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Prolactin-releasing peptide improved leptin hypothalamic signaling in obese mice

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Abstract

The situation following anti-obesity drug termination is rarely investigated, even though a decrease in body weight needs to be sustained. Therefore, this study examined the impact of twice-daily peripheral administration of 5 mg/kg [N-palm- γ Glu-Lys¹¹] prolactin-releasing peptide 31 (palm¹¹-PrRP31) in mice with diet-induced obesity (DIO from consuming a high-fat diet) after 28 days of treatment (palm¹¹-PrRP31 group) and after 14 days of peptide treatment followed by 14 days of discontinuation (palm¹¹-PrRP31 + saline group). At the end of the treatment, cumulative food intake, body weight and subcutaneous fat weight/body weight ratio and leptin plasma level were reduced significantly in both the palm¹¹-PrRP31 group and the palm¹¹-PrRP31 + saline group compared to the saline control group. This reduction correlated with significantly increased FOSB, a marker of long-term neuronal potentiation, in the nucleus arcuatus and nucleus tractus solitarius, areas known to be affected by the anorexigenic effect of palm¹¹-PrRP31. Moreover, activation of leptin-related hypothalamic signaling was registered through an increase in phosphoinositide-3-kinase, increased phosphorylation of protein kinase B (PKB, AKT) and enhanced extracellular signal-regulated kinase 1/2 phosphorylation. Besides, lowered apoptotic markers c-JUN N-terminal kinase and c-JUN phosphorylation were registered in the hypothalamus of both palm¹¹-PrRP31-treated groups. This study demonstrates that palm¹¹-PrRP31 positively affects feeding and leptin-related hypothalamic signaling, not only after 28 days of treatment but even 14 days after the termination of a 14-day long treatment without the yo-yo effect.

Key Words

- ▶ prolactin-releasing peptide
- ▶ FOSB
- ▶ leptin signaling
- ▶ apoptotic activation
- ▶ diet-induced obese mice

*Journal of Molecular
Endocrinology*
(2018) **60**, 85–94

Introduction

Obesity is a common metabolic disease, but its effective noninvasive therapy remains scarce (Bray et al. 2016). Several anti-obesity drugs – analogs of neurotransmitters decreasing appetite or craving for food – were withdrawn from the market because of their severe psychiatric or cardiovascular side effects (for reviews, see

Rodgers et al. 2012, Bray & Ryan 2014, Manning et al. 2014). Conversely, analogs of anorexigenic peptides promise minimal side effects during long-term anti-obesity treatment (Arch 2015, Patel 2015, Bray et al. 2016). Liraglutide, a peptide analog of glucagon-like peptide-1 (GLP1) used to treat type 2 diabetes, has been

recently approved for obesity treatment in the USA (Saxenda). A selective peptide agonist of receptor MC4R, a receptor of anorexigenic neuropeptide α -melanocyte-stimulating hormone (α -MSH), was shown to attenuate body weight in both rats and pigs with DIO (diet-induced obesity) (Fosgerau *et al.* 2014).

Both liraglutide and the α -MSH analog mentioned have palmitic acid attached in order to improve the pharmacokinetics of the drugs. Similarly, palmitoylation of anorexigenic neuropeptide prolactin-releasing peptide (PrRP) increased the stability and half-life of the peptide. In addition, unlike natural PrRP, palmitoylated PrRP accomplished a central anorexigenic effect both in mice and rats with DIO after peripheral administration (Maletínská *et al.* 2015, Holubová *et al.* 2016). Likewise, myristoylated and palmitoylated PrRP31 analogs with C-terminal Phe³¹ modified with non-coded amino acids showed *in vitro* biological activity and an acute effect on food intake after peripheral administration similar to natural PrRP31 (Pražienková *et al.* 2016). Similar biological properties were registered also with [N-palm- γ Glu-Lys¹¹] PrRP31 (palm¹¹PrRP31) with palmitoyl positioned in the middle of the peptide chain (Pražienková *et al.* 2017).

Central effect of PrRP was demonstrated after its intracerebroventricular administration that inhibited food intake and body weight gain and induced c-FOS in brain areas, such as PVN, regulating food intake (Lawrence *et al.* 2002). In PVN, and also in periventricular nucleus and dorsomedial nucleus (DMN) of the hypothalamus, reticular nucleus of the thalamus, nucleus tractus solitarius (NTS) and area postrema PrRP receptor, GPR10 is expressed (Roland *et al.* 1999).

PrRP neurons in the brainstem and hypothalamus were reported to express leptin receptors (Ellacott *et al.* 2002). In DMH PrRP neurons, leptin activated signal transducer and activator of transcription protein 3 (STAT3) (Takayanagi *et al.* 2008) while anorexigenic effect of leptin was impaired in PrRP- or GPR10-knockout mice (Dodd & Luckman 2013). It connected leptin signaling to the anorexigenic effect of PrRP, but the mechanism has not been explained yet.

After termination of long-term administration of an anti-obesity drug, it is necessary to sustain the decrease in body weight in order to minimize the yo-yo effect. However, the situation after anti-obesity drug termination is rarely investigated thoroughly. Therefore, in this study, food intake, body and organ weight and biochemical and hormonal parameters were examined in mice with DIO after a 28-day treatment with palm¹¹-PrRP31 and 28 days after the beginning of a 14-day treatment followed by

14 days without peptide treatment. In addition, the impact of such discontinued treatment was studied in the hypothalamic nuclei and nucleus tractus solitarius that were activated by lipidized PrRP in our previous studies (Maletínská *et al.* 2015, Pražienková *et al.* 2017). In addition, the effect of cessation of the treatment on hypothalamic signaling related to food intake regulation was determined.

Materials and Methods

Synthesis of palm¹¹-PrRP31

Human palmitoylated PrRP31 (SRTHRHSMEI K (N- γ -E (N-palm)) TPDINPAWYASRGIRPVGRF-NH₂), palm¹¹-PrRP31, was synthesized and purified as described previously (Maletínská *et al.* 2015). Palmitoylation in position 11 of the PrRP31 analog was performed on a fully protected peptide on resin as the last step (Pražienková *et al.* 2017). The purity and identity of the peptide was determined by analytical high-performance liquid chromatography and using a Q-TOF micro MS technique (Waters, Milford, MA, USA).

Study in DIO mice administered with palm¹¹-PrRP31 for 28 or 14 days and tissue dissection

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 Czech Republic.

Male C57BL/6 mice from Charles Rivers Laboratories were housed at a temperature of 23°C with a daily 12-h light/darkness cycle (lights on at 06:00 h). The mice were given *ad libitum* water and a standard rodent chow diet (Ssniff Spezialdiäten GmbH, Soest, Germany). Beginning at 8 weeks of age, mice were fed with a high-fat (HF) diet for 12 weeks to induce obesity. The energy content of the HF diet was 5.3 kcal/g, with 13, 60 and 27% of the calories derived from protein, fat and carbohydrate, respectively (Maletínská *et al.* 2015). Food intake and body weight were monitored weekly. Mice resistant to the HF diet were withdrawn from the experiment (approximately 10% of the original number). At the age of 19 weeks, the mice were divided into three groups ($n=14$ animals) and placed into separate cages with free access to HF diet and water.

Mice were subcutaneously (SC) injected twice a day with saline for 28 days (saline group), with palm¹¹-PrRP31 (5 mg/kg) for 28 days (palm¹¹-PrRP31 group) or with

palm¹¹-PrRP31 (5 mg/kg) for 14 days and for the following 14 days with saline (palm¹¹-PrRP31+saline group). The last injection was on afternoon of day 28, before the food was removed. On day 29, at the end of the treatment, overnight-fasted mice were killed by decapitation ($n=10$). The trunk blood was collected, and plasma was separated and stored at -20°C . The intraperitoneal adipose tissue (IPAT), the subcutaneous adipose tissue (SCAT), the brown adipose tissue (BAT) and the liver were dissected, weighed and stored at -70°C . The brains were removed, and the hypothalami were dissected and lysed in lysis buffer (Špolcová *et al.* 2015). The remaining four mice from each group were used for immunohistochemistry.

Determination of hormonal and biochemical parameters in blood and plasma

Glucose levels were measured in whole blood using a Glucocard glucometer (Arkray, Kyoto, Japan). In the plasma, insulin concentrations were measured with radioimmunoassay (RIA) and leptin concentrations were measured with enzyme-linked immunoassay (ELISA) (Millipore). The plasma triglyceride levels were measured by quantitative enzymatic reaction (Sigma), and the free fatty acids (FFA) levels were determined using a colorimetric assay (Roche). Cholesterol was determined by colorimetric assay (Erba Lachema, Brno, Czech Republic). All measurements were performed according to the manufacturer's instructions.

FOSB immunohistochemistry

After the treatment, 4 mice from each group were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and perfused transcardially as previously described (Pirnik *et al.* 2015). The brains were dissected and cut into coronal sections of 30 μm thickness at -22°C using a Cryocut (Leica Microsystems Nussloch GmbH, Germany) and processed as described previously (Pirnik *et al.* 2015) using a monoclonal mouse FOSB protein antibody (1:2000, Abcam 11959). Quantitative assessment of immunostained cells was performed under light microscope (Prima Star, Carl Zeiss), and representative pictures were captured using an Olympus DP 70 digital camera. The FOSB immunoreactive cells were counted separately on each side of the appropriate coronal sections (2–3 sections per animal) within the arcuate nucleus (ARC) (from -1.58 mm bregma to bregma -1.94 mm), paraventricular nucleus (PVN) (from bregma

-0.7 mm to bregma -0.94 mm), the dorsomedial nucleus (DMN) (from bregma -1.82 mm to bregma -1.94 mm) and the nucleus tractus solitarius (NTS) (from bregma -7.48 mm to bregma -7.32 mm) according to Franklin and Paxinos' atlas of the mouse brain (Franklin & Paxinos 1997).

Western blotting

Hypothalami were processed, and western blotting was performed as previously described (Špolcová *et al.* 2015).

The following primary antibodies were used for western blotting: insulin receptor substrate 1 (IRS1), phospho-IRS1 (Ser612), phosphoinositide-3-kinase (PI3 kinase), phospho-AKT (Ser473), total Akt, phospho-signal-regulated kinase 1/2 phosphorylation (MAPK/ERK1/2), total MAPK/ERK1/2, c-JUN, phospho-c-JUN and c-JUN N-terminal kinase (JNK), total STAT3, phospho-STAT3, SOCS3 (Cell Signaling Technology); leptin receptor (long form OB-R) and phospho-leptin receptor (Tyr1141) (Thermo Fisher Scientific) 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

IPAT, SCAT, BAT and liver were processed as described previously (Maletínská *et al.* 2015). Determination of the mRNA expression of *Lep* (leptin) in IPAT and SCAT, *Ucp1* (uncoupling protein-1) in BAT and *Srebp1* (sterol regulatory element-binding protein-1), *Acaca* (acetyl CoA carboxylase), *Lpl* (lipoprotein lipase), *Cpt1a* and *Cpt1b* (carnitine palmitoyltransferase 1a and 1b) in the liver was performed using an ABI PRISM 7500 instrument (Applied Biosystems). The expression of *B2m* (beta-2-microglobulin) was used to compensate for variations in input RNA amounts and the efficiency of reverse transcription.

Statistics

Statistical analysis of the DIO model was performed using unpaired *t*-tests or one-way ANOVA with a Bonferroni's *post hoc* test as indicated in the figure and table legends. The differences between the control and treated groups were considered to be significant at $P<0.05$. Linear regression analysis was used to test the relationship between leptin plasma levels and particular fat tissues.

Figure 1

Chronic effect of palm¹¹-PrRP31 on (A) food intake and (B and C) body weight in DIO mice. Mice were subcutaneously injected twice a day with saline for 28 days (saline group), with palm¹¹-PrRP31 (5 mg/kg) for 28 days (palm¹¹-PrRP31 group) or with palm¹¹-PrRP31 (5 mg/kg) for 14 days and for the following 14 days with saline (palm¹¹-PrRP31+saline group). Data are presented as means $\pm$ s.e.m. Statistical analysis was performed by One-way ANOVA with Bonferroni *post hoc* test, significance is *** P < 0.001 vs the saline treated group (n = 14). No significant difference was found between the groups treated with palm¹¹-PrRP31 for 14 or 28 days.

Results

Effect of the treatment on organ weight and biochemical parameters

In both the palm¹¹-PrRP31 group and the palm¹¹-PrRP31+saline group, cumulative food intake was lowered similarly and significantly from day 9 to day 28 compared to the saline control group (Fig. 1A). Moreover, at the end of the experiment, body weight and SCAT weight/body weight ratio of the palm¹¹-PrRP31 group and the palm¹¹-PrRP31+saline group were similar and were significantly lower than those of the saline control

group (Fig. 1B and Table 1). IPAT weight/body weight ratio remained unaffected by both methods of palm¹¹-PrRP31 treatment (Table 1).

A decrease in liver weight/body weight ratio was similar in both palm¹¹-PrRP31-treated groups but was not significant compared to the saline group (Table 1). Blood glucose, as well as insulin, triglycerides, FFA and cholesterol, in plasma were not affected significantly by both methods of palm¹¹-PrRP31 treatment (Table 1). However, plasma leptin was attenuated significantly in both palm¹¹-PrRP31- and palm¹¹-PrRP31+saline-treated mice compared to saline controls (Table 1).

It was demonstrated that leptin plasma levels correlated positively with subcutaneous adipose tissue (r = 0.852, P < 0.001) in all three groups studied. This was not due to palm¹¹-PrRP31 treatment because a similar relationship was seen even in control group (r = 0.642, P < 0.001). However, there was no relationship between leptin and IPAT (r = 0.003, non-significant).

Activation of neurons in brain areas connected with food intake regulation

Neuronal activity was followed at the end of the experiment in three hypothalamic nuclei connected with food intake regulation – PVN, ARC, DMN and in the NTS by FOSB immunohistochemistry. Significantly increased FOSB was registered in ARC and NTS but not PVN and DMN in the palm¹¹-PrRP31 group compared to saline controls (Fig. 2). FOSB in the palm¹¹-PrRP31+saline group was increased significantly only in NTS in comparison with the saline control group.

Table 1 Metabolic parameters analyzed in fasted blood plasma and organs weights of DIO mice at the end of experiment.

GROUP/parameter	Palm ¹¹ -PrRP31		
	Saline	PrRP31+saline	PrRP31
Glucose (mmol/L)	8.06 $\pm$ 0.45	8.37 $\pm$ 0.30	8.65 $\pm$ 0.40
Insulin (ng/mL)	1.55 $\pm$ 0.30	1.28 $\pm$ 1.16	1.10 $\pm$ 0.17
Leptin (ng/mL)	75.86 $\pm$ 7.70	42.40 $\pm$ 7.01**	35.29 $\pm$ 7.94**
Triglycerides (mg/mL)	0.22 $\pm$ 0.28	0.36 $\pm$ 0.04*	0.26 $\pm$ 0.06
FFA (mmol/L)	0.52 $\pm$ 0.02	0.54 $\pm$ 0.03	0.49 $\pm$ 0.04
Cholesterol (mmol/L)	3.94 $\pm$ 0.22	3.70 $\pm$ 0.21	3.20 $\pm$ 0.25*
SCAT/bw (%)	7.44 $\pm$ 0.50	5.25 $\pm$ 0.36**	4.74 $\pm$ 0.49**
IPAT/bw (%)	4.37 $\pm$ 0.31	4.42 $\pm$ 0.20	5.04 $\pm$ 0.29
Liver/bw (%)	3.63 $\pm$ 0.35	3.30 $\pm$ 0.08	3.22 $\pm$ 0.14

Data are presented as means $\pm$ s.e.m. Statistical analysis was performed by unpaired *t*-test. Significance is * P < 0.05, ** P < 0.01, *** P < 0.001 vs the saline-treated group (n = 10). No significant difference was found between the groups treated with palm¹¹-PrRP31 for 14 or 28 days.

FFA, free fatty acids; IPAT, intraperitoneal adipose tissue; SCAT, subcutaneous adipose tissue.

A The FOSB immunoreactive cells activation

B The amount of immunoreactive cells

Figure 2

(A) The FOSB immunoreactive cells activation and (B) the amount of immunoreactive cells in selected brain nuclei (ARC, NTS, PVN, DMN) involved in food intake regulation. Mice were subcutaneously injected twice a day with saline for 28 days (saline group), with palm¹¹-PrRP31 (5 mg/kg) for 28 days (palm¹¹-PrRP31 group) or with palm¹¹-PrRP31 (5 mg/kg) for 14 days and for the following 14 days with saline (palm¹¹-PrRP31 + saline group). After the treatment, mice were deeply anesthetized and perfused transcardially. The brains were dissected and cut into coronal sections. The FOSB immunoreactive cells were counted separately on each side of the appropriate coronal sections. Data are presented as means $\pm$ s.e.m. Statistical analysis was performed by unpaired t-test. Significance is ** P < 0.01, *** P < 0.001 vs the saline treated group, ++ P < 0.01, +++ P < 0.001 palm¹¹-PrRP31 + saline vs palm¹¹-PrRP31 (n = 4). ARC, nucleus arcuatus; NTS, nucleus of solitary tract; PVN, paraventricular nucleus; DMN, dorsomedial nucleus.

Impact of the treatment on leptin-related signaling pathways in hypothalamus

Signaling pathways were measured in hypothalamic lysates using Western blotting in all three experimental groups (n = 8). As demonstrated in Fig. 3, the palm¹¹-PrRP31 group showed a significant increase in phosphorylation of IRS1 and AKT, and both the palm¹¹-PrRP31 and the palm¹¹-PrRP31 + saline groups showed PI3 kinase activation. Phosphorylation of MAPK/ERK1/2 was increased in both peptide-treated groups compared to the saline-treated group (Fig. 3).

The apoptotic pathway in the hypothalami of both palm¹¹-PrRP31 and palm¹¹-PrRP31 + saline groups was attenuated as shown by decreased levels of JNK and phospho-c-JUN/c-JUN ratio; however, total c-JUN was

increased by the treatment (Fig. 4). There was no significant change in leptin STAT3 signaling and inhibition of this signaling through SOCS3 was attenuated in palm¹¹-PrRP31-treated compared to saline-treated group (Fig. 4). Significant increase of phosphorylated leptin receptor/total leptin receptor was observed in palm¹¹-PrRP31-treated group and decreased total leptin receptor in the same group compared to saline-treated group (Fig. 5).

Impact of the treatment on mRNA expression of selected genes

BAT *Ucp1* mRNA expression was increased in palm¹¹-PrRP31 mice but not palm¹¹-PrRP31 + saline mice compared to saline controls (Fig. 6A). *Lep* mRNA expression tended toward a non-significant decrease in SCAT (Fig. 6B) and

Leptin-related signaling pathways in hypothalamus – western blotting

kDa	saline	palm ¹¹ -PrRP31 + saline	palm ¹¹ -PrRP31	antibody
180				IRS1
85				PI3 kinase
60				p-AKT Ser473
60				AKT
42, 44				p-ERK
42, 44				ERK
42				β-actin

Figure 3

Signaling pathways in hypothalamus. Signaling pathways were measured in hypothalamic lysates using Western blotting. Densitometric quantification of Western blots is normalized to β-actin. Leptin-related signaling pathways – total IRS1, total PI3 kinase, phospho-AKT (Ser473), total AKT, phospho-ERK1/2 and total ERK. IRS1, insulin receptor substrate 1; PI3 kinase, phospho-inositol-3 kinase; AKT, protein kinase B.

was significantly attenuated in IPAT in both the palm¹¹-PrRP31 group and the palm¹¹-PrRP31+saline group compared to saline-treated controls (Fig. 6C).

In the liver, mRNA expression of *Srebf*, a transcription factor of enzymes regulating *de novo* lipogenesis, showed a non-significant decrease in both palm¹¹-PrRP31-treated groups compared to the saline control group (Fig. 6D), as did *Acaca* mRNA, which catalyzes the rate limiting step of *de novo* lipogenesis (Fig. 6E). Liver *Lpl* mRNA was significantly attenuated in the palm¹¹-PrRP31 group and non-significantly reduced in the palm¹¹-PrRP31+saline group (Fig. 6F). Carnitine palmitoyl transferase I (*Cpt1*) is a key enzyme of fatty acid oxidation in the liver. Expression of the liver-specific *Cpt1a* mRNA was non-significantly decreased (Fig. 6G) and that of liver-nonspecific *Cpt1b* mRNA was significantly lowered in both the palm¹¹-PrRP31 group and the palm¹¹-PrRP31+saline group compared to the saline control group (Fig. 6H).

Discussion

As shown in our recent studies, lipidization of PrRP31 not only increased its stability (Maletínská et al. 2015) but also enabled PrRP31 to accomplish a central anorexigenic effect both in mice and rats with DIO (Maletínská et al. 2015, Holubová et al. 2016, Kuneš et al. 2016). In our previous studies, DIO in mice was shown to be associated with high levels of leptin (Matysková et al. 2008) and leptin resistance (Matysková et al. 2010). In this study, the situation after discontinuation of treatment with PrRP31 was investigated because we did not find any data in the literature on anti-obesity drugs, either in current use or following withdrawal. Only Henderson and colleagues (Henderson et al. 2016) followed body weight of cynomolgus monkeys after the cessation of an 8-week treatment with a dual agonist of GLP1 and glucagon and found it to be at the pretreatment level 2 weeks after the

The apoptotic pathway in hypothalamus – western blotting

kDa	saline	palm ¹¹ -PrRP31 + saline	palm ¹¹ -PrRP31	antibody
46, 54				JNK
48				p-c-JUN
48				c-JUN
42				β-actin

JAK2/STAT3 pathway in hypothalamus – western blotting

kDa	saline	palm ¹¹ -PrRP31 + saline	palm ¹¹ -PrRP31	antibody
26				SOCS3
79, 86				p-STAT3
79				STAT3
42				β-actin

Figure 4

The apoptotic pathway in hypothalamus – total JNK, phospho-c-JUN and total c-JUN. JAK2/STAT3 pathway in hypothalamus – total SOCS3, phospho-STAT3 and total STAT3. JNK, c-JUN N-terminal kinase.

treatment. We considered a study on the palm¹¹-PrRP31 post-treatment situation useful regarding a possible yo-yo effect after the drug termination.

In our previous studies (Maletínská et al. 2015, Holubová et al. 2016), repeated treatment of DIO rodents with palm-PrRP31 caused food intake lowering followed by gradual decrease in body weight compared to the quick drop in body weight observed after liraglutide treatment (Hansen et al. 2014). In this study, DIO mice treated for 28 days with palm¹¹-PrRP31 (palm¹¹-PrRP31 group) and those treated with palm¹¹-PrRP31 for 14 days and then 14 days with saline (palm¹¹-PrRP31 + saline group) reached a similar decrease in body weight and SCAT weight to body

weight ratio. However, *lep* mRNA expression in SCAT in both palm¹¹-PrRP31 mice and palm¹¹-PrRP31 + saline mice did not significantly differ from that in saline control mice. On the other hand, *lep* mRNA expression in both palm¹¹-PrRP31 mice and palm¹¹-PrRP31 + saline mice was significantly attenuated compared to saline control mice in IPAT whose IPAT/body weight were not changed by the treatment. It is generally accepted that plasma leptin is directly proportional to its mRNA expression (Wajchenberg 2000, Zhang et al. 2002) and that a decrease in plasma leptin results either from a lowering of particular fat depot(s), an attenuated leptin mRNA expression or both. Therefore, decreased body weight could be a consequence

Phosphorylation of leptin receptor – western blotting

kDa	saline	palm ¹¹ -PrRP31 + saline	palm ¹¹ -PrRP31	antibody
125				Leptin receptor
100				p-leptin receptor Y1141
42				β-actin

Figure 5

Phosphorylation of leptin receptor in hypothalamus. Data are presented as means $\pm$ S.E.M. Statistical analysis was performed by unpaired t-test. Significance is * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs saline treated group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ palm¹¹-PrRP31 + saline vs palm¹¹-PrRP31 ($n = 8$).

of decreased SCAT and tendency of leptin mRNA decrease, despite the discontinuation of treatment. The well-known synergism between the effects of leptin and PrRP (Dodd & Luckman 2013) could suggest possible improvement of leptin resistance by lipidized PrRP.

Changes in metabolic parameters were complemented with genes related to fatty acid metabolism in fat and liver. Lipogenesis in the liver could be attenuated regarding to the non-significant decrease in mRNA expression of both *Acaca*, the enzyme catalyzing the rate limiting step of lipogenesis and *Srebf* that regulates *Acaca* transcription. In addition, we show that mRNA expression of lipolytic enzymes *Lpl* and *Cpt1b* were significantly lowered by the action of palm¹¹-PrRP31. This is in accordance with our previous study with palm-PrRP31, a PrRP31 analog with palmitic acid attached to the N-terminus of the peptide that complexly attenuated liver lipid metabolism (Maletínská et al. 2015). Increased UCP1 in BAT points to the increased energy expenditure and has already been described in our previous study (Pražienková et al. 2017).

Increased neuronal activity determined by FOSB activation, a marker of long-term neuronal potentiation (Nestler et al. 2001), in both the arcuate nucleus and NTS in the palm¹¹-PrRP31 group in this study correlated with our previous results, where c-FOS, a marker of short-term neuronal potentiation, increased after acute administration of palm-PrRP31 (Maletínská et al. 2015). Interestingly, increased FOSB was also found in NTS in the palm¹¹-PrRP31 + saline group; we could hypothesize

that a decrease in the leptin level was rather durable and increased leptin-related signaling was the driving force for maintaining body weight and protecting against the yo-yo effect.

Palm¹¹-PrRP31 attenuated HF feeding that impacted signaling in the hypothalamus, namely, increased IRS1 and AKT through an increase in PI3 kinase and enhanced ERK phosphorylation. Leptin receptor-induced PI3 kinase pathway is more sensitive to be lowered by DIO than STAT3 (Balland & Cowley 2015). Also, in our study, STAT3 signaling was not significantly changed and SOCS3 significantly decreased after the peptide treatment. It seems that the PI3 kinase pathway was restored by the body weight-lowering effect of palm¹¹-PrRP31. Increased ERK1/2 phosphorylation was a result of either leptin receptor or PrRP receptor signaling. Phosphorylation of leptin receptor in hypothalamus was increased only after palm¹¹-PrRP31 treatment.

JNK is generally activated by HF feeding in DIO mice, both in the periphery and the brain, namely, in the hypothalamus (De Souza et al. 2005). The precise mechanism of this JNK effect is still vague. Food intake and body weight in DIO mice were shown to be attenuated by a pan-JNK inhibitor administered both peripherally and intracerebroventricularly (Gao et al. 2017), and neuron-specific JNK inactivation increased hypothalamic insulin sensitivity and peripheral tolerance to glucose and insulin (Belgardt et al. 2010). In this study, palm¹¹-PrRP31 attenuated DIO and in the hypothalamus lowered both JNK and c-JUN phosphorylation related to c-JUN that is

Figure 6

Chronic effect of palm¹¹-PrRP31 on mRNA expressions in DIO mice. (A) *Ucp1* in BAT, (B) *Lep* in SCAT, (C) *Lep* in IPAT, (D) *Srebf*, (E) *Acaca*, (F) *Lpl*, (G) *Cpt1a* and (H) *Cpt1b* in liver. Mice were subcutaneously injected twice a day with saline for 28 days (saline group), with palm¹¹-PrRP31 (5 mg/kg) for 28 days (palm¹¹-PrRP31 group) or with palm¹¹-PrRP31 (5 mg/kg) for 14 days and for the following 14 days with saline (palm¹¹-PrRP31 + saline group). After the treatment the mRNA expressions were determined. Data are presented as mean $\pm$ s.e.m. The data were normalized to beta-2-microglobulin and analyzed by unpaired *t*-test, significance is *P<0.05, **P<0.01 vs the saline treated group, +*P*<0.05 palm¹¹-PrRP31 + saline vs palm¹¹-PrRP31 (*n*=10). *Ucp1*, uncoupling protein-1; *Lep*, leptin; *Lpl*, lipoprotein lipase; *Cpt1b*, carnitine palmitoyltransferase 1b; BAT, brown adipose tissue; IPAT, intraperitoneal adipose tissue; SCAT, subcutaneous adipose tissue

directly regulated by JNK. This connects palm¹¹-PrRP31's body weight-lowering effect to the hypothalamic JNK/c-JUN signaling.

In conclusion, palm¹¹-PrRP31 induced a significant decrease in body weight, SCAT weight and IPAT *Lep* mRNA expression resulting in an attenuated plasma leptin that continued even 14 days after termination of its 14-day-long administration and maintained increased neuronal activity in food intake-regulating neurons of the NTS and in the hypothalamus, and activated PI3 kinase and ERK1/2 pathways but deactivated the apoptotic JNK/c-JUN pathway. We conclude that normalized leptin levels retained the decrease in body weight even after the termination of treatment and prevented the yo-yo effect. Moreover, leptin-related signaling in the hypothalamus was ameliorated, and apoptotic signaling pathways were attenuated by treatment with palm¹¹-PrRP31. Thus, palm¹¹-PrRP31 is highly promising as a possible obesity treatment when a long-term effect is needed.

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.

Funding

This work was supported by GA CR grant No. 16-009185, RVO:61388963 of AS CR and RVO:67985823 of AS CR. The authors would like to thank H Vysušilová for excellent technical assistance, Z Lacinová for mRNA analyses and M Blechová for synthesis of PrRP analog.

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Received in final form 21 November 2017

Accepted 11 December 2017

Accepted Preprint published online 12 December 2017