Lipoprotein Lipase Expression, Serum Triacylglycerol and Tissue Lipid Deposition in Rats Fed on Switching Type of Diet and Treated with Glycyrrhizic Acid

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Authors’ contributions

Author HPY wrote the first draft of the manuscript and performed some analysis of the results. Author SHT also helped to write the article as well as wrote the protocol. Author SWN did the laboratory works and analysis. Author KAK planned and also helped to write the protocol. All authors read and approved the final manuscript.

ABSTRACT

Aims: To determine the anti-hyperglycaemic and anti-hyperlipidaemic effects of glycyrrhizic acid (GA), the root extract of Glycyrrhiza glabra in rats with switching type of diet i.e. between normal diet (ND) and high-fat diet (HFD).

Study Design: In vivo study.

Place and Duration of Study: School of Science, Monash University Sunway Campus between January 2010 and October 2010.

Methodology: Sixteen Sparague-Dawley rats were divided into two groups having eight animals each i.e. group A- ND+GA→ HFD+GA; group B- ND+GA→HFD+no GA. Rats were fed with the corresponding diet and GA (100 mg/kg) for 28 days. The blood glucose, insulin and triacylglycerol levels, lipoprotein lipase expression in the liver, kidney, heart, abdominal muscle (AM), quadriceps femoris (QF), subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) and tissue lipid deposition were measured.

Results: No significant difference in blood glucose levels between groups. GA
significantly lowered ($P=.05$) triacylglycerol in rats fed on a ND with GA and later switched to a HFD without GA (group B) compared to rats fed on a ND with GA and later switched to a HFD with continuous GA supplementation (group A). For LPL expression, group B had significant lower ($P=.05$) LPL expression in the liver, AM, kidney and heart than group A. Group A had significantly smaller ($P=.05$) size of VAT than group B.

**Conclusion:** These findings may indicate the role of GA in lowering blood glucose and triacylglycerol in subjects who had a switch from a ND with GA to a HFD without GA but not in those who had a switch from a ND with GA to a HFD with continuous GA supplementation.

**Keywords:** Glycyrrhizic acid; lipoprotein lipase; tissue lipid deposition; triacylglycerol; high-fat diet.

### 1. INTRODUCTION

In recent decades, advances in science and technology have led to massive increase in global food production. Together with increased availability of fast food chains and processed foods that are high in refined carbohydrate and fat content, these have caused changes in the eating habits of most people worldwide. The switch towards obesogenic diet and adaptation of sedentary lifestyle will eventually contribute to obesity epidemic and the associated chronic diseases [1].

Due to the different storage mode of fats as compared to protein and carbohydrate, the unlimited storage capacity of fats allows it to be stored and incorporated into the adipose tissues [2]. Excessive storage of fats results in the expansion of adipose tissues in the body. Adipose tissues have been recognized as an endocrine organ which secretes various hormones and cytokines such as adiponectin and TNF-α which are related to the overall body energy status [3]. Storage of fats especially in the visceral region has been shown to exert detrimental effects due to its strategic anatomical position near to the liver [4]. Obesity, especially accumulation of fats in the visceral region has been associated with the development of metabolic syndrome (MetS) which is a cluster of metabolic derangements that contribute to the development of type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD) [5].

Lipoprotein lipase (LPL) is an enzyme involved in lipid metabolism. LPL is secreted by the parenchyma cells of extra hepatic tissues such as adipose tissues, heart and skeletal muscles into the circulation [6,7]. LPL is involved in the metabolism of lipoproteins and acts as a central enzyme involved in lipid metabolism. It has been related to several pathophysiological conditions characterized by hypertriglyceridaemia which include obesity, diabetes mellitus and insulin resistance (IR) [6]. Reduced LPL activity has been reported in T2DM patients and diabetic animal models which can be related to the increased production of cytokines such as TNF-α and IL-6 in the adipose tissues [8].

Glycyrrhizic acid (GA) is the active compound of licorice shrub Glycyrrhiza glabra [9]. GA has been shown to improve glucose homeostasis and lipid profile through inhibition of both isoforms of 11β-hydroxysteroid dehydrogenases (11β-HSD) and up-regulation of LPL expression [10,11,12,13]. To determine whether GA could elucidate such effects in rats on a normal diet but later switch to a high-fat diet and given GA, this work was undertaken. The parameters measured were blood glucose, serum insulin and triacylglycerol levels and tissue lipid deposition using rats as the animal models.
2. MATERIALS AND METHODS

2.1 Animals and Treatment

The use of rats had been approved by Monash University Animal Ethics Committee (AEC Approval number SOBSB/MY/2010/06) following NHMRC 1997 Australian Code of Practice for the Care and Use of Animal for Scientific Purposes and Relevant Victorian Legislation. Sixteen male albino rats (Rattus norvegicus) (180–200g) were supplied by University Kebangsaan Malaysia (UKM) Animal House. The rats were randomly assigned into two groups (n=8). Group A rats were fed with normal diet (ND) supplemented with GA for fourteen days, followed by a high-fat diet (HFD) with GA for the subsequent fourteen days. Group B rats were fed with a ND supplemented with GA for fourteen days before switching to a HFD without GA in the following fourteen days (Fig. 1). The treatment lasted for twenty-eight days. The ND was normal rat chow (Gold Coin, Malaysia) while HFD was composed of 60% (vegetable shortening) plant fat mixed with rat chow. GA was given orally to the rats at concentration of 100 mg/kg through feeding bottles. All rats were fed ad libitum. The rats were kept in individual polypropylene cages at 27°C with 12 hour light/ 12 hour dark lighting cycle.

![Fig. 1. Rats assigned into different treatment groups](image)

2.2 Blood and Tissue Sampling

Blood was withdrawn from the apex of the heart with a 22G needle. Part of the blood was collected into tubes containing anticoagulant while the remaining blood was allowed to clot at room temperature. Serum was obtained by centrifuging the clotted blood at 12,000 xg for 10 minutes at 4°C. Serum was stored at -70°C until required for further analysis. Subcutaneous and visceral adipose tissues (SAT and VAT), abdominal muscle (AM), quadriceps femoris (QF), liver, kidney and heart were harvested for analysis. Part of the SAT, VAT and liver were immersed in 10% neutral-buffered formalin in universal bottles for histological analysis. The remaining tissues were snap-frozen in liquid-nitrogen and kept at -70°C until further analysis.
2.3 Determination of Blood Glucose, Serum Insulin Levels and Homeostatic Model Assessment of Insulin Resistance Index (HOMA-IR)

Blood glucose concentrations were measured using the Accu-chek glucometer based on the glucose oxidase method (Roche, Manheim, Germany). Serum insulin levels were determined using the rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) (Linco research, St. Charles, MO). The HOMA-IR index indicates the degree of insulin resistance. A higher HOMA-IR index denotes low insulin sensitivity and vice versa. The HOMA-IR index was calculated based on the calculation given below [14]:

$$\text{HOMA-IR index} = \frac{\text{insulin (µU/mL)} \times \text{glucose (mmol/L)}}{22.5}$$

2.4 Determination of Serum Triacylglycerol (TG) Level

The serum TG level was determined using Wako Triglyceride E kit (Wako Pure Chemical Industries) based on the GPO-DAOS method.

2.5 RNA Extraction and cDNA Synthesis

Total RNA extraction was carried out by using Qiagen RNeasy Mini Kit (Qiagen, USA) for the liver and kidney; QIAshredder spin column (Qiagen, USA) followed by Qiagen RNeasy Mini Kit (Qiagen, USA) for AM, QF and heart while Qiagen RNeasy Lipid Mini Kit (Qiagen, USA) was used for SAT and VAT. RNA purity was determined by measuring the absorbance of RNA at 260 and 280 nm. RNase-free DNase treatment was performed using Promega RQ1 RNase-free DNase (Promega, USA) and cDNA synthesis was performed using Qiagen Omniscript Reverse Transcriptase kit (Qiagen, USA).

2.6 Real Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The LPL expression was determined by qRT-PCR using LPL forward and reverse primers 5'-CAGCAAGGCATACAGGTT-3' and 5'-CGAGTCTCTCCAGTGACATCT-3' and the probe 5'-6-FAM-TTCTCTTGCTCTGACC (BHQ1)-3' (specific for Rattus norvegicus LPL mRNA) [GenBank: BC081836] and normalized to the β-actin (BAC) gene with the forward and reverse primers 5' GTAGGGTGAGGACAGCTCC-3' and 5'-GTTCATAGGTTCTCCAGG-3' and the probe 5'-TET-CTCTCTCTGTCTGGGC (BHQ1)-3' (specific for Rattus norvegicus BAC mRNA) [GenBank: BC063166] [10]. The comparisons of LPL expression between the groups of rats were performed using the Comparative Ct (ΔΔCt) Method, with BAC as reference. Agarose gel electrophoresis was carried out on amp icons generated from qRT-PCR reaction to ensure primer specificity.

2.7 Tissue Lipid Staining and Morphometric Analysis of Adipocytes

SAT, VAT and liver samples with size of approximately 0.5-1 cm³ were fixed in 10% neutral-buffered formalin and embedded in paraffin. 6 µm tissue sections were then stained with Haematoxylin and eosin (H&E) followed by the measurement of 100 adipocytes per field per (µm²) per tissue section at 400 x magnification. Periodic acid Schiff's (PAS) stain was performed on liver tissues only.
2.8 Statistical Analysis

Statistical analysis of blood glucose concentration, serum insulin, HOMA-IR and serum TG levels were performed by using Statistical Package for the Social Science (SPSS) Version 16.0 except for LPL expression. Data distribution was determined using Kolmogorov-Smirnov test. Mann-Whitney U test was used to analyze the blood glucose, serum insulin, HOMA-IR and serum triacylglycerol levels. Independent-T test was used to compare the mean area of adipocyte between subcutaneous and visceral adipose tissues. The data for LPL expression was analysed using Relative Expression Software Tool (REST ©) as reported by Pfaffl and co-workers (2002) [15]. In all analyses, \( P = .05 \) was statistically significant.

3. RESULTS AND DISCUSSION

3.1 Blood Glucose, Serum Insulin and TG Levels and HOMA-IR Index

Group A (6.49±1.25 mmol/L) had higher mean blood glucose levels than group B (5.34±0.81 mmol/L) (Fig. 2 (I)). Group A (2.07±0.29 ng/mL) showed higher mean serum insulin level than group B (1.32±0.42 ng/mL) (Fig. 2(II)). A higher HOMA-IR index indicates lower insulin sensitivity and vice versa (Wallace et al., 2004). Group A (0.74±0.42) had higher mean HOMA-IR index than group B (0.56±0.49) (Fig. 2 (III)). However, there was no significant difference between groups A and B for all the above parameters. The mean serum TG levels of group A (104.6±14.92 mg/dL) was significantly higher than group B (52.65±9.80 mg/dL) \( (P = .05) \) (Fig. 2 (IV).

There was no significant difference observed for blood glucose level, serum insulin and HOMA-IR index between groups of rats. Rats from group A (normal diet for two weeks followed by switching to high fat diet with GA) had higher mean serum TG level than group B (normal diet for two weeks followed by switching to high fat diet without GA for another two weeks). It has been reported that hyperinsulinaemia causes down-regulation of LPL expression in the adipose tissues and skeletal muscles [16]. The hyperinsulinaemic condition as observed in group A could be related to over-production of insulin to overcome the insulin-resistant state due to the presence of high fatty acids content that competes with glucose as the primary substrate for metabolism especially in the skeletal muscles and liver [17]. Group A rats had higher insulin level and lower LPL expression in both the SAT and VAT compared to group B rats. This leads to reduced TG-rich lipoprotein lipolysis in the target tissues (e.g. adipose tissues and skeletal muscles) and high TG remains in the circulation. This contributes to the elevated TG level observed in group A rats.
Fig. 2. Blood glucose (I), serum insulin (II), HOMA-IR index (III) and serum TG level (IV) in rats from groups A and B after 28 days of treatment

A= Rats fed on a ND supplemented with GA for two weeks followed by a HFD supplemented with GA for another two weeks, B= Rats fed on a ND supplemented with GA for two weeks before switching to a HFD and withdrawal of GA for another two weeks. Results expressed as Mean ± SD for eight rats in each group. Values differ significantly among each other according to Mann-Whitney U Test at P =.05

3.2 LPL Expression in Different Tissues

When comparison was made between groups A (calibrator) and B (target), down-regulation of LPL was seen in all tissues with significant results in the liver (0.48-fold difference), kidney (0.17-fold difference), AM (0.11-fold difference), and heart (0.36-fold difference) (P=.05). Non-significant down-regulation was found in the SAT (0.46-fold difference), VAT (0.51-fold difference) and QF (0.34-fold difference) (Fig. 3).

Administration of GA has been shown to improve lipid profile and insulin sensitivity in high-fat and high-sucrose fed rats through improved glucose and lipid homeostasis via peroxisome proliferator-activated receptors (PPAR) activation [10,11,12,13]. With the presence of peroxisome proliferator-respon element (PPRE) in the promoter region of LPL, binding of PPAR ligand e.g. GA up-regulates LPL expression [18]. The LPL expression in both the SAT and VAT of group B was lower compared to group A. Group A rats were supplemented with GA continuously for four weeks while rats from group B were given GA
only at the initial two weeks of the treatment period. These results may indicate that only long-term administration of GA (e.g. two weeks vs four weeks in the present study) would be able to increase LPL expression in the adipose tissues. The effects of four weeks of GA administration was prominently shown from the comparison between groups A and B as rats from both groups were given the same diet and the higher LPL expression seen in group A may be solely due to the effect of GA. The increased LPL expression as seen in group A may also imply that early and continual supplement of PPARγ ligand i.e. GA may aid in the up-regulation of LPL expression in the adipose tissues. Increased LPL expression in the adipose tissues may exert beneficial effects as more TG is broken down and FFA was taken up by the adipose tissues [19]. This will aid in the clearance of TG from the circulation thus preventing increased FFA uptake and ectopic lipid storage in insulin-sensitive organ e.g. liver that eventually leads to IR.

Down-regulation of LPL in the liver prevents uptake of excessive fatty acids into the hepatocytes hence preventing lipotoxicity [20]. The increased LPL expression in the liver of group A rats may due to regulation of LPL expression by multiple dietary factors via nuclear receptors, for example, in the liver, fatty acids may induce LPL expression via activation of liver X receptor (LXR) [7]. This suggested that the increased LPL expression observed in group A rats (normal diet for two weeks followed by switching to high fat diet with GA for another two weeks) may be mediated through activation of PPARγ and LXR via synergistic effects of fatty acids and GA. Both groups A and B rats shared the same diet but GA supplementation was withdrawn from group B after the first two weeks, thus the higher LPL expression in group A could be related to PPARγ-mediated up-regulation of LPL expression.

Increased fatty acids content in the skeletal muscles causes lipotoxicity as aforementioned and interferes with insulin signaling that impairs glucose disposal rate and promotes IR [17,21]. Therefore, an agent that stimulates LPL expression particularly in the skeletal muscles promotes IR. LPL expression was higher in group A rats compared to group B. This may indicate that administration of GA induced LPL expression as GA works synergistically with high fatty acids content to increase LPL expression in the skeletal muscles and leads to IR. This can be seen in group B where the rats shared the same diet as group A but GA was removed in later weeks. Without GA, the induction of LPL by via synergistic effects of GA and fatty acids is reduced. This explained the higher blood glucose, serum insulin and HOMA-IR index in group A rats than group B which could be related to the development of IR in the insulin-sensitive organs in group A rats (similar observations were obtained when comparisons were made between groups A and C). LPL expression in the AM of group B rats was lower than group A rats. This showed that continuous GA supplementation for four weeks may selectively up-regulates LPL expression only in the AM but not QF which could be associated with fiber-type selective transcriptional activity of PPAR-α agonism. Administration of PPAR-α agonist e.g. fenofibrates selectively activates PPAR-α expression in type I (slow twitch) but not type II (fast twitch) skeletal muscle fibers [22]. AM is composed of higher proportion of type I muscle fibers than QF [23] hence potential PPAR-α agonist e.g. GA may selectively induce PPAR-α expression in the AM instead of QF which further leads to a higher LPL expression level in group A rats.

Although both adipose tissues and skeletal muscles act as the main site of hydrolysis of TG-rich lipoproteins, several evidences suggested that cardiac muscle is an important site of regulation of plasma TG levels [24]. Cardiac muscle is the tissue that exhibits the highest LPL expression level/gram of tissue [24]. It was also found that the loss of LPL in the heart leads to defective metabolism of plasma TG. This showed the essential role played by the heart in the regulation of lipid homeostasis. A lower LPL expression level was observed in
group B rats compared to group A. This may indicate that GA supplementation for 4 weeks had aided in the increased LPL expression in the heart similar to the other tissues e.g. kidney. Since PPARα is expressed abundantly in the heart, the elevated LPL expression level may be related to GA-mediated activation of PPARα which further induces LPL expression.

Fig. 3. Fold difference of LPL expression in different tissues with BAC as the reference, group B as target and group A as the calibrator

A= Rats fed on a ND supplemented with GA for two weeks followed by a HFD supplemented with GA for another two weeks, B= Rats fed on a ND supplemented with GA for two weeks before switching to a HFD and withdrawal of GA for another two weeks. Results expressed as relative LPL expression (Fold difference), value>1.0 indicates up-regulation and <1.0 indicates down-regulation. Values differ significantly among each other according to Relative Expression Software Tool (REST ©) at P =.05

3.3 Quantitative and Morphometric Analysis of H&E-stained Subcutaneous and Visceral Adipose Tissues and H&E- and PAS-stained Liver

3.3.1 Mean adipocytes size (area) in subcutaneous and visceral adipose tissues

The mean cross-sectional surface area of adipocytes (100 cells/field/slide) of SAT from groups A and B was 1685.43±757.91 and 1633.22 ±1390.67, respectively. No significant difference was observed between groups (Fig. 4), while the mean cross-sectional surface area of adipocytes of VAT of group A (2582.75±1339.88) rats was significantly smaller than group B (3008.69±777.45) (P=.05). In addition, it was found that the mean cross-sectional surface area of adipocytes of SAT was smaller than the VAT between groups (P=.05) (Fig. 4).

The adipocytes of VAT from group A rats were significantly smaller than that of group B rats. Besides, the VAT of group A rats also displayed prominent irregular-shaped adipocytes (Fig. 5 (II)) accompanied by dissolution and folding of cell membrane which indicated lipolysis [25]. This resulted in the release of the content of adipocytes including FFA that leads to the cytoplasmic shrinkage of the adipocytes. This showed that continuous GA supplementation for four weeks was effective in reducing the size of adipocytes and promotes lipolysis which was in accordance to the higher LPL expression in the VAT of group A rats (Fig. 3). The shrinkage of adipocytes may also indicate apoptosis of the adipocytes to prevent over-spill
and ectopic lipid deposition in the peripheral organs e.g. liver [26]. Due to the strategic anatomical position of VAT and greater anti-lipolytic effects of VAT towards insulin, the increased visceral depots, as observed in visceral obesity, is related to increased FFA into the portal circulation that impairs hepatic metabolism and resulted in systemic IR. The reduced visceral depots as observed in group A rats with continuous GA supplementation is expected to exert beneficial effects as GA may aid in the reduction of visceral fat depots and prevents the development of IR.

It has been reported that the size of the adipocytes in SAT is 50% bigger than VAT [27]. However, our results showed that the adipocytes of SAT from all groups of rats were smaller than VAT which may be related to re-distribution and remodeling of adipocytes. Smaller adipocytes are more insulin sensitive due to larger surface area and are able to take up more glucose [28]. Besides, smaller adipocytes have higher rate of glucose oxidation and this may aid in reducing blood glucose level.

![Fig. 4. Mean cross-sectional surface area of adipocytes from subcutaneous and visceral adipose tissues between rats from groups A and B after 28 days of treatment](image)

A= Rats fed on a ND supplemented with GA for two weeks followed by a HFD supplemented with GA for another two weeks, B= Rats fed on a ND supplemented with GA for two weeks before switching to a HFD and withdrawal of GA for another two weeks. Results expressed as Mean ± SD for eight rats in each group. Values differ significantly among each other according to Independent-T Test at P = .05
Fig. 5. Micrographs of H&E-stained subcutaneous (I) and visceral (II) adipose tissues from rats in groups A and B at 400 x magnification. Red arrow heads showed irregular-shaped adipocytes. C: cytoplasm; N: nuclei and P: tangential slicing of the top or bottom of a cell.

A= Rats fed on a ND supplemented with GA for two weeks followed by a HFD supplemented with GA for another two weeks, B= Rats fed on a ND supplemented with GA for two weeks before switching to a HFD and withdrawal of GA for another two weeks.

3.3.2 Morphometric analysis of H&E- and PAS-stained liver

H&E stain revealed the presence of lipid droplets in all groups with large unstained areas within the cytoplasm that resemble lipid droplets in the liver tissues (Fig. 6 (I)). Periodic acid Schiff’s staining is to identify the presence of polysaccharide molecules. Polysaccharide molecules are stained magenta while the nuclei are stained blue. No significant difference was seen for PAS-stained liver tissues between groups A and B (Fig. 6 (II)).

The liver is an important organ involved in metabolism, detoxification and storage of vitamins and minerals [29]. As the major target site of insulin, it plays an essential role especially in carbohydrate metabolism that determines glucose homeostasis. The liver stores glucose as glycogen when stimulated by insulin and release glucose from the glycogen store when glucose level decreases. The presence of high fatty acid content interferes with the insulin-signaling pathway and prevents insulin-stimulated glucose uptake by the liver and causes hyperglycaemia. Lipid deposition was found in all groups of rats with the higher amount of lipid droplets found in group A rats. Fat accumulation within the hepatocytes takes place when there is an imbalance between the rate of lipid uptake (e.g. synthesis) and disposal (e.g. lipoprotein secretion and fatty acids oxidation). Increased TG accumulation in the hepatocytes is the hallmark of fatty liver disease [30]. The hyperinsulinaemic condition as observed in group A rats may imply insulin-resistant state and this promotes lipolysis in the peripheral tissues (due to the oppose actions of glucocorticoids against insulin). Together with the increased LPL expression in the liver, these promote increased influx and breakdown of TG and FFA uptake into the liver and resulted in the increased lipid deposition in the hepatocytes.
Fig. 6. Micrographs of H&E-stained (I) and PAS-stained (II) liver tissues from rats in groups A and B at 400 x magnification. H: Haematocytes; L: lipid droplet and N: nuclei.

A= Rats fed on a ND supplemented with GA for two weeks followed by a HFD supplemented with GA for another two weeks, B= Rats fed on a ND supplemented with GA for two weeks before switching to a HFD and withdrawal of GA for another two weeks.

4. CONCLUSION

GA regulates lipid metabolism via LPL expression in the liver, skeletal muscles and adipose tissues. A switch towards high-fat diet from normal diet while maintaining continuous GA supplementation for four weeks (group A) leads to elevated blood glucose, serum insulin and TG and HOMA-IR index due to the synergistic effects of GA and fatty acids in inducing LPL expression via PPAR-activation. GA, via modulation of LPL expression, may act as a potential blood glucose and triacylglycerol lowering agent in subjects who have been on a normal diet with GA and switch to a high-fat diet without GA (group B) but not in subjects who had a switch from normal diet to high-fat diet with continuous GA consumption (group A).

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.
COMPETING INTERESTS

Authors have declared that no competing interests exist.

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