High-Fat Diet Induces Resistance to Ghrelin and LEAP2 Peptide Analogs in Mice

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Summary

Recent data suggest that the orexigenic peptide ghrelin and liver-expressed antimicrobial peptide 2 (LEAP2) have opposing effects on food intake regulation. Although circulating ghrelin is decreased in obesity, peripheral ghrelin administration does not induce food intake in obese mice. Limited information is available on ghrelin resistance in relation to LEAP2. In this study, the interplay between ghrelin and LEAP2 in obesity induced by a high-fat (HF) diet in mice was studied. First, the progression of obesity and intolerance to glucose together with plasma levels of active and total ghrelin, leptin, as well as liver *LEAP2* mRNA expression at different time points of HF diet feeding was examined. In addition, the impact of switch from a HF diet to a standard diet on plasma ghrelin and LEAP2 production was studied. Second, sensitivity to the stable ghrelin analogue [Dpr^3]Ghrelin or our novel LEAP2 analogue palm-LEAP2(1-14) during the progression of HF diet-induced obesity and after the switch for standard diet was investigated. Food intake was monitored after acute subcutaneous administration. HF diet feeding decreased both active and total plasma ghrelin and increased liver *LEAP2* mRNA expression along with intolerance to glucose and the switch to a standard diet normalized liver *LEAP2* mRNA expression and plasma level of active ghrelin, but not of total ghrelin. Additionally, our study demonstrates that a HF diet causes resistance to [Dpr^3]Ghrelin, reversible by switch to St diet, followed by resistance to palm-LEAP2(1-14). Further studies are needed to determine the long-term effects of LEAP2 analogues on obesity-related ghrelin resistance.

Key words

LEAP2 • Ghrelin • Ghrelin resistance • LEAP2 resistance • Diet-induced obesity

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Introduction

Obesity is strongly associated with an increased risk of health problems such as type 2 diabetes, cardio-vascular diseases, gastrointestinal disorders, and other comorbidities. Given that obesity is frequently caused by hyperphagia, a comprehensive understanding of food intake regulation is required in order to treat this chronic disease.

Ghrelin is the only known peripheral peptide that increases food intake and acts directly in the hypothalamus [1] stimulating secretion of the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY) in AgRP/NPY neurons [2]. Ghrelin entry to the brain is nowadays envisioned through the choroid plexus and the hypothalamus tanycytes, which form the blood-cerebrospinal fluid (CSF) barrier [3].

Ghrelin is octanoylated on the Ser^3 of the 28 amino acid peptide chain, which makes ghrelin biologically active [1]. Ghrelin receptor, growth hormone secretagogue receptor (GHSR), has the constitutive activity which is almost 50 % of activity reached by ghrelin, which is important for constitutive stimulation of basal food intake [4]. Liver-expressed antimicrobial peptide 2 (LEAP2) inhibits the high constitutive activity of GHSR as well as ghrelin-induced actions [5].

LEAP2 is a 40-amino acid-long peptide expressed mainly in the liver and jejunum [5]. LEAP2 acts as an endogenous antagonist as well as an inverse agonist of GHSR [6,7]. Subcutaneously (SC) administered LEAP2 alone does not affect food intake in *ad libitum*-fed mice [6], but inhibits the ghrelin-induced release of growth hormone (GH) [5]. Mice deficient in LEAP2 display increased sensitivity to the acute effects of ghrelin on food intake and GH secretion [8]. In healthy men, LEAP2 attenuates food intake and postprandial glucose excursions [9].

Blood plasma levels of ghrelin and LEAP2 exhibit opposite trends during fasting and feeding/refeeding in both humans and mice. Plasma LEAP2 rises with body mass, body fat, blood glucose, serum triglycerides (TAG), and intrahepatocellular lipid content in humans and mice [10,11]. In mice, liver *LEAP2* mRNA expression is selectively downregulated at fasting by ketone bodies and is upregulated by HF diet feeding [11]. *LEAP2* mRNA expression in mice even selectively correlated with stores of hepatic glycogen and jejunal lipids [12]. LEAP2 to ghrelin ratio is an indicator of obesity [10]. It also increases during pregnancy in humans and rats, which may be associated with pregnancy weight gain [13].

Obesity lowers ghrelin secretion and its availability in the brain [14]. Fasting in mice with diet-induced obesity (DIO) does not increase ghrelin levels [15] and plasma ghrelin does not drop after the meal in obese humans [16]. Moreover, ghrelin administered peripherally does not acutely induce food intake in DIO mice [15] or agouti mice [17], and has no effect at chronic administration in DIO mice [18]. The orexigenic effect of ghrelin in obesity is lowered *via* inefficient activation of AgRP/NPY neurons [19]. In mice, 12 weeks of a HF diet has been shown to decrease not only plasma ghrelin and *GHRL* mRNA (GHRL – ghrelin and obestatin prepropeptide) expression in the stomach, but also *GHSR* mRNA expression in the hypothalamus, indicating suppression of the neuroendocrine ghrelin axis. Neither peripherally nor centrally administered ghrelin induces food intake, *NPY* and *AgRP* mRNA expression, and NPY and AgRP peptide secretion in DIO mice. However, intracerebroventricular administration of NPY stimulates food intake in both lean and DIO mice, indicating that downstream ghrelin signaling is not affected by obesity [20].

Ghrelin resistance is reversible by a low-calorie diet causing weight loss in obese individuals. However, an increase in ghrelin blood level indicating restoration of ghrelin sensitivity promotes rebound weight gain [21]. There is still no knowledge if resistance to ghrelin is associated with resistance to LEAP2.

In this study, we hypothesized that switching from a HF diet to a St diet could not only improve metabolic and morphometric parameters, but also restore sensitivity to ghrelin and LEAP2 in mice. In the first experiment, the time course of HF diet-induced obesity related parameters were linked to active and total plasma ghrelin and liver *LEAP2* mRNA expression. As obesity is associated with the development of metabolic diseases connected with chronic low-grade inflammation and increased risk of liver steatosis and oxidative stress in the liver, CRP in blood, and peroxides and lipid droplets in the liver were observed in this sense. In the second experiment, sensitivity to ghrelin and LEAP2 was evaluated by monitoring food intake after acute SC administration of either ghrelin analogue [Dpr^3]Ghrelin [22,23] or our recently published palmitoylated LEAP2 analogue palm-LEAP2(1-14) [7] at particular times of feeding HF diet and after the switch to St diet. [Dpr^3]Ghrelin and palm-[LEAP2(1-14) are stable analogs of the natural peptides with affinity to and activation of GHSR similar to natural peptides. Besides, palm-LEAP2(1-14) attenuated food intake after acute SC administration in St diet fed mice [7].

Materials and Methods

Peptides

[Dpr^3]Ghrelin (GS Dpr (N-octanoyl)FLSPEHQ-KAQQRKESKKPPAKLQPR) was synthesised and purified as previously described [24]. Lipidization with the corresponding fatty acid was performed on a fully protected peptide on resin as the last step [25]. Synthesis of palm-LEAP2(1-14) (Nle-TPFWRGVSLRPIG-βAla-Lys(Palm)-NH_2) was assembled using solid-phase peptide synthesis [7]. Peptide purification and identification were carried out using analytical HPLC and the Q-Tof micro® MS technique (Waters, Milford, MA, USA). The purity of the synthesised peptides was greater than 95 % (Dpr – diaminopropionic acid, palm – palmitoyl).

Experimental animals

All experiments followed ethical guidelines for animal experiments, met the regulations stipulated in Act No. 246/1992 of the Czech National Council, and were approved by the Committee for Experiments with

Laboratory Animals of the Academy of Sciences of the Czech Republic (decision no. 96/2020, issued 10/12/2020).

Male C57Bl/6N mice (Charles River, Sulzfeld, Germany) were housed at a temperature of 23 °C with a daily cycle of 12-h light- and dark (light on at 6:00 AM). The mice were given *ad libitum* water and fed either a HF diet containing 13 %, 60 %, and 27 % of calories from protein, fat, and carbohydrate, respectively [26], or a standard chow diet (St) (ssniff® R/M-H; cat. no. V1534; Spezialdiäten GmbH, Soest, Germany) containing 33 %, 9 %, and 58 % of calories from protein, fat, and carbohydrate, respectively.

Experiment 1: Effect of HF diet on mice metabolic parameters – experimental design

An overview of the study design is described in Figure 1A. At the age of 8 weeks, mice were divided into 12 groups (n=8), housed in groups of four animals per cage and fed either HF or St diet. After 8 weeks on HF diet, group 12 was switched from HF to St diet. Body weights were monitored weekly. Mice were sacrificed at different time points according to Figure 1A. One week before sacrificing, an oral glucose tolerance test (OGTT) was performed after 6 h of fasting in each group. Free-fed mice were sacrificed by decapitation and trunk blood was then collected; plasma was separated and stored at -20 °C. Plasma pre-treated with Pefabloc® (Carl Roth, Karlsruhe, Germany) and acidified using HCl was used for ELISA detection of ghrelin according to the manufacturer's protocol.

Epididymal white adipose tissue (eWAT), the liver, and the hypothalamus were dissected and weighed. Tissue samples were frozen in liquid nitrogen and then stored at -80 °C. Morphometric and biochemical analyses, liver histology, and mRNA analysis of tissues were subsequently performed.

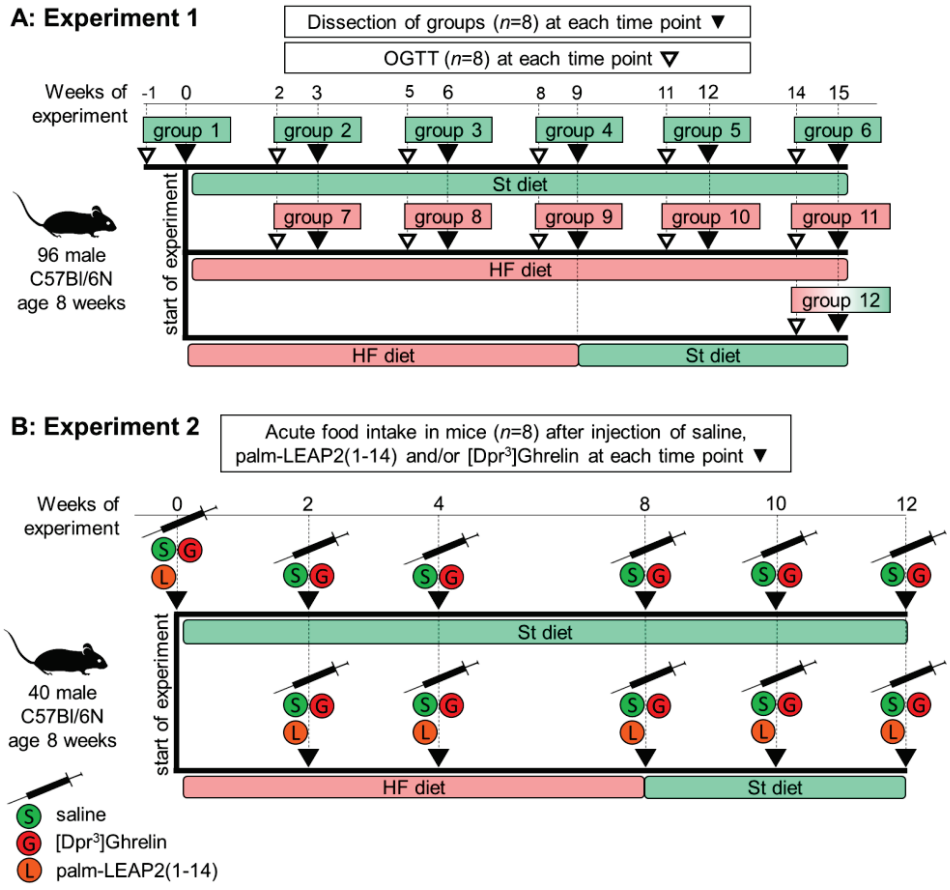

Fig. 1. Scheme of experimental designs. (**A**) Experiment 1. Ninety-six C57Bl/6N male mice were divided into 12 groups. Group 1 was sacrificed at the beginning of the experiment as a control group. Every three weeks, one group fed a St diet (from groups 2-6) and one group fed a HF diet (from groups 7-11) were sacrificed until week 15. Group 12 was fed a HF diet for the first 9 weeks and then switched to a St diet for a further 6 weeks before being sacrificed at week 15. The white triangles indicate the week of the experiment when the OGTT was performed. The black triangles indicate the week of the experiment when the dissection was performed. (**B**) Experiment 2. Forty C57Bl/6N male mice were divided into 5 groups. 2 groups were fed a St diet and 3 groups were fed a HF diet. In the 8th week of the experiment, the diet of all mice was changed to St diet. Food intake was monitored after SC injection of saline, palm-LEAP2(1-14), or [Dpr^3]Ghrelin at 0th, 2nd, 4th, 8th, 10th, and 12th week of experiment (marked by black triangle).

Oral glucose tolerance test

An OGTT was performed after 6 h of fasting one week before sacrificing in each experimental group. At time point 0 (09:00 h), blood was collected from the tail vein to measure insulin, cholesterol, and TAG. The animals were then gavaged with glucose at a dose of 2 g/kg body weight. Concentrations of blood glucose were determined in whole blood at 15, 30, 60, 120, and 180 min after glucose gavage using a glucometer (LifeScan, Inc., Milpitas, CA, USA).

Experiment 2: Effect of HF diet on development of ghrelin resistance – experimental design

The study design is shown in Figure 1B. At the age of 8 weeks (week 0 of the experiment), mice were divided into 5 groups (n=8), and housed in separate cages. Two groups were fed a St diet and 3 groups a HF diet. Since week 8, all mice were fed St diet. Body weight was monitored weekly. The effect of SC administered [Dpr^3]Ghrelin and palm-LEAP2(1-14) on feeding behavior was tested at weeks 0, 2, 4, 8, 10, and 12 in free-fed mice.

On the day of the food intake experiment, at 8:00 AM, the mice were SC injected with 150 µl of saline, palm-LEAP2(1-14) (dissolved in saline) at a dose of 5 mg/kg of body weight, or [Dpr^3]Ghrelin (dissolved in saline) at a dose of 1 mg/kg of body weight in order to achieve a significant change in food intake. Dose of [Dpr^3]Ghrelin was chosen based on the ED_{50} determined in our previous study [25]. Dose of palm-LEAP2(1-14) was chosen based on acute food intake experiment after SC administration of palm-LEAP2(1-14) to lean animals [7]. Fifteen minutes after the injection, the mice were given pre-weighed food pellets. Food intake was monitored every 30 min for 270 min. The animals had free access to water during the experiment.

Determination of biochemical parameters in plasma

Fasted plasma was used to detect insulin on the Sensitive Rat Insulin RIA kit (MilliporeSigma, Burlington, MA, USA), and TAG and cholesterol using colorimetric assays (Erba Lachema, Brno, Czech Republic). Free-fed plasma was used to measure leptin, total ghrelin, active ghrelin (Millipore, St. Charles, MI, USA), and C-reactive protein (CRP) with mouse ELISA kits (Thermo Fisher Scientific, Waltham, MA USA). All measurements were carried out according to the manufacturers' instructions.

Determination of mRNA expression

Samples of the hypothalamus and liver for mRNA determination were processed as previously described [26,27]. The mRNA expressions of *AgRP*, *CART*, *GHSR*, *NPY*, and *POMC* in the hypothalamus, and *LEAP2* in the liver were determined using the ABI PRISM® 7500 instrument (Applied Biosystems, Foster City, CA, USA). Data were normalized to the expression of the reference genes beta-2-microglobulin (*B2m*) or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Oxidative stress

Liver samples were homogenised in ice-cold lysis buffer (62.5 mM Tris-HCl buffer with pH 6.8, 1 % deoxycholate, 1 % Triton X-100, 50 mM NaF, 1 mM Na_3VO_4 and complete protease inhibitor (Roche Applied Science, Mannheim, Germany)) using the Bullet Blender® tissue homogenizer (Next Advance, Inc., Averill Park, NY, USA). Lysates were sonicated for 1 min and centrifuged for 15 min at 13500× g at 4 °C. Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Lysates were diluted to a final concentration of 10 µg/µl in lysis buffer. The Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA USA) was used to measure H_2O_2 concentration. All measurements were carried out according to the manufacturers' instructions.

Haematoxylin and eosin staining of the liver

The right lobe of each liver was carefully removed, fixed with 4 % paraformaldehyde (PFA), and embedded in paraffin. Sections were cut with the Leica ASP200S Tissue Processor (Leica Biosystems, Buffalo Grove, IL, USA) at a thickness of 5 µm (n=3) as described previously [28]. Samples were covered with DPX mounting medium (MilliporeSigma, Burlington, MA, United States). Photomicrographs of liver sections stained with haematoxylin and eosin were taken using the Olympus IX83 inverted microscope (Olympus Europa SE & Co. KG, Hamburg, Germany).

Statistical analysis

Data are presented as the mean ± SEM and analysed with GraphPad 8 Software (San Diego, CA, USA). Data were evaluated by two-way ANOVA with Bonferroni's *post hoc* test or one-way ANOVA with Tukey's test or multiple *t*-test with Bonferroni-Dunn's method for multiple comparisons as described in the

figure legends. Outliers were identified by Grubbs test. $P<0.05$ was considered statistically significant.

Results

Experiment 1

Switching from a HF diet to a St diet decreases body weight and eWAT weight and normalizes LEAP2 mRNA expression in liver as well as active plasma ghrelin and leptin in plasma

Mice were fed a HF diet from the 8th week of age (week 0 of the experiment). Body weight was monitored weekly over the following 15 weeks. Consumption of a HF diet caused higher body weight as well as eWAT weight and leptin level (Fig. 2A-C) compared to a St diet. These differences became significant as early as after 3 weeks of HF diet feeding. In mice that were switched to a St diet after 9 weeks on a HF diet (group 12), we observed a significant reduction in body weight as early as after 2 weeks St diet feeding and their final body weight was similar to that of control mice fed exclusively a St diet (Fig. 2A). Their eWAT weight (Fig. 2B) and plasma leptin (Fig. 2C) followed a similar trend.

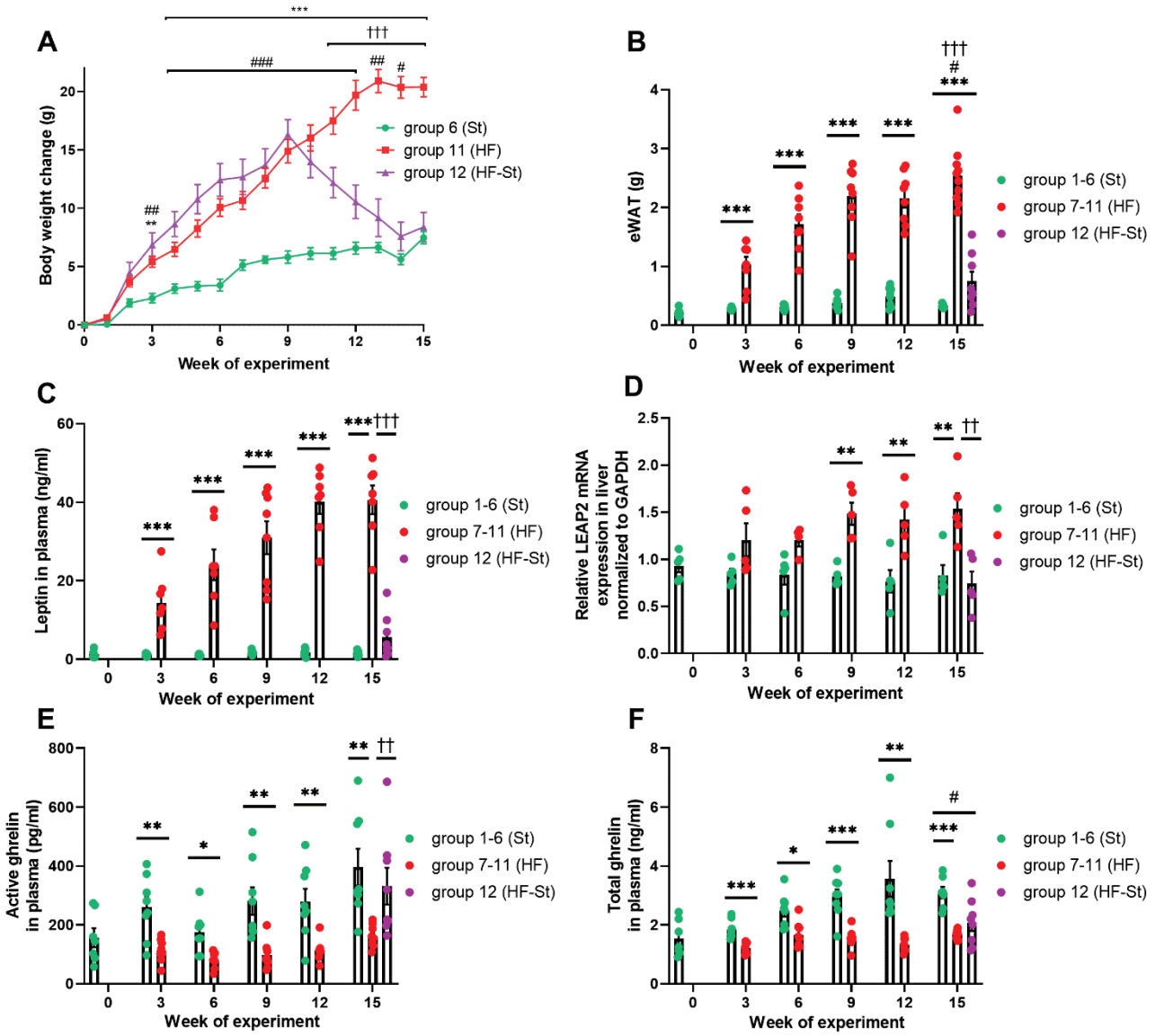

Fig. 2. Effect of HF diet on body weight change (**A**), eWAT weight (**B**), leptin in plasma (**C**), *LEAP2* mRNA in the liver (**D**) and active (**E**), and total (**F**) ghrelin in plasma in C57Bl/6N mice. Data are presented as means ± SEM. Statistical analysis was performed by two-way ANOVA with Bonferroni's *post hoc* test (A) and multiple *t*-test with Bonferroni-Dunn's method for multiple comparisons (B-F). eWAT – epididymal white adipose tissue. Significance is * $P<0.05$, ** $P<0.01$, *** $P<0.001$ HF vs. St; $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.01$, $^{\#\#\#}$ $P<0.001$ HF-St vs. St; † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ HF-St vs. HF (n=8).

The level of *LEAP2* mRNA in the liver (Fig. 2D) increased with higher body weight. In the group fed a 9-week HF diet followed by a 6-week St diet, *LEAP2* mRNA in the liver proved similar to the group fed exclusively a St diet.

Levels of active (Fig. 2E) and total ghrelin (Fig. 2F) in plasma exhibited opposite trends to liver *LEAP2* mRNA expression. Mice fed a HF diet had lower active and total ghrelin compared to those fed a St diet. Switching from a HF to a St diet caused an increase in active ghrelin levels to the levels in mice fed exclusively a St diet. Interestingly, the total ghrelin level in mice fed a 9-week HF diet followed by a 6-week St diet was similar to the level in those fed exclusively a HF diet.

Switching from a HF diet to a St diet improves glucose tolerance

Glucose tolerance was assessed by the OGTT after glucose gavage. Three weeks of HF diet consumption increased glucose levels significantly over the course of oral glucose tolerance testing (Fig. 3A-F). In mice switched from a 9-week HF diet to a 6-week St diet,

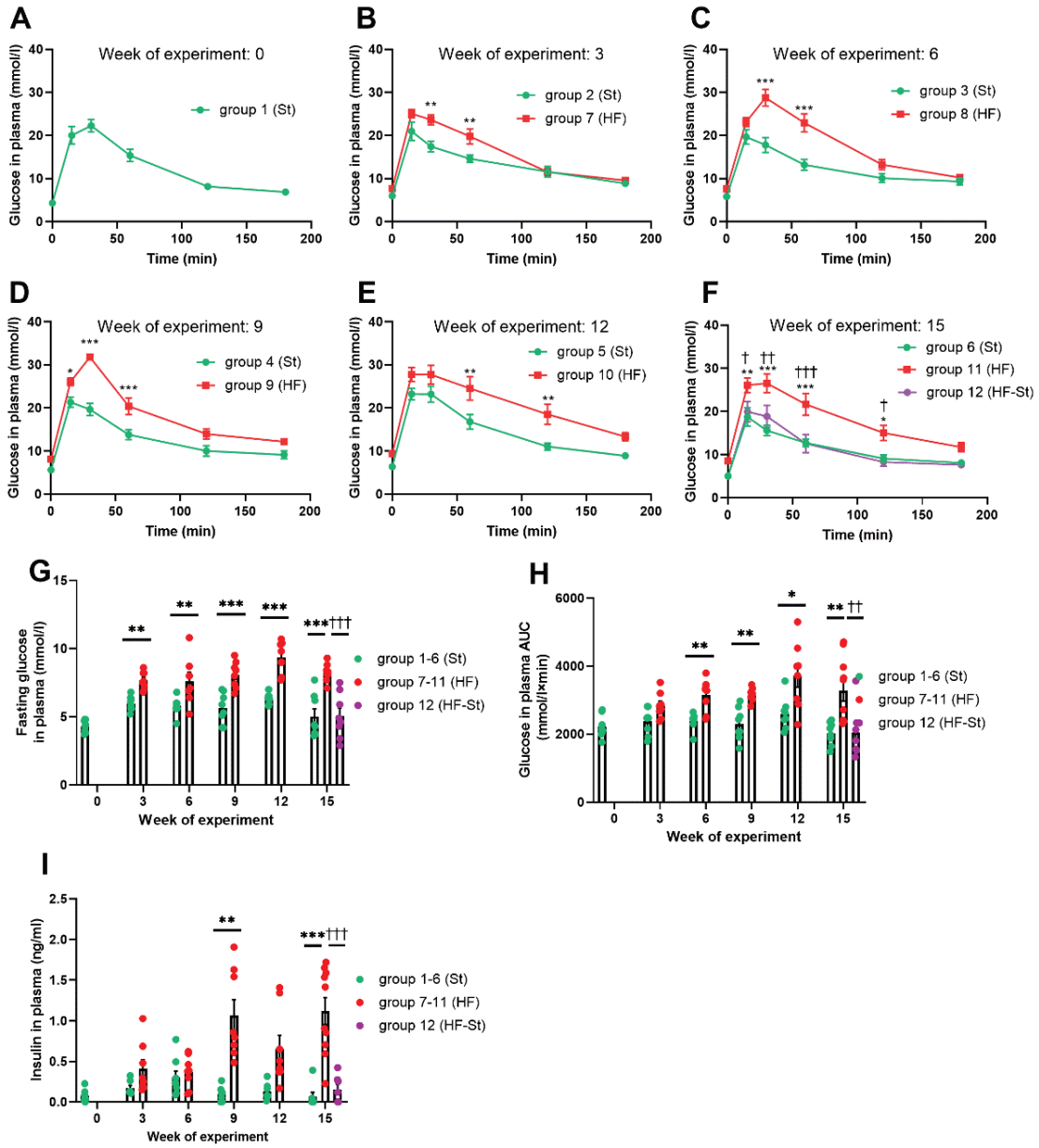

Fig. 3. Blood glucose levels (**A-F**) after oral glucose gavage (dose 2 g/kg) and corresponding plasma levels of fasting glucose (**G**), AUC (**H**), and insulin plasma levels (**I**). OGTT at 0 weeks (A), 3 weeks (B), 6 weeks (C), 9 weeks (D), 12 weeks (E), and 15 weeks of the experiment are expressed as means ± SEM and determined by two-way ANOVA with Bonferroni's *post hoc* test (A-F) and multiple *t*-test with Bonferroni-Dunn's method for multiple comparisons (G and H). Fasting glucose, area under the OGTT curves, and insulin plasma levels are expressed as means ± SEM and determined by the multiple *t*-test. Significance is * $P<0.05$, ** $P<0.01$, *** $P<0.001$ HF vs. St; † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ HF-St vs. HF (n=8).

glucose levels were similar to mice fed exclusively a St diet over the course of oral glucose tolerance testing.

Fasted glucose plasma levels (Fig. 3G) and OGTT area under the curve (AUC) values (Fig. 3H) confirmed results from the courses of OGTT curves.

Fasted plasma insulin levels (Fig. 3I) were significantly higher in mice fed a HF diet for 9 weeks compared to mice fed a St diet. Plasma insulin was similar in mice fed a 9-week HF diet followed by a 6-week St diet and mice fed exclusively St diet.

Switching from a HF diet to a St diet lowers cholesterol and CRP plasma levels, liver steatosis, and oxidative stress in the liver

Fifteen weeks on a HF diet significantly increased the level of cholesterol in plasma (Fig. 4A) and switching from a HF diet to a St diet decreased cholesterol as well as CRP levels (Fig. 4C) to levels observed in mice fed exclusively St diet. Liver weight (Fig. 4D) tended toward a non-significant increase in the group of mice fed a 15-week HF diet compared to other groups. Oxidative stress expressed as H_2O_2 concentration (Fig. 4E) was significantly increased in groups fed a HF diet for 9 and 15 weeks and switching from a HF diet to a St diet tended to decrease it. 15 weeks of HF diet feeding induced reversible steatosis in the liver (Fig. 4F). Switching from a HF diet to a St diet decreased the amount of visible lipid droplets in the liver to those observed in mice fed a 15-week St diet.

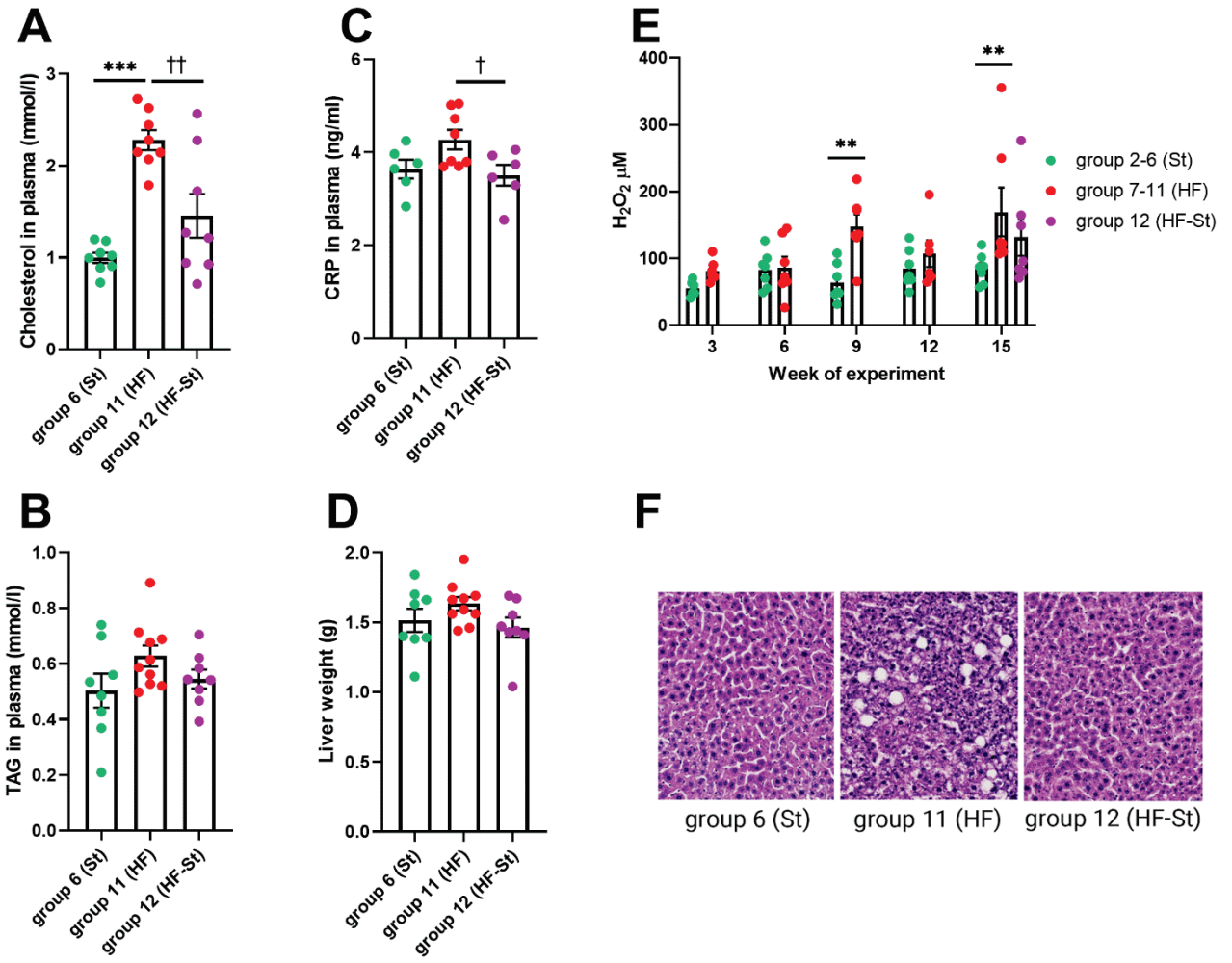

Fig. 4. Liver weight and metabolic parameters at the end of the experiment. Cholesterol (**A**), TAG (**B**), and CRP (**C**) in plasma, liver weight (**D**), oxidative stress in the liver (**E**), and morphological changes in liver tissue (**F**). Data (A-E) are presented as means ± SEM. Statistical analysis was performed by one-way ANOVA with Tukey's method for multiple comparisons. Significance is * $P<0.05$, ** $P<0.01$, *** $P<0.001$ HF vs. St; # $P<0.05$; † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ HF-St vs. HF (n=8). (F) Representative microphotographs of a liver stained by haematoxylin and eosin are magnified 200×.

Switching from a HF diet to a St diet does not affect hypothalamic mRNA expression of neuropeptides and GHSR

Hypothalamic mRNA expression of selected genes was compared between groups 6, 11, and 12 (Fig. 5). *POMC* and *CART* mRNA expression in the hypothalamus tended toward a non-significant increase in the group of mice fed a 15-week HF diet compared to other groups. *AgRP*, *GHSR*, and *NPY* mRNA levels were not affected by the diet in free fed mice.

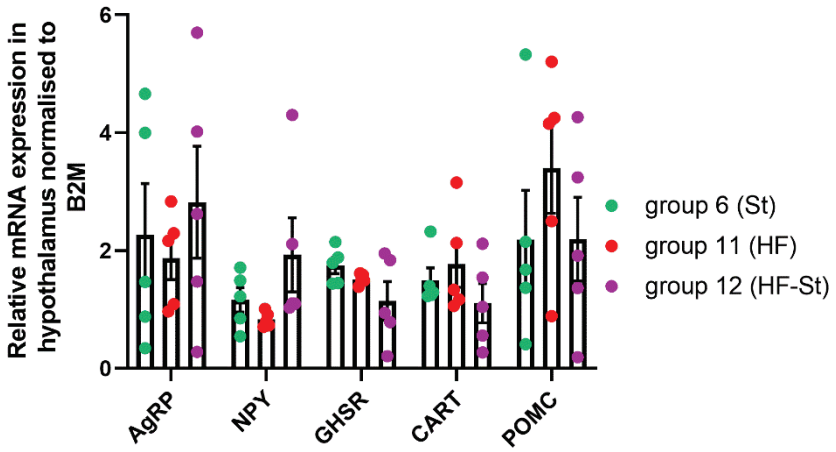

Fig. 5. Effect of HF diet on hypothalamic mRNA expression of *AgRP*, *CART*, *GHSR*, *NPY*, and *POMC* in mice fed a HF diet or a St diet. Data were normalized to B2M and presented as means ± SEM. Statistical analysis was performed by one-way ANOVA with Tukey's method for multiple comparisons (n=5). *AgRP* – agouti-related peptide, *CART* – cocaine- and amphetamine-regulated transcript, *GHSR* – growth hormone-secretagogue receptor, *NPY* – neuropeptide Y, *POMC* – pro-opio-melanocortin.

Experiment 2

HF diet attenuates food intake response to [Dpr^3]Ghrelin and palm-LEAP2(1-14)

The effect of acute SC administration of [Dpr^3]Ghrelin or palm-LEAP2(1-14) on feeding in mice fed a HF diet is shown in Figure 6. Mice fed a St diet were used as controls. The results are expressed as grams of food consumed per 270 min. Curves of cummulative food intake are shown in supplements (Fig. S1). As early as two weeks after HF diet feeding, mice had lost sensitivity to acutely administered [Dpr^3]Ghrelin regarding to increase in food intake, while food intake had fallen below the basal level of food intake due to acute palm-LEAP2(1-14) administration. Resistance to palm-LEAP2(1-14) (i.e. the ability of palm-LEAP2(1-14) to decrease the basal level of food intake developed after 4 weeks of HF diet feeding. Four weeks after the switch of HF diet to a St diet, sensitivity to [Dpr^3]Ghrelin had been restored.

Discussion

Due to the key role, ghrelin plays in regulating food intake and energy expenditure, the pharmaceutical industry has been developing anti-obesity drugs that target the ghrelin receptor GHSR [29]. As ghrelin receptor is a constitutively active G-protein-coupled receptor [30], attention has turned to inverse agonists that are able to reduce the high constitutive activity of GHSR. However, no drug that reduces body weight through GHSR has yet been developed. This may be due to ghrelin resistance, which reduces sensitivity to ghrelin in obese individuals even though their circulating ghrelin is lower than in lean individuals. Switching from a HF diet to a St diet enhances both ghrelin level and sensitivity to ghrelin and normalizes metabolic parameters [21], but whether LEAP2 is also affected remains inconclusive. The plasma level of LEAP2, which is both an endogenous inverse agonist and an antagonist of GHSR, increases during obesity. However, it is not clear whether obesity affects sensitivity to LEAP2 or whether obesity-induced resistance to ghrelin is accompanied by resistance to LEAP2.

Previous studies have shown that plasma ghrelin levels are reduced in obesity [31,32]. Three weeks of HF diet feeding decreased the levels of both active ghrelin and total ghrelin in Experiment 1, which is consistent with the work of Briggs and colleagues [33]. They demonstrated that switching to a control diet after 12 weeks of HF diet feeding increased levels of active ghrelin but did not lead to a re-increase in total ghrelin levels [21]. Nonetheless, we consider an increase in active ghrelin much more important for ghrelin sensitivity than an increase in total ghrelin.

While the plasma level of LEAP2 is increased in obese mice and humans [34], it is decreased during diet-induced weight loss [10]. Even though higher liver mRNA expression was reported in mice with HF diet-induced obesity [11], experiment 1 is the first to compare a time course of *LEAP2* mRNA expression in the livers

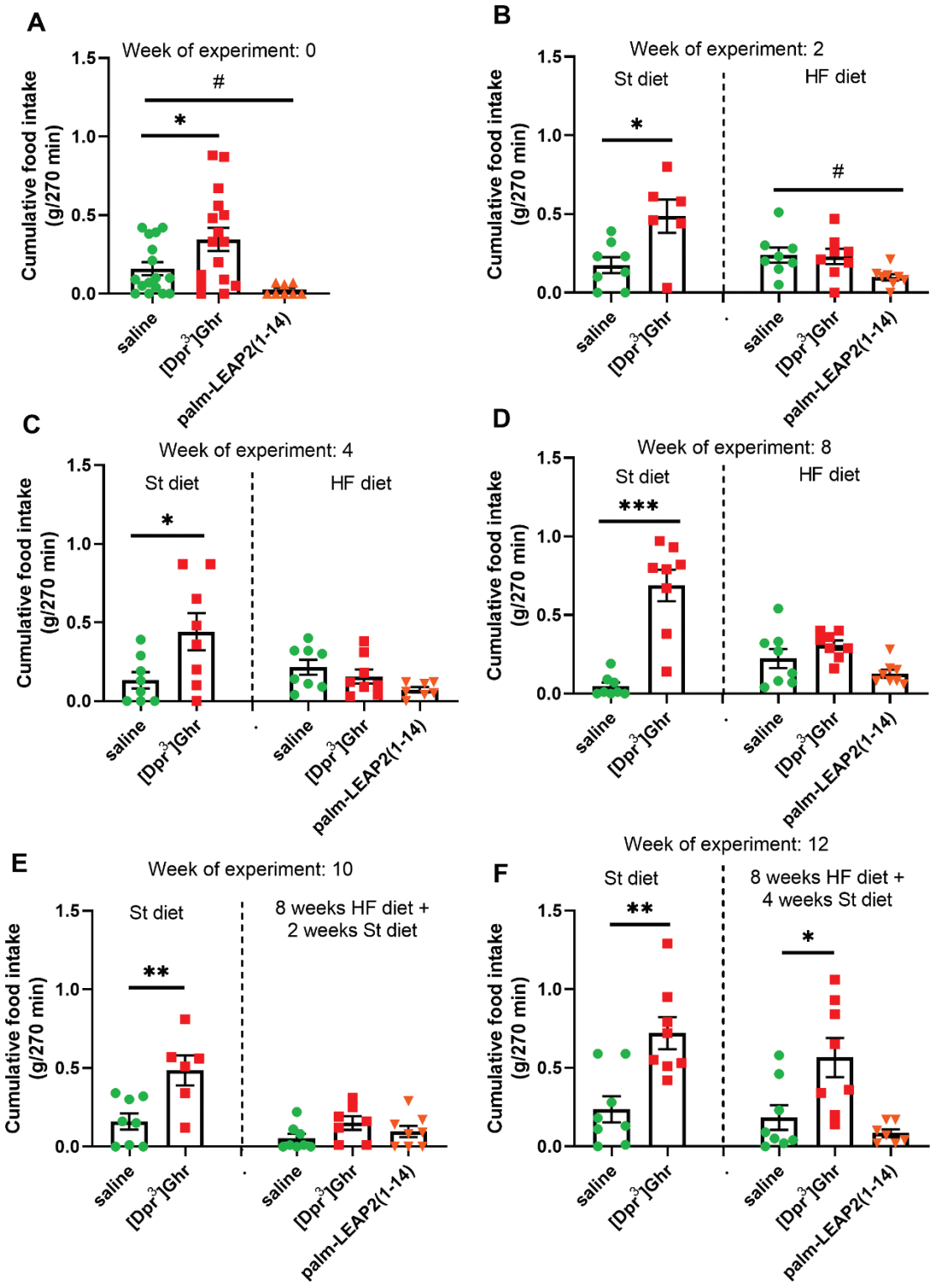

Fig. 6. Cumulative food intake 270 min after SC [Dpr^3]Ghrelin (1 mg/kg) or palm-LEAP2(1-14) (5 mg/kg) administration in mice fed a HF diet or a St diet for 0 (**A**), 2 (**B**), 4 (**C**), and 8 (**D**) weeks followed by a St diet only for a further 2 (**E**) and 4 (**F**) weeks. Data are presented as means of AUC ± SEM. Statistical analysis was performed by *t*-test. Significance is * P<0.05, ** P<0.01, *** P<0.001 [Dpr^3]Ghrelin vs. saline; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001 palm-LEAP2(1-14) vs. saline (n=6-8).

of mice fed a HF diet, a St diet, or a diet that alternates between the two. After 3 weeks of HF diet feeding, we observed a non-significant increase in liver *LEAP2* mRNA, which became significant after 9 weeks of HF diet feeding. Switching from a HF diet to a St diet completely restored liver *LEAP2* mRNA expression to the level observed in mice exclusively fed a St diet. Here it is proper to mention two limitations of this study. The

first is that we did not determine *LEAP2* mRNA expression in jejunum, another significant LEAP2 producer. We rather simplistically assumed that the liver LEAP2 production in mouse is the biggest one similarly as in rat [35]. The second is that due to technical problems with LEAP2 ELISA kits and limited volume of plasma, we were not able to determine plasma LEAP2.

In Experiment 1, we observed significantly increased glucose excursion at OGTT in mice fed a HF diet after only 3 weeks that were restored after switching to a St diet. Similarly, Reynolds and colleagues showed that 6 weeks of HF diet feeding led to glucose intolerance in mice, but after switching to a St diet, glucose tolerance was restored [36]. The time course of increase in body weight owing to the exclusive HF diet feeding was mirrored by increase in plasma leptin and cholesterol level, intolerance to glucose and also LEAP2 liver production, and decrease in active ghrelin level. Analogously, increase in active ghrelin and decrease in *LEAP2* mRNA expression were accompanied by normalized body weight, plasma cholesterol level, and tolerance to glucose after the switch to St diet.

Low levels of active ghrelin in plasma have been demonstrated in individuals with non-alcoholic steatohepatitis [37]. On the other hand, CRP levels were found to correlate with LEAP2 plasma level [38]. Similarly, obesity-related chronic low-level inflammation is characterized by increased circulating CRP and permanently increased oxidative stress [39]. A negative correlation between ghrelin and CRP levels in plasma was proven [40]. Decreased plasma ghrelin correlates not only with increased immunoglobulin production, often observed in patients with chronic liver disease [41] but also with liver inflammation [42].

Weight loss then could attenuate the low-level inflammation as was seen in Experiment 1. An increase in active ghrelin and a decrease in LEAP2 liver production after the switch from HF to St diet was accompanied by a decrease in plasma CRP, liver oxidative stress, and steatosis. Clearly visible lipid droplets in the livers of mice fed a HF diet disappeared after switching to a St diet. Then LEAP2/ghrelin ratio could become a measure of low-grade obesity-related systemic and liver inflammation.

In our study, mRNA expression of neuropeptides did not differ between the St diet-fed group and the HF diet-fed group. Briggs and colleagues proved that hypothalamic *AgRP* and *NPY* expression in free-fed mice is similar in both HF- and control-fed groups. However, in fasted mice, mRNA expression of *AgRP* and *NPY* was higher in their control diet-fed group than in their HF diet-fed group [43]. In agreement with Kohsaka and colleagues, mRNA encoding *POMC* and *CART* tended to increase under diet-induced obesity conditions in our study [44].

Previous studies have indicated that both peripheral and central administration of ghrelin is not adept at inducing food intake in HF diet-fed mice [15,18,19]. It has also been suggested that ghrelin resistance develops as early as after 3-4 weeks of HF diet feeding [33,45] and that diet-induced weight loss restores ghrelin sensitivity [21]. In our previous study [7], we proved that acute SC administration of the LEAP2 analogue palm-LEAP2(1-14) lowered food intake in lean mice. In Experiment 2, we show for the first time that a HF diet induces [Dpr^3]Ghrelin resistance in mice as early as after 2 weeks on a HF diet, while palm-LEAP2(1-14) resistance develops after 4 weeks on a HF diet. Switching from a HF diet to a St diet restored [Dpr^3]Ghrelin sensitivity after 4 weeks. However, palm-LEAP2(1-14) sensitivity was not fully restored. The difference between [Dpr^3]Ghrelin-induced food intake and the control group was much higher than the reduction of food intake after palm-LEAP2(1-14) administration. Therefore, statistical evaluation might not reveal a significant difference in the latter case.

In conclusion, this study offers new insights into the interplay between ghrelin and LEAP2 in HF diet-induced obesity. Our data demonstrate that switching from a HF diet to a St diet restores *LEAP2* liver mRNA expression as well as plasma levels of active ghrelin to values in mice exclusively fed a St diet. Simultaneously, increased body weight due to HF diet feeding mirrored by enhanced leptin level, intolerance to glucose, and liver steatosis was lowered by the switch to St diet. We also show that a HF diet induces not only reversible ghrelin resistance but also palm-LEAP2(1-14) resistance. Further studies are needed to determine the long-term effects of LEAP2 analogues on obesity-related ghrelin resistance.

Conflict of Interest

There is no conflict of interest.

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