


ORIGINAL ARTICLE

Linoleic acid in diets of mice increases total endocannabinoid levels in bowel and liver: modification by dietary glucose

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Summary

Aim

Linoleic acid (LA) is an essential fatty acid involved in the biosynthesis of arachidonic acid and prostaglandins. LA is known to induce obesity and insulin resistance. In this study, two concentrations of LA with or without added glucose (G) were fed to mice to investigate their effects on endocannabinoid (EC) biology.

Materials and Methods

Four groups of C57BL/6 mice were provided with diets containing 1% or 8% LA with or without added G (LAG) for 8 weeks. Body weights, food intake, circulating glucose and insulin levels were measured throughout the study. Following euthanasia, plasma, bowel and hepatic ECs, monoacylglycerol lipase and fatty acid amide hydroxylase protein levels (enzymes responsible for EC degradation) and transcriptional activity of PPAR α in liver were quantified. Liver was probed for evidence of insulin receptor activity perturbation.

Results

Increasing dietary LA from 1% to 8% significantly increased circulating, small bowel and hepatic ECs. 1%LAG fed mice had lowest feed efficiency, and only liver levels of both ECs were reduced by addition of G. Addition of G to 1% LA diets resulted in elevated monoacylglycerol lipase and fatty acid amide hydroxylase protein levels ($p < 0.001$ and $p < 0.001$, respectively) in liver due to increased transcriptional activity of PPAR α ($p < 0.05$). The reduced EC levels with addition of G also correlated with a measure of enhanced insulin action.

Conclusion

In conclusion, body weight of mice is influenced by the source of calorie intake. Furthermore, tissue EC/g are dependent on tissue-specific synthesis and degradation that are modulated by dietary LA and G which also influence food efficiency, and down-stream insulin signalling pathways. The findings could potentially be useful information for weight management efforts in humans.

Keywords: Endocannabinoids, high fat, high glucose, linoleic acid.

Introduction

Obesity results when more calories are ingested than are expended, with both genetic (1) and environmental factors (2) playing a role. Not as well appreciated may be that changing dietary habits to align with dietary

guidelines on composition of foods, especially types of fat, has exacerbated the environmental aspects of obesity. Current guidelines recommend replacing saturated fats with polyunsaturated fats, the rationale being to reduce the incidence of cardiovascular disease that would result from lowering low-density lipoprotein levels

in circulation and consequently consumption of vegetable oils skyrocketed (3,4). The American Heart Association guidelines, e.g. recommend consumption of 5–10% energy intake from linoleic acid (LA) (5). LA is the predominant 18 carbon n-6 polyunsaturated fatty acid in the Western diet and is primarily obtained from vegetable oils. Although some intake of LA is essential to prevent its deficiency (6), current meta analyses from three research group provide conflicting data regarding levels of actual dietary intake of LA for health (3,7–9).

Worldwide there has been an increase in the consumption of LA (4,10,11). Consumption of soybean oil (a chief source of LA), for instance, increased ~1,000-fold per capita over the past century represented 7.38% of energy in American diets (4), and investigators reported on a highly significant correlation between the prevalence of soybean oil consumption, LA amounts and obesity in the United States (12,13). In a recent study, it was found that increasing LA amounts in diets from 1% to 8% of the total energy intake in order to reflect human LA consumption over the past century furthered obesity in mice (14). Consumption of LA, a chief constituent of vegetable oils such as soybean, safflower and canola oils, has increased due to commercial availability and dietary recommendation. Other sources of LA are nuts, eggs and meats. Apart from nutritional transition (shift towards plant based fat vs. animal fat) that has resulted in increased human consumption of LA (15), changes in animal feed practices have also resulted in increased animal LA consumption (16). Moreover, LA is rarely consumed alone, and carbohydrate intake, especially from sugars, has also increased concurrently. There has been an increase in consumption of refined sugar from 55.5 to 69.1 kg per capita between 1970 and 2000 in the United States (17), e.g. Thus, along with the consumption of LA in the form of fried food, there is an increase in the consumption of sweetened beverages which contain sugar (18), as often soda and fried foods are consumed together as a meal (6,19). Additionally, availability of vending facilities has facilitated consumption of sweetened beverage (20). Thus, it is imperative to understand the effect of combined consumption of sugar and LA to investigate for any concomitant interactions.

LA consumption may have a second unintended consequence besides being an inexpensive source of unnecessary calories: it is converted to arachidonic acid from γ -LA and eicosatetraenoic acid (21). Arachidonic acid in membrane phospholipids (ARA-PL) is the precursor to 2-arachidonoylglycerol (2-AG) (22) and arachidonylethanolamine (AEA, also known as anandamide) (22), the two best characterized endocannabinoids (ECs), and increasing dietary LA concentrations leads to increasing amounts of hepatic ARA-PL and ECs in

rodents (14). The EC system consists of the EC synthesizing enzymes, ECs themselves (23,24), cannabinoid receptors (CB1 and 2) (25,26) and the enzymes responsible for EC degradation, namely, monoacylglycerol lipase (MAGL) (27) and fatty acid amide hydroxylase (FAAH) (28). Activation of cannabinoid 1 receptor (CB1R/CB1bR) centrally and peripherally favours increased food intake, insulin resistance, fatty acid and triglyceride synthesis and fatty liver (29–31). Although blockade of CB1R by rimonabant was effective as an appetite suppressant in rodents and humans (32,33), it led to serious side effects such as irritability, anxiety and suicidal ideation which resulted in its withdrawal from marketplace (34). To better combat the problem of obesity, understanding the underlying mechanism that produce EC hyperactivity is thus important.

The effects of two concentrations of dietary LA, alone and in combination with glucose (G), on EC concentrations in bowel and liver, and the metabolic consequences of the diets in mice are reported here. It is hypothesized that the alterations in EC levels, metabolic parameters and glucose signalling pathways are to be differentially perturbed in LA and LAG fed mice.

Methods

Animals handling and tissue collection

Male C57BL/6 mice were obtained at 6 weeks of age from the Jackson Laboratory (Bar Harbour, ME). After 1 week of acclimatization, they were randomly assigned to four food groups, $n = 8$ per group: 1%LA, 8%LA, 1%LAG and 8%LAG (LA = linoleic acid, G = 22% glucose). The food was provided as pellets (Dyets Inc., Bethlehem, PA: see Table 1 for components) and was designed to be isocaloric per gram of the diet. Briefly, diets contained 49% kcal/kg from fat, 36% kcal/kg from carbohydrate, and 15% kcal/kg from protein. In diets containing added G, 22% energy (en) is derived from G in the form of dextrose. Cornstarch was used as a neutral source of carbohydrate in 1% and 8% LA diets. The 8%LA is within the concentration recommended by American Heart Association. The sources of LA in the diets were soybean and safflower oils. The remaining fat calories were saturated fats derived from hydrogenated coconut oil, lard and whole butter. The mice were maintained on a 12-h day/night light cycle and had *ad libitum* access to food and water. Body weight and food intake were measured weekly for 8 weeks. Fasting blood was collected a day before sacrifice for assay of glucose, insulin and leptin levels in circulation. After 8 weeks of study, mice were sacrificed, blood was obtained and serum was immediately separated by centrifugation at 4 °C, followed by freezing at –80 °C for

Table 1 Composition of mouse diets used in the study

	1%LA	8%LA	1%LAG	8%LAG
High nitrogen casein (g/kg)	200	200	200	200
L cysteine	3	3	3	3
Sucrose	90	90	90	90
Dyetrose	70.02	90	50.59	50
Cornstarch	270.466	270.466	0	0
Dextrose	0	0	290	310.556
Safflower oil	-	48.9	-	48.9
Soybean oil	9.7	-	9.7	-
Hydrogenated coconut oil	250.3	21.1	250.3	21.1
t Butylhydroquinone	0.01	0.01	0.01	0.01
Whole butter	-	181	-	181
Ethoxyquin	-	0.024	-	0.024
Lard	-	20	-	20
Cellulose	50	50	50	50
Mineral mix	35	35	35	35
Vitamin mix	10	10	10	10
Supplement	10	10	10	10
Choline chloride	1.4	1.4	0	1.4
Fatty acid profile				
Myristic acid	48.7	23.7	48.7	23.7
Palmitic acid	24.3	61.1	24.3	61.1
Palmitoleic acid	0	5.2	0	5.2
Stearic acid	32.2	30.2	32.2	30.2
Oleic acid	2.2	63.3	2.2	63.3
Capric acid	0	4.8	0	4.8
Decanoic acid	13.5	1.1	13.5	1.1
Lauric acid	117.9	15.4	117.9	15.4
Myristoleic acid	0	2.9	0	2.9
Butyric acid	0	6.2	0	6.2
Caproic acid	0	3.6	0	3.6
Caprylic acid	0	2.2	0	2.2
Octanoic acid	0	1.3	0	1.3
Arachidic a	0	2	0	2
Linoleic acid	5.3	44.8	5.3	44.8
Linolenic acid	0.7	3.0	0.7	3.0

later analyses. Rinsed small bowel and liver were collected and frozen in liquid nitrogen and subsequently at -80°C for processing.

Tissue levels of endocannabinoids

Endocannabinoids (ECs) extracted from serum, small bowel and liver were based on a previously described protocol (35,36). Separation of the AEA and 2-AG was accomplished using a Waters XBridge C18 guard column (Milford, USA) and Zorbax Eclipse XDB-C18 column (4.6 mm \times 50 mm, 1.8 μm Agilent, Santa Clara, CA, USA) at 20°C using Shimadzu prominence system (Shimadzu, Japan) consisting LC-20AB binary pump, SIL-20AC-HT autosampler and DGU-A3 degasser. Mobile phase A consisted of water with 0.1% formic acid

and mobile phase B was acetonitrile containing 0.1% formic acid. Linear gradient was run as follows: 0 min 60% B; 1 min 60% B; 7 min 90% B; 10 min 90% B; 10.1 min 90% B; 14.9 min 60% B at a flow rate of 0.8 mL min^{-1} with a total run time of 15 min. MS/MS was performed in API 5500 QTRAP (Applied Biosystems, Foster city, USA) equipped with electrospray ionization. Calibration curve was prepared in methanol for tissues with a serial dilution of AEA ($0.07\text{--}150\text{ ng mL}^{-1}$) and 2-AG, ($100\text{--}0.048\text{ }\mu\text{g mL}^{-1}$) and spiked in plasma from $0.158\text{--}16\text{ ng mL}^{-1}$ for both AEA and 2-AG. Internal standards were added prior to tissue and serum extraction as well as for calibration. AEA d^4 and 2-AG d^5 were used as internal standards for AEA and 2-AG.

Semiquantitative values for the metabolites were determined using area ratios of the targeted metabolites and the corresponding internal standard. The data were acquired and analysed using Analyst version 1.5.1 (Applied Biosystems). Positive electrospray ionization data were acquired using multiple reactions monitoring. The TIS instrumental source settings for temperature, curtain gas, ion source gas 1 (nebulizer), ion source gas 2 (turbo ion spray) and ion spray voltage were 550°C , 30 psi, 60 psi, 60 psi and 5,500 V, respectively. The standards were characterized using the following multiple reactions monitoring ion transitions and TIS compound parameters (declustering potential, collision energy and cell exit potential): AEA: 348.2/62.1 (80, 18 and 10 V); AEA d^4 : 353.1/66.1 (80, 18 and 10 V); 2-AG: 379.4/287.2 (80, 20 and 10 V); 2-AG d^5 : 384.4/287.4 (80, 20 and 10 V).

Glucose and hormone determination

Fasting blood glucose was assayed using an Easy Gluco glucometer (U.S. Diagnostics, New York, USA), fasting plasma insulin was assayed using a mouse-specific insulin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem, Elk Grove Village, IL) and fasting plasma leptin was also assayed using a mouse-specific leptin ELISA kit (Crystal Chem). Fasting blood glucose and insulin was measured as opposed to random blood test in order to have uniform readings not affected by the time of food ingestion.

Triglyceride analysis

Neutral lipids were extracted from homogenized liver tissues in 50-mM NaCl using chloroform/methanol (2:1 v/v). After centrifugation, the interphase was washed with homogenizing buffer (50-mM NaCl) and mixed with methanol. Subsequently, the organic phase was introduced with 10% Triton X-100 (Sigma, St. Louis, MO)

and dried under fume hood overnight. Triglyceride content was analysed on dried pellet directly using commercial colorimetric reagents (Wako, Mountain View, CA).

Haematoxylin and eosin stain

The haematoxylin and eosin stain (H&E) was performed on the sectioned frozen optimal cutting temperature compound (OCT) liver tissue as previously described (37). The image analysis was performed in Olympus 1X51.

Western blotting

The remaining liver portions and bowel were flash frozen and then stored at -80°C . Approximately 40 mg of frozen tissue (bowel and liver) was homogenized in RIPA buffer (Boston Bioproducts, Ashland, MA) containing phosphatase and protease inhibitors (Sigma, St. Lois, MO) using an OMNI Bead Ruptor 24 (Kennesaw, GA). Protein quantification was performed using a Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Equal amounts of protein were separated on 4–12% SDS-polyacrylamide gels (Thermo Fisher Scientific) and transferred to polyvinylidene difluoride membranes using an iBlot (Thermo Fisher Scientific). The membranes were blocked with 5% milk in Tris Buffer saline Tween 20, incubated overnight with primary antibodies, rabbit anti-MAGL (1:200), mouse anti-FAAH (1:200) and rabbit anti- β -actin (1:1,000) from Abcam (Cambridge, MA), and rabbit anti-phospho-insulin receptor substrate 1 (IRS1) (1:500), rabbit anti-IRS1 (1:500) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (1:3,000) (Cell Signalling, Danvers, MA). Blots were then incubated with secondary antibodies (1:2,500; Cell Signalling) for 1 h at room temperature and then developed using a chemiluminescent ECL substrate (GE healthcare, Chicago, IL). Densitometry of bands was analysed using Image J software (NIH). The relative amount of each protein was normalized to β -actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunoprecipitation

Total liver extracts (500 μg) were immunoprecipitated with mouse anti-IRS2 antibody (Cell Signalling) using Protein G Dynabeads (Thermo Fisher Scientific), according to the manufacturer's protocol. Western blots for IRS2 were performed as described earlier using primary rabbit anti-IRS2 (1:500, Cell Signalling) antibody. A separate gel was run with equal amounts of total liver protein extract and stained using coomassie blue (Sigma) to demonstrate protein levels in each lane.

Transcriptional activity of PPAR α

The transcriptional activity of PPAR α in liver was assayed using the commercially available ELISA kit (Abcam ab133107). Briefly, nuclear extracts were prepared from livers (Abcam) and subsequently subjected to this assay as per manufacturer instruction.

Statistical analysis

All data were analysed by ANOVA and Fisher's least significant difference *post hoc* test using Graph Pad Prism. Data are presented as mean \pm standard error of mean. When comparing 1% to 8% within the same group (LA or LAG), * designates statistical significance. When comparing LA to LAG (1% or 8%), # designates statistical significance. \ddagger , # $p \leq 0.05$; **, ## $p \leq 0.01$, ***, ### $p \leq 0.001$, ****, #### $p < 0.0001$.

Results

Increasing linoleic acid (LA) and adding glucose (G) resulted in increased body weight in mice

At study initiation, 7 week-old mice weighed 20.0 ± 0.2 g. They were randomized into four different diet groups: 1%LA, 8%LA, 1%LAG or 8%LAG. Mice from all food groups gained weight throughout the study duration (Figure 1A). After 8 weeks of study duration, mice given 8%LA were significantly heavier when compared to 1%LA-fed mice ($p \leq 0.05$; Figure 1B), and addition of G to either 1%LA or 8%LA caused both groups to gain even more weight than corresponding LA-only fed mice ($p \leq 0.05$ and $p \leq 0.01$, respectively; Figure 1B). Average daily food intake (weekly food intake/7) was quantified as well as total calorie intake for the 8-week duration (Figure 1C,D). Average daily food intake of 8%LA-fed mice declined by week 5, it remained constant in the other three groups. 1%LAG and 8%LAG-fed mice consumed similar numbers of calories, but yet 8%LAG fed mice were heavier (Figure 1B). Interestingly, 1%LAG-fed mice consumed significantly more total calories than 8%LA-fed mice ($p \leq 0.01$) yet had similar body weights (Figure 1A,B). Addition of G in both 1%LA and 8%LA diets lead to greater calorie consumption than LA-only diets ($p \leq 0.0001$ and $p \leq 0.05$, respectively; Figure 1D). In sum, the feed efficiency (body weight gain over 8 weeks/total calorie intake over 8 weeks) was significantly less in 1%LAG-fed mice, i.e. gained less weight for total calorie consumed, compared to the other three groups ($p \leq 0.0001$; Figure 1E). Circulating fasting leptin levels mirrored the total calorie intake in that leptin levels was highest in 1%LAG and 8%LAG fed mice (Figure 1F).

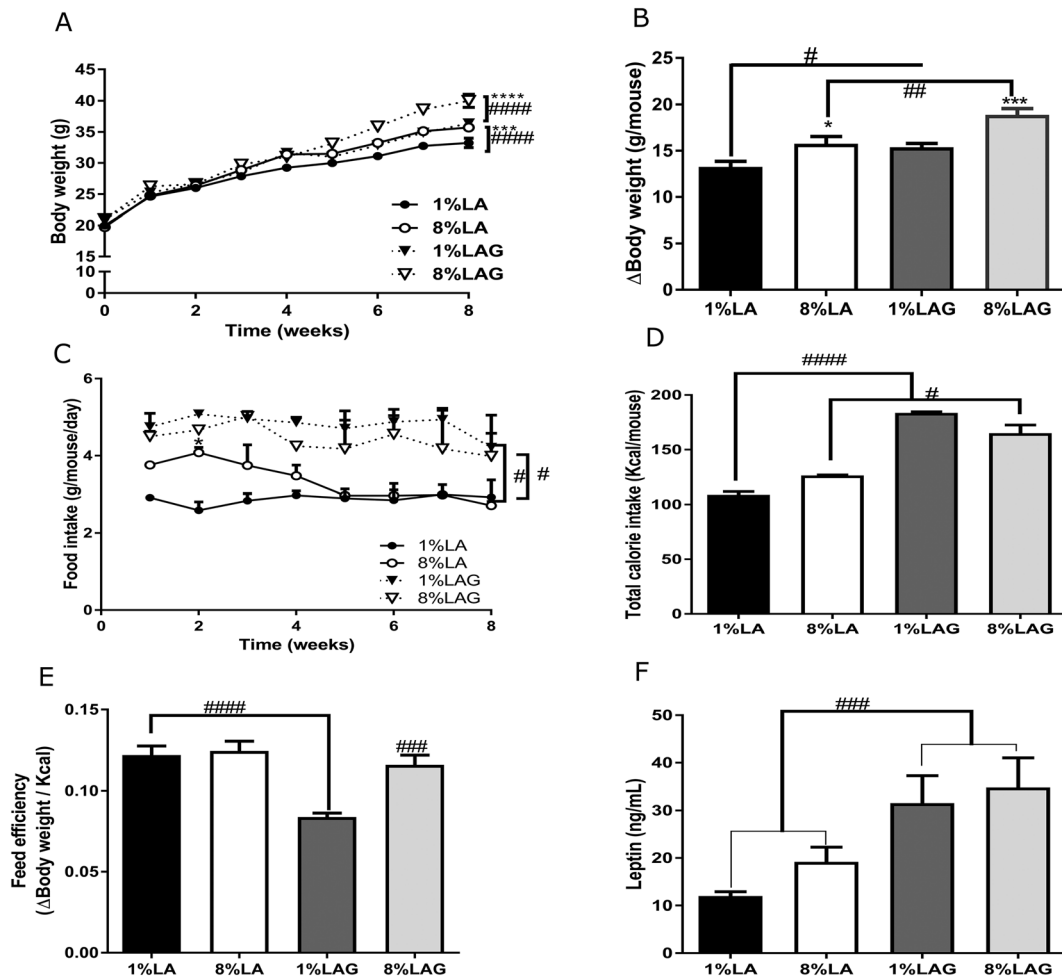


Figure 1 Increasing linoleic acid (LA) and adding glucose (G) resulted in increased body weight and food intake in mice over 8 weeks of study. (A) Weight profile of the mice over 8 weeks of diet – (1%LA, 8%LA, 1%LAG and 8%LAG). (B) Change in body weight (final body weight – initial body weight). (C) Average daily food intake (weekly food intake/7) (in grams) for all study groups. (D) Total calorie intake for each study group. (E) Feed efficiency (body weight gain over 8 weeks/total calorie intake over 8 weeks). (F) Plasma leptin levels after 8 weeks for all four diet groups. Figures are represented as mean \pm standard error of mean. When comparing 1% to 8% within the same group (LA or LAG), * designates statistical significance. When comparing LA to LAG (1% or 8%), # designates statistical significance. # $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$; #### $p \leq 0.0001$.

Tissue endocannabinoid levels are modulated by LA and G in small bowel, liver and circulation

Endocannabinoids (ECs) in circulation were increased by increasing LA levels from 1% to 8% in both diets ($p \leq 0.01$ and $p < 0.05$, respectively, for AEA; $p \leq 0.01$ for 2-AG; Figure 2A,B), but levels were not altered by addition of G. The levels of AEA and 2-AG in bowel were significantly elevated in mice fed 8%LA compared to mice fed 1%LA ($p \leq 0.01$, $p \leq 0.05$; Figure 2C,D): however, addition of G to either 1%LA or 8%LA diets resulted in less accumulation of AEA/g of tissue ($p \leq 0.05$), while 2-AG/g accumulation did not appear to be affected by G. Similar to bowel, AEA and 2-AG levels in liver were significantly elevated by 8%LA compared to 1%LA diet

($p \leq 0.05$), while unlike the case in bowel, G addition to both 1%LA and 8%LA caused reduction in AEA ($p < 0.05$, $p < 0.06$; Figure 2E,F) and 2-AG ($p < 0.05$; Figure 2E,F). Also of note is that concentrations of both ECs/g of tissue were many fold higher in bowel compared to liver.

Oral glucose modulates endocannabinoids in liver by regulating endocannabinoid-degrading enzymes

Monoacylglycerol lipase (MAGL) and FAAH are enzymes mainly responsible for degradation of 2-AG and AEA, respectively, and were further investigated if G might be regulating their expression to account for reduced levels of ECs when added to either 1% or 8% LA (Figure 3). It

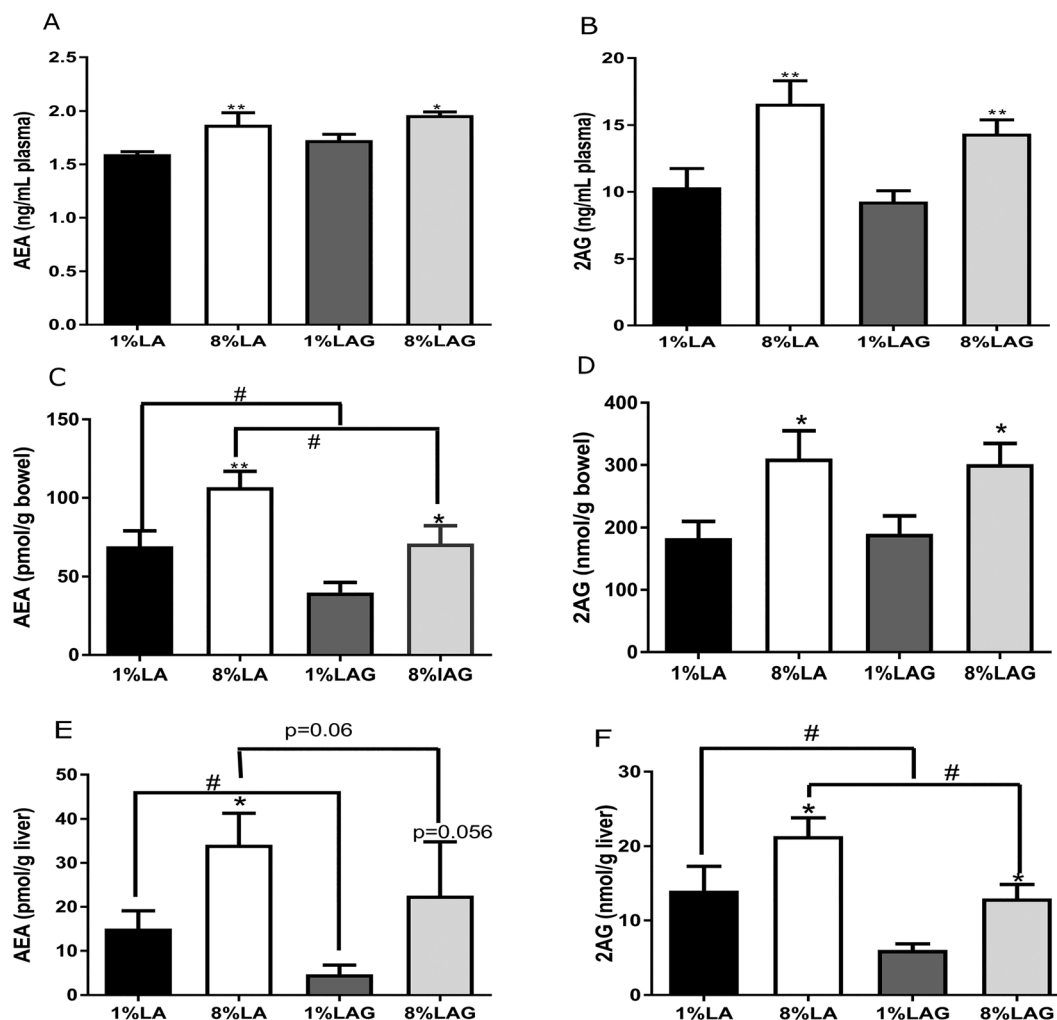


Figure 2 Anandamide (AEA) and 2-arachidonyl glycerol (2-AG) levels in serum, bowel and liver are modulated by high LA and oral G in mice after 8 weeks of diet. Anandamide (AEA) levels were measured in plasma (A), bowel (C) and liver (E) by LC-MS/MS for all four diet groups. 2-Arachidonyl glycerol (2-AG) levels were measured in plasma (B), bowel (D) and liver (F) also by LC-MS/MS for all four diet groups. Figures are represented as mean \pm standard error of mean. When comparing 1% to 8% within the same group (LA or LAG), * designates statistical significance. When comparing LA to LAG (1% or 8%), # designates statistical significance. * $p \leq 0.05$; ** $p \leq 0.01$.

has been found that protein levels of both enzymes (MAGL and FAAH) increased significantly in liver on addition to G to 1%LA ($p \leq 0.001$ and $p \leq 0.0001$, respectively; Figure 3A–D). Similar trend follows on addition of G to 8%LA ($p < 0.06$ and $p < 0.01$, respectively; Figure 3A–D). Degrading enzymes in the bowel extracts by western blot analysis were not detected with the same methodology used for liver tissue: it is already reported that these enzymes are present in very low amounts in bowel (38). The degrading enzymes are transcriptionally controlled, at least in part, by PPAR α activity (39,40), and G and its metabolites, G-6-phosphate and G-1-phosphate, are endogenous ligands of PPAR α , with G itself having the highest affinity (41,42). Nuclear extracts of livers was assayed from mice using an ELISA-based

transcriptional assay for PPAR α activation. G added to both 1%LA and 8%LA diets increased the transcriptional activities of PPAR α ($p \leq 0.05$, $p < 0.01$; Figure 3E), while changing the LA amounts alone did not impact the activity.

Combined dietary LA and G causes insulin resistance in liver and influences fasting insulin levels

Fasting blood glucose and fasting plasma insulin levels were measured in the mice. Fasting blood glucose was 26% lower in the 1%LA-fed mice ($p \leq 0.05$), compared to the other three groups (Figure 4A), while fasting plasma insulin was significantly elevated only in the mice given 1%LAG ($p \leq 0.05$; Figure 4B). In our study, it was found

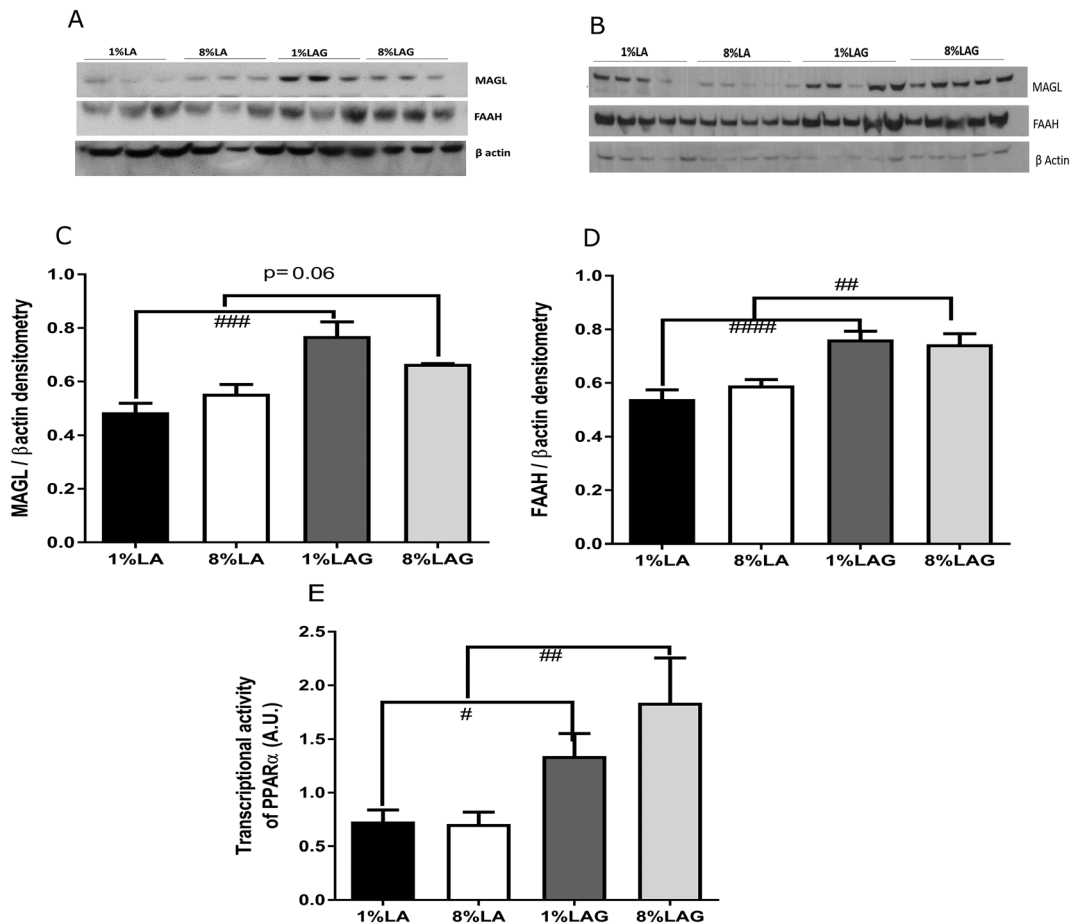


Figure 3 Monoacylglycerol lipase (MAGL), fatty acid amide hydrolase (FAAH) levels and transcriptional activity of nuclear PPAR α are altered in the liver of mice fed 1% and 8% LAG diet. (A) and (B) Western blot analysis of MAGL and FAAH in mouse livers after 8 weeks of diet. β -actin was used as a loading control. Densitometric analysis of protein bands of MAGL (C) and FAAH (D) using Image J software. (E) Transcriptional activity of PPAR α in nuclear extracts from mouse livers after 8 weeks on diet. Figures are represented as mean \pm standard error of mean. When comparing LA to LAG (1% or 8%), # designates statistical significance. # $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$; #### $p \leq 0.0001$.

that the mice in which G was added to both LA diets had significant suppression of liver IRS2 protein levels, compared to mice consuming 1%LA and 8%LA diets ($p \leq 0.001$, $p < 0.01$; Figure 4C,D), while IRS2 levels were similar with both 1%LA and 8%LA diets; total protein inputs for the western blot were similar in all four groups (Figure 4E). Furthermore, protein levels of total IRS1 in the liver of animals supplemented with G were significantly decreased compared to both 1%LA and 8%LA diets ($p \leq 0.05$; Figure 4F,G). However, pIRS1 (Ser307) were increased in animals that had G added to their diets ($p \leq 0.01$; Figure 4H).

Liver weight normalized to total body weight increased when G was added to LA diets ($p < 0.001$; Figure 5A). H&E staining of the liver denotes fat deposition in the liver (Figure 5B). Liver triglycerides/g liver was elevated in 8%LAG fed mice compared to 8%LA fed mice ($p < 0.05$; Figure 5C).

Discussion

This is the first report of a study in mice evaluating the effects on bowel EC levels of increasing dietary LA and whether glucose (G) addition to LA impacts tissue EC levels. Increasing LA content from 1% to 8% did not increase total calorie intake in mice. This observation is similar to previous published results (14). Inclusion of G to both 1%LA and 8% LA diets increased hyperphagia, as noted earlier (43).

Our study suggests that both the nature and source of calories, which are modulating EC levels, are instrumental to weight gain in mice and not just the total calorie intake. This is corroborated by the fact that feed efficiency was similar in 1%LA, 8% LA and 8%LAG fed mice and significantly less in 1%LAG fed mice, which also had the lowest intrahepatic ECs. This is also reflected by the fact that 1%LAG fed mice ate similar numbers of calories as

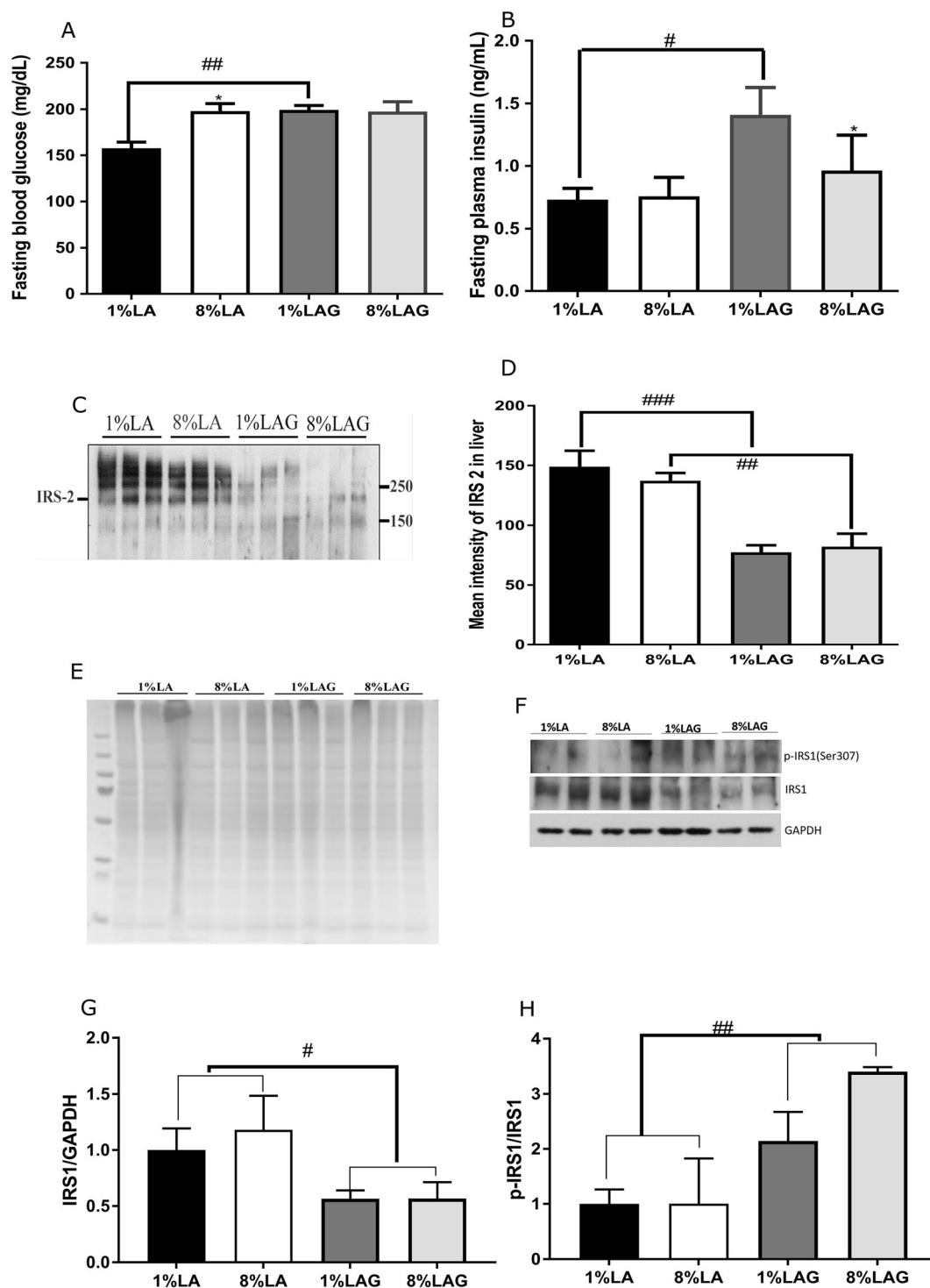


Figure 4 1% and 8% LAG diet elevated fasting plasma insulin levels, caused hepatic insulin resistance and altered down-stream insulin substrates in liver. (A) Fasting blood glucose levels measured after 18 h of fasting in mice. (B) Fasting plasma insulin levels measured by enzyme-linked immunosorbent assay. (C) Western blot of immunoprecipitated IRS2 in mouse liver. (D) Densitometric analysis of protein bands of IRS2. (E) Coomassie stained total protein levels are shown for equal loading. (F) Western blot of IRS1 and p-IRS1 (ser 307) in mouse livers after 8 weeks of diet. Glycereraldehyde 3-phosphate dehydrogenase was used as a loading. (G) Densitometric analysis of IRS1. (H) Densitometric analysis of pIRS1 (ser207)/IRS1. Figures are represented as mean \pm standard error of mean. When comparing 1% to 8% within the same group (LA or LAG), * designates statistical significance. When comparing LA to LAG (1% or 8%), # designates statistical significance. *, # $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$.

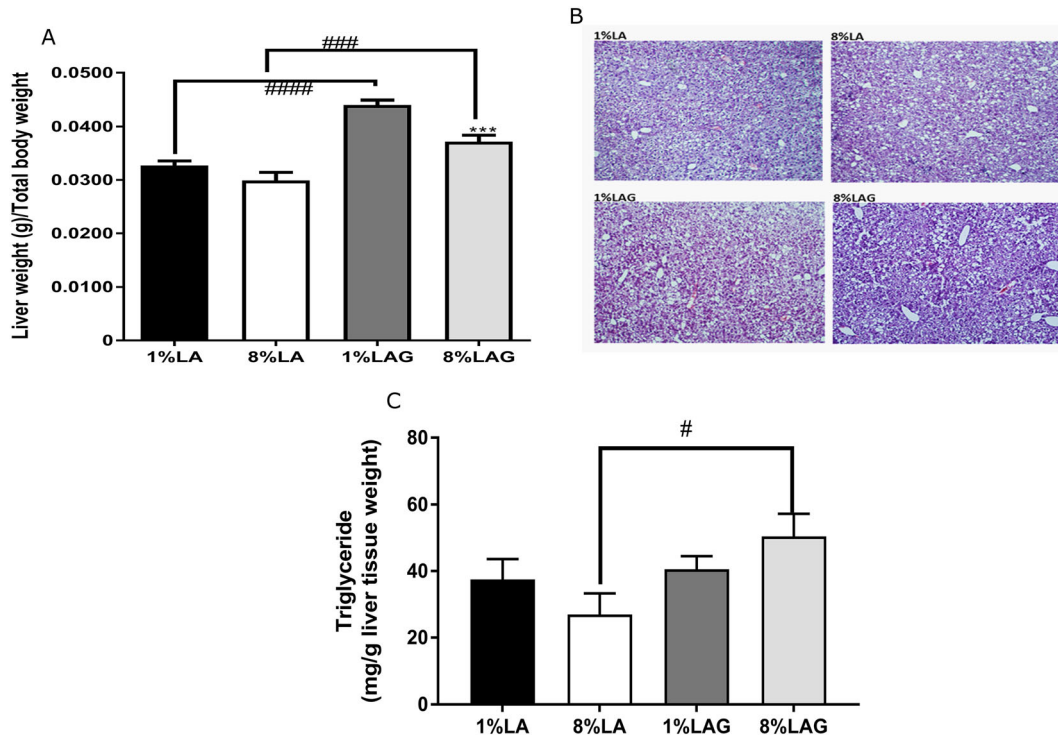


Figure 5 Oral G increases triglycerides deposition in liver when added to 8%LA diets. G increase liver weight in mice. (A) Total liver weight of mice normalized to total body weight at the termination of the study for all four groups of diet. (B) Haematoxylin and eosin (H&E) stained sections. Frozen liver tissues stained with H&E from the mice in all diet groups. (C) Triglycerides (mg)/liver weight(g) measured in the liver at the termination of the study for all four groups of diet. Figures are represented as mean \pm standard error of mean. When comparing 1% to 8% within the same group (LA or LAG), * designates statistical significance. When comparing LA to LAG (1% or 8%), # designates statistical significance. # $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

8%LAG fed mice yet gained less weight. Altered intrahepatic ECs in 1%LAG fed mice likely implicated this phenotype.

Increasing the content of LA in diets led to increased EC tissue concentrations in bowel and liver, as well as in circulation. Previous studies reported an increase in hepatic ECs with high LA intake in mice (12,14). However, it was noticed that hepatic AEA/g and 2-AG/g were actually reduced upon addition of G to both 1%LA. A similar trend in AEA/g was observed when G was added to 8%LA, while a decrease in 2-AG/g occurred when G was added to 8%LA diets. This was likely due to increased amounts of degrading enzymes, MAGL and FAAH, secondary, at least in part, to increased glucose-induced PPAR α activity: PPAR α is known to regulate transcription of EC degrading enzymes (39,40). The slight variability in MAGL and AEA can be due to analytical sample handling/extraction procedure.

Hepatic AEA levels in mice fed 1%LA were similar to the values reported in literature for medium fat diet with 1%LA (35% energy derived from fat). Liver 2-AG levels were elevated compared to medium fat diets with 1%LA. This discrepancy could be because the diet used

in our study resulted in 49% of the energy content being from fat as opposed to the more usual 35% reported in the literature (14). Plasma EC levels are very similar to the values reported with consumption of western diets (44). EC levels in duodenum have not previously been reported in LA diets for comparison.

Glucose and its metabolites, glucose-1-phosphate and glucose-6-phosphate, are endogenous ligands of PPAR α with glucose itself having the highest affinity. Interaction with these ligands via ligand binding domain causes a conformational change within PPAR α resulting in the recruitment of coactivators and increase in transactivation potential (42). Previous studies reported an increase in FAAH mRNA in hypothalamic regions of rat brain with fructose and sugar ingestion (45,46). Increased circulating leptin levels in mice fed high G amount was also reported previously (47). The elevated leptin levels failed to lessen calorie intake in LAG mice which is in line with previous studies (47,48). Thus, leptin is a marker of body fat mass and not a suppressor of feeding (49).

The calculated concentrations of both AEA/g and 2-AG/g of tissue were higher in duodenum compared to liver in LA diets. To our knowledge, this finding has not

been previously reported. But while this difference is significant, and while the relative changes within a matrix are accurate, the differences between matrices (liver vs. bowel) may be confounded by the matrix itself; therefore, further studies are needed to confirm this finding. This finding is interesting nevertheless as EC levels in circulation are thought to be derived from adipose tissue (50) and not directly subject to dietary G regulation, as they appear to be in liver and bowel. Our data suggest that ECs released from bowel add to the mass of ECs in circulation after LA(G) consumption. Higher EC/g in bowel may be because protein levels of EC degrading enzymes would appear to be very low in bowel, as has been noted previously (38), and, unlike the case in liver, were too low for detection by western blotting. In previous studies, it has been demonstrated that hypothalamic FAAH is increased with fructose consumption (47). However, recently it was reported that FAAH activity is not itself under the influence of AEA in hepatocytes (51). Further investigations are required to understand the role of dietary LA and glucose towards FAAH activity.

Increasing LA from 1% to 8% caused greater weight gain and increased circulating glucose levels. However, fasting insulin levels did not increase in response to increasing fasting glucose in animals on diets that contained 8%LA. Likely that the chronic 8%LA diet suppressed β -cell responsiveness to circulating glucose, but when glucose was added to the 1%LA diet, β -cell glucose metabolism likely increased insulin transcription and translation that became permissive to increased insulin secretion but not in the presence of 8%LA. While it is not known if increasing dietary LA from 1% to 8% increased EC levels in islets, this is almost certainly the case and would have resulted in suppression of glucose-mediated insulin secretion, based on prior work (52,53).

Insulin receptor activity in liver is negatively modulated by the EC system, as had been previously shown by us and others (54,55), due to direct downregulation of receptor tyrosine phosphorylation. Insulin binding causes autophosphorylation of insulin receptors, and IRS proteins are next phosphorylated by the activated insulin receptor, which in turn lead to activation of several downstream signalling molecules necessary for glucose, fat and protein disposal, and the trophic properties of insulin (56). Ablation of IRS2 is associated with severe insulin resistance and hyperglycaemia, and hepatic IRS2 and IRS1 protein levels have been reported to decrease when liver insulin resistance occurs as a result of high fat feeding (HFF) (57). Our study allows separation of effects of HFF alone from HFF with added G.

Mice in which G was added to both LA diets had significant suppression of liver IRS2 protein levels, compared

to mice consuming 1% and 8% LA only diets; IRS2 levels were similar with both 1% and 8% LA diets – both diets contain 49% fat and are therefore HFF diets. Total IRS1, which is thought to be the primary IRS in action in liver in mice in the postprandial period, was also further suppressed by addition of G. It can therefore be concluded that hepatic ECs are not involved in carbohydrate suppression of IRS protein levels because both AEA and 2-AG liver levels were actually lower in animals given added dietary G with either 1% or 8% LA.

However, pIRS1 (ser307) levels, which are regulated by insulin, were increased by addition of G to the LA diets. It has been reported that this particular serine phosphorylated site on IRS1 is actually a regulatory site that positively modulates the severity of insulin resistance *in vivo*: initially based on *in vitro* culture conditions, this specific serine phosphorylated site was thought to worsen insulin action that was not borne out in *in vivo* conditions (58). The findings also imply that there is a factor in the *in vivo* situation that is absent in culture conditions and that pIRS1 (ser307) phosphorylation is actually an adaptive mechanism responding to insulin resistance. Our data would suggest that ECs are involved in this adaptive response because when EC levels were decreased by addition of dietary G, pIRS1 (ser307) levels increased due to improved insulin action. Our data suggest that both triglyceride level/g of liver and liver weight normalized to body weight did not change in 8%LA versus 1%LA fed mice. Our data corroborates previous studies, which demonstrated that while difference in LA consumption altered liver EC levels, mRNA levels of liver lipogenic genes did not follow a similar pattern to ECs (12) under high LA consumption conditions. In a previous study, even after 14 weeks of feeding, 8%LA diet failed to cause an increase in steroyl regulatory element binding protein (SREBP-1c), acetyl coA carboxylase (ACC1) or fatty acid synthase (FAS) mRNA in liver (12). This could therefore result in similar triglycerides level with 8%LA(G) versus 1%LA(G) diets, suggesting *de novo* lipogenesis not being perturbed with high LA intake. Increase in liver weight in LAG fed mice mirrors their total calorie intake. Interestingly, 8%LAG fed mice had higher triglyceride/g of liver compared to 8%LA fed mice, while this was not the case with 1%LAG versus 1%LA fed mice.

In summary, the metabolic parameters of body weight and degree of fat storage in liver are dependent on dietary content and the nature of calorie intake and also total calories consumed. Furthermore, it appears that EC levels are subject to tissue-specific synthesis and degradation that are influenced by dietary content, and this in turn influences the down-stream signalling pathways involved in insulin receptor signalling.

Conflict of Interest Statement

The authors declare no conflict of interest.

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References

- Walley AJ, Blakemore AIF, Froguel P. Genetics of obesity and the prediction of risk for health. *Hum. Mol. Genet.* 2006; **15**: R124–R130. <https://doi.org/10.1093/hmg/ddl215>.
- Brantley PJ, Myers VH, Roy HJ. Environmental and lifestyle influences on obesity. *J. La. State Med. Soc.* 2005; **157** Spec N(0024–6921 (Print)): S19–S27.
- Jandacek RJ. Linoleic acid: A nutritional quandary. *Healthcare.* 2017; **5**<https://doi.org/10.3390/healthcare5020025>.
- Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *Am. J. Clin. Nutr.* 2011; **93**: 950–962.
- Harris WS, Mozaffarian D, Rimm E, et al. Omega-6 fatty acids and risk for cardiovascular disease: A science advisory from the American Heart Association nutrition subcommittee of the council on nutrition, physical activity, and metabolism; council on cardiovascular nursing; and council on epidem. *Circulation* 2009; **119**: 902–907. <https://doi.org/10.1161/CIRCULATIONAHA.108.191627>.
- O'Connor SG, Koprowski C, Dzibur E, Leventhal AM, Huh J, Dunton GF. Differences in mothers' and children's dietary intake during physical and sedentary activities: An ecological momentary assessment study. *J. Acad. Nutr. Diet.* 2017; **117**: 1265–1271. <https://doi.org/10.1016/j.jand.2017.02.012>.
- Farvid MS, Ding M, Pan A, et al. Dietary linoleic acid and risk of coronary heart disease: A systematic review and meta-analysis of prospective cohort studies. *Circulation* 2014; **130**: 1568–1578. <https://doi.org/10.1161/CIRCULATIONAHA.114.010236>.
- Chowdhury R, Warnakula S, Kunutsor S, et al. Association of dietary, circulating, and supplement fatty acids with coronary risk: A systematic review and meta-analysis. *Ann. Intern. Med.* 2014; **160**: 398–406. <https://doi.org/10.7326/M13-1788>.
- Ramsden CE, Zamora D, Majchrzak-Hong S, et al. Re-evaluation of the traditional diet-heart hypothesis: Analysis of recovered data from Minnesota Coronary Experiment (1968-73). *BMJ* 2016<https://doi.org/10.1136/bmj.i1246>.
- Naughton SS, Mathai ML, Hryciw DH, McAinch AJ. Australia's nutrition transition 1961–2009: A focus on fats. *Br. J. Nutr.* 2015; **114**: 337–346. <https://doi.org/10.1017/s0007114515001907>.
- Sanders TAB. Polyunsaturated fatty acids in the food chain in Europe. *Am. J. Clin. Nutr.* 2000; **71**: 176s–178s.
- Alvheim AR, Malde MK, Osei-Hyiaman D, et al. Dietary linoleic acid elevates endogenous 2-AG and anandamide and induces obesity. *Obesity* 2012; **20**: 1984–1994. <https://doi.org/10.1038/oby.2012.38>.
- Ailhaud G, Massiera F, Weill P, Legrand P, Alessandri JM, Guesnet P. Temporal changes in dietary fats: Role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. *Prog. Lipid Res.* 2006; **45**: 203–236. <https://doi.org/10.1016/j.plipres.2006.01.003>.
- Alvheim AR, Torstensen BE, Lin YH, et al. Dietary linoleic acid elevates the endocannabinoids 2-AG and anandamide and promotes weight gain in mice fed a low fat diet. *Lipids* 2014; **49**: 59–69. <https://doi.org/10.1007/s11745-013-3842-y>.
- Naughton SS, Mathai ML, Hryciw DH, McAinch AJ. Linoleic acid and the pathogenesis of obesity. *Prostaglandins Other Lipid Mediat.* 2016; **125**: 90–99. <https://doi.org/10.1016/j.prostaglandins.2016.06.003>.
- Butler G. Manipulating dietary PUFA in animal feed: Implications for human health. *Proc. Nutr. Soc.* 2014; **73**: 87–95. <https://doi.org/10.1017/S0029665113003790>.
- Gross LS, Li L, Ford ES, Liu S. Increased consumption of refined carbohydrates and the epidemic of type 2 diabetes in the United States: An ecologic assessment. *Am. J. Clin. Nutr.* 2004; **79**: 774–779. <https://doi.org/10.1093/ajcn/79.5.774>.
- Deshpande G, Mapanga RF, Essop MF. Frequent sugar-sweetened beverage consumption and the onset of cardiometabolic diseases: Cause for concern? *J Endocr Soc.* 2017; **1**: 1372–1385. <https://doi.org/10.1210/js.2017-00262>.
- Sharma S, Dortch KS, Byrd-Williams C, et al. Nutrition-related knowledge, attitudes, and dietary behaviors among head start teachers in Texas: A cross-sectional study. *J. Acad. Nutr. Diet.* 2013; **113**: 558–562. <https://doi.org/10.1016/j.jand.2013.01.003>.
- Hipp JA, Becker HV, Marx CM, Tabak RG, Brownson RC, Yang L. Worksite nutrition supports and sugar-sweetened beverage consumption. *Obes Sci Pract.* 2016; **2**: 144–153. <https://doi.org/10.1002/osp4.44>.
- Salem N, Pawlosky R, Wegher B, Hibbeln J. In vivo conversion of linoleic acid to arachidonic acid in human adults. *Prostaglandins Leukot. Essent. Fatty Acids* 1999; **60**: 407–410. [https://doi.org/10.1016/S0952-3278\(99\)80021-0](https://doi.org/10.1016/S0952-3278(99)80021-0).
- Naughton SS, Mathai ML, Hryciw DH, McAinch AJ. Fatty acid modulation of the endocannabinoid system and the effect on food intake and metabolism. *Int J Endocrinol.* 2013; **2013**: 1–11. <https://doi.org/10.1155/2013/361895>.
- Lu HC, MacKie K. An introduction to the endogenous cannabinoid system. *Biol. Psychiatry* 2016; **79**: 516–525. <https://doi.org/10.1016/j.biopsych.2015.07.028>.
- Sugiura T, Kondo S, Sukagawa A, et al. 2-arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* 1995; **215**: 89–97. <https://doi.org/10.1006/bbrc.1995.2437>.
- Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* 1988; **34**: 605–613. doi:88/050605–09\$02.00/O.
- Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993; **365**: 61–65. <https://doi.org/10.1038/365061a0>.
- Blankman JL, Simon GM, Cravatt BF. A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem. Biol.* 2007; **14**: 1347–1356. <https://doi.org/10.1016/j.chembiol.2007.11.006>.
- Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 1996; **384**: 83–87. <https://doi.org/10.1038/384083a0>.
- Osei-Hyiaman D, DePetrillo M, Pacher P, et al. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis

- and contributes to diet-induced obesity. *J. Clin. Invest.* 2005; **115**: 1298–1305. <https://doi.org/10.1172/JCI200523057>.
30. González-Mariscal I, Krzysik-Walker SM, Doyle ME, et al. Human CB1 receptor isoforms, present in hepatocytes and β -cells, are involved in regulating metabolism. *Sci. Rep.* 2016; **6**: <https://doi.org/10.1038/srep33302>.
 31. Ghosh S, González-Mariscal I, Egan JM, Moaddel R. Targeted proteomics of cannabinoid receptor CB1 and the CB1b isoform. *J. Pharm. Biomed. Anal.* 2017; **144**: 154–158. <https://doi.org/10.1016/j.jpba.2016.11.003>.
 32. Gonalons N, Ravinet Trillou C, Delgorge C, et al. Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *Am J Physiol Integr Comp Physiol.* 2015; **284**: R345–R353. <https://doi.org/10.1152/ajpregu.00545.2002>.
 33. Després J-P, Golay A, Sjöström L. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N. Engl. J. Med.* 2005; **353**: 2121–2134. <https://doi.org/10.1056/nejmoa044537>.
 34. Christensen R, Kristensen PK, Bartels EM, Bliddal H, Astrup A. Efficacy and safety of the weight-loss drug rimonabant: A meta-analysis of randomised trials. *Lancet* 2007; **370**: 1706–1713. [https://doi.org/10.1016/S0140-6736\(07\)61721-8](https://doi.org/10.1016/S0140-6736(07)61721-8).
 35. Wang L, Liu J, Harvey-White J, Zimmer A, Kunos G. Endocannabinoid signaling via cannabinoid receptor 1 is involved in ethanol preference and its age-dependent decline in mice. *Proc. Natl. Acad. Sci. U. S. A.* 2003; **100**: 1393–1398. <https://doi.org/10.1073/pnas.0336351100>.
 36. González-Mariscal I, Montoro RA, Doyle ME, et al. Absence of cannabinoid 1 receptor in beta cells protects against high-fat/high-sugar diet-induced beta cell dysfunction and inflammation in murine islets. *Diabetologia* 2018; **61**: 1470–1483. <https://doi.org/10.1007/s00125-018-4576-4>.
 37. Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. *Cold Spring Harb. Protoc.* 2008; **2008**: <https://doi.org/10.1101/pdb.prot4986>.
 38. Chon SH, Douglass JD, Zhou YX, et al. Over-expression of monoacylglycerol lipase (MGL) in small intestine alters endocannabinoid levels and whole body energy balance, resulting in obesity. *PLoS ONE* 2012; **7**: e43962. <https://doi.org/10.1371/journal.pone.0043962>.
 39. Labar G, Wouters J, Lambert DM. A review on the monoacylglycerol lipase: At the interface between fat and endocannabinoid signalling. *Curr. Med. Chem.* 2010; **17**: 2588–2607. <https://doi.org/10.2174/092986710791859414>.
 40. Rakhshandehroo M, Sanderson LM, Matilainen M, et al. Comprehensive analysis of PPAR α -dependent regulation of hepatic lipid metabolism by expression profiling. *PPAR Res.* 2007; **2007**: 1–13. <https://doi.org/10.1155/2007/26839>.
 41. Baes M, Peeters A. Role of PPAR α in hepatic carbohydrate metabolism. *PPAR Res.* 2010; **2010**: 1–12. <https://doi.org/10.1155/2010/572405>.
 42. Hostetler HA, Huang H, Kier AB, Schroeder F. Glucose directly links to lipid metabolism through high affinity interaction with peroxisome proliferator-activated receptor ?? *J. Biol. Chem.* 2008; **283**: 2246–2254. <https://doi.org/10.1074/jbc.M705138200>.
 43. Gaysinskaya VA, Karatayev O, Shuluk J, Leibowitz SF. Hyperphagia induced by sucrose: Relation to circulating and CSF glucose and corticosterone and orexigenic peptides in the arcuate nucleus. *Pharmacol. Biochem. Behav.* 2011; **97**: 521–530. <https://doi.org/10.1016/j.pbb.2010.10.008>.
 44. Perez PA, DiPatrizio NV. Impact of maternal western diet-induced obesity on offspring mortality and peripheral endocannabinoid system in mice. *PLoS ONE* 2018; **13**: e0205021. <https://doi.org/10.1371/journal.pone.0205021>.
 45. Erlanson-Albertsson C, Lindqvist A. Fructose affects enzymes involved in the synthesis and degradation of hypothalamic endocannabinoids. *Regul. Pept.* 2010; **161**: 87–91. <https://doi.org/10.1016/j.regpep.2010.01.003>.
 46. Lowette K, Roosen L, Tack J, Vanden Berghe P. Effects of high-fructose diets on central appetite signaling and cognitive function. *Front. Nutr.* 2015; **2**: <https://doi.org/10.3389/fnut.2015.00005>.
 47. Lindqvist A, Baelemans A, Erlanson-Albertsson C. Effects of sucrose, glucose and fructose on peripheral and central appetite signals. *Regul. Pept.* 2008; **150**: 26–32. <https://doi.org/10.1016/j.regpep.2008.06.008>.
 48. Huang BW, Chiang MT, Yao HT, Chiang W. The effect of high-fat and high-fructose diets on glucose tolerance and plasma lipid and leptin levels in rats. *Diabetes Obes. Metab.* 2004: <https://doi.org/10.1111/j.1462-8902.2004.00323.x>.
 49. Lindqvist A, De La Cour CD, Stegmark A, Håkanson R, Erlanson-Albertsson C. Overeating of palatable food is associated with blunted leptin and ghrelin responses. *Regul. Pept.* 2005; **130**: 123–132. <https://doi.org/10.1016/j.regpep.2005.05.002>.
 50. Blüher M, Engeli S, Klötting N, et al. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. *Diabetes* 2006; **55**: 3053–3060. <https://doi.org/10.2337/db06-0812>.
 51. Mc Intosh AL, Huang H, Landrock D, et al. Impact of Fabp1 gene ablation on uptake and degradation of endocannabinoids in mouse hepatocytes. *Lipids* 2018; **53**: 561–580. <https://doi.org/10.1002/lipd.12071>.
 52. González-Mariscal I, Krzysik-Walker SM, Kim W, Rouse M, Egan JM. Blockade of cannabinoid 1 receptor improves GLP-1R mediated insulin secretion in mice. *Mol. Cell. Endocrinol.* 2016; **423**: 1–10. <https://doi.org/10.1016/j.mce.2015.12.015>.
 53. Jourdan T, Godlewski G, Kunos G. Endocannabinoid regulation of β -cell functions: Implications for glycaemic control and diabetes. *Diabetes. Obes. Metab.* 2016; **18**: 549–557. <https://doi.org/10.1111/dom.12646>.
 54. Kim W, Doyle ME, Liu Z, et al. Cannabinoids inhibit insulin receptor signaling in pancreatic β -cells. *Diabetes* 2011; **60**: 1198–1209. <https://doi.org/10.2337/db10-1550>.
 55. Wang X, Yu Q, Yue H, Zhang J, Zeng S, Cui F. Circulating endocannabinoids and insulin resistance in patients with obstructive sleep apnea. *Biomed. Res. Int.* 2016; **2016**: 1–8. <https://doi.org/10.1155/2016/9782031>.
 56. White MF. IRS proteins and the common path to diabetes. *Am. J. Physiol. Endocrinol. Metab.* 2002; **283**: E413–E422. <https://doi.org/10.1152/ajpendo.00514.2001>.
 57. Withers DJ, Gutierrez JS, Towery H, et al. Disruption of IRS2 causes type 2 diabetes in mice. *Nature* 1998; **391**: 900–904. <https://doi.org/10.1038/36116>.
 58. Copps KD, Hancer NJ, Opere-Ado L, Qiu W, Walsh C, White MF. Irs1 serine 307 promotes insulin sensitivity in mice. *Cell Metab.* 2010; **11**: 84–92. <https://doi.org/10.1016/j.cmet.2009.11.003>.