Low-arginine and low-protein diets induce hepatic lipid accumulation through different mechanisms in growing rats

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Research

Keywords: arginine deficiency, low-protein diet, hepatosteatosis, apolipoprotein A-IV, insulin signaling

Posted Date: January 22nd, 2020

DOI: https://doi.org/10.21203/rs.2.21577/v1

Version of Record: A version of this preprint was published on August 3rd, 2020. See the published version at https://doi.org/10.1186/s12986-020-00477-5.

Abstract

Background Dietary protein deficiency and amino acid uimbalance cause hepatic fat accumulation. We previously demonstrated that only arginine deficiency as well as total amino acid deficiency in a diet caused significant hepatic triglyceride (TG) accumulation in young Wistar rats. In this study, we explored the mechanisms of this fatty liver formation using these two models.

Methods A low-total-amino acid diet (equivalent to 5% protein) and a low-arginine diet (solely the arginine content alone is as low as the low-total-amino acid diet) to the rats for 2 weeks.

Results There was substantially greater hepatic TG accumulation in the low-arginine group than in the low-total-amino acid group. The low-total-amino-acid diet potentiated insulin signals in the liver and enhanced de novo lipogenesis. By contrast, the low-arginine diet inhibited hepatic very-low-density lipoprotein secretion, without affecting hepatic insulin signaling and lipogenesis.

Conclusions We conclude that, although the arginine intake of the low-arginine group was as low as that of the low-total-amino-acid group, these two diets developed a fatty liver via completely different mechanisms. The potentiation of insulin signaling and resultant increases in fatty acid synthesis seem to drive the effects of a low-protein diet, whereas lower VLDL secretion may be the main causes of low-arginine diet-induced TG accumulation in the liver.

1. Background

Dietary protein has a strong impact on growth, body composition, and energy homeostasis. Protein overload results in a lower body weight and promotes insulin resistance in humans [1, 2] and rats [3–5]. By contrast, dietary protein malnutrition induces hepatic fat accumulation, a symptom of kwashiorkor, in rats and humans [6, 7]. The dietary amino acid balance also affects fat deposition in the liver. For example, rats fed low-protein diets supplemented with one or more indispensable amino acids such as threonine or methionine experience excessive fat deposition in the liver [8], and supplementation of a low-protein diet with DL-threonine prevents hepatic fat accumulation [8].

Non-alcoholic fatty liver disease (NAFLD) represents a series of hepatic disorders triggered by excessive liver fat accumulation, and is thus associated with central obesity and insulin resistance. Although the effects of dietary fat and carbohydrates on liver fat accumulation have been extensively studied, the effects of dietary protein and amino acids on the modulation of energy homeostasis are also important considerations in the pathogenesis of NAFLD, but are less well understood [1]. For example, insulin is a major anabolic hormone that is intimately involved in hepatic metabolism, and its secretion is affected by dietary protein and amino acid intake. Protein malnutrition suppresses insulin secretion from the pancreas in response to glucose intake [2, 3]. Some amino acids such as arginine and leucine are also known to stimulate insulin secretion [4, 5]. Moreover, our group previously demonstrated that dietary protein malnutrition decreased plasma insulin and insulin-like growth factor (IGF)-1 concentrations [6, 7], and upregulated the protein expression of IRS2 in the liver, one of the key mediators of insulin signaling in

growing rats, resulting in increased insulin sensitivity [7]. These results implied that enhanced insulin sensitivity via dietary protein deficiency might mediate fatty liver development.

We previously investigated the impacts of various amino acids on fat accumulation in the liver using amino acid-deficient diets, and the results showed that the liver triglyceride (TG) content could be increased only by arginine deficiency out of the 20 major amino acids tested [8]. Therefore, we hypothesized that lipid accumulation in the liver induced by dietary amino acid deficiency could be attributed to increased insulin sensitivity, which might be specifically derived by dietary arginine deficiency. Thus, we tested the hypothesis by evaluating the metabolic status and hepatic lipid flux using models of total amino acid and arginine deficiency.

2. Methods And Materials

2.1. Materials

Anti-insulin receptor subunit β (IRβ) and anti-CD36 antibodies for immunoblotting were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-IRS2 (clone 9.5.2) antibody for immunoblotting was purchased from Merck Millipore (Billerica, MA, USA). Anti-phospho-p70 S6K (Thr389), anti-p70 S6K (49D7), anti-phospho-4E-BP1 (Thr37/46), anti-4E-BP1, and anti-phospho-AMPKα (Thr172) (40H9) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The anti-GAPDH (6C5) antibody was purchased from Abcam (Cambridge, UK). Anti-rabbit and mouse IgG horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (Little Chalfont, UK).

2.2. Animals

The experiments were approved by the Animal Usage Committee of the Faculty of Agriculture of the University of Tokyo and performed in accordance with its guidelines (Permission No. P09-375). Male Wistar rats were purchased from Charles River Laboratories International (Kanagawa, Japan). The animals were housed individually in wire cages with free access to food and water. The rats were maintained at a room temperature of 23°C ± 1°C with 50–60% relative humidity under a 12-h light/dark cycle (light from 08:00 to 20:00). In the pre-experimental period, the rats were fed a purified diet containing 15% protein from casein.

2.3. Comparison of the effects of the low-total-amino acid and low-arginine diets

Six-week-old male Wistar rats were randomly divided into three groups with different diets: the amino acid mixture control diet (equivalent of 15% protein in the diet; 15PAA), low-arginine diet in which the concentration of arginine in young rodents was 33% of that in the 15PAA diet (low Arg, n = 8), and the low-total-amino acid diet (equivalent of 5% protein in the diet; 5PAA). This amino acid mixture simulated

casein composition [9]. The diet compositions are shown in Tables 1 and 2.Rats were given *ad libitum* access to tap water and food. Body weight and food intake were measured daily. Fourteen days after initiation of the experimental diets, the rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg) 1 h after removing the diet, and a postprandial blood sample was collected from the carotid artery. This blood samples were obtained at feeding condition. The liver, longissimus muscles, and abdominal fats were removed and weighed. The livers and muscle tissues were soaked in RNAlater (AMBION, Austin, TX, USA) or snap-frozen in liquid nitrogen and stored at −80°C until use. These organ samples were used to analyses tissue TG concentration, western blotting, and RNA expression.

We performed oral glucose tolerance tests (OGTTs) 12 days after initiation of the experimental diets. After 16 h of overnight fasting, glucose (2 g/kg) was orally administered to the rats. Blood was collected from the tail vein into heparinized tubes that were chilled on ice, and preparandial blood samples were obtained at this fasting condition. The blood samples were subjected to centrifugation at 3,000 ×*g* for 5 min at 4°C and the supernatants were transferred to new tubes. Plasma samples were stored at −80°C until analysis.

2.4. Blood biochemistry

Blood biochemical parameters, including total cholesterol, TG, free fatty acids (FFA), and glucose concentrations, were determined using commercial kits (Cholesterol E-test, Triglyceride E-test, NEFA C-test, and Glucose CII test, respectively; Wako Pure Chemical Industries, Osaka, Japan). The plasma insulin concentration was measured using an insulin measurement kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to the manufacturer's instructions.

2.5. Lipid extraction and TG measurements

Lipids were extracted from frozen livers and longissimus muscles by a modified Folch method [10] in a 2:1 (vol/vol) mixture of chloroform/methanol. The extracts were washed with 0.5 volumes of 0.8% KCl and centrifuged at 1,500 ×*g* for 10 min, and the organic phases were recovered. The TG content in the liver and plasma was also determined using a commercial kit (Wako Pure Chemical Industries) according to the manufacturer's instructions.

2.6. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from homogenized livers using NucleoSpin® RNA (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The total RNA concentration was measured with a NanoDrop® spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA). The quality of the RNA

was determined by assessing the A260/280 ratio and by agarose gel electrophoresis. The RNA was reverse-transcribed into cDNA using PrimeScript® RT Master Mix (Takara Bio, Shiga, Japan). cDNA was amplified using SYBR® Premix Ex Taq II (Takara Bio) according to the manufacturer's protocol. We designed the primers for RT-PCR with the design software Primer 3. β-actin was used as an endogenous control. The following PCR primers were used: β-actin (*Actb*) forward, 5'-GGAGATTACTGCCCTGGCTCCTA-3', and reverse, 5'-GACTCATCGTACTCCTGCTTGCTG-3'; MTP (*Mttp*) forward, 5'-AGCAACATGCCTACTTCTTACAC-3', and reverse, 5'-TCACGGGTTCACTTTCACTG-3'; apolipoprotein A-IV (*ApoA4, Apoa4*) forward, 5'-ACCCTCTTCCAGGACAAACTTG-3', and reverse, 5'-CCTTGGTTAGATGTCCACTCAGTTG-3'; and apolipoprotein B (*ApoB, Apob*) forward, 5'-CCTGTCCATTCAAAACTACCACA-3', and reverse 5'-CAATGAACGAATCAGAAGGTGA-3'.

2.7. Western blotting

Western blotting analysis was performed as previously described [7, 11]. In brief, frozen livers were homogenized in homogenizing buffer and centrifuged at 100,000 ×*g* for 1 h at 4°C. The protein content in the supernatant was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes. The membranes were blocked with blocking buffer, and then incubated at 4°C overnight with primary antibodies against IRβ, CD36, and FFA synthase at 1:200, and against PI3 kinase p85, phospho-p70 S6K (Thr389), p70 S6K, phospho-4E-BP1 (Thr37/46), 4E-BP1, phospho-AMPKα (Thr172), AMPKα, phospho-acetyl-CoA carboxylase (Ser79), acetyl CoA carboxylase, and acetyl CoA carboxylase 1 at 1:1,000. Primary mouse anti-IRS2 and anti-GAPDH antibodies were used at dilutions of 1:1,000 and 1:3,000, respectively. We visualized the blots by chemiluminescence after incubating with donkey anti-rabbit IgG or sheep anti-mouse IgG conjugated to horseradish peroxidase (1:2,500). The immunoreactive bands were exposed and the signals were quantified using a cooled charge-coupled device camera system (LAS-3000 Mini; Fujifilm, Kanagawa, Japan).

2.7. Very-low-density lipoprotein (VLDL) excretion test

Male Wistar rats were fed a casein control diet between 10:00 and 18:00 for 7 days prior to the experiment. After habituation, the rats (7.5 weeks of age, 208–229 g) were assigned to the 15PAA (n = 8), 5PAA (n = 8), and low Arg (n = 9) groups. The diet compositions are same as in above section (Table 1 and 2). The experimental diets were provided for 5 h from 9:00 to 14:00. We then administered tyloxapol (200 mg/kg, dissolved in 0.9% NaCl; Triton WR-1339, Sigma-Aldrich, St. Louis, MO, USA) to all rats under isoflurane anesthesia (3–4%, 5 l/min; Dainippon Sumitomo Pharma, Osaka, Japan) via the tail vein. Blood was collected from the tail vein into chilled, heparinized tubes prior to tyloxapol injection, and at 30, 60, 120, and 240 min after injection. The TG concentrations in the plasma were measured with a commercial kit as described in section 2.5 above. Tyloxapol inhibits endogenous lipoprotein lipase and

accumulates plasma TG [12, 13]. Therefore, we monitored an increase in plasm TG concentration from0 time. The TG secretion rate was expressed in mg TG /dl/min.

2.8. Respiratory exchange ratio (RER)

Six-week-old male Wistar rats were assigned to the 15PAA (n = 6), 5PAA (n = 6), and low Arg (n = 6) groups. Six days after initiation of the experimental diets, animals were individually placed in a metabolic chamber for 24 h, and VO_2 and VCO_2 were monitored every 10 min with an OXYMAX system (Columbus Instruments, USA). Rats were allowed free access to water and food during the experiment.

2.9. De novo lipogenesis assay

De novo lipogenesis was measured based on the method described previously with small modifications[14]. Six-week-old male Wistar rats were assigned to the 15PAA (n = 8), 5PAA (n = 8), and low Arg (n = 8) groups (240–280 g) for one night (from 18:00 to 10:00). Rats were allowed free access to food. The following day, rats of each group were further divided into D_2O (Sigma Aldrich, USA) or H_2O injection group (5 ml/kg body weight, i.p.). Liver samples were collected under isoflurane anesthesia (3–4%, 5 l/min; Dainippon Sumitomo Pharma, Osaka, Japan) 24 hours after injection and stored at -80°C until used for analysis. Lipids were extracted as described in section 2.5 above[10]. The extracted lipids were hydrolyzed and methylated by FFA Methylation Kit (Nacalai Tesque, Japan), and the obtained products were purified by Fatty Acid Methyl Ester Purification Kit (Nacalai Tesque, Japan). Purified FFA esters were analyzed with gas chromatography-mass spectrometry (GCMS-QP2010 Plus, SHIMADZU, Japan) to quantify palmitate isotopomers. Relative amount of fatty acids was normalized by tissue weight and the difference in the total amount of methyl palmitate isotopomers between the D_2O-injected group and H_2O-injected group was interpreted as the *de novo* lipogenesis rate.

2.10. Hepatic FFA uptake assay

In vivo FFA uptake assay was conducted according to the previous reports [15, 16].Six-week-old male Wistar rats were assigned to the 15PAA (n = 8), 5PAA (n = 8), and low Arg (n = 8) groups (240–280 g) for one night (from 18:00 to 10:00). The following day, rats of each group were further divided into two groups; one was treated with fluorescent FFA analog (0.1 mg/kg, BODIPY® FL C12, Invitrogen, USA) and the other was treated with only vehicle (0.1% bovine serum albumin in phosphate-buffered saline) by intravenous injection under isoflurane anesthesia (3–4%, 5 l/min; Dainippon Sumitomo Pharma, Osaka, Japan). The liver samples were collected 10 min after injection and stored at -80°C until used for analysis. Lipids were extracted[10] and reconstituted in isopropanol,. Then its fluorescence was measured by the ARVO X3 microplate reader (PerkinElmer, USA). All values were normalized by tissue weight and

the value of vehicle-administered group was subtracted as background fluorescence from that of BODIPY-administered group.

2.11 Statistical analysis

All data are presented as the mean ± standard error. The data were statistically analyzed with the Ekuseru-Toukei 2010 software package (Social Survey Research Information, Tokyo, Japan). Statistical significance was calculated using one-way analysis of variance (ANOVA) with the Bonferroni and Tukey–Kramer post-hoc tests to assess differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Diet-induced changes in the body composition of rats

Body weight gain was slightly lower in the 5PAA group than in the 15PAA group, whereas the weight gain of the low Arg group was comparable to that of the control 15PAA group (Table 3). Liver TG levels were significantly higher in the 5PAA group than those in the 15PAA group (Fig. 1B, $p < 0.01$) as previously reported [7, 11]; however, even higher TG levels than the 5PAA group were observed in the low Arg group (Fig. 1B, $p < 0.01$ vs. 15PAA and 5PAA). Macroscopic observations also supported this result, as the livers of the 5PAA and low Arg groups were whiter than those of the 15PAA group (Fig. 1A). The TG content in the longissimus muscle and the visceral fat volume were significantly higher in the 5PAA group compared to those of the 15PAA group, whereas these parameters were not affected by the low Arg diet (Fig. 1C, E). Liver weight was significantly higher in the low Arg group than that of the 15PAA group, but this was not the case for the 5PAA group (Fig. 1D). These data suggested that, in contrast to our expectation, a low-total-amino acid diet and low-arginine diet might lead to different phenotypes, given that the low Arg diet did not completely recapitulate the effects of 5PAA administration.

3.2. Diet-induced changes in blood biochemical parameters

The blood glucose and insulin levels were over 190 mg/dl and 1.9 ng/ml, respectively, in the postprandial blood, confirming the postprandial condition (Table 3, Supplemental Table1). No differences were observed in free FFA concentrations in the preprandial and postprandial conditions among the three groups (Fig. 2A and B). By contrast, postprandial plasma TG and total cholesterol levels were significantly lower in the low Arg group than in the 15PAA group (Fig. 2D, Table 3), whereas the 5PAA diet did not affect plasma TG levels in the pre and postprandial condition (Fig. 2C and D). The high-density lipoprotein cholesterol concentrations were similar among the three groups in the preprandial and postprandial blood (Table 3).

3.3. Glucose tolerance

When the OGTT was performed at 12 days after initiation of the experimental diets, plasma glucose levels of the 5PAA group were found to be lower than those of the 15PAA group at 30 and 60 min after glucose loading (Fig. 3A). In addition, the plasma insulin levels were significantly lower in the 5PAA group than those in the 15PAA group at 30 min after glucose loading (Fig. 3B). The fasting plasma glucose levels in the low Arg group were higher than those in the 15PAA group (Fig. 3A), whereas the fasting plasma insulin levels did not differ significantly between the low Arg and 15PAA groups (Fig. 3B). The plasma glucose level after glucose loading was not affected by the low Arg diet. The plasma insulin levels increased less in the low Arg group after glucose loading than in the 5PAA and 15PAA groups. The plasma insulin levels fell at 60 min after glucose loading in the 15PAA group but did not decrease in the low Arg group.

3.4. Hepatic insulin receptor signaling

Western blotting analysis revealed that the expression of IRβ was slightly, but not significantly, higher in the livers of 5PAA-fed rats than in those of the 15PAA-fed rats (Fig. 4A and B). Rats fed the 5PAA diet exhibited markedly higher expression levels of IRS2 (Fig. 4A and C) and 4E-BP1 (Fig. 4A and D) in the liver than rats fed the 15PAA or low Arg diet. The threonine phosphorylation of 4E-BP1 was significantly higher in the 5PAA group than in the 15PAA or low Arg group (Fig. 4A and E). Low Arg-fed rats showed a lower total IRβ level than the 15PAA- and 5PAA-fed rats, but IRS2, total 4E-BP1, and phosphorylated 4E-BP1 levels were comparable to those of 15PAA-fed rats.

CD36, a scavenger FFA receptor, is known to mediate FFA uptake into the metabolic tissues and has been reported to be associated with non-alcoholic fatty liver development [16, 17]. Hepatic CD36 expression in the low Arg group was significantly lower than that in the 15PAA and 5PAA groups (Fig. 4A and F).

3.5. TG secretion, de novo lipogenesis, FFA uptake, and energy consumption

We next assessed hepatic lipid flux in rats fed the 5PAA and low Arg diets. For the hepatic TG secretion assay, the rats were fed the indicated experimental diets for 5 h after fasting for 15 h, and then tyloxapol was injected into the tail vein. The rats in the three groups consumed approximately equal amounts of chow during the 5 h of refeeding (15PAA: 13 ± 1 g; 5PAA: 14 ± 1 g; low Arg: 14 ± 0 g). When plasma TG concentrations were measured at 0, 30, 60, 120, and 240 min after tyloxapol injection, every group showed time-dependent increases, but the rate of increase of the low Arg group was significantly lower than that of the 15PAA group (Fig. 5A). Remarkably, the hepatic TG secretion rate of the 5PAA group did not significantly change compared to that of the 15PAA group (Fig. 5B). Analysis of hepaticFFA uptake

activity using a fluorescent FFA analog showed comparable levels among the three groups (Fig. 5C). In addition, the *de novo* lipogenesis assay demonstrated that the lipid synthetic activity in the liver was enhanced only in the 5PAA group, whereas it was unchanged in the low Arg group (Fig. 5D).

We also measured the RER of these three groups (Fig. 5E, Supplementary Figure 2). RER is a stoichiometric ratio of expirated CO_2 to inspirated O_2, which roughly reflects the oxidized energy source. Theoretically, an RER of 1.0 represents the dominant consumption of carbohydrates, and a decrease of the RER to approximately 0.7 represents an increasing portion of lipid consumption. RERs of the three groups measured during the night were almost comparable at around 1.0, whereas RERs measured during the day showed just slightly lower levels in the 5PAA and low Arg groups compared to that of the 15PAA group.

3.6. Hepatic TG secretion-related gene expression in the liver

Microarray analysis using the liver samples from rats of the three groups was conducted to evaluate the effects of these diets on hepatic transcripts (Supplemental Table 2). The results showed a marked decrease (0.47-fold) in the expression of the *ApoA4* gene in the liver of the low Arg group compared with that of the 15PAA group, in both microarray and quantitative PCR analysis. ApoA4 is one of the apolipoproteins primarily expressed in the intestine in mammals, which is believed to be involved in chylomicron formation [18]. However, in rodents, it is reported to be expressed also in the liver and was associated with VLDL formation and TG secretion [19].

There were no significant differences in *ApoB* and *Mttp* mRNA levels, which are also important genes for hepatic VLDL formation, between the 15PAA and low Arg groups (Fig. 6A, B), whereas there was a tendency of *Mttp* mRNA levels to be slightly increased in the livers of the rats in the 5PAA group. However, the low Arg diet caused a dramatic decrease in *ApoA4* mRNA levels compared with the 15PAA diet, while the 5PAA diet had no effect (Fig. 6C).

4. Discussion

We previously reported that both dietary total amino acid deficiency and only arginine deficiency caused fatty liver, and activation of insulin receptor signaling is known to stimulate lipid accumulation [7, 11]. Therefore, we speculated that the phenotypes caused by a low Arg diet simply reflected those caused by the 5PAA diet, which could be mediated through enhanced insulin sensitivity. However, in the present study, we identified several completely different phenotypes between the 5PAA and low Arg groups, including the lipid accumulation level, serum lipid profile, glucose tolerance, and insulin signal activities. Furthermore, because it is generally considered that the liver TG content is determined by the integration of de novo lipogenesis, TG secretion, FFA uptake, and lipid oxidation [20], we evaluated these activities in rats fed the 5PAA or low Arg diet. Contrary to our expectation, these parameters also completely differed from each other: only the 5PAA group exhibited increased lipogenesis, and only the low Arg group showed

attenuated TG secretion, while FFA uptake and RER were not very different. Regarding RER, it was slightly lower particularly during the day time in the 5PAA and low Arg groups than the 15PAA group, which indicated that lipid oxidation rates of the both groups were almost the same or slightly higher than that of the 15PAA group. But, because it was an opposite effect to lipid accumulation, it could not be responsible for fatty liver development. Collectively, these results indicate that distinct mechanisms underlie the fatty liver formation induced by total amino acid deficiency and only arginine deficiency.

IRβ and IRS2 are pivotal mediators of insulin activity in the liver. We found that the low-protein diet (5PAA group) increased (or tended to increase) the protein expression levels of total IRβ, IRS2, and phosphorylation of their downstream effectors 4E-BP1 and 4E-BP1 in the liver. This further suggests that the 5PAA diet potentiates insulin signaling. The activation of this pathway is known to up-regulate FFA synthesis [11, 21–23]; indeed, increased de novo lipogenesis was observed in 5PAA diet-fed rats. We recently reported that the intracellular TG level was increased by amino acid deficiency without addition of any lipids or hormones, accompanied by enhanced lipid synthesis, when hepatocytes were cultured in an amino acid-sufficient or -deficient medium [8]. These results clearly indicate that hepatocytes themselves monitor the extracellular amino acid concentrations to induce lipid accumulation in a cell-autonomous manner. Both amino acid deficiency-dependent potentiation of insulin signaling followed by enhancement of lipogenesis and amino acid deficiency-dependent lipogenesis must lead to accumulation of lipids in the liver in response to 5PAA feeding. In contrast, the low Arg diet decreased IRβ and did not have any statistically significant effects on IRS2 and 4E-BP1 protein levels. These findings further supported that the 5PAA and low Arg diets induced hepatic fat accumulation via different mechanisms; that is, 5PAA diet-induced TG accumulation might be implemented through insulin signaling, while low Arg diet-induced accumulation is insulin-independent.

We also demonstrated that only the 5PAA diet induced visceral and muscular fat accumulations, in addition to the liver. Some reports have shown that a low-protein diet leads to intramuscular and other fat deposition in piglets [24–26], and a high-protein diet reduces visceral fat and hepatic TG levels in humans [27, 28]. In addition, we previously found that activation of insulin signaling in response to 5PAA diet feeding was specific to the liver and was not observed in the white adipose tissue or muscle [11]. Taken together, these results suggest that the dietary protein content and amino acid balance can provoke a tissue-specific reaction, and are thus important determinants of body fat deposition in mammals.

The assembly and secretion of VLDL are the key regulatory steps of plasma and intrahepatic TG and FFA levels owing to its glyceride-rich structure, and are closely linked to the progression of NAFLD. The 5PAA diet had no effect on postprandial plasma TG and preprandial free FFA levels, whereas the low Arg group had lower postprandial plasma TG levels than the control group, although their preprandial free FFA levels did not differ. By contrast, FFA uptake into the liver was not influenced by either the 5PAA diet or the low Arg diet, suggesting that the change in serum lipid concentrations was unlikely to be a direct reason for the fatty liver formation.

In this study, the VLDL secretion rate was analyzed by a tyloxapol injection. Tyloxapol is a non-ionic detergent that prevents the catabolism of triacylglycerol-rich lipoproteins by lipoprotein lipase [29, 30], and is widely used for the in vivo determination of the secretion and clearance rates of VLDL [31]. We found that VLDL secretion in the 5PAA group was not significantly changed, while that of the low Arg group significantly decreased. These findings suggested that the hepatic lipid accumulation caused by the low Arg diet, but not the 5PAA diet, was attributable to downregulated VLDL secretion. Because the inhibition of VLDL secretion could be observed at the early stage of feeding the low Arg diet, inhibition of VLDL secretion might be one of the causes of fatty liver formation.

Arginine deficiency has been reported to raise plasma orotic acid levels [32, 33] and dietary supplementation of orotic acid caused a fatty liver with liposomal ApoB deficiency in rats[34], suggesting that orotic acid may be a key metabolite mediating the arginine-deficient phenotype by inhibiting VLDL assembly. We have also been measuring plasma orotic acid concentrations in rats exposed to a low-arginine diet over different lengths of time, and our findings will be presented in a separate report.

Although Mttp and ApoB are the most important components in VLDL assembly [35], neither the 5PAA nor the low Arg diet affected the expression of these genes significantly. Instead, we discovered a decrease in the expression level of ApoA4. ApoA4 is a 46-kDa protein with a lipid-binding site that is expressed in the intestine and liver of rodents [36], and is generally believed to be expressed primarily in the small intestine in humans, although minor expression in the human liver and human hepatocyte lines was also reported [37, 38]. ApoA4 expression is positively correlated with VLDL secretion; adenoviral overexpression of ApoA4 in the liver increases VLDL secretion and decreases the hepatic TG level, and ApoA4-knockout mice show lower VLDL secretion than wild-type controls [13]. Thus, ApoA4 can be another important apolipoprotein for VLDL formation. Based on these findings, attenuation of VLDL secretion in response to the low Arg diet could possibly be achieved through down-regulation of ApoA4 expression.

We demonstrated that a mild deficiency in dietary total amino acids induces fat accumulation in visceral and ectopic, but not subcutaneous, fat. Increases in ectopic fat accumulation and intra-abdominal fat are thought to occur in parallel [39, 40]. Feeding a low-protein diet to piglets leads to deposition of intramuscular and other fat [24, 25]. In healthy humans, a high-protein diet reduces visceral fat [27], and a high-protein/low-carbohydrate diet also lowers hepatic TG levels [28]. Therefore, dietary protein content is an important determinant of body fat deposition in mammals.

5. Conclusions

Our present results demonstrated that a mild deficiency in dietary total amino acids and only in dietary arginine caused a large increase in TG accumulation in the liver by different mechanisms, even though both diets were lacking in arginine to the same extent. We previously showed that the serum amino acid profile of only arginine-deficient rats was completely different from that of total amino acid-deficient rats [8]. We conclude that the low-arginine diet causes hepatic lipid accumulation by attenuating VLDL

secretion, which is caused, at least in part, by the down-regulation of ApoA4 expression. Although the concrete molecular mechanisms remain elusive, elucidating these differences can be the key to explain the tissue-specific responses to dietary protein malnutrition regarding insulin-like activity and lipid metabolism.

Abbreviations

4E-BP1; eukaryotic initiation factor 4E-binding protein

5PAA; diet containing amino acids from casein equivalent to 5% protein

ApoA4; apolipoprotein A-IV

ApoB; apolipoprotein B

IRβ; insulin receptor subunit β

MTP; microsomal triglyceride transfer protein

NAFLD; non-alcoholic fatty liver disease

PCR; polymerase chain reaction

RT; reverse transcription

S6K; S6 kinase

TG; triglyceride

VLDL; very-low-density lipoprotein

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Declarations

1. *Ethics approval and consent to participate*

All experiments using animals were approved by the Animal Usage Committee of the Faculty of Agriculture of the University of Tokyo and performed in accordance with the Guidelines for Animal Experiments of The University of Tokyo (Permission No. P09-375).

2. *Consent for publication*

Not applicable.

3. *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

4. *Competing interests*

The authors declare that they have no competing interests.

5. *Funding*

This work was supported by the Program for the Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (awarded to Takahashi S-I and Kato H.). In addition, we thank the support from R&D matching funds on the field for Knowledge Integration and innovation from Bio-oriented Technology Research Advancement Institution, NARO, Grant- in-Aid for Scientific Research (S) #25221204 and Scientific Research (A) #18H03972, Core-to-core program A. A. Advanced Research Networks from JSPS.

6. *Authors' contributions*

O.L., N.H., T.S-I, and K.H. were responsible for the study concept and design. O.L., N.H., K.A. and A.Y., contributed to the acquisition of animal data. T.Y. and T.Y. performed western blotting and analyzed these data. O.L., N.H., T.S-I and K.H. assisted with data analysis and interpretation of findings. O.L. and N.H. drafted the manuscript with input from all authors. T.Y., T.Y., Y.D., H.F. and J.H. contributed to the interpretation of the results. K.H. provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

7. *Acknowledgments*

We thank Dr. Susan Hall (University of North Carolina) for critically reading the manuscript.

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Supplementary Information

Additional file 1.

Supplementary Figure 1 Insulin signaling-related protein levels in rats fed the low-protein and low-arginine diets. Six-week-old male Wistar rats were fed the 15PAA (n = 8), low Arg (n = 8), and 5PAA (n = 8) diet for 14 days. Whole-liver lysates were analyzed by immunoprecipitation and immunoblotting with antibodies against p85 (B), S6K (C), pS6K (D), AMPK (F),and pAMPK(G) and S6K as an internal control , pS6K/S6K(E)and pAMPK (H)and GAPDH as an internal control.

Supplementary Figure 2 Average values of RER of light period (8am-8pm) and dark period (8pm-8am) were calculated individually.

Additional file 2.

Supplemental Table 1 Plasma glucose and insuline levels n rats fed the low-protein and low-arginine diets.

Supplemental Table 2 List of apolipoprotein genes.

Tables

Table 1 Composition of diet

Component	15P AA	5PAA	Low Arg
	g/kg diet		
Amino acid mixture[1]	150.0	50.0	146.5
DL-methionine	2.5	0.8	2.5
Corn starch	620.5	747.2	649.0
Soy bean oil	50.0	50.0	50.0
Vitamin mixture[2]	10.0	10.0	10.0
Mineral mixture[2]	40.0	40.0	40.0
Choline chloride	2.0	2.0	2.0
Cellulose powder	100.0	100.0	100.0

1 Composition of amino acid mixture were described in Table 2

2 AIN-76 prescription (Oriental Yeast Co., ltd)

Table 2 The amino acid content of each diet

	15PAA	5PAA	Low Arg
	g/kg diet		
Alanine	4.08	1.36	4.08
Arginine	5.25	1.75	1.75
Asparagine-H_20	5.76	1.92	5.76
Aspartate	5.05	1.68	5.05
Cystine	0.8	0.27	0.80
Glutamate	14.65	4.88	14.65
Glutamine	14.65	4.88	14.65
Glycine	2.59	0.86	2.59
Histidine	4.06	1.35	4.06
Isoleucine	7.12	2.37	7.12
Leucine	13	4.33	13.00
Lysine-HCl	14.1	4.70	14.10
Methionine	3.89	1.30	3.89
Phenylalanine	7.2	2.40	7.20
Proline	14.98	4.99	14.98
Serine	8.09	2.70	8.09
Threonine	6.09	2.03	6.09
Tryptophan	7.76	0.58	1.73
Tyrosine	1.73	2.59	7.76
Valine	9.16	3.05	9.16
Total volume	150.00	50.00	146.50

Table 3 Blood biochemical parameters in the postprandial condition after feeding the low-protein and low-arginine diets for 14 days

	15PAA			5PAA			Low Arg		
n	8			8			9		
Body weight (g)	286	+/-	5^{a}	267	+/-	5^{b}	283	+/-	$5^{a\,b}$
Total cholesterol (mg/dl)	69.9	+/-	2.8^{a}	65.0	+/-	2.0^{a}	50.8	+/-	2.3^{b}
HDL cholesterol (mg/dl)	14.8	+/-	0.6	11.6	+/-	0.7	13.7	+/-	0.5
Glucose (mg/dl)	202	+/-	8	196	+/-	9	209	+/-	9
Insulin (ng/ml)	3.2	+/-	0.2^{ab}	1.9	+/-	0.4^{a}	4.6	+/-	0.6^{b}

Data are shown as the mean +/- S.E.M.

Different superscript letters denote significant differences (p<0.05)

Figures

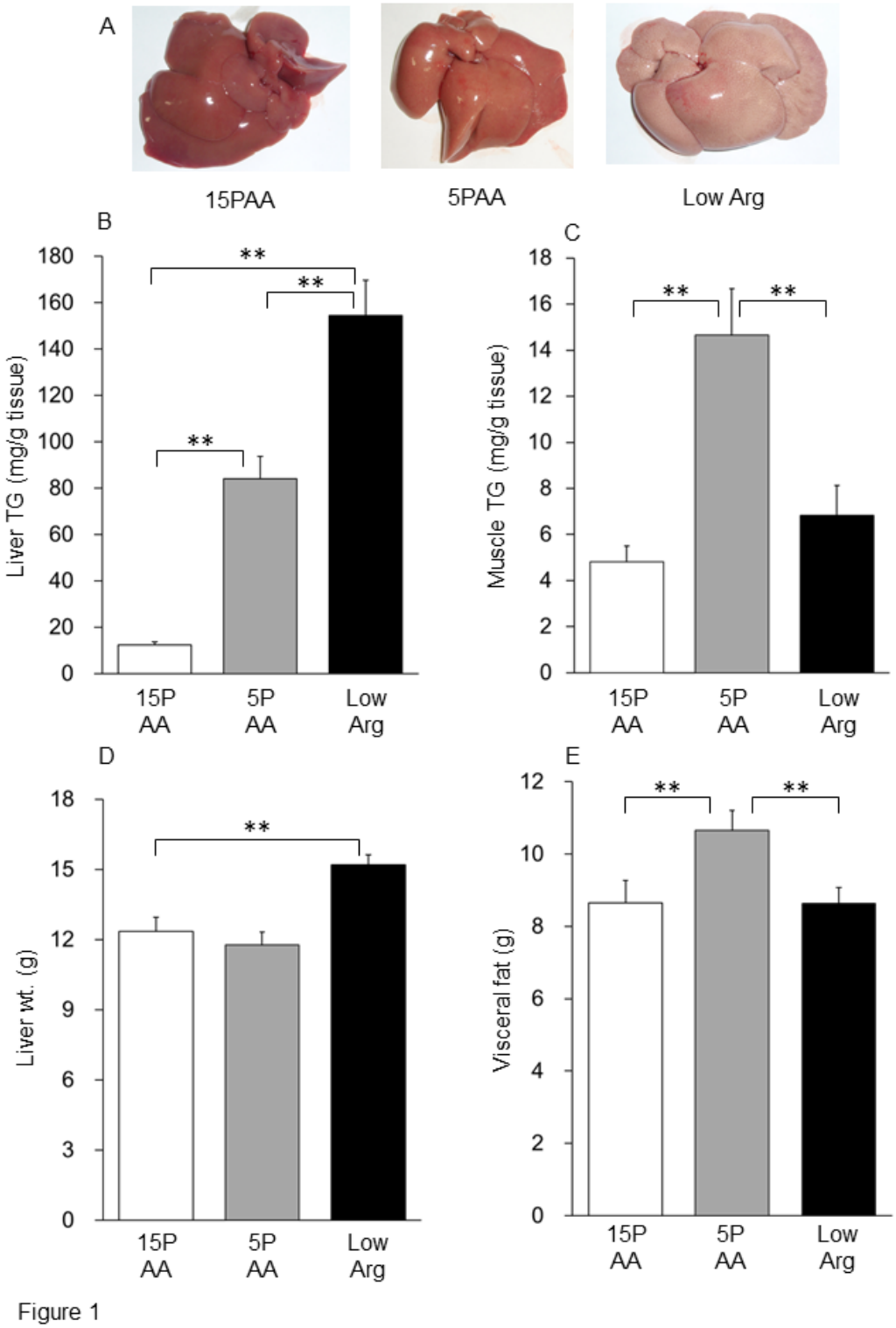

Figure 1

Figure 1

Effects of the low-protein and low-arginine diets on the liver. Macroscopic liver characteristics (A), hepatic (B) and muscle (C) TG contents, liver weight (D), and visceral fat weight (E) in 6-week-old male Wistar rats fed the 15PAA (n = 8), low Arg (n = 8), and 5PAA (n = 8) diet for 14 days. The values are means ±

standard errors. Asterisks indicate significant differences as assessed by one-way ANOVA with Bonferroni and Tukey–Kramer post-hoc tests (*p < 0.05; **p < 0.01).

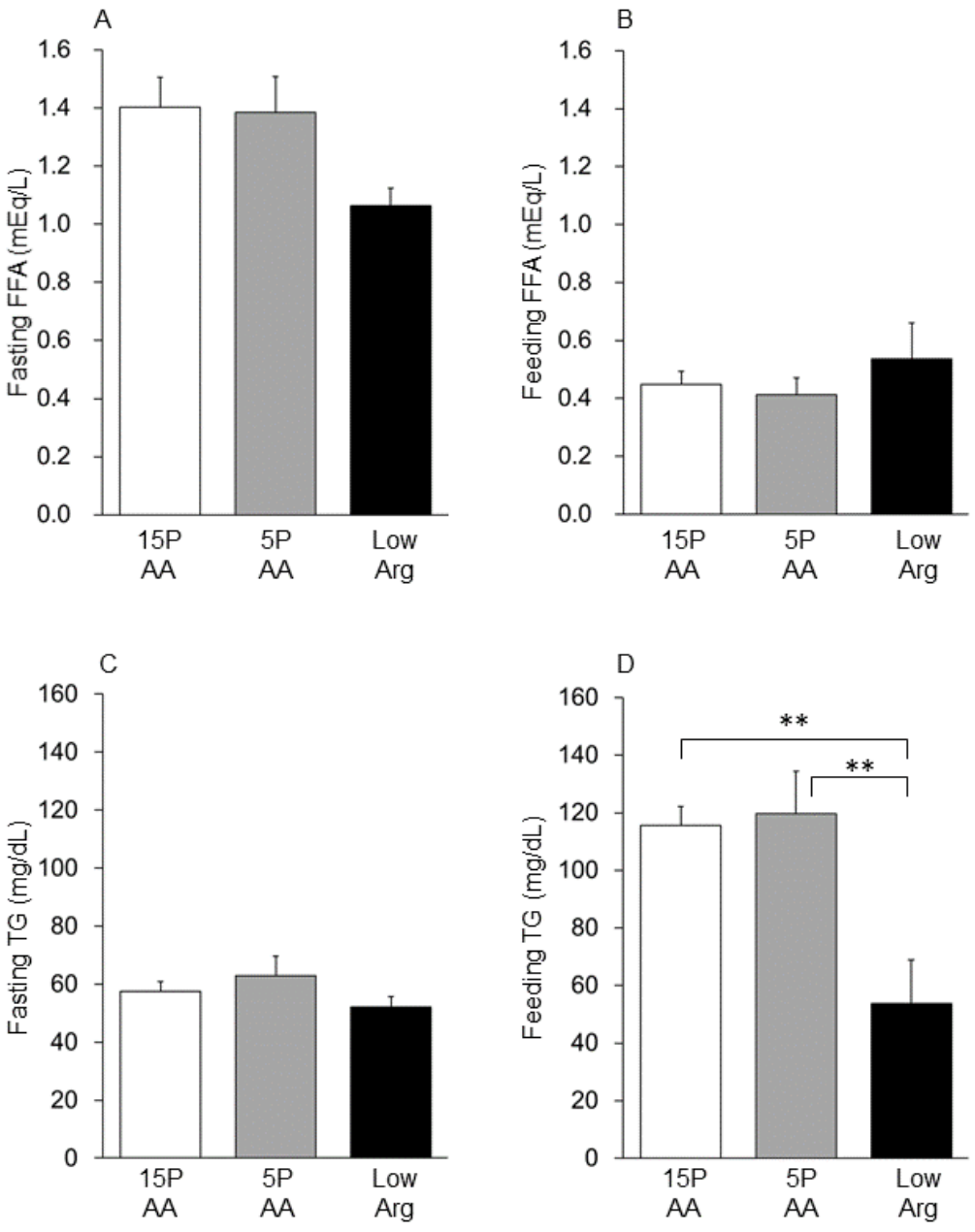

Figure 2

Figure 2

Plasma free fatty acid and TG levels in preprandial or postprandial conditions after feeding the low-protein and low-arginine diets. Six-week-old male Wistar rats were fed the 15PAA (n = 8), low Arg (n = 8), and 5PAA (n = 8) diet for 14 days. Preprandial free fatty acid (A) and TG (C) levels and postprandial free

fatty acid (B) and TG (D) levels are shown. The values are the means ± standard errors. Asterisks indicate significant differences as assessed by one-way ANOVA with Bonferroni and Tukey–Kramer post hoc tests (**p < 0.01).

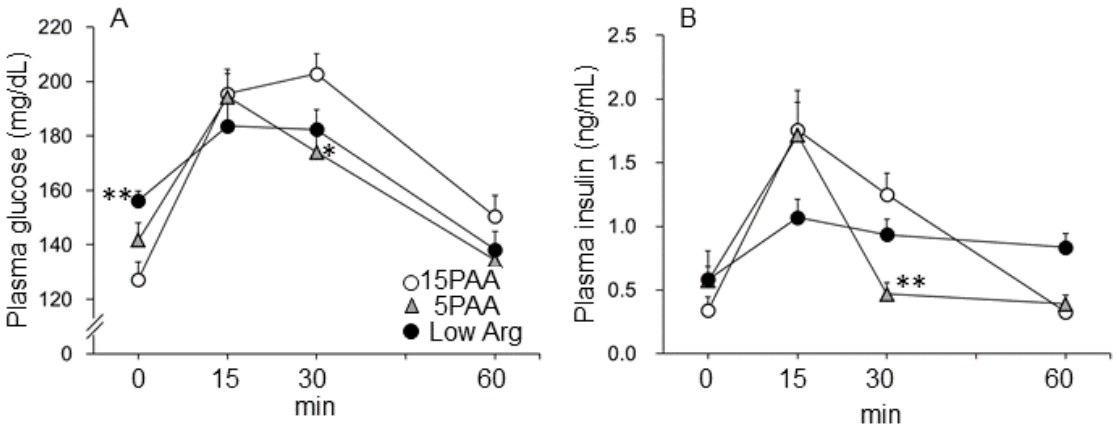

Figure 3

Figure 3

Effects of the low-protein and low-arginine diets on blood glucose and insulin levels after glucose loading. Six-week-old male Wistar rats were fed the 15PAA (n = 8), low Arg (n = 8), and 5PAA (n = 8) diet,

and then OGTTs were performed on day 12 after initiation of the experimental diets. Plasma glucose (A) and insulin (B) concentrations are shown. The values are the means ± standard errors. Asterisks indicate significant differences as assessed by one-way ANOVA with Bonferroni and Tukey–Kramer post-hoc tests (*p < 0.05; **p < 0.01).

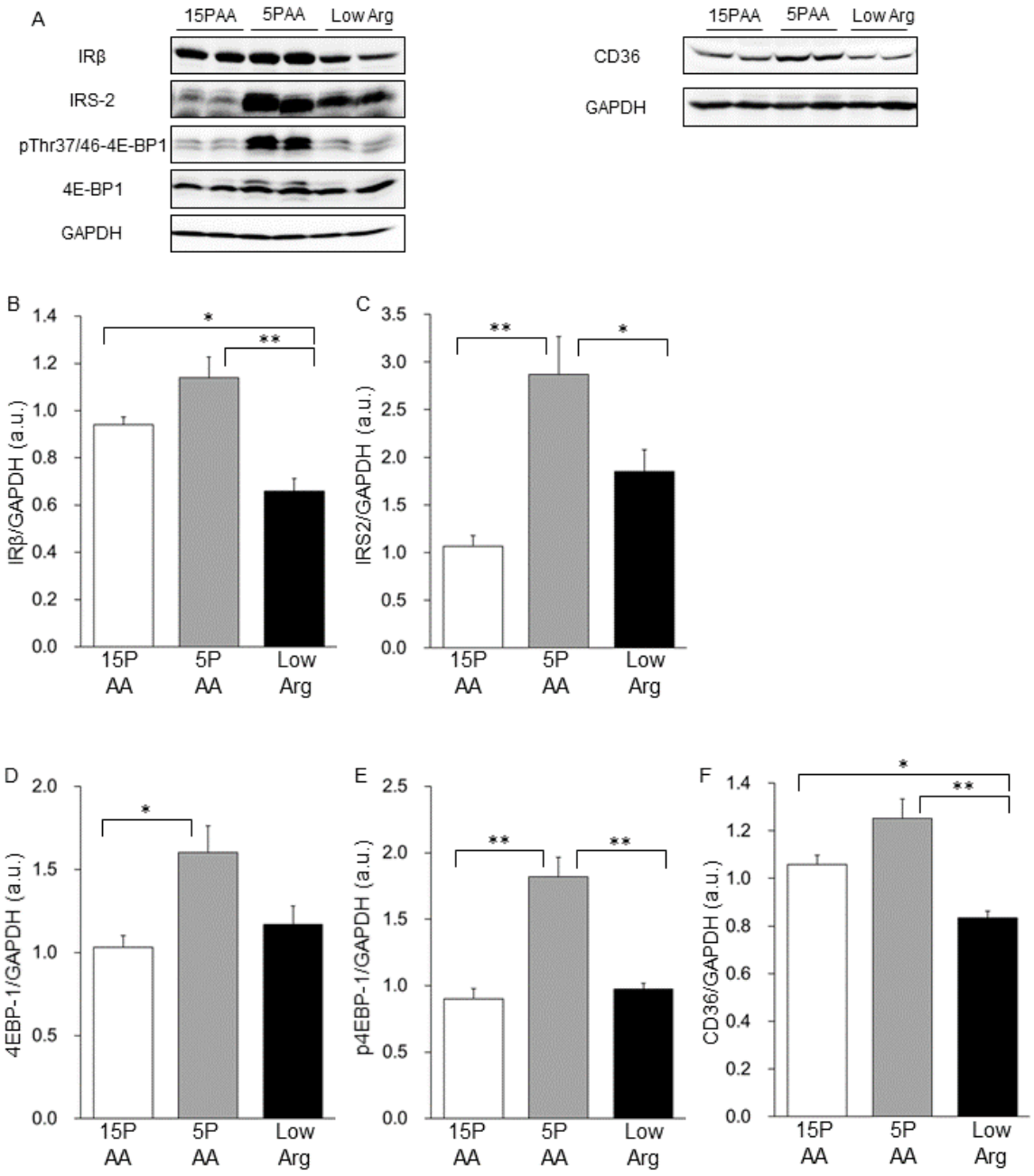

Figure 4

Figure 4

Insulin signaling-related protein levels in rats fed the low-protein and low-arginine diets. Six-week-old male Wistar rats were fed the 15PAA (n = 8), low Arg (n = 8), and 5PAA (n = 8) diet for 14 days. Whole-liver lysates were analyzed by immunoprecipitation and immunoblotting with antibodies against IRβ (B), IRS-2 (C), 4E-BP1 (D), phosphorylated 4E-BP1 (Thr37/46) (E), CD36 (F), and GAPDH as an internal control. Representative immunoblots (A) are shown. The values are the means ± standard errors. Asterisks indicate significant differences as assessed by one-way ANOVA with Bonferroni and Tukey–Kramer post-hoc tests (*p < 0.05; **p < 0.01).

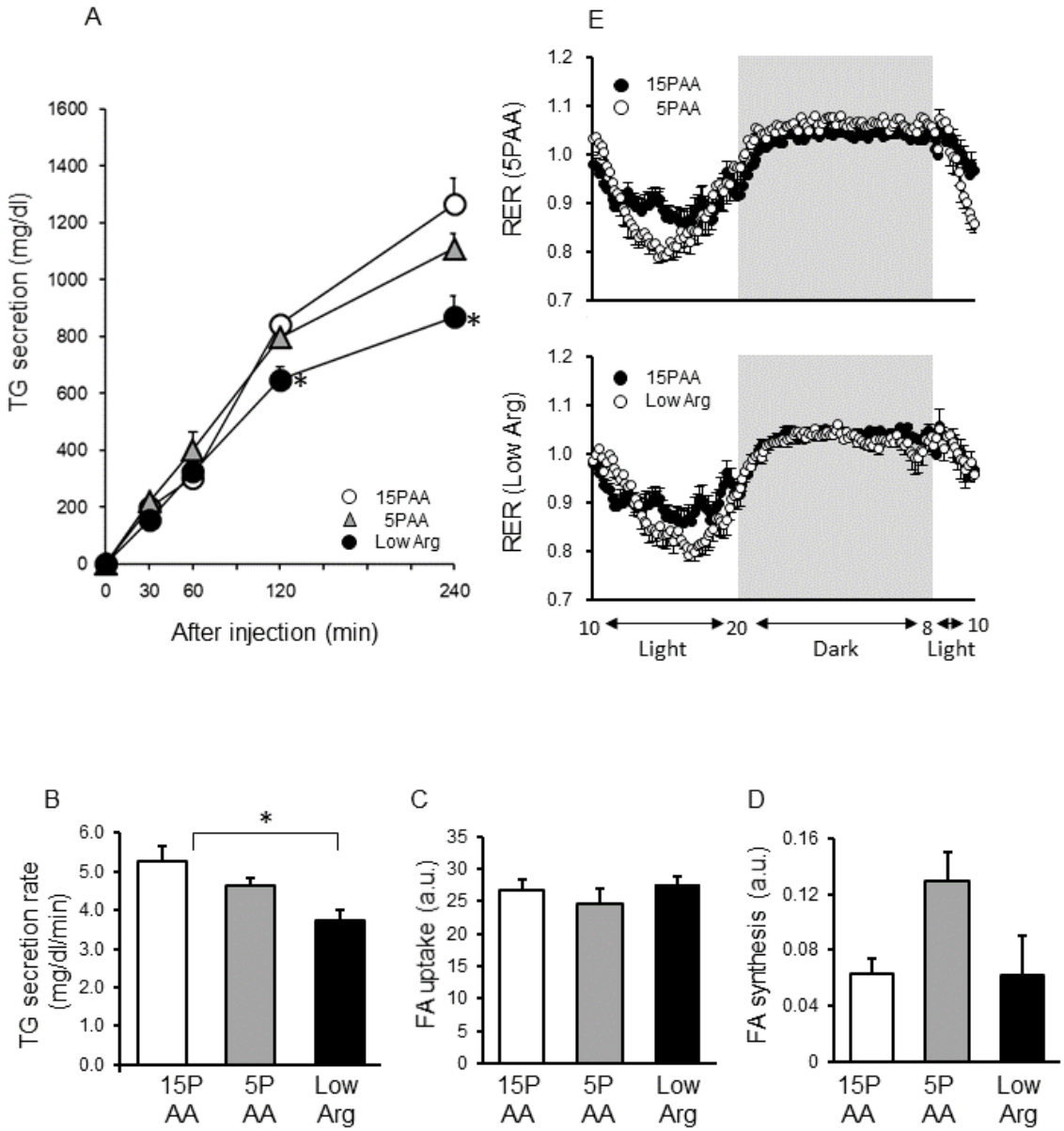

Figure 5

Figure 5

VLDL secretion from the livers, lipogenesis, fatty acid uptake, and respiratory exchange ratio (RER) of rats fed the low-protein and low-arginine diets. Rats were fed the 15PAA (n = 8), 5PAA (n = 8), and low Arg (n = 8) diet. After 5 h of feeding, rats were injected with tyloxapol (200 mg/kg), and then TG concentrations (A and C) were measured at 0, 30, 60, 120, and 240 min post-injection. The RER (B) of each rat (n = 6) was measured for 24 h. De novo lipogenesis assay (D) was performed by measuring palmitate isotopomers after intraperitoneal injection of D2O or H2O to rats fed the 15PAA (n = 8), 5PAA (n = 8), and low Arg (n = 8) for one night (18:00¬–10:00). Fatty acid uptake (E) was evaluated by incorporation of a fatty acid analog, BODIPY® FL C12, to the liver of rats fed 15PAA (n = 4), 5PAA (n = 4), and low Arg (n = 4) for one night (18:00–10:00), and fluorescence was measured in the liver lipid extractions. The values are the means ± standard errors. Asterisks indicate significant differences assessed by one-way ANOVA with Bonferroni and Tukey–Kramer post-hoc tests (*$p < 0.05$).

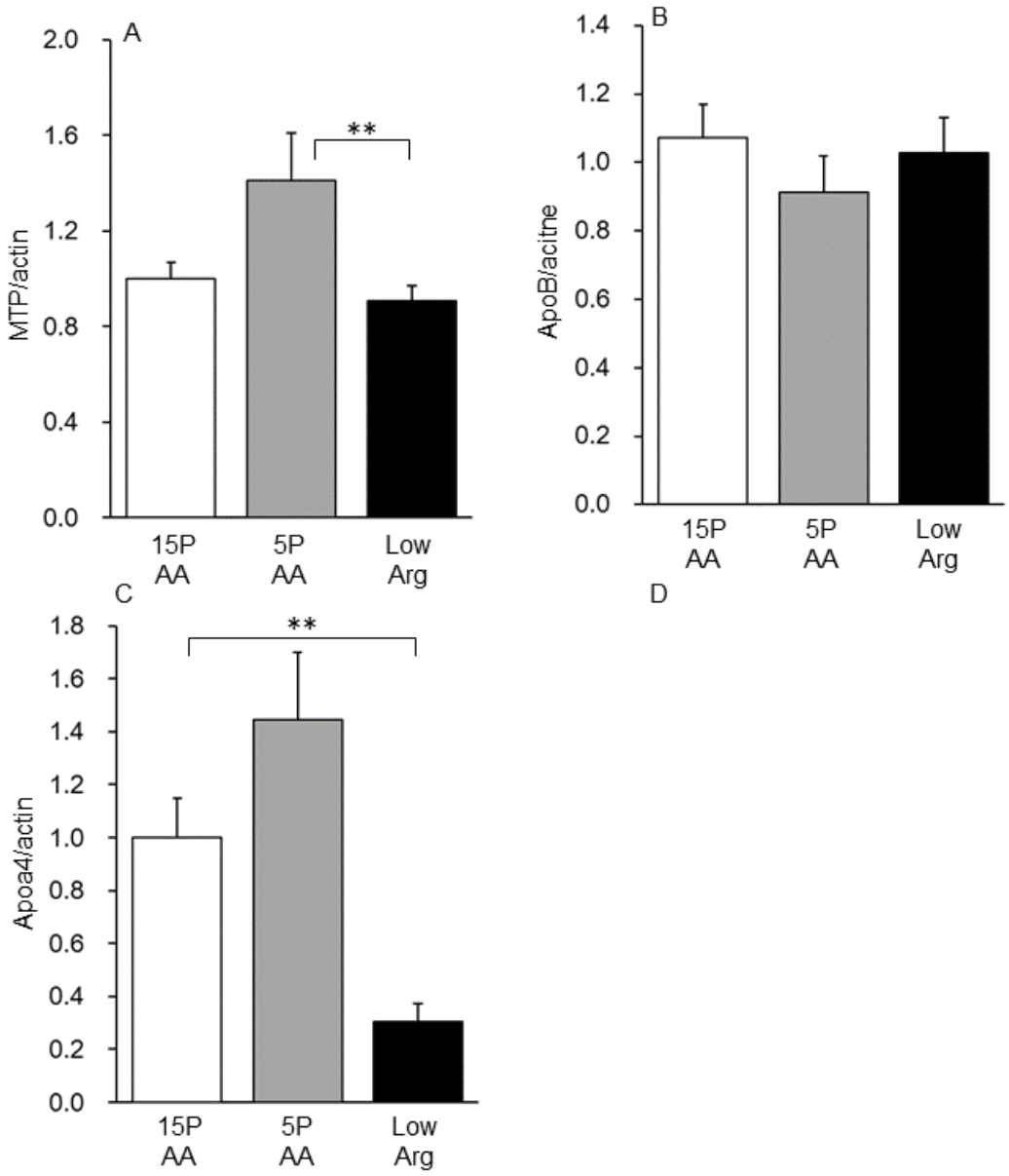

Figure 6

Figure 6

Effects of the low-protein and low-arginine diets on the expression of VLDL assembly-related genes. Six-week-old male Wistar rats were fed the 15PAA (n = 8), low Arg (n = 8), and 5PAA (n = 8) diet for 14 days. Total RNA was isolated from the liver and then subjected to quantitative RT-PCR. The transcript expression levels for Mttp (A), ApoB (B), and ApoA4 (C) are shown. Asterisks indicate significant

differences as assessed by one-way ANOVA with Bonferroni and Tukey–Kramer post-hoc tests (**p < 0.01).

Supplementary Files

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