Dietary protein restriction and lipid metabolism

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Title: Dietary protein dilution limits dyslipidemia in obesity through FGF21-driven fatty acid clearance.

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Abstract

Recent studies have demonstrated that dietary protein dilution (PD) can promote metabolic inefficiency and improve glucose metabolism. However, whether PD can promote other aspects of metabolic health, such as improve systemic lipid metabolism, and mechanisms therein, remains unknown. Mouse models of obesity such as high fat diet fed C57Bl/6N mice, and New Zealand Obese mice were fed normal (i.e. 20%P) and protein-dilute (i.e. 5%EP) diets. Fgf21-/- and Cd36-/- and corresponding littermate +/+ controls were also studied to examine gene-diet interactions. Here we show that chronic PD retards the development of hypertrigylceridemia and fatty liver in obesity, and that this relies on the induction of the hepatokine fibroblast growth factor 21 (FGF21). Furthermore, PD greatly enhances systemic lipid homeostasis, the mechanisms by which include FGF21-stimulated, and cluster of differentiation 36 (CD36) mediated, fatty acid clearance by oxidative tissues such as heart and brown adipose tissue. Taken together our pre-clinical studies demonstrate a novel nutritional strategy, as well as highlight a role for FGF21-stimulated systemic lipid metabolism, in combatting obesity-related dyslipidemia.

Keywords: nutrition, transport, metabolism, fatty liver

Introduction

Dietary protein dilution (PD), where dietary protein intake is reduced and replaced by calories from carbohydrate and/or fat, has emerged as an influential environmental variable affecting ageing and age-related disease such as type 2 diabetes (T2D). Indeed several studies have demonstrated that PD promotes longevity in rodents [1-3] and can retard age-related disease such as cancer [4] and type 2 diabetes [5]. Furthermore, protein intake rates have been positively associated with increased risk for type 2 diabetes and all-cause mortality in humans [6, 7].

While metabolic health pursuant to dietary PD are likely to involve numerous mechanisms, we recently demonstrated that dietary PD promotes glucose homeostasis in murine T2D via induction of the liver-derived hormone fibroblast-growth factor 21 (FGF21) [5]. Indeed, it has been reproducibly shown that FGF21 is an endocrine signal of PD in rodents [3, 5, 8, 9] and in humans [5, 8, 10, 11]. While the downstream mechanisms by which FGF21 improves metabolic health with PD are yet to be fully resolved, FGF21 does confer the increased energy expenditure and glucose disposal upon PD, at least in mice [5, 8], and heightened energy expenditure is thought to be a promising strategy to combat obesity-related metabolic disease [12, 13].

Other than worsened glucose homeostasis, another major metabolic pathology associated with obesity is dyslipidemia, which often ultimately manifests in such diseases as fatty liver disease [14] and atherosclerosis-related cardiovascular disease [15]. Importantly, in humans and non-human primates, FGF21 treatment has been shown to promote weight loss and improve biomarkers of dyslipidemia in obesity [16, 17]. Similar results have been found in mice, where FGF21 lowers plasma triglycerides [18, 19] by various mechanisms including the clearance of both TG-rich lipoproteins and non-esterified fatty acids into various tissues [19] as well as affecting adipose tissue lipolysis [20, 21], ultimately culminating in reduced arteriosclerosis [22]. Hence, here we tested whether and how PD improves lipid metabolism in mice and demonstrate that PD promotes fatty acid clearance in peripheral tissues, and reduces fatty liver in obesity, at least in part via FGF21.

Methods

Mouse experiments.

Male mice, aged 7 weeks upon arrival, were acclimated to the housing facility (12-12h light-dark cycle, 22-24°C) and fed their respective low fat experimental control diet for at least one week prior to experimentation. Mice used for experiments were C57Bl/6NCrl mice (#027, Charles River Laboratories), as well as New Zealand Black (NZB, #000993, NZB/BlNJ) and New Zealand Obese (NZO, #002105, NZO/HlLtJ, Jackson Laboratories) strains. We also studied diet-gene activity interactions in male Fgf21 -/- [20] and Cd36-/- [23] mice which were on the C57Bl/6 background.

For studies of protein restriction in the setting of HF diet-induced obesity, diet compositions are outlined in Supplementary Table 1. Following acclimation on the low-fat control diet, some C57Bl/6NCrl mice were switched to a diet containing low protein, or maintained on the control diet, and some mice were sacrificed after 3d, 14d, and 112d to examine the time-effect of diet treatment on metabolic phenotypes. We tested the effects of dietary protein restriction in the setting of murine obesity-driven insulin resistance and T2D using nutritional and polygenic experimental mouse models: C57Bl6/NCrl mice fed a high fat (HF) diet and the NZO mouse strain. While both models share obesity and insulin resistance in common, male NZO mice display hyperphagia and random fed hyperglycemia with reduced levels of physical activity and energy expenditure [24]. Following acclimation, C57Bl/6NCrl mice were switched to a diet containing low protein, or a HF diet containing either normal or low protein for 16 weeks prior to euthanizing (in the random fed state) by cervical dislocation, with subsequent trunk blood collection and tissue harvesting. For studies involving NZO vs lean NZB mice, half of the mice from each genotype were switched to a low protein diet for 10 weeks followed by tissue collection as described above. These studies have been previously reported [5].

We also examined diet-gene activity interactions in Fgf21+/+ and Fgf21-/- mice. For diet feeding studies, we conducted both low-fat diet and high-fat diet studies with either 20%E or 5%E from protein, as reported previously [5]. Furthermore, we examined diet-gene activity interactions in Cd36+/+ and Cd36-/- mice. Here we examined we fed mice with the diets for 4 weeks with the OLTT and tracer study conducted at week 2 and 4, respectively.

Inclusion criteria were mice of a certain age (i.e. 8-9 weeks) at the beginning of the experiment. Criteria for exclusion of mice from study groups were obvious infections/wounds which would impact on feeding behavior as well as metabolic profile. These criteria were pre-established. Animal experiments were conducted according to local, national, and EU ethical guidelines and approved by local regulatory authorities (Regierungspräsidium Karlsruhe, DEU), and conformed to ARRIVE guidelines.

Metabolic Phenotyping.

Blood samples were collected from the tail vein in the 5-6h fasted state (ZT6-7) for assessment of blood glucose and serum insulin levels for the calculation of an insulin sensitivity index (ISI; 1000/(glucose (mM) x insulin (pM)), which is a good surrogate measure of whole-body insulin action in mice [5].

An oral lipid (100µL/mouse olive oil delivered by oral gavage; O1514, Sigma-Aldrich, DEU) tolerance test (OLTT) was conducted in the fasted (i.e. 5-6h) state as we have conducted previously [25], with blood samples collected from a tail vein nick before and at 1, 3 and 6h thereafter.

To assess in vivo tissue-specific long-chain fatty acid uptake we adapted a method previously described [26] where we injected a small amount of R-^{3}H-bromopalmitic acid (dried and then dissolved in 10% mouse serum) into the tail vein of mice after at 3-4h fast. Blood samples were collected from the tail vein at 2 and 15 min after injection and mice were then sacrificed at 16 min by cervical dislocation and tissues were rapidly harvested, carefully weighed and frozen in LN_2. Known amounts of tissues were homogenized in Solvable (6NE9100, Perkin-Elmer) and tissue homogenates and serum samples were then subjected to liquid-scintillation counting ((Packard 2200CA Tri-Carb Liquid Scintillation analyzer; Packard Instruments). Tissue uptake rates were calculated based upon the weighted average of the specific activity of the administered ^{3}H-NEFA (cpm/mol), along with tissue counts (cpm) and mass (g), and time (h) of collection after administration.

Blood metabolites & hormones. Commercially available kits were used to measure serum non-esterified fatty acids (NEFA; NEFA-HR, Wako), glycerol/triglyceride (TG; TR-0100; Sigma-Aldrich), cholesterol (CH200, Randox), insulin (80-INSMS-E01, Alpco) and FGF21 (MF2100, R&D systems)

essentially according to manufacturer's instructions. All samples were loaded in order to fit within the assay range of the reagents supplied.

Tissue metabolite extraction and assay. For tissue lipid determinations, frozen tissue samples were pulverized, weighed and extracted. Lipid analyses were conducted according to established guidelines [27] using glycerol/triglyceride (TR-0100; Sigma-Aldrich) assay kits. Values were calculated as molar concentration per gram wet tissue as well as amounts per organ by multiplying by total tissue mass.

Tissue histology. For oil red O lipid staining, 5 μm cryosections of liver embedded in Tissue Tek OCT compound (Sakura, NL) were fixed in Baker's formol for 5 mins, followed by 5 mins in 60% isopropanol. Lipids were stained for 10 mins with Oil Red O (Sigma Aldrich). After brief rinses in 60% isopropanol and water, sections were counterstained with hematoxylin and coverslipped using Dako mounting medium (Agilent Technologies). For Sudan Red staining, cryo sections (5 μm) were cut and stained with Sudan Red (0.25% Sudan IV in ethanolic solution).

Study design criteria and statistical analyses

Based upon preliminary data showing the expected effect size of major outcome variables, a power analysis was conducted in order to determine the minimal number of animals to be used for each experiment. For genotype difference studies, offspring mice from Het x Het breeding's were initially randomized to each experiment group. Afterwards, counterbalancing was done in order to realize equal sample sizes per experimental group. When conducting studies, the investigators were aware of which mouse was in which experimental group due to prior genotyping and allocation. However, the technical assistants involved in the studies were blinded.

Statistical analyses were performed using t-tests (two-sided), or 2-way analysis of variance (ANOVA) with or without repeated measures, where appropriate, with Holm-Sidak-adjusted post-tests. All analyses were carried out with SigmaPlot v.13 software (Systat Software GmbH, DEU).

Results

As we previously demonstrated that dietary protein dilution (PD) improves glucose homeostasis [5], here we chose to examine another important outcome of obesity-related metabolic dysfunction, namely dyslipidemia. To investigate this further, we conducted a time-course study to distinguish the acute and chronic effects of PD, and demonstrate that while there were rapid effects of PD on body mass and composition, with a rapid and sustained lower accrual of body mass, lean mass, and fat mass over time in PD fed mice (Fig. 1A-C). Furthermore, the difference in fat mass was more pronounced over time, and eventually became a major influence on total body mass. The effects of PD on systemic glucose homeostasis, as determined by an insulin sensitivity index (Fig. 1D) based upon fasting insulin and glucose levels (Fig. 1E-F), are rapid and sustained. Similarly, serum FGF21 levels were also higher (Fig. 1G), and the effects of the diet were rapid and sustained. Similarly, serum triglyceride (Fig. 1H), but not non-esterified fatty acids (NEFA, Fig. S1A) or cholesterol (Fig. S1B), are reduced rapidly upon PD feeding. On the other hand, liver triglyceride levels were profoundly lower, but only after chronic PD feeding (Fig, 1I; Fig. S1C-D).

Given that serum and liver triglyceride levels are thought to be a pre-disposing factor for cardiovascular and non-alcoholic fatty liver disease, respectively [14, 15], particularly in the context of obesity-driven metabolic complications, we then investigated whether PD would retard the effects of obesity to promote a dyslipidemic phenotype. To this end, we employed two mouse models of obesity, a dietary model (i.e. high fat diet feeding) and a polygenetic model of hyperphagia-induced obesity [5]. Chronic PD in both of these models reduced both blood serum (Fig. 2A-B) and liver (Fig. 2C-E; Fig. S2A) triglyceride levels. The effects of PD were particularly pronounced in the obese models, with triglyceride levels returning to those of the control diet feeding, independent of body mass and fatness [5].

To then obtain insight into the mechanisms by which PD mediates this improved lipid metabolism, we employed an oral lipid tolerance test, and could demonstrate that PD feeding enhances lipid homeostasis as reflected by a substantially blunted serum triglyceride (Fig. 2F) and NEFA (Fig. 2G) excursion following the lipid dose. While this enhanced lipid tolerance may be explained by several factors, we chose to investigate whether there is enhanced NEFA clearance, which ultimately might also explain the lower serum triglyceride levels as well. Of note, *in vivo* NEFA uptake was enhanced in liver, heart, as well as brown (BAT) and white (WAT) adipose tissues, but not skeletal muscle (Fig. 2H; Fig. S2B).

As the metabolic response to PD is partially explained by an induction and secretion of liver-derived fibroblast growth factor 21 (FGF21) [5, 8] we chose to investigate whether FGF21 was involved in the lipid metabolic phenotype we observe with PD (Fig. 1-2). Indeed, studies in rodents [18, 19, 28] and humans [16, 17] have demonstrated that FGF21 *per se* has an effect on lipid metabolism and can reduce biomarkers of lipid metabolic dysfunction in obesity. In response to chronic PD feeding, the reductions in both serum and liver triglyceride levels were abrogated in the absence of FGF21, both in response to low (Fig. 3A-B) and high (Fig. 3C-E) fat diet feeding, results which corroborate former studies of mice showing that FGF21 can retard hepatic steatosis [29-33]. This aligned with an abrogation of downregulated triglyceride stores upon PD in BAT, skeletal muscle and heart (Fig. S3A-C), suggesting a clear systemic effect of PD to affect lipid homeostasis via FGF21. In line with this, in further relatively acute diet administration experiments, the effects of PD to enhance lipid homeostasis during an oral lipid challenge were completely absent in Fgf21 knockout mice (Fig. 3F-G). To understand which tissues contribute to this effect we then performed in vivo NEFA uptake tracer experiments, and could show that while the upregulation in NEFA clearance into BAT, heart and inguinal WAT (iWAT) was absent in Fgf21 KO mice, the uptake in liver and abdominal WAT (aWAT) was conversely enhanced (Fig. 3H & S3D).

Since FGF21 is known to promote enhanced systemic lipid disposal via CD36 [19], and CD36 is known to promote FA uptake in highly oxidative tissues such as heart and BAT [34] of which we observe to be affected by PD (Fig. 2), we then investigated whether the effects of PD to promote enhanced lipid metabolism relies on FA clearance by CD36. To this end we employed Cd36 knockout mice, and could observe that the effect of PD to lower serum triglyceride levels was completely abrogated with systemic CD36 loss-of-function (Fig. 4A). Furthermore, the effects of PD to enhance lipid homeostasis during an oral lipid challenge were completely absent in Cd36 knockout mice (Fig. 4B-C). To understand the tissues which contribute to this effect we then performed in vivo NEFA uptake tracer experiments, and could show that while the upregulation in NEFA clearance in BAT, heart and iWAT was absent in Cd36 KO mice, the uptake in liver was conversely enhanced (Fig. 4D & S4).

Discussion

Here we demonstrate that feeding a protein diluted diet (PD) to mice reduces obesity-related hypertriglyceridemia and hepatosteatosis (Fig. 2). Since these parameters are closely linked to obesity-related pathologies such as cardiovascular disease [15] and non-alcoholic steatohepatitis [14] and hepatocellular carcinoma [35], feeding a protein reduced diet may be a potential nutritional

strategy to retard the development of such diseases. Clearly this is a potential area for future investigations. Importantly, our studies align with prior studies demonstrating that single essential amino acid restriction affects systemic lipid homeostasis [36], however whether the amino acid component of dietary protein, and if so, which particular amino acid(s), is involved remain elusive. This is an important area of investigation, and an in silico predictive approach similar to that taken by Piper and colleagues [37] may lead us in the right direction for deciphering key nutrient-phenotype interactions.

In terms of the molecular mechanisms, we demonstrated that the induction of FGF21 [5] upon PD, is related to and it required for the effects of PD to retard hypertriglyceridemia and hepatosteatosis (Fig. 3). This is in agreement with prior studies demonstrating that FGF21 can lower hypertriglyceridemia in both rodents [18, 19] and humans [16, 17] and can retard the development of hepatosteatosis and non-alcoholic steatohepatitis in rodent models [29-31, 33, 38]. In terms of the mechanisms by which FGF21 can revert hypertriglyceridemia and ectopic lipid deposition in obesity, we show that PD feeding increases systemic lipid clearance and promotes non-esterified fatty acid uptake into various tissues such as heart and brown adipose tissue, of which is dependent on FGF21 (Fig. 2 & 3). This result is partly in line with the results of Sheja and colleagues [19], who demonstrated that FGF21 can promote TG-rich lipoprotein and FA uptake in adipose tissues of mice and our former studies demonstrating that PD promotes increased energy expenditure and systemic FA oxidation via FGF21 [5]. Furthermore, we demonstrated that the effects of PD to enhance lipid tolerance and tissue NEFA clearance were dependent on CD36 (Fig. 4). Importantly, this result was very similar to those from the experiments using FGF21 knockout mice (Fig. 3), strengthening the prior evidence [19] that FGF21 stimulates lipid clearance thorough mechanisms involving CD36. While the precise mechanisms by which FGF21 stimulates FA uptake remain elusive in the context of PD, it is likely that FGF21 operates via an indirect action via the brain and the sympathetic nervous system [39-42] or via direct action on adipose tissues [43-45] and perhaps liver [46]. Indeed, FGF21 has been shown to stimulate CD36 translocation to the surface membranes of adipose tissue in vivo [19], and, along with other functions, is known to operate as a facilitative peptide transporter of long-chain fatty acids at cell-surface membranes [47]. On the other hand, others have reported that CD36 is necessary for coenzyme Q uptake and hence proper mitochondrial function in tissues [48], which thus may explain the former [34] and current data that CD36 is responsible for FA disposal in highly oxidative tissues, particularly when they are stimulated to enhance FA oxidation.

As the major metabolic source of the increase in hepatocellular triglycerides in fatty liver disease results from direct uptake of adipose-derived NEFAs [14, 49, 50], we propose that the reduction in

fatty liver and perhaps improved glucose metabolism [51] by chronic PD may relate to enhanced FA clearance and burning by oxidative tissues such as heart and BAT. Indeed, when enhanced clearance by such tissues was disrupted by either FGF21 or CD36 loss-of function, FA clearance by the liver was exacerbated (Fig. 3 & 4). Furthermore, our previous work showed that glucose uptake into these tissues as well as whole-body glucose and fatty acid oxidation were enhanced in dietary PD [5], suggesting a general switch away from nitrogenous- and toward fat- and carbohydrate-carbon fuel oxidation. Lastly, as increased serum and liver triglyceride are reliable biomarkers for metabolic disease such as fatty liver disease and atherosclerosis, our results point towards the potential application of PD as a prophylaxis for such obesity-driven disease outcomes including non-alcoholic steatohepatitis and cardiovascular dysfunction.

In summary, dietary protein dilution retards dyslipidemia and hepatosteatosis in obesity and this may relate to enhanced FGF21-mediated lipid clearance in oxidative tissues.

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AM, AZ, & AJR performed experiments and analyzed samples. MH conducted and coordinated the liver Sudan red staining’s. SA-B & HGA provided the Cd36 knockout mice for experimentation. AV & SH contributed to experimental advice and data discussions as well as infrastructure and support. AM & AJR analyzed data. AJR wrote the manuscript and AJR, AM, SA-B, HGA, SH and MH edited the manuscript. All authors declare no conflict of interest pertaining to this work.

Figure legends

Figure 1. Time-effects of dietary protein dilution on body composition and metabolic parameters in mice.

(A): Body mass after 3d, 14d or 112d in mice fed a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. N = 5-6/group.

(B): Lean mass as determined by ECHO-MRI from mice as in (A).

(C): Fat mass as determined by ECHO-MRI from mice as in (A).

(D): Insulin sensitivity index (ISI(f)) from mice as in (A).

(E): Fasting blood serum insulin levels from mice as in (A).

(F): Fasting blood glucose levels from mice as in (A).

(G): Blood serum fibroblast growth factor 21 (FGF21) levels from mice as in (A).

(H): Serum triglyceride (TG) levels from fibroblast growth factor 21 (Fgf21) sufficient (Fgf21+/+) and deficient (Fgf21-/-) mice fed a low-fat (LFD) diet (HFD) with 20% protein (CD) or 5% calories from protein (PD) for 12 weeks. N = 7-8/group.

(I): Liver triglyceride (TG) amounts from mice as in (A).

Data are mean ± SEM. A-K: Significant effect of time: *P<0.05, **P<0.01, ***P<0.001. Significant effect between CD and PD: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

Figure S1. Time-effects of dietary protein dilution on body composition and metabolic parameters in mice.

(A): Blood serum non-esterified fatty acids (NEFA) levels from mice after 3d, 14d or 112d in mice fed a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. N = 5-6/group.

(B): Blood serum cholesterol levels from mice as in (A).

(C): Liver triglyceride (TG) levels per unit liver mass from mice as in (A).

(D): Liver mass from mice as in (A).

Data are mean ± SEM. A-K: Significant effect of time: *P<0.05, **P<0.01, ***P<0.001. Significant effect between CD and PD: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

Figure 2. Dietary protein dilution reduces serum and liver triglyceride levels in obesity and enhances systemic lipid homeostasis.

(A): Serum triglyceride (TG) levels from mice fed a low-fat (LFD) or high-fat diet (HFD) with 20% protein (CD) or 5% calories from protein (PD) for 16 weeks. N = 7-8/group.

(B): Serum triglyceride (TG) levels from lean New Zealand Black (NZB) or New Zealand Obese (NZO) mice fed a high-carbohydrate diet with 20% calories protein (CD) or 5% calories from protein (PD) for 10 weeks. N = 6-8/group.

(C): Liver triglyceride (TG) amounts from mice as in (C).

(D): Liver triglyceride (TG) amounts from mice as in (D).

(E): Neutral lipid (i.e. Oil-red-O) staining of liver cryosections from mice as in (C). Shown are 2 representative images of different individual mice. Scale bar: 50µm.

(F): Blood serum triglyceride levels during an oral lipid tolerance test in C57Bl/6N mice after 1 week of pre-feeding a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. N = 4/group.

(G): Blood serum NEFA levels from mice as in (H).

(H): Tissue long-chain fatty acid (LCFA) uptake rate during the *in vivo* fatty acid uptake experiment from C57Bl/6N mice pre-treated for 2 weeks with a high-carbohydrate diet with 20% calories protein (CD) or 5% calories from protein (PD). BAT: brown adipose tissue; GCM: gastrocnemius complex muscle; iWAT: inguinal white adipose tissue; aWAT: abdominal WAT. N=4/group.

Data are mean ± SEM. A-H: Significant effect of HFD/genotype: *P<0.05, **P<0.01, ***P<0.001. Significant effect between CD and PD: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

Figure S2. Dietary protein dilution reduces serum and liver triglyceride levels in obesity and enhances systemic lipid homeostasis.

(A): Neutral lipid (i.e. Oil-red-O) staining of liver cryosections from lean New Zealand Black (NZB) or New Zealand Obese (NZO) mice fed a high-carbohydrate diet with 20% calories protein (CD) or 5% calories from protein (PD) for 10 weeks. Shown are 3 representative images of different individual mice. Scale bar: 50µm.

(B): Non-esterified fatty acid (NEFA) specific activity of ^{3}H-bromopalmitic acid during the *in vivo* fatty acid uptake experiment from mice pre-treated with a high-carbohydrate diet with 20% calories protein (CD) or 5% calories from protein (PD). N=4/group.

Data are mean ± SEM. Significant effect between CD and PD: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

Figure 3. Dietary protein dilution retards obesity-driven lipid metabolic dysfunction via FGF21-driven NEFA clearance.

(C): Serum triglyceride (TG) levels from fibroblast growth factor 21 (Fgf21) sufficient (Fgf21+/+) and deficient (Fgf21-/-) mice fed a high-fat diet (HFD) with 20% protein (CD) or 5% calories from protein (PD) for 12 weeks. N = 7-8/group.

(D): Liver triglyceride (TG) amounts from mice as in (C).

(E): Neutral lipid (i.e. Sudan red) staining of liver cryosections from mice as in (C). Shown are 3 representative images of different individual mice. Scale bar: 50µm.

(F): Blood serum triglyceride (TG) levels during an oral lipid tolerance test (OLTT) in mice as in (A) but treated with the diets for 1 week.

(G): Serum non-esterified fatty acids (NEFA) from the same mice as in (F).

(H): Tissue uptake rates of long-chain fatty acids (LCFA) by ^{3}H-bromopalmitic acid tracing in the same mice as in (F) but treated with the diets for 2 weeks. N=5/group.

Data are mean ± SEM. A-H: Significant effect of dietary protein: *P<0.05, **P<0.01, ***P<0.001. Significant effect of genotype: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001. See also Figure S2.

Figure S3. Dietary protein dilution retards obesity-driven lipid metabolic dysfunction via FGF21-driven NEFA clearance.

(A): Brown adipose tissue (BAT) triglyceride amounts from fibroblast growth factor 21 (Fgf21) sufficient (Fgf21+/+) and deficient (Fgf21-/-) mice fed a low-fat (LFD) diet with 20% protein (CD) or 5% calories from protein (PD) for 12 weeks. N = 7-8/group.

(B): Gastrocnemius skeletal muscle (GCM) triglyceride amounts from mice as in (A).

(C): Heart triglyceride amounts from mice as in (A).

(D): Non-esterified fatty acid (NEFA) specific activity of ^{3}H-bromopalmitic acid during the *in vivo* fatty acid uptake experiment from fibroblast growth factor 21 (Fgf21) sufficient (Fgf21+/+) and deficient (Fgf21-/-) mice fed a low-fat diet (LFD) with 20% protein (CD) or 5% calories from protein (PD). N=5/group.

Data are mean ± SEM. A-D: Significant effect of dietary protein: *P<0.05, **P<0.01, ***P<0.001. Significant effect of genotype: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001. See also Figure 2.

Figure 4. Dietary protein dilution enhances lipid metabolism and NEFA clearance by heart and BAT via CD36.

(A): Blood serum triglyceride (TG) levels from cluster of differentiation 36 (Cd36) sufficient (Cd36+/+) and deficient (Cd36-/-) mice fed a low-fat diet (LFD) with 20% protein (CD) or 5% calories from protein (PD) for 4 weeks. N = 7-8/group.

(B): Blood serum triglyceride (TG) levels during an oral lipid tolerance test (OLTT) in mice as in (A) after 2 weeks diet treatment.

(C): Blood serum non-esterified fatty acid (NEFA) levels from mice as in (B).

(D): Tissue uptake rates of long-chain fatty acids (LCFA) by ^{3}H-bromopalmitic acid tracing in mice as in (A) after 4 weeks diet treatment. N=3-4/group.

Data are mean ± SEM. A-D: Significant effect of dietary protein: *P<0.05, **P<0.01, ***P<0.001. Significant effect of genotype: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001. See also Figure S3.

Figure S4. Dietary protein dilution enhances lipid metabolism and NEFA clearance by heart and BAT via CD36.

Non-esterified fatty acid (NEFA) specific activity of ^{3}H-bromopalmitic acid during the *in vivo* fatty acid uptake experiment from cluster of differentiation 36 (Cd36) sufficient (Cd36+/+) and deficient (Cd36-/-) mice fed a low-fat diet (LFD) with 20% protein (CD) or 5% calories from protein (PD). N=3-4/group.

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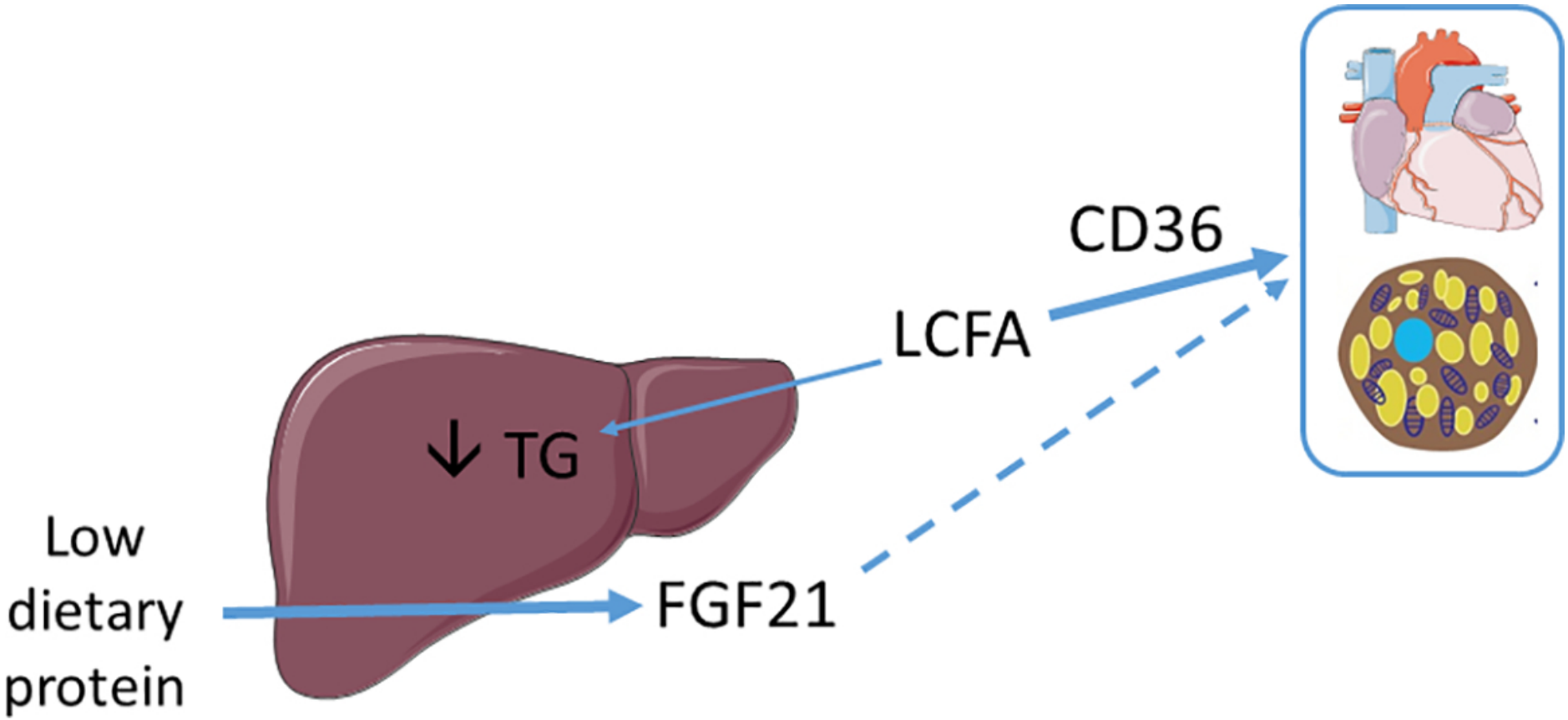
Highlights

- Dietary protein dilution lowers serum and liver triglycerides and improves systemic fatty acid handling, especially in obese mice
- The effects of dietary protein dilution require FGF21
- Dietary protein dilution enhances CD36-mediated fatty acid clearance in brown adipose tissue and heart



Graphics Abstract

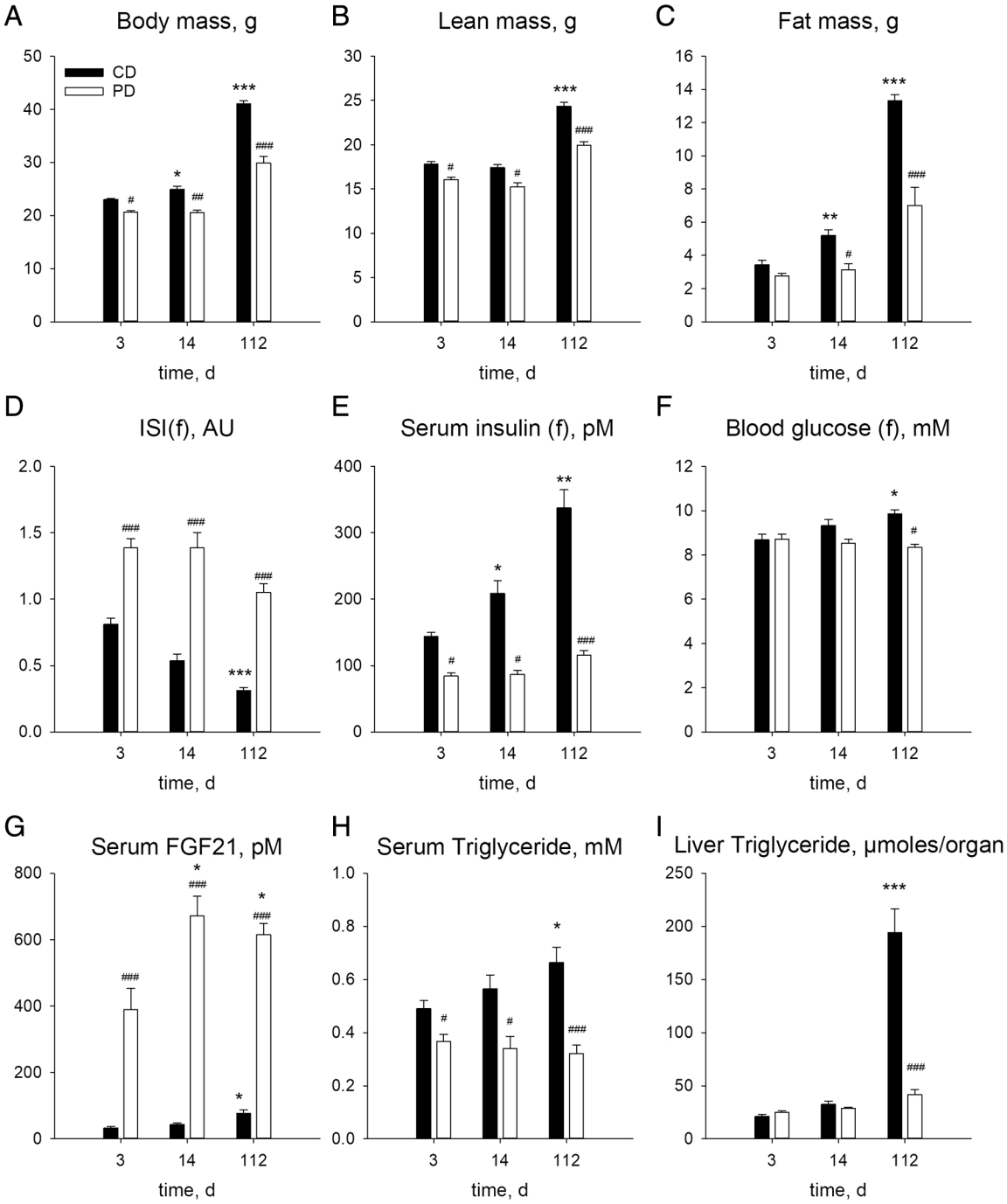

A
Body mass, g
CD
PD
time, d
B
Lean mass, g
time, d
C
Fat mass, g
time, d
D
ISI(f), AU
time, d
E
Serum insulin (f), pM
time, d
F
Blood glucose (f), mM
time, d
G
Serum FGF21, pM
time, d
H
Serum Triglyceride, mM
time, d
I
Liver Triglyceride, µmoles/organ
time, d

Figure 1

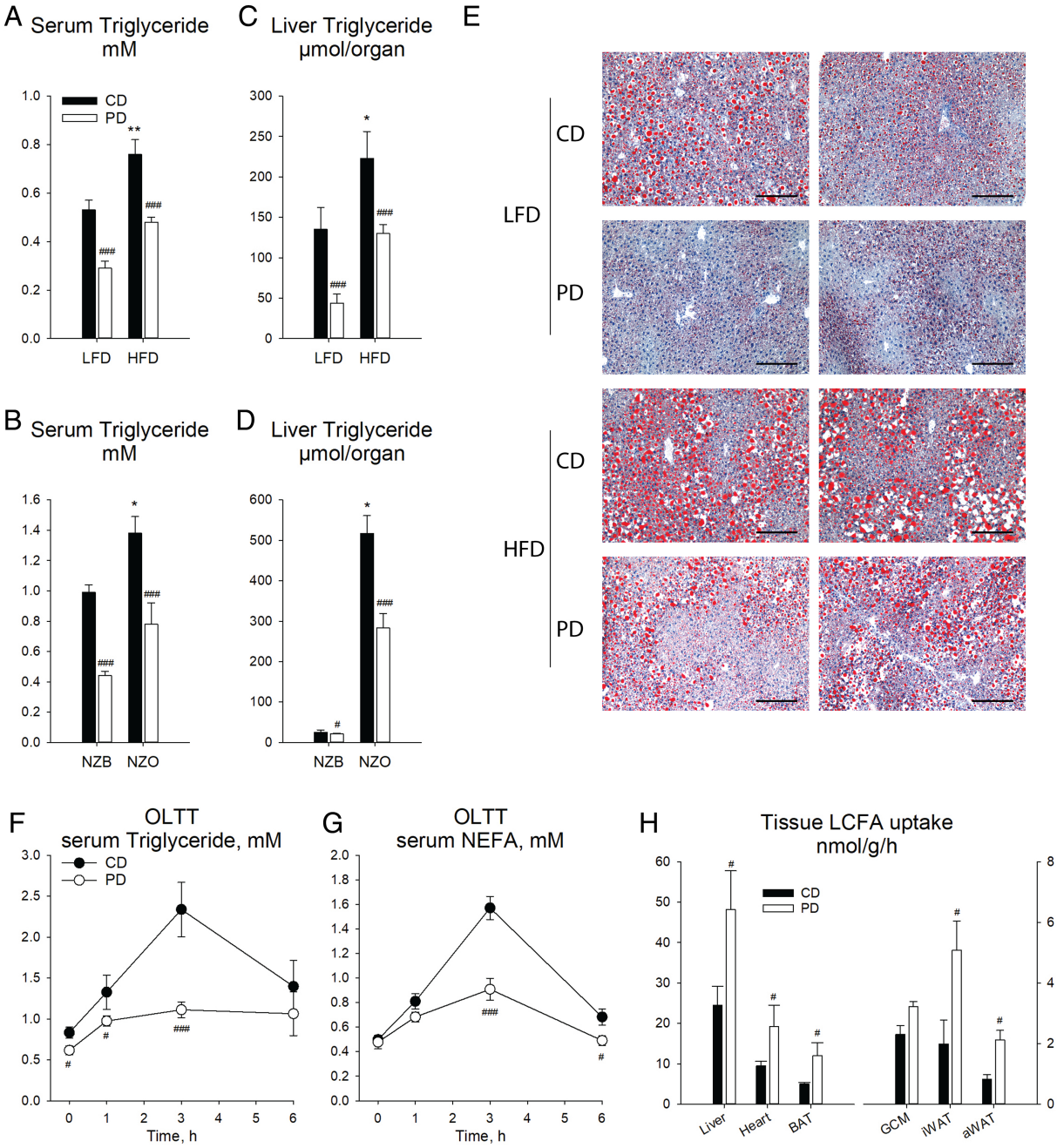
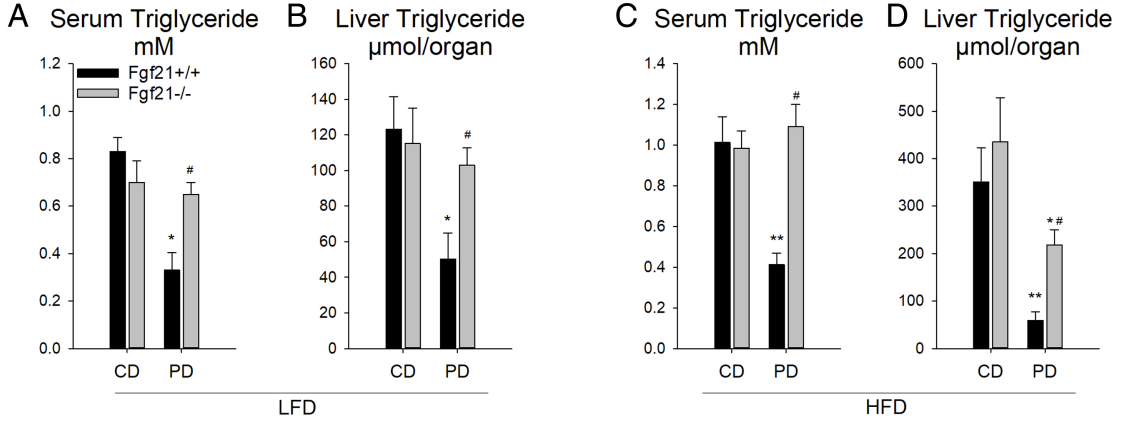

A
Serum Triglyceride
mM
CD
PD
LFD
HFD
B
Serum Triglyceride
mM
NZB
NZO
C
Liver Triglyceride
µmol/organ
D
Liver Triglyceride
µmol/organ
E
LFD
HFD
CD
PD
F
OLTT
serum Triglyceride, mM
Time, h
G
OLTT
serum NEFA, mM
Time, h
H
Tissue LCFA uptake
nmol/g/h
Liver
Heart
BAT
GCM
iWAT
aWAT

Figure 2


A
Serum Triglyceride
mM
Fgf21+/+
Fgf21-/-
CD
PD
LFD
B
Liver Triglyceride
μmol/organ
C
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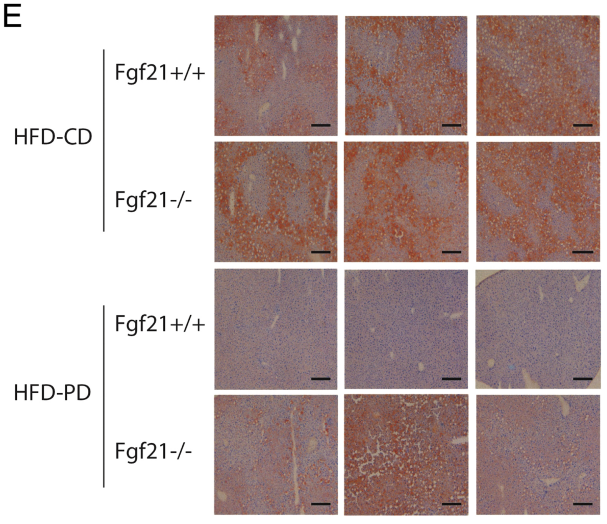
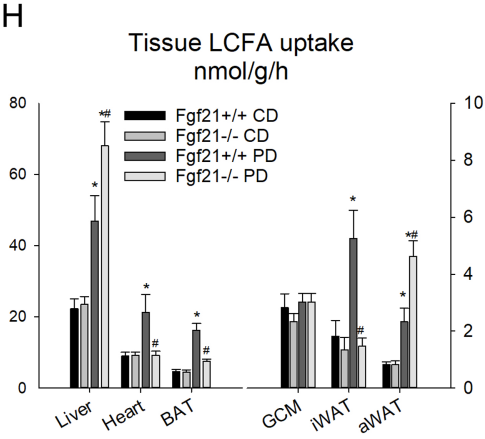
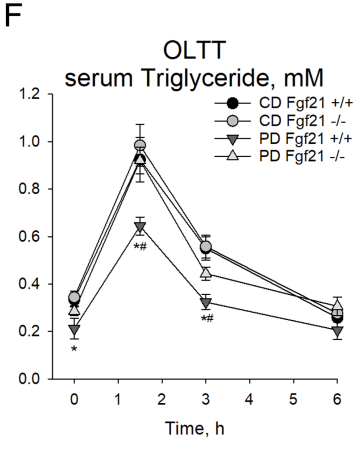
mM
HFD
D
Liver Triglyceride
μmol/organ


E
HFD-CD
HFD-PD
Fgf21+/+
Fgf21-/-


H
Tissue LCFA uptake
nmol/g/h
Fgf21+/+ CD
Fgf21-/- CD
Fgf21+/+ PD
Fgf21-/- PD
Liver
Heart
BAT
GCM
iWAT
aWAT


F
OLTT
serum Triglyceride, mM
CD Fgf21 +/+
CD Fgf21 -/-
PD Fgf21 +/+
PD Fgf21 -/-
Time, h

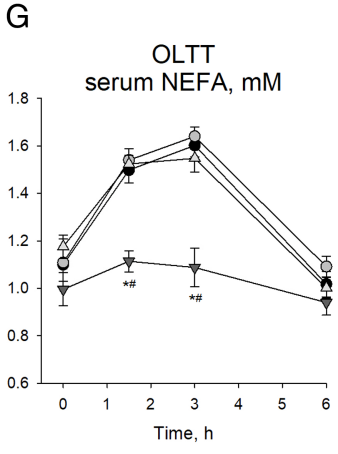

G
OLTT
serum NEFA, mM
Time, h

Figure 3

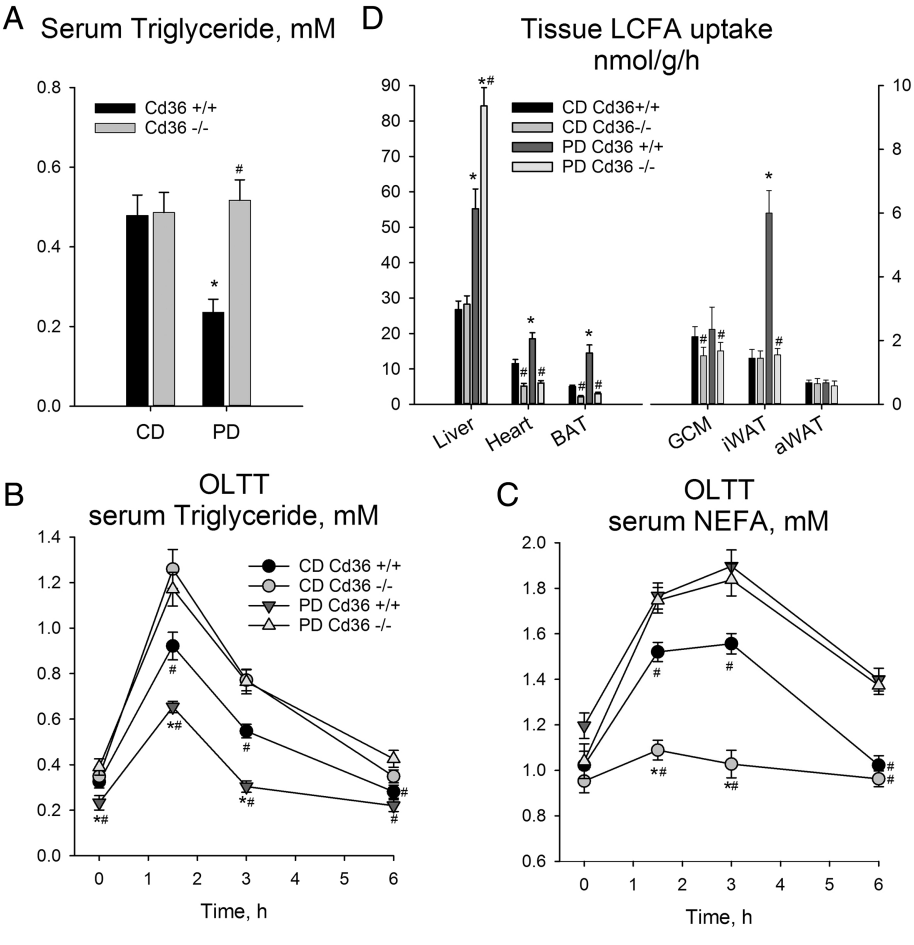

A
Serum Triglyceride, mM
Cd36 +/+
Cd36 -/-
CD
PD
D
Tissue LCFA uptake
nmol/g/h
CD Cd36+/+
CD Cd36-/-
PD Cd36 +/+
PD Cd36 -/-
Liver
Heart
BAT
GCM
iWAT
aWAT
B
OLTT
serum Triglyceride, mM
CD Cd36 +/+
CD Cd36 -/-
PD Cd36 +/+
PD Cd36 -/-
Time, h
C
OLTT
serum NEFA, mM
Time, h

Figure 4

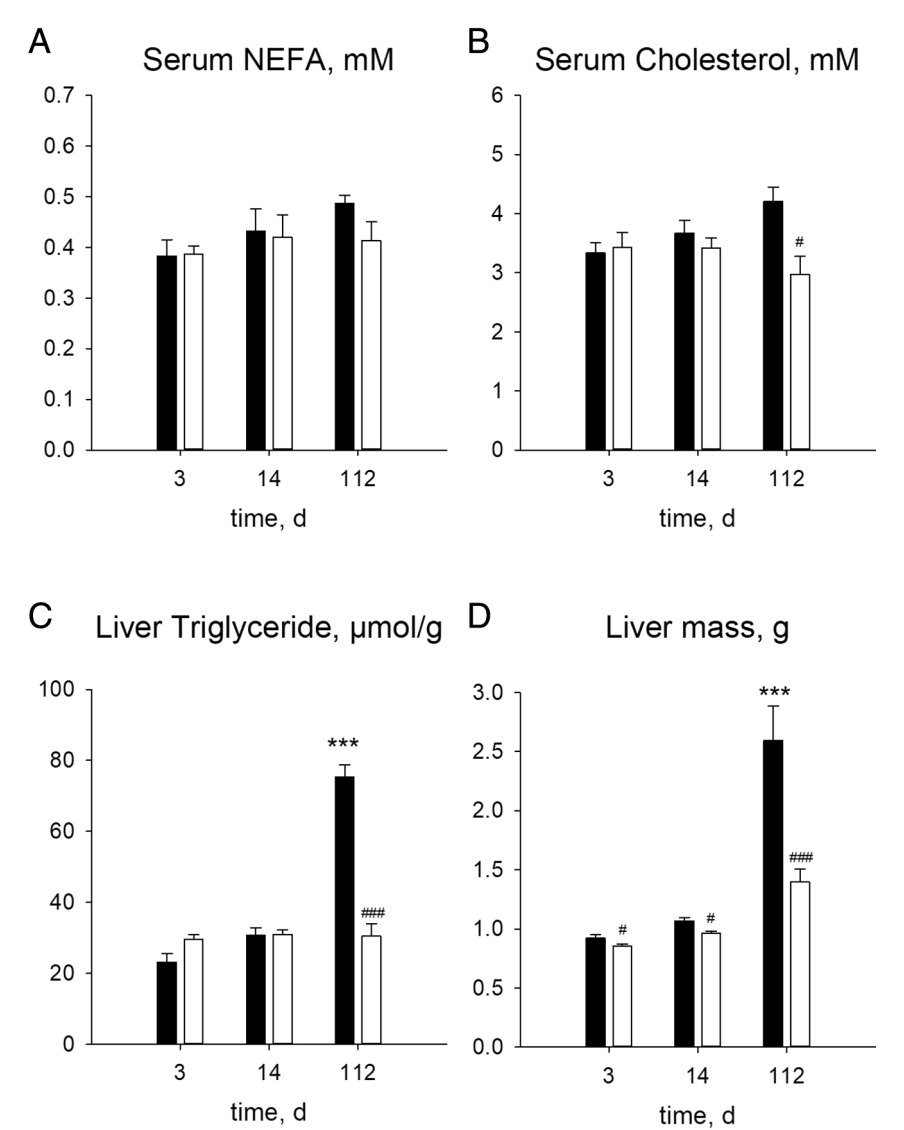

A
Serum NEFA, mM
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
3
14
112
time, d
B
Serum Cholesterol, mM
6
5
4
3
2
1
0
#
3
14
112
time, d
C
Liver Triglyceride, µmol/g
100
80
60
40
20
0
***
###
3
14
112
time, d
D
Liver mass, g
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
#
#
###
3
14
112
time, d

Figure 5

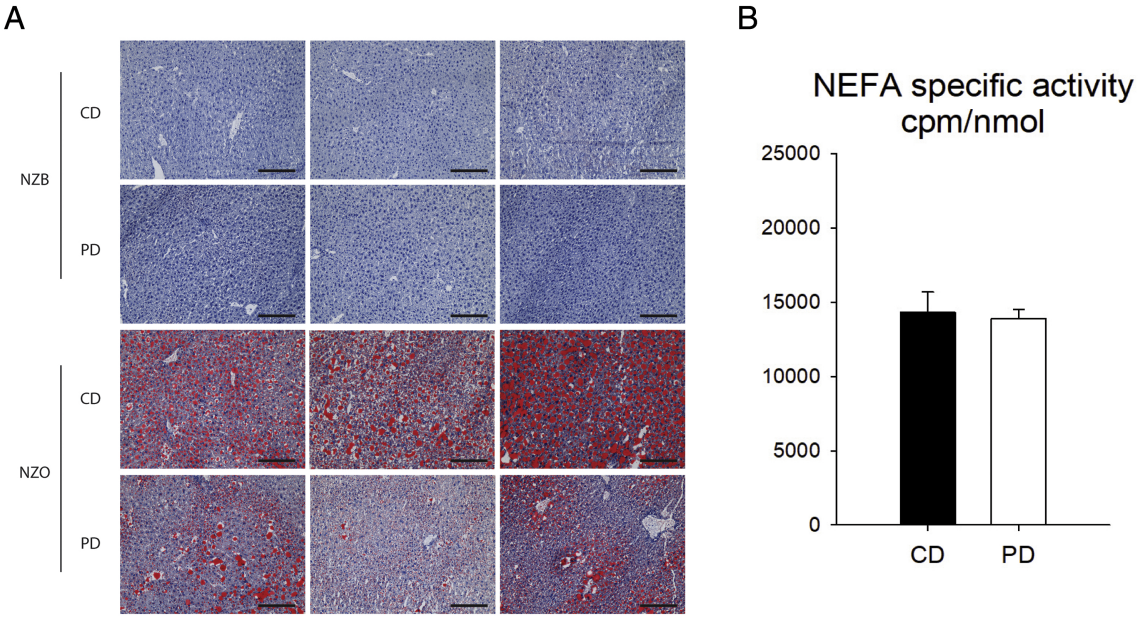

A
NZB
CD
PD
NZO
CD
PD
B
NEFA specific activity
cpm/nmol
25000
20000
15000
10000
5000
0
CD
PD

Figure 6

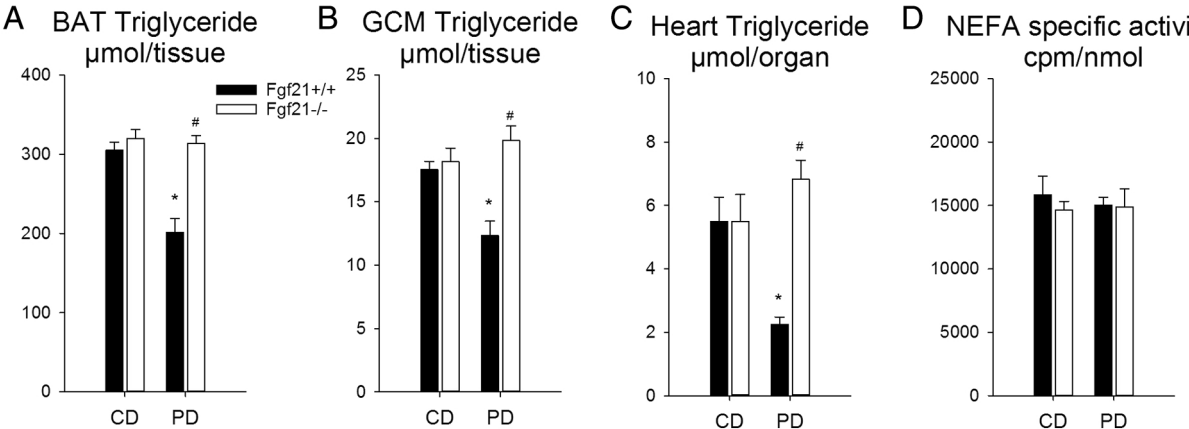

A
BAT Triglyceride
µmol/tissue
400
300
200
100
0
Fgf21+/+
Fgf21-/-
*
#
CD
PD
B
GCM Triglyceride
µmol/tissue
25
20
15
10
5
0
*
#
CD
PD
C
Heart Triglyceride
µmol/organ
10
8
6
4
2
0
*
#
CD
PD
D
NEFA specific activity
cpm/nmol
25000
20000
15000
10000
5000
0
CD
PD

Figure 7

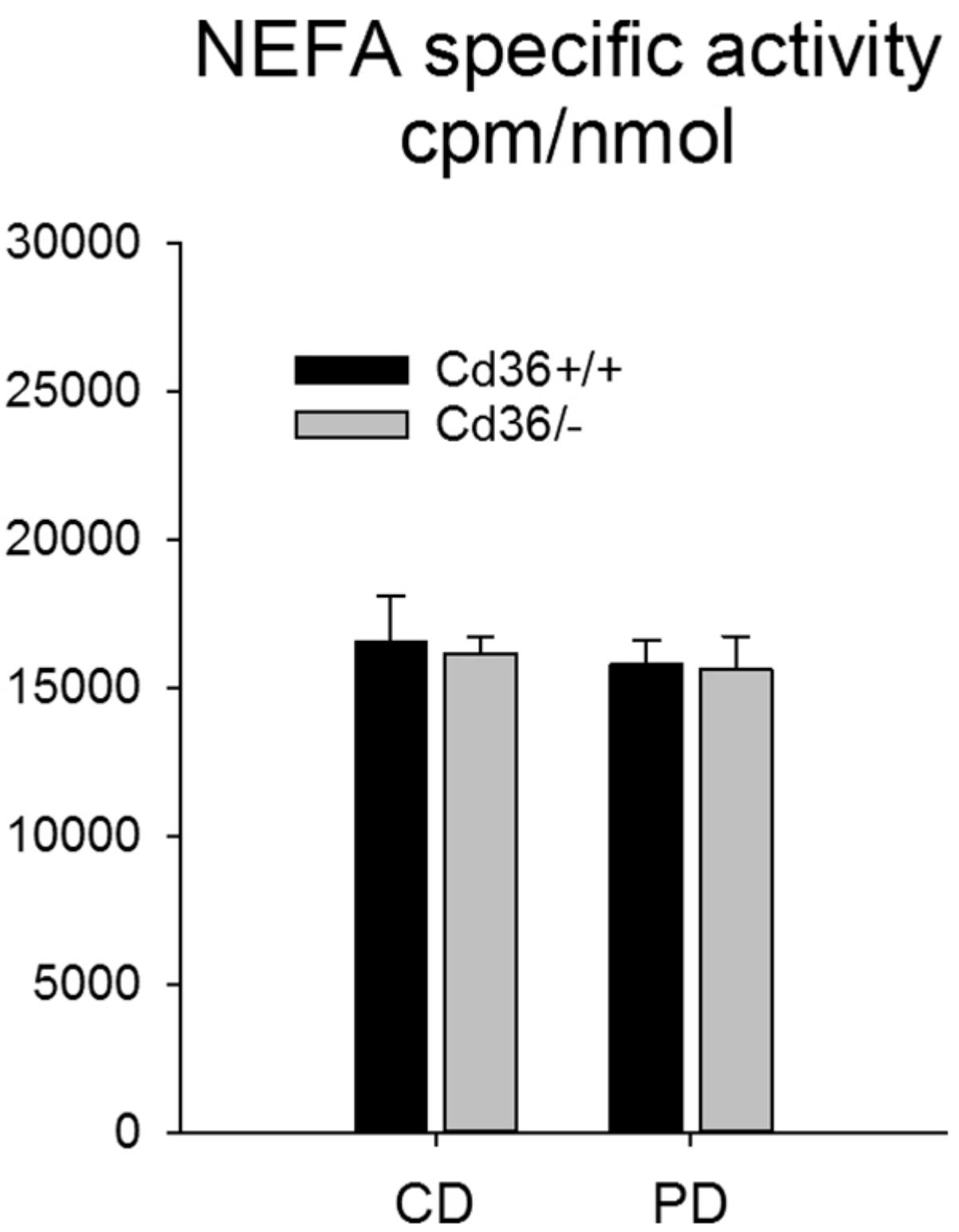

NEFA specific activity
cpm/nmol
30000
25000
20000
15000
10000
5000
0
Cd36+/+
Cd36/-
CD
PD

Figure 8