) group while GA increased its expressions in the kidney of the short-term stressed (14-day exposure) group and AM and QF of the long-term stressed group.

With regards to LPL, stress did not affect LPL expressions in all the studied tissues from both the 14- and 28-day exposure groups except significant up-regulation of LPL was detected in the QF of the latter group (p<0.05) (**Figure 2E**, **2F**). GA did not affect LPL expressions in both the 14- and 28-day exposure groups except significant up-regulation of LPL was found in the heart of the latter group (p<0.05). Stress and GA increased LPL expression only in the QF and heart of the long-term exposure group respectively.

As for the elongases and desaturases expressions in the liver, stress down-regulated ELOVL5 in the long-term exposure group while up-regulated ELOVL6 in the short-term exposure group while hepatic desaturases were unaffected by stress. Neither hepatic elongases nor desaturases expressions were affected by GA (**Figure 2G**, **2H**).

Fatty acid profiles in the liver, SAT, VAT and pancreas

Together with hepatic elongases and desaturases, the analysis of fatty acids in different tissue compartments was undertaken to understand how changes in the chain length and degree of saturation of fatty acids may affect the overall lipid metabolism status.

Liver

For the 14-day exposure group, exposure to stress induced by light significantly reduced the amount of the saturated fatty acids (SFA) i.e. palmitic and stearic acid and monounsaturated fatty acids (MUFA) i.e. oleic acid (p<0.01) (**Table 3**). GA treatment significantly elevated the amount of stearic acid (p<0.01) but did not affect the amount of palmitic and oleic acids (p>0.05). The amount of palmitic acid, stearic acid and oleic acid for Groups A, B and C were as shown in **Table 3**. As for the PUFA i.e. linolenic, docosahexaenoic acid (DHA), arachidonic acid (AA) and linoleic acids, similar trend was found in response to stress and GA treatment. Rats exposed to stress without given GA (Group B) had reduced linolenic, DHA, AA and linoleic acids compared to rats fed on a HCD only (Group A). However, significant results were

Table 4. Fatty acid profiles in the SAT, VAT and pancreas of Groups A, B and C for 14-day exposure and 28-day exposure

The amount of fatty acid (ng/mL/mg tissue)		14-day exposure			28-day exposure		
		SAT	VAT	Pancreas	SAT	VAT	Pancreas
SFA							
Palmitic acid	A	85.36 ± 7.73	34.98 ± 2.44	64.26 ± 10.82	204 ± 8.43	181.25 ± 6.24	72.89 ± 5.19
	B	96.53 ± 2.62	31.27 ± 1.32	64.90 ± 1.28	218 ± 3.58	188.42 ± 2.75	49.73 ± 3.39*
	C	86.11 ± 3.80	36.68 ± 7.43	93.26 ± 4.26#	175.00 ± 12.76#	138.51 ± 1.29##	56.50 ± 6.66
MUFA							
Oleic acid	A	29.41 ± 1.73	24.26 ± 2.62	23.50 ± 2.00	73.5 ± 4.00	49.45 ± 3.76	20.13 ± 1.56
	B	29.32 ± 1.37	35.29 ± 0.72*	18.57 ± 0.07	55.9 ± 2.52*	49.30 ± 0.67	13.81 ± 0.57*
	C	22.89 ± 1.23#	27.81 ± 3.23	28.09 ± 2.43#	61.3 ± 2.75	37.80 ± 1.94#	13.80 ± 0.96
PUFA							
Linoleic acid	A	10.39 ± 0.43	6.96 ± 0.82	4.66 ± 0.38	14.90 ± 1.51	10.46 ± 0.57	4.71 ± 0.30
	B	9.92 ± 0.84	9.68 ± 0.41	4.69 ± 0.20	12.01 ± 0.33	11.41 ± 0.60	3.54 ± 0.27*
	C	8.13 ± 0.15	8.41 ± 0.70	6.25 ± 0.47#	13.58 ± 0.95	10.28 ± 0.48	3.70 ± 0.17

*indicates $p<0.05$ comparison between Group A and B; # & ##indicates $p<0.05$ and $p<0.01$ respectively for comparison between Group B and C.

only found for the linoleic ($p<0.01$) and linolenic acids ($p<0.05$). GA-treated rats (Group C) had significantly higher DHA ($p<0.05$), AA ($p<0.01$) and linoleic acids ($p<0.05$) but not linolenic acid ($p>0.05$) than rats without given GA (Group B). The amount of linolenic acid, DHA, AA and linoleic acids for Groups A, B and C were as shown in **Table 3**. In the 28-day exposure group, neither stress nor GA affected the amount of palmitic and oleic acids ($p>0.05$). Rats exposed to stress (Group B) had lower amount of stearic acid than Group A ($p>0.05$) while GA-treated rats (Group C) had a further reduction of stearic acid when compared to Group B rats ($p>0.05$). Linoleic acid was significantly elevated by stress ($p<0.05$) but was unaffected by GA ($p>0.05$). The amount of DHA, AA and linolenic acids was also unaffected by stress or GA as indicated by the insignificant difference when comparisons were made between Groups A and B and Groups B and C ($p>0.05$). The amount of the constituents of fatty acids for 28-day exposure groups is as shown in **Table 3**.

SAT

For the 14-day exposure group, the amount of oleic acid in the GA-treated rats (Group C) was significantly lower than Group B ($p<0.05$) (**Table 4**). No difference was found between Groups A and B ($p>0.05$). No difference was found between Groups A and B and Groups B and C for both palmitic and linoleic acids ($p>0.05$). For the 28-day exposure group, rats fed on a HCD and exposed to stress (Group B) had significantly lower amount of oleic acid than Group A ($p<0.01$) (**Table 4**). No difference was found between Groups B and C ($p>0.05$). Stress did not affect the amount of palmitic acid but GA was found to reduce palmitic acid significantly when compared to stressed rats ($p<0.05$). Neither stress nor GA had effect on the linoleic acid across all groups ($p>0.05$). The values for oleic acid, linoleic acid and palmitic acid were shown in **Table 4**.

VAT

Table 4 showed the amount of palmitic, oleic and linoleic acids found in the VAT of rats subjected to 14-day and 28-day light stress. For the 14-day exposure group, neither stress nor GA affects the amount of palmitic and linoleic acids ($p>0.05$) (**Table 4**). However, stress was found to elevate the oleic acid ($p<0.05$). No difference was found between Groups B and C ($p>0.05$). For the 28-day exposure group, despite the absence of the effect of stress on the oleic and palmitic acids ($p>0.05$), GA-treated rats (Group C) had significantly reduced amount of both fatty acids than Group B ($p<0.05$ for oleic acid and $p<0.01$ for palmitic acid) (**Table 4**). Neither stress nor GA affect the amount of linoleic acid ($p>0.05$).

Pancreas

Table 4 showed the amount of palmitic, oleic and linoleic acids found in the pancreas of rats subjected to 14-day and 28-day light stress. For the 14-day exposure group, stress did not affect the oleic, palmitic and linoleic acids as shown by the insignificant difference between Groups A and B ($p>0.05$) (**Table 4**). GA-treated group (Group C) had significantly higher amount of all of the fatty acids than the group without GA (Group B) ($p<0.05$). For the 28-day exposure group, stress significantly reduced the amount of oleic, palmitic and linoleic acids ($p<0.05$ between Groups A and B) (**Table 4**). GA treatment did not affect the amount of these fatty acids ($p>0.05$ between Groups B and C).

Discussion

Stress together with over-consumption of high-calorie foods has been recognized as the primary contributor to the development of various metabolic diseases [32] while elongases and desaturases have been shown to play an important role in T2DM and MetS [12, 13]. GA administration has been shown to improve lipid metabolism in rats fed on either a high-sucrose or high-fat diet via regulation of LPL and PPAR-γ expressions [7, 8, 20, 33]. Furthermore, GA-treated rats fed on a high-fat diet were found to have higher LPL expressions in tissues characterized with high PPAR-α expressions i.e. liver and skeletal muscles [7, 8]. Therefore, it is hypothesized that GA-mediated regulation of PPAR and LPL together with modification of fatty acid content in different tissues by elongases and desaturases could also improve lipid metabolism caused by a combination of stress and a HCD.

Overall, stress did not affect PPAR-α expressions in both the 14- and 28-day exposure groups (**Figure 2A**, **2B**). As for PPAR-γ, only the heart of the rats exposed to long-term stress showed elevated PPAR-γ expressions (**Figure 2C**), however, the involvement of PPAR-γ in cardiac metabolic homeostasis remains elusive. This is because cardiac effects following PPAR-γ activation takes place via the indirect pathways [34]. These include altered glucose and fatty acids transportation to the heart via actions on adipose metabolism and also the effects of extra-cardiac derived circulating factors upon PPAR-γ activation. Studies from our laboratory showed that oral-administration of GA for 24 hours induced significant up-regulation of PPAR-γ expression in both the AM and QF of rats fed-on a normal diet [26]. Hence, it is expected to see significant changes of PPAR expressions at least for PPAR-γ in rats receiving a HCD and exposed to short- and long-term stress as stipulated in this study. However, the regulatory effect of GA on PPAR-α and PPAR-γ expressions in rats exposed to short-term stress (14-day exposure) was not apparent in the type of treatment used in the present study as shown by the insignificant difference in PPAR expressions in various types of tissues in the GA-treated rats.

The dosage of GA used in the present study may be insufficient to cause significant changes in the PPAR-γ expression in most of the tissues (**Figure 2D**) due to increased GA clearance. Stress mediators e.g. GCs and catecholamines have shown to reduce activity of GA thus causing inhibition of GA actions in certain tissues [35, 36]. *In vivo* GA clearance is proportional to the transportation rate of hepatic bile acid sulfates and glucuronides of GA across the multidrug resistance associated protein 2 (MRP2) transporter found in the canalicular membrane of the hepatocyte [37]. Administration of dexamethasone (a synthetic GC) was found to increase MRP2 in the hepatocytes of rats thus promoting GA clearance [35]. The stress treatment used in the present study could have activated the stress response and promoted GA clearance which then contributed to the insignificant effects of GA on PPAR expressions in most of the studied tissues.

Significant increase in PPAR-γ expressions were found in the skeletal muscles, both AM and QF of the long-term exposure group given GA (Group C) (**Figure 2D**). PPAR-γ activation has been found to enhance insulin-stimulated glucose uptake via increased insulin-stimulated PI3-K activity and translocation of GLUT-4 to the cell membrane [38]. Furthermore, PPAR-γ activation, particularly in the muscles aids in the diversion of FFA into adipose tissues especially SAT [39]. This prevents ectopic lipid deposition in the muscles thus protecting the muscles from the lipotoxic effects of FFA. In addition, elevated TAG content is also linked to IR through altered muscle fiber composition [39]. PPAR-γ activation also increases activation of PPAR-γ-coactivator 1α (PG1α) expres-

sion which is able to induce fiber-type switch from type 2 to type 1 muscle fibers with greater insulin sensitivity and oxidative capacity [40]. This is important as individuals with MetS have higher proportions of type 2 muscle fibers [41]. Constant exposure to high circulating FFA levels were found to increase intramyocellular TAG [42]. The subsequent accumulation of toxic lipid metabolites e.g. diacylglycerol (DAG) and ceramides from TAG synthesis interfere with the insulin signaling pathway that eventually leads to IR [42]. The low LPL expression in the liver and kidneys (**Figure 2E**, **2F**) was in agreement with various studies which have shown that LPL expression is almost non-existent in the liver and very low in the kidneys [43-45].

The rats exposed to short-term stress and given GA had significant up-regulation of PPAR-α and PPAR-γ in their kidneys (**Figure 2B**, **2D**). Although the underlying mechanisms of PPAR-α/PPAR-γ-mediated renoprotection remain elusive, it has been reported these effects could be brought about via protection against diabetic nephropathy via multiple pathways (Guan and Breyer, 2001; Iglesias and Diez, 2006; Park *et al*., 2006b; Zheng and Guan, 2007). Administration of PPAR-α and PPAR-γ agonists was found to reduce albuminuria in diabetic nephropathy which may be mediated through promotion of albumin uptake and degradation in the proximal tubules (Park *et al*., 2006a; Yano *et al*., 2007; Zheng and Guan, 2007). Administration of PPAR-α agonists was found to attenuate renal lipotoxicity through an increased gene expression of ATP binding cassette transporter-A1 and enhanced apolipoprotein A1-mediated cholesterol efflux from lipid-loaded mesangial cells (Ruan *et al*, 2003; Varghese, 2003). The activation of PPAR-α and PPAR-γ also exerts their anti-fibrotic and anti-inflammatory effects on the kidneys which are mediated through attenuation of oxidative and nitrative stresses via the suppression of oxidative-stress induced growth factors such as collagen I and fibronectin that induce ectopic glomerular matrix production (Wilmer *et al.* 2002; Bagi *et al*., 2004). PPAR-α and PPAR-γ activation also promote anti-apoptotic effects on the kidneys (Chung *et al*., 2005; Chen *et al*., 2009). In addition, it has been reported that the renoprotective effects conferred by PPAR-α and PPAR-γ activation on the kidneys are mediated through improved glucose metabolism (Guan, 2004). Activation of PPAR-α and PPAR-γ in the kidneys has also been implicated in sodium homeostasis and blood pressure (BP) regulation and confers protection against the development of renal injury in insulin-resistant rats (Park *et al*., 2006a; Galan *et al*., 2009).

When comparing between Groups A, B and C within the short- and long-term exposure groups, despite the absence of the effects of stress in both SAT and VAT, rats exposed to long-term stress (28-day exposure) treated with GA (Group C) had a significantly reduced amount of palmitic acid (**Table 4**). Among all the fatty acids studied in both human and rodent models, saturated palmitic acid has been shown to exert the most toxic effects on pancreatic-islets by preventing proliferation, inducing oxidative stress and promoting β-cell apoptosis [46]. In the present study, oleic acid is the major species that made up TAG found in the adipose tissues. Oleic acid was found to be lower in the SAT for 14-day exposure and VAT for 28-day exposure treated with GA. Monounsaturated oleic acid was found to prevent palmitate-induced apoptosis via up-regulation of anti-apoptotic protein Bcl_2 [47]. Since adipose tissues act as the primary TAG storage site, these may indicate reduced delivery of palmitic acid and increased oleic acid to the pancreas which is especially important under excess caloric intake conditions. Neither stress nor GA affects linoleic acid content in the SAT and VAT of both 14- and 28-day exposure groups. Linoleic acid found in the adipose tissues only acts as the reservoir for highly-unsaturated fatty acids (HUFA) [48]. These include arachidonic acid (AA) and docosahexaenoic acid (DHA) which are the main components of the phospholipids in maintaining membrane fluidity [49].

Although the pancreas had the same fatty acids content as SAT and VAT, the long-term exposure group (28-day exposure) had lower amount of fatty acids than the short-term exposure group (14-day exposure). This could be related to the protective mechanism of the body against transportation of fatty acids to the organs which are more vulnerable to excess fatty acids e.g. pancreas [50, 51]. Non-adipose tissues e.g. liver and skeletal muscles are able to remove excess FA as aforementioned. Pancreatic β-islet cells on the other hand have limited fatty acid oxidative capacity to remove excess fatty acids. Hence, despite the elevated

fatty acids content in the SAT and VAT of the long-term exposure group, the fatty acids content in the pancreas appeared to be lower than the short-term exposure group (**Table 4**). However, GA-treated group (Group C) of the short-term exposure group had significantly higher FA content of palmitic, oleic and linoleic acid in the pancreas. Changing the intensity or periodicity of light illumination may induce stress and consequently, significant changes of FA content in the pancreas.

In the liver, both palmitic and oleic acids which are the main fatty acids in the phospholipids are higher in the rats exposed to long-term stress (28-day exposure) than short-term stress (14-day exposure) (**Table 3**). This may indicate higher rate of fatty acid and hence TAG synthesis. This was in accord with the significantly higher TAG level of the long-term exposure group than the short-term exposure group (**Table 2**). The LDL-cholesterol was too low to be detected in the long-term exposure group. This could be associated with the limitations of using an indirect method which is the determination of LDL-cholesterol using mathematical equation. For example, the equation is not applicable when TAG exceeds certain level e.g. 400 mg/mL [27]. Indeed, while total cholesterol and HDL-cholesterol remain comparable between the short and long-term exposure group, the latter had generally higher TAG level than the former (**Table 2**).

Lipid metabolism is tightly controlled by insulin, glucagon and adrenaline [10]. A higher insulin level inhibits fatty acids oxidation and activates the fatty acid synthesis pathway that generates palmitic acid as the end product, while a higher adrenaline level induces fatty acid oxidation (FAO) and lowers fatty acids synthesis. Such hormonal profiles promote fatty acids (especially palmitic acid) production. This is followed by increased elongation of palmitic acid and further desaturation to produce oleic acid. Since the liver is the primary site of *de novo* lipogenesis [52], these may indicate increased production of oleic acid transported to the adipose tissues to be stored as TAG. Similarly, higher palmitic and oleic acids were found in the SAT and VAT.

When comparisons were made within groups of either 14- or 28-day exposure, for the 14-day exposure group, rats on a HCD (Group B) and exposed to stress had significantly lower palmitic and oleic acid than the group given HCD only (Group A) (**Table 4**). The stress response induced in the short-term exposure rats may induce increased glucose production, which in turn inhibits fatty acid synthesis pathway that generates palmitic acid as the end-product. The amount of oleic acid also decreases following the decrease in the amount of its precursor, palmitic acid.

ELOVL6 is an important enzyme involved in the formation of LCFA e.g. stearic acid through elongation of saturated and monounsaturated fatty acids with 12, 14 and 16 carbons [14, 53]. In the present study, rats exposed to short-term stress (14-day exposure) (Group B) had significantly higher ELOVL6 expressions in the liver than the controls (Group A) which could possibly increase the abundance of LCFA. Accumulation of the lipid metabolites of LCFA e.g. acyl-CoAs, diacylglycerol (DAG) and ceramides were found to be a more prominent determinant of IR than TAG itself [54-56]. DAG accumulation has been associated with increased protein kinase C epsilon ε (PKCε) and impaired activation and phosphorylation of insulin receptor 1 and 2 (IRS-1 and IRS-2) tyrosine by insulin [54, 55]. However, the amount of stearic acid of the short-term exposure group was unaffected by the increase in ELOVL6 expression. These could again be associated with the compensatory mechanism against lipotoxicity. ELOVL5, on the other hand is an enzyme that elongates mono- and polyunsaturated fatty acids (from 16 to 20 carbons) [15]. Rats exposed to long-term stress (28-day exposure) had significant down-regulation of hepatic ELOVL5 (**Figure 2G**). ELOVL5-/- rats have been shown to be associated with reduced DHA and AA and elevated C18 FA e.g. stearic acid accompanied by increased TAG synthesis [57]. However, the FA profile of the 28-day exposure rats suggested otherwise. Rats exposed to 28-day of light had reduced stearic acid while no changes were found for AA and DHA (**Table 3**).

Both *in vivo* and *in vitro* studies have shown that administration of PUFA lowers the risk of developing T2DM via improved insulin sensitivity. For example, male Wistar rats given fish oils showed significant improvements in dyslipidaemia and insulin sensitivity. Furthermore, treatment with PUFA significantly increases the number of insulin receptors in Ehrlich cells

(cells that display mammalian insulin receptor binding characteristics). HUFA e.g. AA and DHA particularly have been shown to play an important role in PUFA-related improvement in T2DM. HUFA are involved in many physiological functions in mammals [17]. Mammals are unable to synthesize HUFA from acetyl-CoA due to the absence of w3 desaturase and Δ12 desaturase [17]. However, mammals are able to synthesize HUFA from PUFA. D5D and D6D are involved in the synthesis of AA acid and DHA from linoleic and linolenic acids (essential fatty acids that must be obtained from diets) [58]. Rats exposed to short-term stress (14-day exposure) and treated with GA (Group C) had significantly higher linoleic acids, AA and DHA in the liver (**Table 3**). The primary role of AA in improving T2DM was found to be associated with its anti-oxidative capacity where it counteracts the increased oxidative damage caused by fatty acids to the beta-cells and its ability to divert FFA storage into less toxic TAG. Since lipotoxicity is mainly associated with oxidative damage caused by free radical generation from FFA metabolites e.g. ceramides, these roles of AA are beneficial in preventing the destruction of beta-cells which can lead to IR and subsequent T2DM. AA is readily converted into metabolites i.e. leukotrienes and prostaglandins. What is more interesting is AA itself is sufficient to provide the anti-oxidative effect and is independent of its metabolites i.e. eicosanoids. Administration of palmitic acid was found to increase incorporation of saturated fatty acids into neutral lipids i.e. TAG [59]. Upon cellular uptake, FFA is converted into fatty-acyl CoA which then leads to increased ROS and ceramide synthesis that could lead to apoptosis of pancreatic islet-beta cells. Administration of AA was found to increase superoxide dismutase which functions to inactivate superoxide anions thus protecting the pancreatic islets lipotoxicity [59]. Different from the protective role of AA against T2DM which is mediated through the anti-oxidative mechanism, the beneficial effects of DHA on T2DM is associated with an overall improved lipid metabolism. These include i.) increased lipolysis of TAG and enhanced fatty acid oxidation, ii.) raised HDL levels and iii.) improved LPL profiles [60]. These are associated with the role of n-3 fatty acids as PPAR activators hence promoting fatty acid utilization via increased FAO by PPAR-α or improved adipogenesis by PPAR-γ [61].

However, despite the induction of HUFA synthesis, possibly by GA, no change was observed for D5D and D6D expressions in the GA-treated rats (14-day exposure) (**Figure 2H**). This may be due to the rapid induction of the other PPAR-α responsive genes such as acyl CoA oxidase and carnitine palmitoyl transferase-I for FAO while desaturases (D5D, D6D and D9D) mRNA was found to take longer to reach maximum induction via indirect mechanisms [62, 63]. Peroxisome proliferators promote degradation of unsaturated fatty acids via increased production of FAO enzymes in both peroxisomes and mitochondria [64]. These events lead to an increase in HUFA demand for the membrane phospholipids, thus resulting in induction of desaturases [17]. However, since the PPAR-α expression in the liver is unaffected by potential peroxisome proliferators- GA, the direct relationship between PPAR-α and desaturases in regulating HUFA synthesis could not be determined in the present study. As for the 28-day exposure group, neither stress nor GA affects the AA and DHA levels.

Conclusion

Our results provide evidence for the role of GA in improving lipid metabolism in rats exposed to stress and on a HCD via selective regulation of PPAR, LPL and hepatic elongase and desaturase expressions and modification of fatty acids. Since hormones produced by the adipose tissues are an important determinant of the energy status, lipid and carbohydrate metabolism, it will also be essential to examine the effects of GA on adipocytokines such as the adiponectin, resistin and TNF-α in rats fed on a HCD and exposed to stress. A more complete understanding of the molecular and biochemical pathways and the interaction between them as regulated by GA is likely to lead to new therapeutic approaches and a greater understanding of the management of obesity, IR, dyslipidaemia and hyperglycaemia.

Disclosure of conflict of interest

None.

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