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Synergistic effect of leptin and lipidized PrRP on metabolic pathways in *ob/ob* mice

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Abstract

Lack of leptin production in *ob/ob* mice results in obesity and prediabetes that could be partly reversed by leptin supplementation. In the hypothalamus, leptin supports the production of prolactin-releasing peptide (PrRP), an anorexigenic neuropeptide synthesized and active in the brain. In our recent studies, the palmitoylated PrRP analog palm¹¹-PrRP31 showed a central anorexigenic effect after peripheral administration. This study investigates whether PrRP could compensate for the deficient leptin in *ob/ob* mice. In two separate experiments, palm¹¹-PrRP31 (5 mg/kg) and leptin (5 or 10 µg/kg) were administered subcutaneously twice daily for 2 or 8 weeks to 8- (younger) or 16- (older) week-old *ob/ob* mice, respectively, either separately or in combination. The body weight decreasing effect of palm¹¹-PrRP31 in both younger and older *ob/ob* mice was significantly powered by a subthreshold leptin dose, the combined effect could be then considered synergistic. Leptin and palm¹¹-PrRP31 also synergistically lowered liver weight and blood glucose in younger *ob/ob* mice. Reduced liver weight was linked to decreased mRNA expression of lipogenic enzymes. In the hypothalamus of older *ob/ob* mice, two main leptin anorexigenic signaling pathways, namely, Janus kinase, signal transducer and activator of transcription-3 activation and AMP-activated protein kinase de-activation, were induced by leptin, palm¹¹-PrRP31, and their combination. Thus, palm¹¹-PrRP31 could partially compensate for leptin deficiency in *ob/ob* mice. In conclusion, the results demonstrate a synergistic effect of leptin and our lipidized palm¹¹-PrRP31 analog.

Key Words

- ▶ prolactin-releasing peptide
- ▶ leptin
- ▶ *ob/ob* mice
- ▶ hypothalamic leptin signaling

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Introduction

Leptin has an appetite-regulating effect that occurs in the arcuate nucleus of the hypothalamus, where it activates anorexigenic proopiomelanocortin (POMC) and inhibits orexigenic neuropeptide Y (NPY) neurons and thus induces a decrease in food intake and an increase in energy consumption (Schwartz *et al.* 1996, Kwon *et al.* 2016). In addition, leptin action in the hypothalamus is

connected with other anorexigenic neuropeptides, such as prolactin-releasing peptide (PrRP) (Ellacott *et al.* 2002).

PrRP was described to be produced in the nucleus tractus solitarius (NTS), ventrolateral medulla (VLM), and dorsomedial hypothalamus (DMH) (Maruyama *et al.* 1999, Dodd & Luckman 2013). It was suggested that PrRP neurons project from the NTS, where PrRP was detected

in neuron bodies, to the hypothalamus, where PrRP was found in neuron fibers (Hinuma *et al.* 1998). In the paraventricular nucleus of the hypothalamus (PVN), PrRP fibers were shown to target anorexigenic corticotropin-releasing hormone (CRH) (Matsumoto *et al.* 1999). CRH production was also proven to be promoted by leptin (Schwartz *et al.* 1996).

Most PrRP neurons in VLM and DMH were shown to express leptin receptor (Ellacott *et al.* 2002); co-localization of PrRP and leptin receptor was questioned in the brainstem (Garfield *et al.* 2012). The combined central administration of leptin and PrRP had an additive anorexigenic effect on nocturnal food intake in freely fed rats. Moreover, in obese Zucker *fa/fa* rats lacking functional leptin receptor signaling, negligible PrRP mRNA expression was detected (Ellacott *et al.* 2002).

Leptin deficiency in *ob/ob* mice causes obesity based on hyperphagia and decreased energy expenditure; in addition, type 2 diabetes and hyperlipidemia are characteristic of *ob/ob* mice (Zhang *et al.* 1994).

Productive binding of leptin to its receptor affects two main anorexigenic pathways in the hypothalamus: JAK-STAT3 signaling (Janus kinase 2, signal transducer and activator of transcription-3) (Vaisse *et al.* 1996, Maniscalco & Rinaman 2014) and AMP-activated protein kinase (AMPK) (Minokoshi *et al.* 2004, Kahn 2019). AMPK is a serine/threonine kinase activated by an increase in the AMP:ATP ratio that reflects energy deficiency (Hardie 2008). In peripheral tissues, leptin-induced activation of AMPK results in lipid oxidation (Minokoshi *et al.* 2004) and attenuation of stored triglycerides (Unger *et al.* 1999). On the other hand, inhibition of hypothalamic AMPK is necessary for the anorexigenic and body weight-lowering effects of leptin; leptin inhibits AMPK specifically in the PVN and arcuate nucleus (Arc) of the hypothalamus (Minokoshi *et al.* 2004).

PrRP-deficient mice displayed late-onset obesity and adiposity, resulting from increased meal size, hyperphagia, and attenuated responses to cholecystokinin and leptin (Takayanagi *et al.* 2008). As PrRP is a brain-born and brain-acting neuropeptide, it cannot be administered peripherally to achieve central anorexigenic effects. Attachment of palmitic acid to PrRP enabled the implementation of the anorexigenic effect of PrRP after peripheral administration and also stabilized the palm¹¹-PrRP31 molecule (Maletinska *et al.* 2015, Prazienkova *et al.* 2017).

In this study, both leptin and palm¹¹-PrRP31 were repeatedly peripherally administered separately or in combination to *ob/ob* mice to explore the potential interaction between leptin and PrRP regarding their

anorexigenic effect and impact on metabolic disturbances in *ob/ob* mice. If the palm¹¹-PrRP31 effect was powered by a subthreshold leptin dose, the combined effect could be considered synergistic. The impact of treatment with leptin, palm¹¹-PrRP31, and their combination on hypothalamic signaling was then investigated to determine whether PrRP followed leptin anorexigenic pathways.

Ob/ob mice of two ages were utilized: younger mice (treatment from 8 to 10 weeks old) in a metabolically active state, when the effect of lipidized PrRP on liver lipid metabolism could be expected, as in our previous study with diet-induced obese mice (Maletinska *et al.* 2015), and older *ob/ob* mice (treatment from 16 to 24 weeks old) with established morbid obesity.

Materials and methods

Animals

Ob/ob male mice and their wild-type (WT) littermates (5 weeks old) were obtained from ENVIGO (Correzzana, Italy). The mice were housed under controlled conditions at a constant temperature of 22±2°C, a relative humidity of 45 – 65% and a fixed daylight cycle (lights on: 6:00 h – 18:00 h), with two mice per cage. The animals were provided free access to water and the standard rodent chow diet Ssniff® R/M-H (Ssniff Spezialdiäten GmbH, Soest, Germany) containing 33, 9 and 58% of calories from proteins, fats and carbohydrates, respectively.

All of the animal experiments followed the ethical guidelines for animal experiments and the Act of the Czech Republic Nr. 246/1992 and were approved by the Committee for Experiments with Laboratory Animals of the Academy of Sciences of the Czech Republic.

Substances

Palm¹¹-PrRP31, an analog of human prolactin-releasing peptide palmitoylated at position 11, was synthesized at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic, as previously described (Pražienková *et al.* 2017). The structure of palm¹¹-PrRP31 was SRTHRHSMEIK(N-γ-E(N-palmitoyl)) TPDINPAWYASRGIRPVGRF-NH₂. Mouse leptin was obtained from Sigma-Aldrich.

Dosing of substances

Leptin doses (5 and 10 µg/kg twice daily) were chosen as a subthreshold according to a daily dose of 1 µg/mouse that was proven to be the minimum to achieve the anorexigenic

effect in *ob/ob* mice (Harris *et al.* 1998). The palm¹¹-PrRP31 dose used (5 mg/kg twice daily) was chosen according to our previous studies, where it consistently decreased food intake in lean C57BL/6J mice after acute administration and food intake and body weight in C57BL/6J mice with diet-induced obesity after sub chronic administration (Prazienkova *et al.* 2017, Holubova *et al.* 2018).

Experimental design

The schema of the experimental design is shown in Fig. 1.

In Experiment 1, *ob/ob* and WT male mice (5 weeks old) were randomized into groups of 8–10 animals. After 8 weeks of age (younger mice), the mice were treated for 2 weeks as follows: 1. WT saline, 2. *ob/ob* saline, 3. *ob/ob* leptin, (5 µg/kg), 4. *ob/ob* palm¹¹-PrRP31 (5 mg/kg), and 5. *ob/ob* leptin+palm¹¹-PrRP (5 µg/kg+5 mg/kg). The compounds were dissolved in saline and administered subcutaneously twice a day. Food intake (FI) and body weight (BW) were monitored daily during the dosing period.

In Experiment 2, *ob/ob* and WT male mice (6–8 weeks old) were randomized into groups of ten animals. After 16 weeks of age (older mice), the mice were treated for 8 weeks as follows: 1. WT saline, 2. *ob/ob* saline, 3. *ob/ob* leptin (10 µg/kg), 4. *ob/ob* palm¹¹-PrRP31 (5 mg/kg), and 5. *ob/ob* leptin+palm¹¹-PrRP (10 µg/kg+5 mg/kg).

The compounds were dissolved in saline and administered subcutaneously twice a day. FI and BW were monitored daily during the dosing period.

The oral glucose tolerance test (OGTT) was measured in Experiment 2 (Fig. 1): 6-h-fasted mice were administered a glucose solution at a dose of 2 g/kg BW by gavage. Blood samples were obtained from the tail vessels. The blood glucose concentrations were measured using a glucometer (Arkray, Tokyo, Japan) at 0, 30, 60, 90, 120, and 180 min after glucose administration.

In the open field test in Experiment 2, fed mice were placed individually in an open field (TSE Systems,

Bad Homburg, Germany), and their locomotor activity (velocity, total distance traveled, percentage of area visited and distance from the closest wall) was measured as described previously (Maletínská *et al.* 2008, 2015).

One week before the end of both experiments, rectal temperature was measured (Rodent thermometer BIO-TK9882, Bioseb, Pinellas Park, FL, USA).

At the end of both experiments, blood samples were collected from the tail veins of 12-h fasted mice, and blood plasma was separated and stored at –80°C. The mice were then deeply anesthetized with pentobarbital (170 mg/kg of body weight, Sigma-Aldrich) and transcardially perfused with ice-cold 0.01 mol/L pH 7.4 phosphate buffered saline (PBS) supplemented with heparin (10 U/mL, Zentiva, Prague, Czech Republic). The brains were removed, and the hypothalami were dissected and lysed in lysis buffer (Špolcová *et al.* 2015). During the dissections, the brains were maintained on ice to prevent tissue degradation. Subcutaneous adipose tissue (SCAT), intraperitoneal adipose tissue (IPAT) and livers of all of the mice were dissected and weighed. The liver was dissected, and the caudate lobes of each liver were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.4. After 24 h of fixation, the liver was stored in 70% ethanol at 4°C until tissue processing in the Leica ASP200S tissue processor (Leica Biosystems Inc.). The paraffin embedding station Leica EG1150H (Leica Biosystems Inc.) was used to create paraffin blocks from the wax-penetrated liver samples. Another part of liver tissue, IPAT and SCAT were flash-frozen in liquid nitrogen and stored at –80°C for later extraction of mRNA.

Determination of hormonal and biochemical parameters

The blood glucose levels were measured using a glucometer (Arkray, Kyoto, Japan). Glycated hemoglobin (HbA1c) was measured using Afinion kits (Afinion AS100, Axis-Shield PoC-AS, Oslo, Norway). The plasma insulin concentrations

Figure 1
Schema of experimental design.

were measured using an RIA assay (Millipore). Cholesterol was determined by colorimetric assay (Erba Lachema, Brno, Czech Republic). The plasma triglyceride (TAG) levels were measured using a quantitative enzymatic reaction (Sigma-Aldrich), and the free fatty acid (FFA) levels were determined using a colorimetric assay (Roche). All measurements were performed according to the manufacturer's instructions.

Liver histology

Liver samples in paraffin blocks were cut on a Leica RM2255 microtome (Leica Biosystems Inc.) to slices of 5 μ m thickness. Deparaffinization in xylene and rehydration in an ethanol range was performed. Slices were stained in hematoxylin using Weigert's iron hematoxylin solution set (HT1079-1Set, Sigma-Aldrich). Slices were washed with tap water and subsequently stained for 1 min in 0.5% eosin Y (C.I. 45380, Carl Roth GmbH+Co. KG, Karlsruhe, Germany). After washing with tap water, the samples were dehydrated and covered with DPX mounting medium (06522, Sigma-Aldrich). Histological images were performed at 200 $\times$ magnification.

Western blotting

Hypothalami were processed, and Western blotting was performed as previously described (Špolcová *et al.* 2015). The following primary antibodies were used: total STAT3, phospho-STAT3 (Y705), phospho-STAT3 (S727), SOCS3, total AMPK, phospho-AMPK, phosphoinositide-3-kinase (PI3 kinase), total AKT, phospho-AKT (S473) (Cell Signaling Technology) and beta-actin (Sigma-Aldrich). The following secondary antibodies were used: anti-mouse IgG HRP-linked antibody and anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology).

Determination of mRNA expression

The mRNA expression of the genes of interest in liver (acetyl-CoA carboxylase 1 (*Acaca*), peroxisome proliferator-activated receptor (*Prarg* and *Ppara*), sterol regulatory element-binding protein 1 (*Srebp1*), fatty acid synthase (*Fasn*), phosphoenolpyruvate carboxykinase 1 (*Pck1*), carnitine palmitoyltransferase 1a (*Cpt1a*), and glucose-6-phosphatase (*G6pc*)) was determined using an ABI PRISM 7500 instrument (Applied Biosystems) in samples from the mouse liver as described previously (Prazienkova *et al.* 2017). The expression of beta-2-microglobulin (*B2m*)

was used to compensate for variations in input mRNA amounts and the efficiency of RT. The formula 2-dCt was used to calculate relative gene expression.

Statistics

The data are presented as the means $\pm$ S.E.M. Statistical analysis was performed using unpaired *t*-test or one-way or two-way ANOVA followed by Bonferroni's *post hoc* test as indicated in Figure legends and Tables with Graph-Pad Prism Software, and $P < 0.05$ was considered statistically significant.

Results

Experiment 1: Treatment with leptin, palm¹¹-PrRP31, and their combination in younger *ob/ob* mice

The leptin + palm¹¹-PrRP31 combination attenuated food intake and body weight in a synergistic manner in younger mice

A decreasing effect of the leptin + palm¹¹-PrRP31 combination on cumulative food intake was significant after the first day of treatment compared to both the *ob/ob* saline and *ob/ob* leptin; between days 11 and 14, the effect of the combined treatment was significant compared to that in the *ob/ob* saline. There were significant differences between phenotypes (*ob/ob* saline versus WT saline) between days 5 and 14 (Fig. 2A and B). The body weight change caused by the treatment was significant only for the leptin + palm¹¹-PrRP31 combination since day 12 compared to both *ob/ob* saline and *ob/ob* leptin (Fig. 2C); it was obvious that *ob/ob* mice did not gain weight during the treatment with the leptin + palm¹¹-PrRP31 combination. The final body weight of *ob/ob* saline was significantly higher than that of WT saline (Fig. 2D); only the leptin + palm¹¹-PrRP31 combination significantly lowered the final body weight compared to *ob/ob* saline (Fig. 2E).

Leptin and palm¹¹-PrRP31 synergistically decreased the liver weight of younger *ob/ob* mice

As expected, WT saline group had significantly lower weights of SCAT and IPAT compared to *ob/ob* saline group. None of the treatments affected adipose tissue weights compared to *ob/ob* saline (Table 1A).

The liver weight of WT saline group was significantly higher than that of *ob/ob* saline group (Fig. 3A and Table 1A). Leptin significantly lowered liver weight in *ob/ob*

Figure 2

Food intake and body weight change of *ob/ob* mice in Experiment 1. (A) Food intake and (B) cumulative food intake at the end of experiment: *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (C) Body weight change of *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (D) Body weight at the end of experiment of WT saline and *ob/ob* saline mice. (E) Body weight at the end of experiment of *ob/ob* treated mice with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. Data are means $\pm$ S.E.M. ($n = 8-10$). Significance is $*/**P < 0.05$, $*/**/+P < 0.01$, $****/+P < 0.0001$ vs *ob/ob* saline or *ob/ob* leptin group, respectively (t -test or one-way ANOVA + Bonferroni *post hoc* test). Magnification was 200 $\times$.

mice; its effect was pronounced in the leptin + palm¹¹-PrRP31 combination (Fig. 3A and Table 1A). As single leptin did not cause any significant effect, the combined action of leptin and palm¹¹-PrRP31 seems synergistic (Fig. 3B). Histology of liver slices demonstrated regression of fat droplets in the liver tissue of all treated *ob/ob* groups compared to *ob/ob* saline toward the image of WT saline (Fig. 3C).

Body temperature was significantly lower in *ob/ob* saline compared to WT saline; none of the treatments affected body temperature in *ob/ob* mice (Table 1A).

Leptin and palm¹¹-PrRP31 synergistically attenuated blood glucose in younger *ob/ob* mice

Hyperglycemia was obvious in *ob/ob* saline compared to WT saline (Table 1B). Blood glucose was significantly lowered by both leptin and palm¹¹-PrRP31, and the leptin + palm¹¹-PrRP31 combination normalized glycemia to the level of WT saline (Table 1B); this result again pointed to a synergistic action of leptin and palm¹¹-PrRP31. *Ob/ob* saline was very significantly hyperinsulinemic compared to WT saline (Table 1B); neither treatment significantly affected the insulin level of *ob/ob* mice (Table 1B).

Table 1 Morphometric and metabolic parameters in Experiment 1.

	WT saline	<i>ob/ob</i> saline	<i>ob/ob</i> leptin	<i>ob/ob</i> palm ¹¹ -PrRP31	<i>ob/ob</i> leptin+palm ¹¹ -PrRP31
(A) Morphometric parameters					
BW (g)	20.38 ± 0.54****	37.28 ± 0.67	35.64 ± 0.75	35.30 ± 0.49	35.34 ± 0.85
SCAT (g)	0.28 ± 0.05****	4.08 ± 0.28	3.74 ± 0.28	3.60 ± 0.29	3.68 ± 0.24
IPAT (g)	0.28 ± 0.04****	2.83 ± 0.11	2.57 ± 0.09	2.51 ± 0.10	2.60 ± 0.10
Liver weight (g)	0.97 ± 0.24****	2.73 ± 0.11	2.36 ± 0.09*	2.36 ± 0.12	2.08 ± 0.09***
Rectal temp (°C)	36.75 ± 0.17****	35.00 ± 0.21	35.64 ± 0.28	35.22 ± 0.32	35.33 ± 0.36
(B) Metabolic parameters					
Glucose (mmol/l)	5.65 ± 0.18***	14.19 ± 1.82	9.38 ± 1.16*	9.71 ± 1.21*	5.97 ± 0.35***
Insulin (ng/ml)	1.47 ± 0.51****	21.02 ± 3.53	15.35 ± 3.34	24.03 ± 4.41	19.27 ± 3.06
CHOL (mmol/l)	2.07 ± 0.10***	3.43 ± 0.22	3.13 ± 0.10	3.18 ± 0.21	3.11 ± 0.12
TAG (mmol/l)	1.02 ± 0.12	0.90 ± 0.09	1.02 ± 0.07	0.79 ± 0.04	1.11 ± 0.08
FFA (mmol/l)	0.12 ± 0.01***	0.32 ± 0.04	0.39 ± 0.05	0.34 ± 0.07	0.46 ± 0.04

(A) Morphometric parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (B) Metabolic parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. Data are means ± s.e.m. ($n = 8-10$). Significance is * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs *ob/ob* saline (t-test or one-way ANOVA + Bonferroni *post hoc* test).

Increased cholesterol but comparable TAG and FFA levels were detected in *ob/ob* saline compared to WT mice. None of the treatments affected cholesterol or TAG or FFA levels in *ob/ob* mice (Table 1B).

The leptin + palm¹¹-PrRP31 combination lowered liver mRNA expression of enzymes regulating lipid metabolism in younger mice

Liver mRNA expression levels of *Acaca* (Fig. 4A), which catalyzes the rate-limiting step in fatty acid synthesis, and *Fasn* (Fig. 4B), which catalyzes the next step of *de novo* lipogenesis, were higher in *ob/ob* saline than those in WT saline and were attenuated similarly by the treatment with the leptin + palm¹¹-PrRP31 combination (Fig. 4A and B). Single leptin treatment also lowered *Fasn* mRNA liver expression in *ob/ob* mice (Fig. 4B). However, no difference in the mRNA expression of *Srebf1*, which controls the expression of *Acaca* and *Fasn*, was found between the *ob/ob* saline and WT saline groups, and no treatment affected it significantly (Fig. 4C). The mRNA expression levels of *Pck1* and *Cpt1a*, both enzymes regulating fatty acid oxidation, were also significantly enhanced in *ob/ob* saline compared to WT saline, but no treatment affected their expression (Fig. 4D and E). A similar pattern was observed for peroxisome proliferator-activated receptors *Ppara* and *Pparg*, which are other regulators of fatty acid oxidation (Fig. 4F and G).

The mRNA expression of *G6pc*, a key enzyme catalyzing gluconeogenesis and glycogenolysis, was enhanced in the *ob/ob* saline compared to WT saline and was attenuated by leptin + palm¹¹-PrRP31 combination treatment (Fig. 4H).

Ucp-1 mRNA expression was determined in Experiment 1. There were no significant differences (Supplementary

Fig. 4A, see section on [supplementary materials](#) given at the end of this article).

Experiment 2: Treatment with leptin, palm¹¹-PrRP31, and their combination in older *ob/ob* mice

Treatment did not affect the behavior of older *ob/ob* mice

The open field test clearly showed lowered velocity, traveled distance and area covered by 16-week-old *ob/ob* mice compared to the age-matched WT mice. Analogously, after treatment at the age of 16–24 weeks, *ob/ob* saline showed a significant decrease in all of the above-mentioned parameters, and no effect of treatment on locomotor activity was found. Since the average distance from the maze wall (wall distance) did not differ between *ob/ob* mice and WT mice at either 16 or 24 weeks, we could assume that anxiety was not a probable cause of locomotor activity attenuation (Supplementary Fig. 1). The lower mobility could be attributed to the morbid obesity of *ob/ob* mice and leptin deficiency-related behavioral changes.

Morphometric and metabolic parameters before the treatment of older mice

Before starting the experiment, at the age of 16 weeks, ten *ob/ob* and ten WT mice were taken out from their particular cohort to characterize them before the treatment. Body, SCAT, and liver weights were significantly lower in WT mice compared to *ob/ob* mice (Supplementary Table 1A).

Analogously to Experiment 1, *ob/ob* mice had high hyperinsulinemia, but unlike in Experiment 1, they were normoglycemic similarly as WT mice (Supplementary Table 1B). Similar to Experiment 1, *ob/ob* mice had a significantly

Figure 3

Liver weight and liver histology of *ob/ob* mice in Experiment 1. (A) Liver weight of WT saline and *ob/ob* saline mice. (B) Liver weight of *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (C) Liver histology of WT saline, *ob/ob* saline, *ob/ob* leptin, *ob/ob* palm¹¹-PrRP31 and *ob/ob* leptin + palm¹¹-PrRP31. Data are means $\pm$ S.E.M. ($n = 8-10$). Significance is * t - $P < 0.05$, *** t - $P < 0.001$, **** t - $P < 0.0001$ vs *ob/ob* saline or *ob/ob* leptin group, respectively (t -test or one-way ANOVA + Bonferroni *post hoc* test).

increased cholesterol level, but TAG and FFA levels did not differ from those of WT mice (Supplementary Table 1B).

Leptin and palm¹¹-PrRP31 synergistically lowered body weight in older *ob/ob* mice

None of the treatments affected cumulative food intake compared to *ob/ob* saline (Fig. 5A and B). Until day 22 of the treatment, the leptin + palm¹¹-PrRP31 combination caused a negative change in body weight in *ob/ob* mice (Fig. 5C). The change in body weight caused by the leptin + palm¹¹-PrRP31 combination was significant compared to that of the *ob/ob* saline after day 20 and compared to that of *ob/ob* leptin after day 22 of the treatment (Fig. 5C). Figure 5C showed a decline in body weight at day 20, this could happen because anorexigenic substances in mice with DIO were effective until certain time of the treatment and then the effect of the substance ceased most probably because of the compensatory mechanisms that attenuated anorexigenic effect of the substances. This is also known from the treatment of obesity in humans as well. The final body weight of *ob/ob* saline was significantly higher than that of WT saline (Fig. 5D); treatment with the leptin + palm¹¹-PrRP31 combination lowered the final body weight significantly compared to *ob/ob* saline (Fig. 5E). All results point to a synergistic effect of leptin and palm¹¹-PrRP31 on body weight in *ob/ob* mice.

Leptin and palm¹¹-PrRP31 normalized body temperature synergistically in older *ob/ob* mice

As expected, SCAT weight was significantly higher in *ob/ob* saline compared to WT saline. Only leptin + palm¹¹-PrRP31 significantly lowered SCAT weight (Table 2A). Similar to Experiment 1, WT saline group had a significantly lower liver weight than *ob/ob* mice. Unlike Experiment 1, no treatment significantly affected the liver weight of *ob/ob* mice (Table 2A).

Similar to Experiment 1, *ob/ob* saline were hypothermic compared to WT saline. Although single leptin and single palm¹¹-PrRP31 did not affect body temperature, the leptin + palm¹¹-PrRP31 combination significantly upregulated the body temperature of *ob/ob* mice (Table 2A); these findings again could point to their synergistic action.

The treatment did not affect glucose resistance but lowered cholesterol in older *ob/ob* mice

The glucose level in *ob/ob* saline did not differ from the normoglycemic WT saline; no treatment affected blood

Figure 4

mRNA expression in liver in Experiment 1. *Ob/ob* mice were treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (A) *Acaca*. (B) *Fasn*. (C) *Srebp1*. (D) *Pck1*. (E) *Cpt1a*. (F) *Ppara*. (G) *Pparg*. (H) *G6pc*. Data are means $\pm$ s.e.m. ($n = 8-10$). Significance is * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs *ob/ob* saline (*t*-test or one-way ANOVA + Bonferroni *post hoc* test).

glucose measured at the end of Experiment 2 (Table 2B). On the other hand, the *ob/ob* saline had significantly higher HbA1c levels compared to WT saline; no treatment affected HbA1c levels (Table 2B). The course of OGTT was similar for all treated and *ob/ob* saline, and compared to that of WT mice, it pointed to glucose intolerance in *ob/ob* mice untreatable by leptin and PrRP analog at doses used (Fig. 6). Again, *ob/ob* mice were hyperinsulinemic compared to WT mice (Table 2B).

Similar to Experiment 1, cholesterol levels were significantly higher in *ob/ob* saline than in WT saline at the end of Experiment 2. Unlike Experiment 1, treatment with palm¹¹-PrRP31 and its combination with leptin

attenuated cholesterol levels toward that of WT saline (Table 2B). The TAG and FFA levels did not differ between *ob/ob* saline and WT saline; neither were affected by any treatment (Table 2B).

Liver mRNA expression of proteins regulating lipid metabolism was not affected by the treatment in older *ob/ob* mice

Unlike Experiment 1, no difference was found in liver mRNA expression of enzymes and transcription factors regulating lipid metabolism between *ob/ob* saline and WT saline, and no treatment affected these mRNA liver expression levels (Supplementary Fig. 2).

Figure 5

Food intake and body weight change of *ob/ob* mice in Experiment 2. (A) Food intake and cumulative food intake at the end of experiment of *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (B) Body weight change of *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (C) Body weight at the end of experiment of WT saline and *ob/ob* saline mice. (D) Body weight at the end of experiment of *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. Data are means $\pm$ S.E.M. ($n = 8-10$). Significance is * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ vs *ob/ob* saline or *ob/ob* leptin group, respectively (t -test or one-way ANOVA + Bonferroni *post hoc* test).

Ucp-1 mRNA expression was determined in Experiment 2, and there were only significant differences between phenotypes (*ob/ob* saline versus WT saline), but the treatment did not cause any significant change compared to *ob/ob* saline (Supplementary Fig. 3B).

Leptin and palm¹¹-PrRP31 synergistically attenuated SOCS3, and both leptin and palm¹¹-PrRP31 lowered AMPK phosphorylation in the hypothalami of *ob/ob* mice

Activation of the anorexigenic hypothalamic pathways JAK-STAT and AMPK was followed after treatment with leptin, palm¹¹-PrRP31, and their combination and compared to *ob/ob* saline group (Fig. 7A). STAT3 protein was enhanced after leptin treatment in *ob/ob* mice (Fig. 7D); Tyr705 p-STAT did not differ significantly between saline

and compound-treated *ob/ob* groups (Fig. 6B), but Ser727 p-STAT was increased by all three treatments (Fig. 7C). SOCS3 protein, which negatively regulates leptin receptor signaling, was significantly lowered by palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31 combination treatment, but not by leptin; this finding could point to leptin + palm¹¹-PrRP31 synergistically attenuating the effect of SOCS3 production (Fig. 7E).

Regarding the common leptin and insulin pathway, PI3K protein was increased significantly by palm¹¹-PrRP31 but not by single leptin or its combination with palm¹¹-PrRP31 in *ob/ob* mice (Fig. 7F); phosphorylation of AKT at Ser473 was not affected significantly by any treatment and only tended to increase (Fig. 7G).

AMPK phosphorylation was significantly lowered after all three treatments (Fig. 7I).

Table 2 Morphometric and metabolic parameters Experiment 2.

	WT saline	<i>ob/ob</i> saline	<i>ob/ob</i> leptin	<i>ob/ob</i> palm ¹¹ -PrRP	<i>ob/ob</i> leptin+palm ¹¹ -PrRP
(A) Morphometric parameters					
BW (g)	32.23 ± 0.80****	60.90 ± 0.93	60.36 ± 1.18	58.15 ± 0.87	56.36 ± 1.16*
SCAT (g)	0.39 ± 0.06****	6.45 ± 0.33	6.23 ± 0.24	5.57 ± 0.31	5.19 ± 0.36*
IPAT (g)	0.49 ± 0.05****	1.98 ± 0.06	1.99 ± 0.13	2.37 ± 0.16	2.31 ± 0.13
Liver weight (g)	1.35 ± 0.04****	4.42 ± 0.14	4.35 ± 0.20	3.93 ± 0.06	4.08 ± 0.18
Rectal temp (°C)	37.84 ± 0.17****	35.42 ± 0.18	35.09 ± 0.26	34.93 ± 0.32	36.52 ± 0.14*
(B) Metabolic parameters					
Glucose (mmol/l)	8.05 ± 0.27	7.96 ± 0.38	6.78 ± 0.49	8.29 ± 0.40	8.99 ± 0.97
Hb1Ac (mmol/mol)	23.8 ± 0.36****	33.2 ± 1.28	35.5 ± 1.09	32 ± 2.04	28.1 ± 2.18
Insulin (ng/ml)	0.11 ± 0.04***	9.43 ± 1.78	9.90 ± 2.82	16.77 ± 4.98	9.74 ± 3.74
CHOL (mmol/l)	1.75 ± 0.07****	5.00 ± 0.24	4.50 ± 0.17	3.66 ± 0.22***	3.82 ± 0.18**
TAG (mmol/l)	0.9 ± 0.09	0.82 ± 0.03	0.98 ± 0.05*	0.82 ± 0.04	0.83 ± 0.04
FFA (mmol/l)	1.33 ± 0.08	1.42 ± 0.06	1.59 ± 0.11	1.64 ± 0.10	1.59 ± 0.12

(A) Morphometric parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (B) Metabolic parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. Data are means ± s.e.m. ($n = 8-10$). Significance is * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs *ob/ob* saline (t-test or one-way ANOVA + Bonferroni *post hoc* test).

Discussion

Leptin and PrRP were shown to cooperate in decreasing food intake and increasing energy expenditure in rodents after their central administration (Ellacott *et al.* 2002). In our previous studies, lipidized PrRP analogs were described to attenuate obesity and beneficially affect related metabolic disturbances in mice with diet-induced obesity (Maletinska *et al.* 2015, Holubova *et al.* 2018) and improve tolerance to glucose in Zucker diabetic (ZDF) rats and Koletsky spontaneously hypertensive obese rats (SHROB) with impaired leptin signaling (Holubova *et al.* 2016, Mikulaskova *et al.* 2018). This study aimed to further elucidate the role of leptin and PrRP in energy metabolism and to identify possible molecular mechanisms underlying these effects.

Two experiments with leptin, palm¹¹-PrRP31, and leptin+palm¹¹-PrRP31 combined with two treatment periods (2 and 8 weeks) were performed in *ob/ob* mice at two ages: a younger one (8–10 weeks), which is metabolically active, and an older one (16–24 weeks), with established morbid obesity. The morphometric and metabolic parameters of single leptin at a subthreshold dose, single palm¹¹-PrRP31, and their combination at the end of both experiments were studied for their potential synergistic effect as well as an evaluation of the impact of both treatments on lipid metabolism and of the longer treatment on the main signaling pathways of leptin. At two ages, *ob/ob* mice differed in metabolic parameters and their susceptibility to treatment.

As hypothesized, in both experiments, food intake and body weight were negatively affected by both leptin

and palm¹¹-PrRP31, but the body weight change was significantly lowered only after combined leptin+palm¹¹-PrRP31 treatment. This indicates an anorexigenic synergistic effect of leptin and palm¹¹-PrRP31 on body weight in *ob/ob* mice and a possible influence of this combination on the metabolic state and signaling pathways.

As *ob/ob* mice are known to be hypothermic (Ohtake *et al.* 1977), both in younger and older age (Gratuze *et al.* 2017), body temperature was also followed at the end of both experiments. *Ob/ob* mice were hypothermic compared to WT mice in both experiments, but only in older mice did the leptin+palm¹¹-PrRP31 combination significantly upregulate the body temperature of *ob/ob* mice, indicating their synergistic action.

Figure 6

Oral glucose tolerance test in Experiment 2. WT mice were treated with saline and *ob/ob* mice were treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. Blood glucose was measured after oral glucose load 2 weeks before the end of treatment. Data are means ± s.e.m. ($n = 8-10$) (Two-way ANOVA + Bonferroni *post hoc* test).

Figure 7

Hypothalamic signalling in Experiment 2. Western blot analyses in hypothalami of *ob/ob* mice treated with saline, leptin, palm¹¹-PrRP31 and leptin + palm¹¹-PrRP31. (A) Overview of Western blots for specific proteins. (B) p-STAT3 (Y705). (C) p-STAT3 (S727). (D) STAT3. (E) SOCS3. (F) PI3K. (G) p-AKT (S473). (H) AKT. (I) p-AMPK. (J) AMPK. Data are means $\pm$ S.E.M. ($n = 8-10$). Significance is $^{*}/P < 0.05$, $^{**}/P < 0.01$, $^{***}/P < 0.001$ vs *ob/ob* saline or *ob/ob* leptin group, respectively (one-way ANOVA + Bonferroni *post hoc* test).

Hyperglycemia was reported in *ob/ob* mice a long time ago (Enser 1972), and later on, it was specifically found only between 5 and 16 weeks of age (Menahan 1983). Moreover, *ob/ob* mice at 26 weeks old were found to be normoglycemic compared to both WT and *ob/+* controls (Gratuze *et al.* 2017). In this study, *ob/ob* saline mice aged 10 weeks were hyperglycemic; 2 weeks of treatment with leptin, palm¹¹-PrRP31 and their combination attenuated blood glucose significantly. On the other hand, at 16 weeks of age (start of Experiment 2), all *ob/ob* mice were normoglycemic, and no treatment affected blood glucose. However, the HbA1c level, which is considered a long-term measure of glucose control (Sacks 2013), was significantly

higher in the older *ob/ob* saline than in WT saline and was not affected by any treatment. In addition, the OGTT demonstrated impaired glucose tolerance in older *ob/ob* mice when compared to WT saline and was not affected by any treatment. Similarly, a glucose tolerance test after an intraperitoneal glucose load showed impaired glucose tolerance in *ob/ob* mice at 26 weeks old (Gratuze *et al.* 2017). This suggests that even though *ob/ob* mice at the older age of 24 weeks were normoglycemic, their regulation of blood glucose was impaired.

A significantly enhanced insulin level in *ob/ob* mice at 8 and 16 weeks of age was reported a long time ago (Beloff-Chain *et al.* 1975), and *ob/ob* mice were found

to be hyperinsulinemic until 63 weeks of age (Menahan 1983). Similarly, in this study, *ob/ob* mice aged 10, 16, and 24 weeks had hyperinsulinemia that was resistant to any treatment. Likewise, hyperinsulinemia and an enhanced HOMA index were reported at 4 weeks and then at 26 weeks of age in *ob/ob* mice (Gratuze *et al.* 2017).

Increased cholesterol but similar TAG levels in *ob/ob* mice compared to WT mice were also reported a while ago (Enser 1972). Similarly, in this study, cholesterol levels were found to be significantly increased in *ob/ob* mice at the ages of 10, 16, and 24 weeks compared to their respective WT controls. Two-week-long treatment did not affect cholesterol levels in younger *ob/ob* mice, but an 8-week-long intervention with palm¹¹-PrRP31 and its combination with leptin attenuated cholesterol levels in 24-week-old *ob/ob* mice very significantly. Analogously to Enser *et al.* (1972), the TAG level did not differ between the *ob/ob* saline and WT saline at 10, 16, and 24 weeks of age and was not affected by any treatment.

As liver weight was significantly higher in 10-week-old *ob/ob* saline compared to WT saline and was attenuated by all treatments and histological images of liver steatosis followed this pattern in Experiment 1, an effect of treatment on liver lipid metabolism came into question. Similarly, the increased liver weight of 14-week-old *ob/ob* mice was linked to *de novo* lipogenesis (Perfield *et al.* 2013). In this study, *ob/ob* controls aged 10 weeks showed increased hepatic *de novo* lipogenesis through *Acaca* and *Fasn* mRNA expression compared to WT controls that was attenuated by treatment with the leptin + palm¹¹-PrRP31 combination. Lipid oxidation as evaluated through mRNA expression of *Cpt1a* and *Pck1*, which catalyze fatty acid oxidation, was enhanced in *ob/ob* saline compared to WT saline but remained unaffected by any treatment. In *ob/ob* mice, the leptin + palm¹¹-PrRP31 combination also lowered hepatic synthesis of glucose, the main substrate for lipogenesis, through attenuation of *G6pc* mRNA, a key enzyme catalyzing gluconeogenesis and glycogenolysis. Although liver weight was also significantly higher at 16 and 24 weeks of age in *ob/ob* mice compared to WT mice in Experiment 2, no differences between *ob/ob* saline and WT saline in liver mRNA expression of genes regulating lipid metabolism were registered, and no effect of the treatment was found. This could be explained by a gradual decrease in hepatic fatty acid synthesis from 7 to 16 weeks of age in *ob/ob* mice (Kaplan & Leveille 1981). Rodriguez *et al.* (2015) found mRNA expression of aquaglyceroporin 9 (AQP9), the channel for glycerol influx into liver, to be positively correlated with hepatic steatosis in *ob/ob* mice.

It points to glycerol abundance as another driving force of liver steatosis, besides *de novo* lipogenesis.

Lipidized PrRP was shown to target and activate the PVN, DMN, arcuate nucleus, and lateral hypothalamic area (Maletinska *et al.* 2015, Pirnik *et al.* 2015), and PI3K activation was detected after sub chronic palm¹¹-PrRP31 administration in the hypothalamus (Holubova *et al.* 2018). In this study, two main anorexigenic hypothalamic leptin pathways, JAK-STAT and AMPK, were followed in hypothalami from Experiment 2, where body weight change after leptin + palm¹¹-PrRP31 treatment was significant compared to *ob/ob* saline and single leptin. Regarding JAK/STAT, phosphorylation of STAT3 was found to be increased only at Ser727 but not at Tyr705 after treatment with leptin, palm¹¹-PrRP31, and the leptin-palm¹¹-PrRP31 combination. Although Tyr705 is considered the primary phosphorylation epitope in STAT3, direct Ser727 phosphorylation by insulin and other ligands was reported (Zhang *et al.* 2001). Positively regulated STAT3 and downregulated SOCS3 at the same time suggested that both leptin and palm¹¹-PrRP31 support JAK/STAT signaling in the hypothalami of *ob/ob* mice.

Hypothalamic AMPK phosphorylation was significantly lowered after all three treatments. This finding suggests that the AMPK anorexigenic pathway in the hypothalami of *ob/ob* mice was restored not only by leptin supplementation but also by palm¹¹-PrRP31 treatment. As inhibition of hypothalamic AMPK activity is necessary for the anorexigenic effects of leptin (Minokoshi *et al.* 2004), this study suggests that palm¹¹-PrRP31 could compensate for deficient leptin in *ob/ob* mice regarding leptin anorexigenic action in the hypothalamus. Moreover, a significant decrease in SOCS3 by palm¹¹-PrRP31 was not reached by a subthreshold dose of single leptin but by the leptin + palm¹¹-PrRP31 combination.

The synergistic effect of leptin and palm¹¹-PrRP31 was proven by several parameters: the decrease in liver weight and glucose levels after a shorter treatment in younger mice, and the body weight change, increase in body temperature, lowered cholesterol level and SOCS3 production with a longer treatment of older mice.

In conclusion, leptin and palm¹¹-PrRP31 synergistically lowered body weight and synergistically increased body temperature in older *ob/ob* mice with established morbid obesity. Regarding PrRP, this finding is novel and could point to other potent beneficial PrRP effects in the brain as obesity and hypothermia were recently linked to neurodegeneration.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0188>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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