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Úloha stabilních analogů peptidu uvolňujícího prolaktin při obezitě a hypertenzi

The role of stable analogs of prolactin-releasing peptide in obesity and hypertension

Disertační práce

Vedoucí disertační práce/Školitel: RNDr. Lenka Maletínská, CSc.

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ABSTRAKT

Peptidy snižující příjem potravy neboli anorexigenní peptidy mají velký potenciál v léčbě obezity a jejích komplikací, jako jsou například vysoké hladiny glukózy nebo vysoký krevní tlak. Nevýhodou těchto látek je neschopnost prostoupit hematoencefalickou bariérou do mozku po periferním podání. V naší laboratoři byly navrženy a syntetizovány lipidizované analogy peptidu uvolňujícího prolaktin (PrRP), u kterých byla prokázána vyšší stabilita v krevní plazmě a centrální biologický účinek po periferním podání. Tento centrální účinek byl navíc potvrzen zvýšenou aktivitou proteinu c-Fos v příslušných hypotalamických jádrech.

Cílem této disertační práce bylo zjistit efektivní dávku palmitovaných PrRP31 analogů v akutních experimentech na příjem potravy a charakterizovat jejich dlouhodobý efekt v myších a potkaních modelech obezity a diabetu. Pro tyto studie byly použity následující modely: dietou indukované obézní (DIO) myši (C57Bl/6J), DIO potkani Sprague Dawley a dva modely s nefunkčním leptinovým receptorem: ZDF (Zucker diabetic fatty) potkani a spontánně hypertenzní obézní (SHROB) potkani.

Příjem vysokotukové diety zvýšil u myší i potkanů tělesnou hmotnost a krevní tlak. Dvoutýdenní intraperitoneální léčba palmitovaným analogem PrRP31 významně snížila příjem potravy i tělesnou hmotnost. Tato léčba dále zlepšila také glukózovou toleranci u DIO potkanů a navrátila krevní tlak k fyziologickým hodnotám u DIO myší. U ZDF a SHROB potkanů intraperitoneální léčba snížila příjem potravy, ale neovlivnila tělesnou hmotnost, pravděpodobně kvůli částečné leptinové rezistenci, která je důsledkem nefunkčního leptinového receptoru. Překvapivě léčba významně zlepšila glukózovou toleranci u SHROB potkanů a byla zde tendence ke zlepšení glukózové tolerance i u ZDF potkanů. Předpokládáme, že funkční leptinová signalizace je důležitá pro anorexigenní účinek a nikoli pro antidiabetický účinek palmitovaného PrRP31.

Lipidizované analogy PrRP31 prokázaly anti-obezitní a anti-diabetické účinky ve zvířecích modelech s obezitou a metabolickými poruchami. Palmitované analogy PrRP jsou díky svým anorexigenním, hmotnost snižujícím a navíc antidiabetickým účinkům atraktivními kandidáty pro léčbu obezity a diabetu.

KLÍČOVÁ SLOVA

peptid uvolňující prolaktin, regulace příjmu potravy, krevní tlak, metabolický syndrom, zvířecí modely obezity a hypertenze

ABSTRACT

Anorexigenic neuropeptides have the potential to decrease food intake and ameliorate obesity and its complications such as high blood glucose or high blood pressure. However, they are not able to cross the blood-brain barrier after peripheral application. Recently, we have designed and synthesized lipidized analogs of prolactin-releasing peptide (PrRP), which resulted in stabilization of the molecule and allowed us to apply the peptide to the periphery to achieve its central biological effect, as it was demonstrated by increased neuronal activity shown by c-Fos in particular hypothalamus nuclei.

The aim of this study was to choose the effective dose in acute food intake experiments and then to characterize the subchronic effect of palmitoylated PrRP analogs in mouse and rat models of obesity and diabetes. Several animal models were used: diet-induced obese (DIO) mice (C57Bl/6J), DIO Sprague-Dawley rats, and two rat models with leptin receptor-deficiency: Zucker diabetic (ZDF) rats and spontaneously hypertensive (SHROB) rats.

Consumption of a high-fat diet in DIO mice and rats increased their body weight and blood pressure. Two-week intraperitoneal treatment with palmitoylated PrRP31 lowered the food intake, body weight, and returned the blood pressure to normal levels. This treatment also improved glucose tolerance in DIO rats. In contrast, in ZDF and SHROB rats, the same treatment lowered the food intake but did not significantly affect the body weight, probably because of severe leptin resistance that was likely due to a non-functional leptin receptor. However, the treatment improved glucose tolerance and reduced blood insulin levels in the SHROB model and tended to improve glucose tolerance in the ZDF model, suggesting that functional leptin is required for the anorexigenic but not for the antidiabetic effects of palmitoylated PrRP31.

Our data showed a good efficacy of lipidized PrRP31 in animal models of obesity and related metabolic complications. Thus, the strong anorexigenic, body weight-reducing and blood glucose-improving effects make palmitoylated PrRP an attractive candidate for anti-obesity and glucose-lowering treatments.

KEY WORDS

prolactin-releasing peptide, food intake regulation, blood pressure, animal models of obesity and hypertension

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CONTENTS

ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone
AgRP	agouti-related peptide
ARC	nucleus arcuatus
BP	blood pressure
BBB	blood-brain barrier
BW	body weight
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CRH	corticotropin-releasing hormone
DIO	diet-induced obesity
FFA	free fatty acids
GLP-1	glucagon-like peptide-1
GPR10	G-protein coupled-receptor 10
HFD	high-fat diet
HR	heart rate
ICV	intracerebroventricular
IP	intraperitoneal
IV	intravenously
LHA	lateral hypothalamic area
MAP	mean arterial pressure
MCH	melanin-concentrating hormone
MetS	metabolic syndrome
NPY	neuropeptide Y
NTS	nucleus tractus solitarii
OGTT	oral glucose tolerance test
palm-PrRP31	prolactin-releasing peptide palmitoylated at the N-terminus
palm^{11}-PrRP31	prolactin-releasing peptide palmitoylated at position 11
POMC	pro-opiomelanocortin
PrRP	prolactin-releasing peptide
PVN	paraventricular nucleus
RVLM	rostral ventrolateral medulla

SBP	systolic blood pressure
SC	subcutaneously
SHR	spontaneously hypertensive rat
SHROB	spontaneously hypertensive obese rat
SNS	sympathetic nervous system
SNA	sympathetic nervous activity
STD	standard diet
TAG	triglycerides
TRH	thyreotropin-releasing hormone
VMN	ventromedial nucleus
ZDF rat	Zucker diabetic fatty rat

1. INTRODUCTION

This PhD thesis is a part of the research of the Lenka Maletínská´s group at the Institute of Organic Chemistry and Biochemistry AS CR and the Experimental Hypertension Group at the Institute of Physiology AS CR. Most parts of the thesis are based on publications accepted in scientific journals. The list of these publications is given on page 64.

1.1. Obesity and its treatment

Obesity is the most prevalent nutrition problem worldwide. The World Health Organization concluded that more than 650 million people were obese in 2016 (http://www.who.int/mediacentre/factsheets/fs311/en/). Obesity is a prerequisite for metabolic syndrome (MetS), clustering of the risk factors such as insulin resistance, dyslipidemia, and hypertension that together culminate in the increased risk of type 2 diabetes mellitus and cardiovascular diseases (O'Neill and O'Driscoll, 2015; Sookoian and Pirola, 2011; Zalesin et al., 2011). Unfortunately, despite tremendous efforts there is still no efficient non-invasive therapy for obesity.

Several antiobesity drugs were withdrawn from the market because of their severe psychiatric or cardiovascular side effects (for reviews, see (Bray and Ryan, 2014; Manning et al., 2014; Rodgers et al., 2012). The promising tool for antiobesity treatment are analogs of peptides that decrease food intake, so called anorexigenic peptides. Anorexigenic peptides have minimal side effects during the long-term antiobesity treatment (Arch, 2015; Bray et al., 2016; Patel, 2015). Liraglutide, a peptide analog of glucagon-like peptide-1 (GLP-1) that was originally developed as a type 2 diabetes mellitus drug, has been recently approved for antiobesity treatment in the USA and Europe (Saxenda). Other peptides evaluated as potential drugs for obesity treatment are pancreatic polypeptide and peptide YY. These peptides, called gut-brain peptides, affect the gastrointestinal tract and have central anorexigenic effects (Karra and Batterham, 2010). Furthermore, there are several neuropeptides originating in the brain that also have an anorexigenic effect, such as prolactin-releasing peptide (PrRP), cocaine- and amphetamine-regulated transcript (CART) peptide, or α-melanocyte-stimulating hormone (α-MSH) (Mikulášková et al., 2016). Peptid CART is not prospective for potential

treatment because its receptor has not yet been identified (Nagelová et al., 2014). The α-MSH analog has been shown to be a stable substance with a strong anorexigenic effect (Fosgerau, Raun, Nilsson, Dahl, & Wulff, 2014); however, its further research was terminated because of its adverse effects on the skin (Royalty et al., 2014).

The currently used pharmacological therapy for obesity has been shown to have great potential to improve or prevent some cardiovascular risks and diseases (Gadde et al., 2011; Kim et al., 2014; Torgerson et al., 2004). To prevent these serious health problems, it is crucial to determine the mechanisms that regulate both food intake and blood pressure (BP) (Mikulášková et al., 2016).

1.2. Food intake regulation and cardiovascular system

The regulation of food intake involves mutual integration of signals from both the central nervous system and the periphery. The key role in the regulation of food intake is played by the hypothalamus, where the action of a large number of peptides and neurotransmitters is integrated to mediate regulation of short-term and long-term dietary intake. The hypothalamus is a brain region containing several nuclei: nucleus arcuatus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventromedial nucleus (VMN), and dorsomedial nucleus (Schwartz et al., 2000). One of the major regions of the hypothalamus considered as involved in feeding and satiety is ARC (Sobrino Crespo et al., 2014). ARC acts as a feeding control center and contains populations of neurons, so called “primary neurons.” The primary neurons express neuropeptides that increase appetite (orexigenic neuropeptides), including neuropeptide Y (NPY) and agouti-related peptide (AgRP), as well as neuropeptides that reduce appetite (anorexigenic neuropeptides), such as α-MSH and CART. These two populations of primary neurons also contain receptors for hormones from the periphery that regulate food intake in the long term such as leptin and insulin, and also for hormones that regulate food intake in the short term such as peptide YY, cholecystokinin (CCK), GLP-1 and orexigenic ghrelin, which regulates food intake in both the long and short term. These neurons send axonal projections to PVN, VMN and LHA second-order neurons, which conduct the related impulses to the thalamus (Arora and Anubhuti, 2006). Another important brain area involved in regulating food

intake is nucleus tractus solitarii (NTS) in the brainstem (Yu and Kim, 2012). NTS receives short-term signals derived from the gastrointestinal tract, such as CCK, which relay through the sensory vagus nerve. NTS also receives neuronal projections from other hypothalamic areas, mainly PVN, indicating that there is communication between the hypothalamus and brainstem. NTS neurons produce GLP-1, NPY and pro-opiomelanocortin (POMC). (Arora and Anubhuti, 2006; Sobrino Crespo et al., 2014; Yu and Kim, 2012). Figure 1 shows a diagram of the interrelationship between the central and peripheral factors regulating food intake.

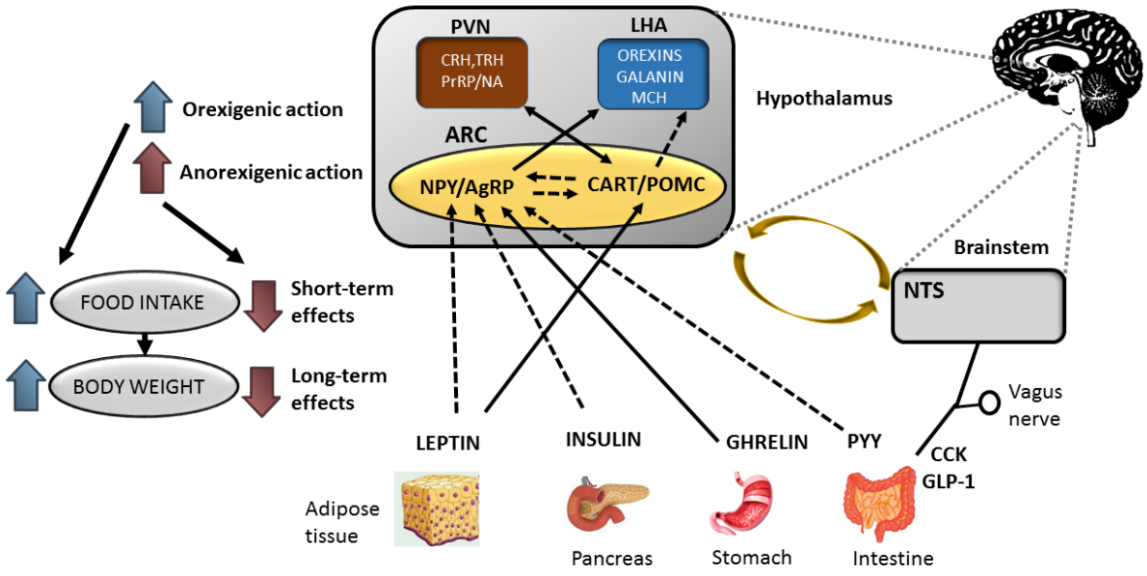


Fig. 1 Diagram of the interrelationship between the central and peripheral factors regulating food intake

The solid lines indicate stimulatory effects and dotted lines indicate inhibitory effects. PVN - paraventricular nucleus, LHA - lateral hypothalamic area, ARC - arcuate nucleus, NTS - nucleus tractus solitarii, CRH - corticotropin-releasing hormone, TRH - thyreotropin-releasing hormone, PrRP - prolactin-releasing peptide, MCH – melanin-concentrating hormone, NPY - neuropeptide Y, AgRP - agouti-related peptide, CART - cocaine- and amphetamine-regulated transcript peptide, POMC - pro-opiomelanocortin, CCK - cholecystokinin, GLP-1 - glucagon-like peptide-1 (Mikulášková et al., 2016)

It is evident that several peptides participating in the regulation of appetite or feeding behavior also cause cardiovascular effects that could be mediated through

the activation or inactivation of the sympathetic nervous system (SNS) (Mikulášková et al., 2016). Increasing evidence points to central nervous system activation as a key contributor to the development of both obesity and hypertension (DiBona, 2013; Hall et al., 2000; Hall et al., 2010). Increased sympathetic nerve activity (SNA) is a characteristic feature of many animal models of obesity (Kassab et al., 1995; Prior et al., 2010; Rahmouni et al., 2005). High SNA has also been documented in human obesity, essential hypertension, as well as heart failure (Grassi et al., 1995; Hall et al., 2010; Vaz et al., 1997; Wier et al., 2009; Wofford et al., 2001). As demonstrated in Fig. 2, we can hypothesize that peptides released from second-order neurons in PVN can modulate SNS in the hypothalamus. This leads to activation of a specific BP set-point in rostral ventrolateral medulla (RVLM) of the brainstem and finally to the modulation of sympathetic outflow (Osborn, 2005).

However, the link between the pathways involved in food intake regulation and BP regulation is not completely known. It is evident that the central sympathetic nervous system is a common denominator in this process (Vaněčková et al., 2014). However, further research is needed to enable more precise identification of the hypothalamic signaling mechanisms of food intake regulating peptides that might be considered as therapeutic targets for obesity treatment and maybe even for influencing blood pressure as well (Mikulášková et al., 2016).

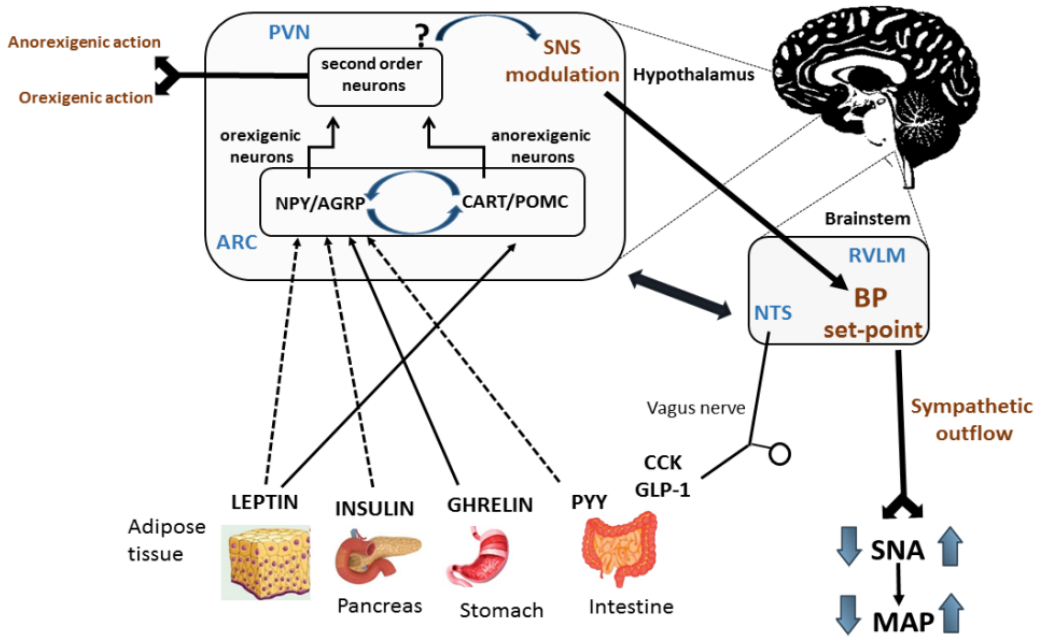


Fig. 2 Potential mechanism by which food-regulating peptides could modulate SNA and thus blood pressure

The activation of second-order neurons in PVN increases SNS modulation, which through the BP specific set point in the brainstem could modulate sympathetic outflow leading to the MAP modulation. The solid lines indicate stimulatory effects and dotted lines indicate inhibitory effects. PVN - paraventricular nucleus, ARC-arcuate nucleus, NTS - nucleus tractus solitarii, RVLM - rostral ventrolateral medulla, SNS - sympathetic nervous system, BP - blood pressure, MAP - mean arterial pressure, SNA - sympathetic nervous activity, CCK - cholecystokinin, GLP-1 - glucagon-like peptide-1, NPY - neuropeptide Y, AgRP - agouti-related peptide, CART - cocaine- and amphetamine-regulated transcript peptide, POMC - pro-opiomelanocortin (Mikulášková et al., 2016)

1.3. Experimental models of obesity and metabolic syndrome

Since metabolic syndrome is a multifactorial disorder, it is difficult to find a suitable experimental model to study this pathology. A representative rat model to study MetS seems to be Zucker diabetic fatty (ZDF) rats, which is a model of impaired leptin receptor signaling (Fellmann et al., 2013). ZDF rats are mainly used for studying obesity, but they also present changes similar to those seen in human MetS and they are frequently used for studying potential antiobesity and antidiabetic peptidic drugs (Skarbaliene et al., 2015). Another rat model with impaired leptin receptor signaling used for studying MetS are spontaneously

hypertensive obese rats (SHROB), so called Koletsky rats (Fellmann et al., 2013; Mikulášková et al., 2018; Shintani et al., 2001) (Fig. 3). SHROB were derived by mating a female spontaneously hypertensive rat (SHR) with a normotensive Sprague-Dawley male rat (Koletsky, 1973). SHROB rats are considered an animal model with phenotypic features of MetS (Koletsky, 1975b). They develop obesity, hyperinsulinemia, hyperlipidemia, and spontaneous hypertension (Koletsky, 1975a).

One of the major risks for development cardiovascular and metabolic dysfunction including obesity and hypertension is high dietary fat intake. Hypercaloric diets, rich in lipids, are widely used in experimental studies to induce metabolic disorders commonly found in humans (Buettner et al., 2007; Dourmashkin et al., 2005; Oliveira et al., 2009). Most rodents tend to become obese on specific diets, and diet-induced obese rodents are therefore suitable for study of pathologies of MetS (Bergman et al., 2006; Shafrir et al., 1999; Varga et al., 2010).

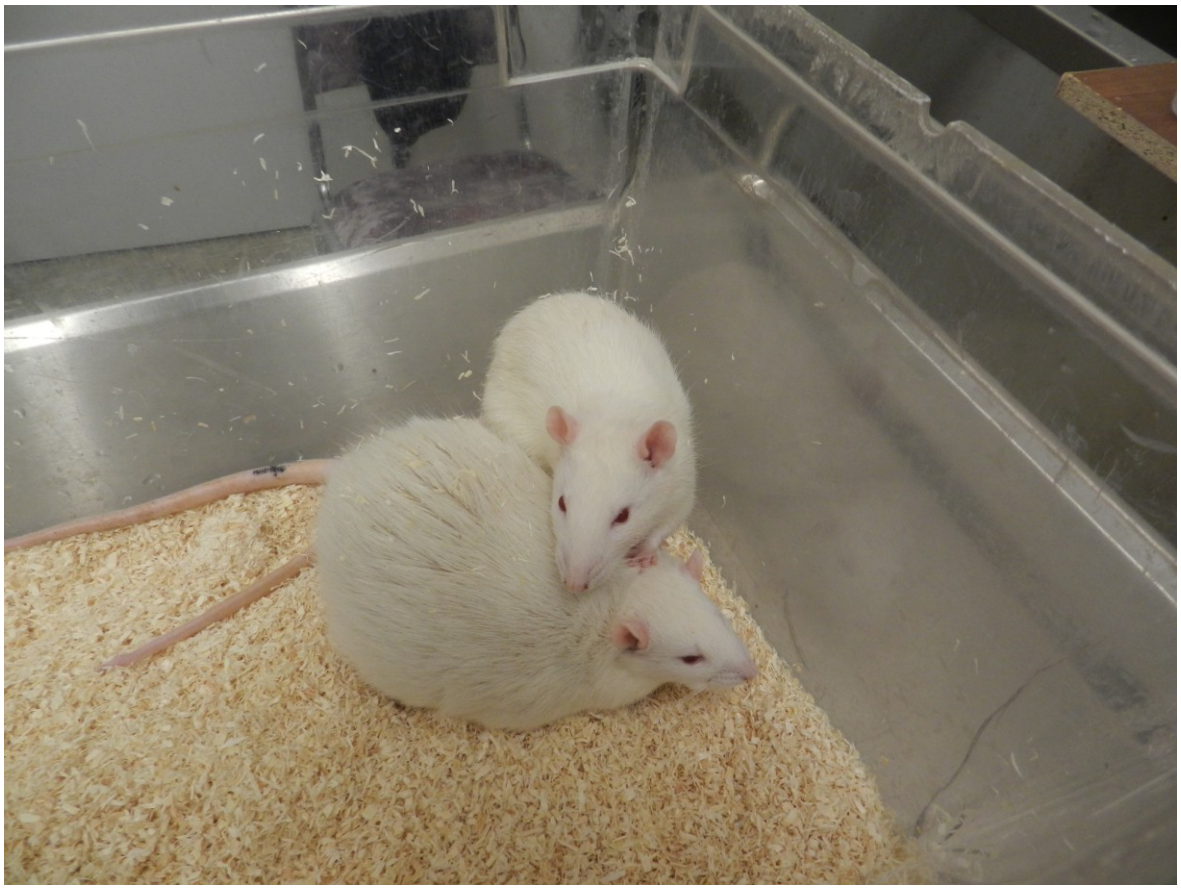


Fig. 3 Koletsky rat and its control - spontaneously hypertensive rat

1.4. Prolactin-releasing peptide

Prolactin-releasing peptide is an anorexigenic neuropeptide initially isolated from the hypothalamus as a ligand for the orphan G-protein-coupled receptor GPR10 (Hinuma et al., 1998). It belongs to the family of so called RF amide peptides with C-terminal arginine and phenylalanine amide sequence. There are two biologically active isoforms of PrRP containing 20 amino acids (PrRP20) or 31 amino acids (PrRP31), as shown in Fig. 4.

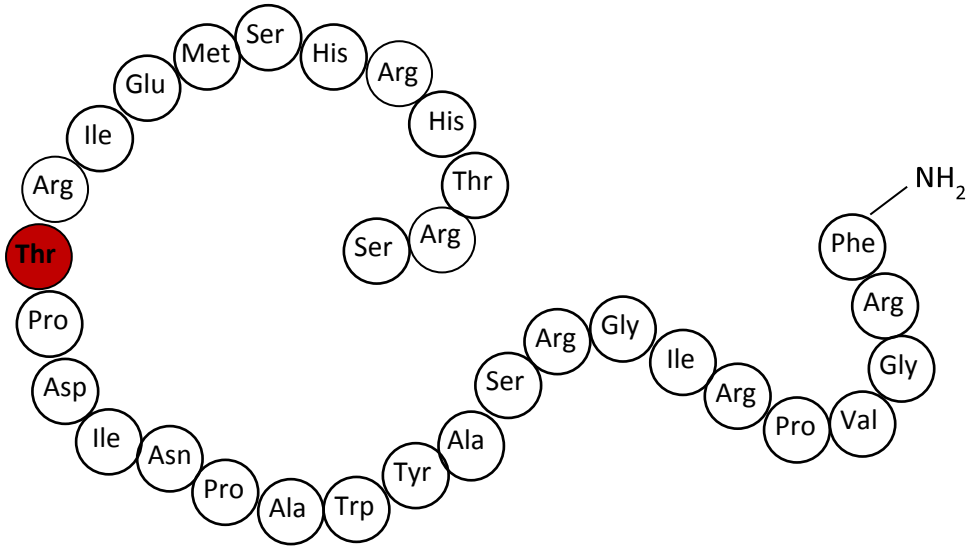


Fig. 4 Structure of human prolactin-releasing peptide

The structure of human prolactin-releasing peptide contains 31 amino acids; threonine in red marks the beginning of PrRP20

The first described effect of PrRP was prolactin release from rat pituitary tumor cells, both primary cells and rat cell line RC-4B/C (Hinuma et al., 1998). However, a direct prolactin-releasing effect of PrRP in mammals was soon questioned (Jarry et al., 2000; Maruyama et al., 1999) and it was established that PrRP has other physiological functions, including the regulation of food intake (Lawrence et al., 2000) and energy expenditure (Takayanagi et al., 2008).

PrRP and its receptor were detected in several hypothalamic nuclei as well as in the brainstem, suggesting an involvement of PrRP in the control of food intake and BW regulation (Ibata et al., 2000; Lawrence et al., 2000; Roland et al., 1999). This function was supported by the fact that both, mice with an inactivated PrRP gene (Mochiduki et al., 2010; Takayanagi et al., 2008) or GPR10 receptor (Bjursell

et al., 2007; Gu et al., 2004) developed late-onset obesity. In rats, centrally administered PrRP was shown to inhibit food intake and BW gain. Moreover, Fos immunoreactivity was enhanced after PrRP administration in PVN (Lawrence et al., 2002).

The effects of PrRP are considered to be associated with the effects of leptin, a long-term acting regulator of energy balance. PrRP neurons in the brain regions involved in food intake regulation, VMN of the hypothalamus, ventrolateral medulla and NTS of the brainstem also contain leptin receptors (Ellacott et al., 2002). Furthermore, in rats, intracerebroventricular (ICV) coadministration of PrRP and leptin resulted in additive reductions in nocturnal food intake and BW gain and an increase in energy expenditure (Ellacott et al., 2002). Moreover, PrRP acting through its receptor may be a key mediator in the central satiating action of CCK. The anorexigenic peptide CCK was shown to have no effect on food intake in GPR10 knockout mice (Bechtold and Luckman, 2006). The hypothetical anorexigenic interaction among PrRP, leptin and CCK is described in our review (Kuneš et al., 2016).

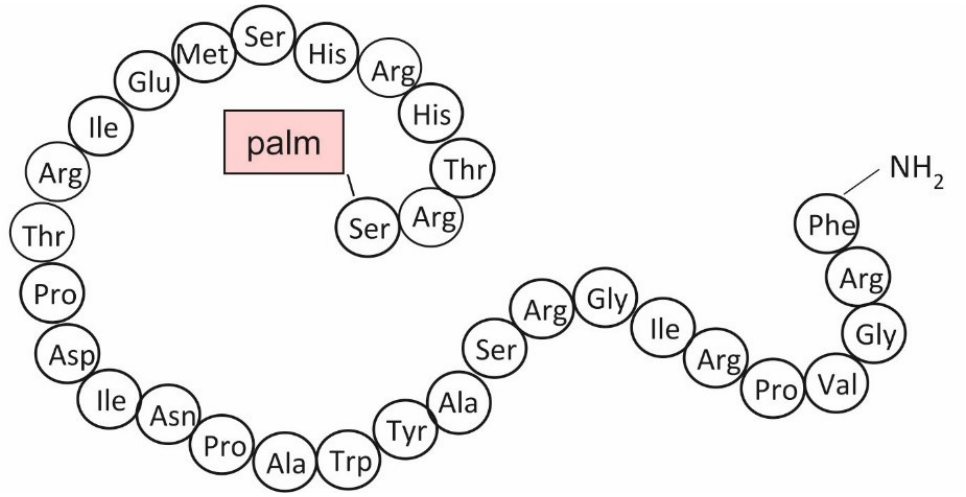
PrRP is suggested to be involved in the central control of BP and to play a role in the regulation of cardiovascular homeostasis; for review see (Mikulášková et al., 2016), although the neuronal pathway for the PrRP effect on the cardiovascular system has yet to be elucidated (Horiuchi et al., 2002; Samson et al., 2000).

1.4.1 Lipidized analogs of PrRP as potential antiobesity drugs

Anorexigenic neuropeptides represent a promising tool for the obesity treatment. These peptides act centrally and food intake is also regulated centrally; therefore, their anorexigenic action after peripheral administration depends on their ability to reach their target brain receptors and exert their central effect. Another important issue in the drug development is peptide stability and prolonged half-life of the neuropeptide in the organism. One of the frequently used strategies for peptidic drug development is lipidization, i.e., attachment of fatty acid to the peptide through an ester or amide bond (Malavolta and Cabral, 2011). Both liraglutide (Gault et al., 2011) and α-MSH (Fosgerau et al., 2014) analog mentioned above have palmitic acid attached to their molecule in order to improve the pharmacokinetics of the drug. Similarly, palmitoylation of PrRP increased stability and half-life of the peptide.

We have previously shown that analogs of lipidized PrRP designed in our laboratory display high affinities for PrRP receptor GPR10 as well as for NPFF2 receptor *in vitro* (Prazienkova et al., 2016; Pražienková et al., 2017). These analogs also showed prolonged anorexigenic effects in different animal models (for review see (Kuneš et al., 2016)). In our previous study, two weeks of twice-daily treatment with PrRP31 palmitoylated at the N-terminus (palm-PrRP31) (the structure is shown in Fig. 5A) and PrRP20 myristoylated at the N-terminus to mice with high-fat (HF) diet-induced obesity (DIO) significantly decreased cumulative food intake and BW. The animals lost BW due to the reduction in the fat mass and we also observed a decrease in circulating leptin levels and decreased lipogenesis in the adipose tissue (Maletínská et al., 2015). Another analog palmitoylated through a linker of gamma-glutamic acid at position 11 (palm^{11}-PrRP31) (the structure is shown in Fig. 5B) showed a central effect by neuronal activation and a decrease in food intake after a single injection to mice. Moreover, two weeks of repeated administration of palm^{11}-PrRP31 revealed not only a significant decrease in BW, but also improvement in multiple metabolic parameters related to obesity and its related diseases (Pražienková et al., 2017). We therefore selected palm-PrRP31 and palm^{11}-PrRP31 as our lead compounds for the following studies.

A/



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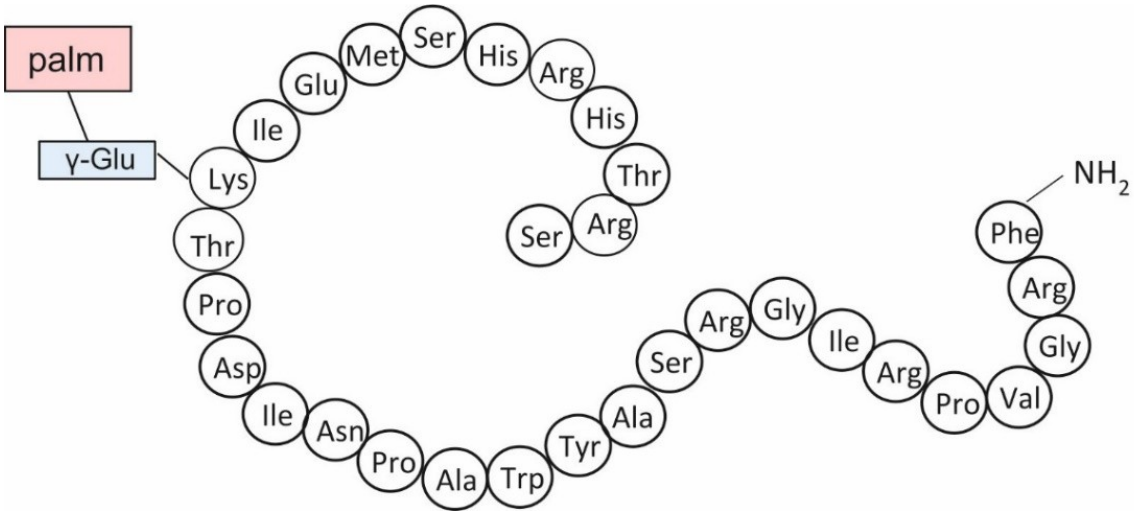


Fig. 5 Structures of palmitoylated analogs of human prolactin-releasing peptide 31

A/ Prolactin-releasing peptide palmitoylated at the N-terminus (palm-PrRP31)

B/ Prolactin-releasing peptide palmitoylated in position 11, γ-Glu – γ-glutamic acid linker (palm^{11}-PrRP31)

2. AIMS OF THE THESIS

Anorexigenic neuropeptides have the potential to decrease food intake and ameliorate obesity. However, they are not able to cross the blood-brain barrier after peripheral application. Recently, in our laboratory at IOCB AS CR we have designed and synthetized lipidized analogs of prolactin-releasing peptide, which resulted in stabilization of the molecule and allowed us to apply the peptide to the periphery to achieve their central biological effect. The aims of my thesis were:

Investigation of the acute effect of palmitoylated analogs of prolactin-releasing peptide on food intake in free-fed rats

The first aim of my thesis was to evaluate the acute anorexigenic effect of different doses and different routes of administration of palmitoylated analogs of prolactin-releasing peptide *in vivo* in free-fed lean Wistar rats.

Characterization of palmitoylated analogs of prolactin-releasing peptide in chronic *in vivo* experiments – mice and rats with metabolic disorders

The second aim, based on the data from acute food intake experiments, was to determine the long-term effect of palmitoylated prolactin-releasing peptide analogs in obese mice and rats with associated metabolic complications by monitoring the metabolic parameters related to food intake regulation and cardiovascular regulation.

Blood pressure measurement after administration of prolactin-releasing peptide - investigation of the acute effect in free-fed rats and the chronic effect in mice and rats on high-fat diet

High blood pressure is related to obesity. Despite the fact that obesity-related hypertension is a very serious health problem, no clear evidence yet exists as to which occurs first, obesity or hypertension, because both diseases can also exist separately. The third aim of my thesis was to evaluate the acute and chronic effect of anorexigenic prolactin-releasing peptide on blood pressure in normotensive Wistar rats and also to determine the effect of high-fat diet on the blood pressure in mice and rats.

3. Methods

3.1. Synthesis of PrRP31

Human palmitoylated PrRP31 analogs (SRTHRHSMEI K (N-γ-E (N-palm)) PDINPAWYASRGIRPVGRF-NH2) (palm^{11}-PrRP31) and (N-palm SRTHRHSMEIRTPDINPAWYASRGIRPVGRF-NH2) (palm-PrRP31) (Fig. 5) were synthesized and purified at the Institute of Organic Chemistry and Biochemistry, Prague, as described in (Maletínská et al., 2015; Mikulášková et al., 2018).

3.2. Experimental animals

To investigate the mechanism by which palmitoylated analogs of PrRP31 decrease food intake and ameliorate metabolic parameters, several animal models of obesity and MetS were used. In chronic experiments, after the treatment with palmitoylated PrRP31 analogs, food intake, BW and metabolic parameters were monitored.

Animals were housed under standard laboratory conditions (temperature 23 ± 1°C, 12 h light/dark cycle) and were fed with a chow described in the respective literature and drank tap water or fructose in water ad libitum. All animal experiments followed the ethical guidelines for work with animals by the Act of the Czech Republic Nr. 246/1992 and were approved by the Committee for Experiments with Laboratory Animals of the ASCR. Regarding ZDF and Sprague Dawley rats, all procedures and experimental protocols conformed to the European Convention on Animal Protection and Guidelines on Research Animal Use.

3.3. Acute food intake experiments

Acute food intake experiments were performed in male Wistar rats (250-300g) (Harlan Laboratories, Correzzana, Italy).

3.3.1. Food intake measurement after single injection

On the day of the food intake experiment, free-fed rats were injected with vehicle (controls) or palm-PrRP31 subcutaneously (SC), intraperitoneally (IP), or intravenously (IV): SC or IP at doses of 1, 5, and 10 mg/kg, or IV into the jugular vein at doses of 0.01 and 0.1 mg/kg (n = 5–6). Rats were injected (0.1 ml/100 g)

two hours before lights were turned off. Food intake was monitored next morning, 14 h after the injection, in grams of food consumed (Mikulaskova et al., 2016).

3.3.2. Food intake measurement after repeated injections

On the day of the food intake experiment, free-fed rats were injected with vehicle (controls), palm-PrRP31 SC, IP or IV : SC or IP at doses of 5 mg/kg, or IV into jugular vein at a dose of 0.01 mg/kg (n = 5–6). Rats were injected (0.1 ml/100 g) two hours before lights were turned off. Food intake was monitored continuously using an automatic feeding system (PhenoMaster, TSESystems, Bad Homburg, Germany). Injection was repeated for three consecutive days at the same time of the day (Mikulaskova et al., 2016).

3.4. Chronic treatment with palmitoylated analogs of PrRP31

3.4.1. Diet-induced obese mice

Inbred C57BL/6 male mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Beginning at nine weeks of age, mice were fed high-fat diet containing 60 % of fat for four months (Maletínská et al., 2015) to induce obesity (HFD groups). The control group was fed with standard rodent chow diet Ssniff1 R/M-H (Ssniff Spezialdiäten GmbH, Soest, Germany) (STD group).

Mice were SC injected twice a day with saline (HFD and STD vehicle) or palm^{11}-PrRP31 (5 mg/kg) (HFD palm^{11}-PrRP31) for 21 days. Food intake and BW was monitored every day. Arterial blood pressure was measured at the end of the experiment by direct puncture of the carotid artery under pentobarbital anesthesia.

3.4.2. Diet-induced obese Sprague Dawley and Zucker diabetic fatty rats

Sprague–Dawley male rats were obtained from Harlan Laboratories (Correzzana, Italy), ZDF-Leprfa/Crl, diabetic fa/fa male rats, and lean controls were obtained from Charles River (Saint-Germain-sur-l'Arbresle, France).

ZDF rats were fed a diet of Purina 5008. Sprague-Dawley rats were fed from 6-8 weeks of age with low-fat (STD) diet D12450B containing 70 % carbohydrate or HFD D12492 containing 60 % of fat. Palm-PrRP31 was admnistred IP at doses of 0.2, 1, and 5 mg/kg for 17 days bi-daily to both ZDF rats and DIO Sprague Dawley rats. Food intake and BW were monitored every day. The oral glucose

tolerance test (OGTT) was performed at the end of the experiment (Holubová et al., 2016).

3.4.3. Koletsky and spontaneously hypertensive rats

Homozygous SHROB male rats (fa^k/fa^k) and their controls, hypertensive lean SHR littermates were purchased from Charles River (Wilmington, USA).

Rats were provided food - Ssniff and water ad libitum. $Palm^{11}$-PrRP31 was IP administered at a dose of 5 mg/kg once a day for 21 days. BW and food intake were monitored every two days during the drug application. At the end of the experiment, fasted blood plasma was colected for determination of metabolic parameters and OGTT was performed. Thereafter, arterial blood pressure was measured by direct puncture of the carotid artery under light ether anesthesia. Metabolic parameters were determined as described in our study (Mikulášková et al., 2018). Hypothalami samples were processed and western blotting was performed as described in our previous papers (Mikulášková et al., 2018; Špolcová et al., 2015).

3.5. Blood pressure measurement

3.5.1. Acute blood pressure measurement

Acute BP experiments were performed with conscious male Wistar rats (250 - 300g) (Harlan Laboratories, Correzzana, Italy). Rats were housed under standard laboratory conditions. Water and pelleted diet Sniff were provided ad libitum. The carotid artery was cannulated for BP and heart rate (HR) monitoring and the jugular vein for IV drug administration, under isoflurane anaesthesia. Eighteen hours after the surgery, $palm^{11}$-PrRP31 or palm-PrRP31 dissolved in saline was administred IV, IP and SC at doses that had decreased food intake in previous experiments – 0.1 mg/kg IV and 5 mg/kg IP and SC. Basal BP values were monitored first. Thereafter, in the IP and SC experiments saline was injected IP or SC to receive control BP. After saline injection, $palm^{11}$-PrRP31 or palm-PrRP31 was injected IP or SC. Blood pressure and HR was monitored online (n=8-9) using PowerLab system (AD Instruments Ltd., Bella Vista, NSW, Australia) in the following intervals for IP and SC experiments: 10 minutes – basal BP; 10 minutes – after saline injection; 60 minutes – after $palm^{11}$-PrRP31 or palm-PrRP31

administration; for IV experments: 10 minutes – basal BP, 10 minutes after the treatment. The experimental setup is shown in Fig. 6.

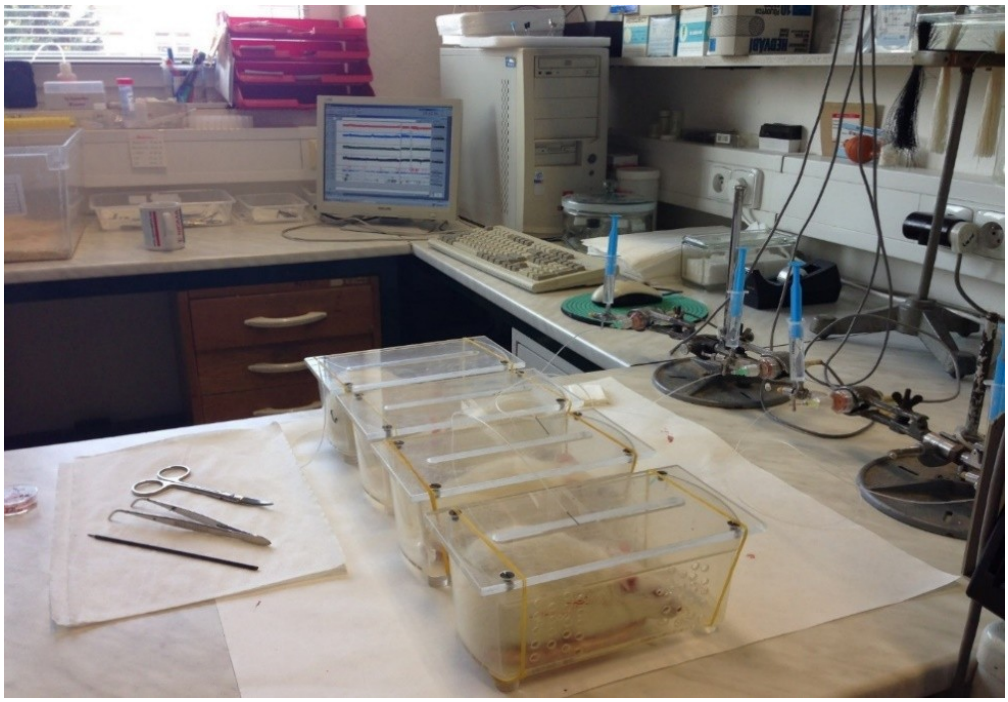


Fig. 6 Experimental setup for online monitoring of BP and HR

3.5.2. Telemetry

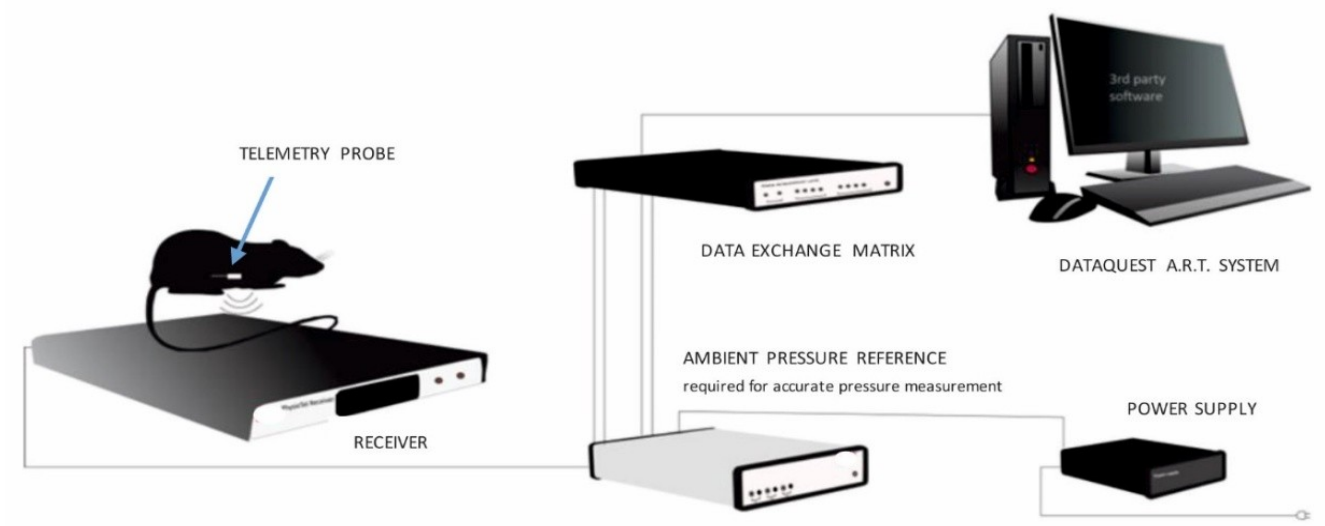
Blood pressure measurement by telemetry (the scheme is shown in Fig. 7A) was performed in two different experiments. To allow BP and HR monitoring, telemetry probes (Fig. 7B) (Data Sciences International, St. Paul, MN) were implanted in the abdominal aorta, under isoflurane anaesthesia. After the recovery, BP and HR were monitored.

In the first experiment, Wistar male rats (250-300 g) (Harlan Laboratories, Correzzana, Italy) were used. Rats were housed under standard laboratory conditions. Water and pelleted diet Sniff were provided ad libitum. Animals were treated SC or IP (as shown in Fig. 15, chapter 4.5.2.1.) with palm^{11}-PrRP31 at a dose of 5 mg/kg once daily, one hour before lights were turned off (n=5-6). Treatment started seven days after implantation of the telemetric probe and blood pressure was measured continuously for 15 folowing days.

To find out whether HFD had any effect on BP and HR, the second experiment was performed. Wistar Kyoto (WKY) rats (Harlan Laboratories, Correzzana, Italy) were fed either the high-fat diet D12492 containing 60 % fat or the low-fat diet Sniff (n=6). Both BP and HR measurement and HFD feeding started one week after

implantation of the telemetric probe. Every week, BP and HR were monitored for 24 hours on Thursdays for 13 weeks.

A/



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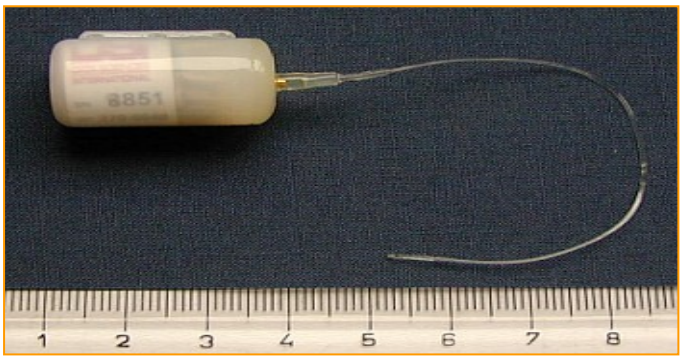


Fig. 7 Telemetry

A/ Telemetry system

B/ Telemetric probe

3.6. Statistical analysis

The results are expressed as the means ± S.E.M. Data were evaluated by unpaired t-test, one-way or repeated measures analysis of variance (ANOVA) with Bonferroni or Dunnett´s post hoc test as indicated in the tables and figures using the GraphPad software (Graph-Pad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

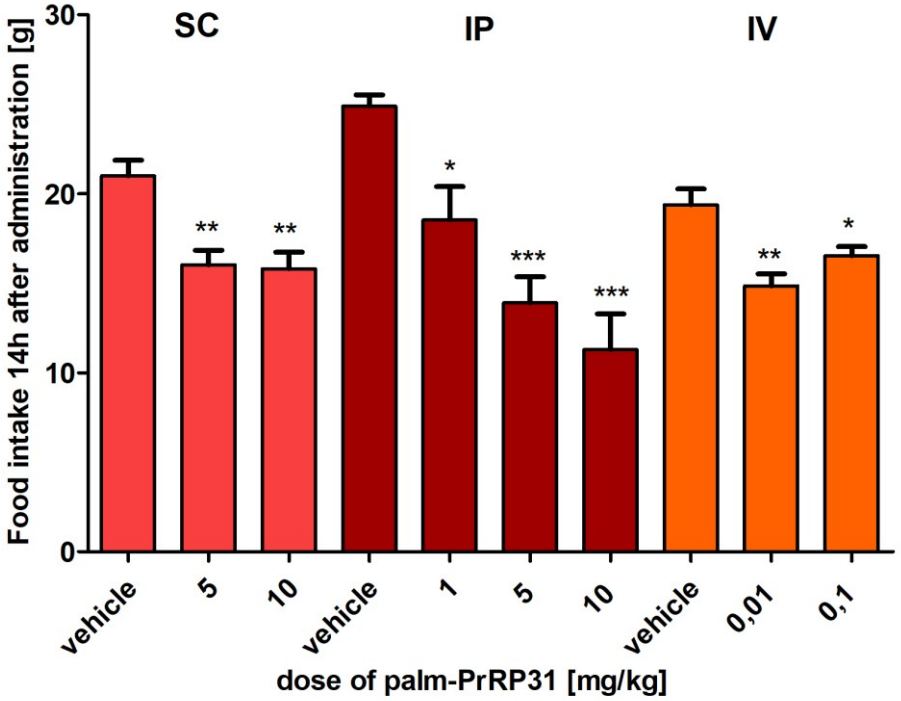
4. RESULTS

4.1. Palm-PrRP31 analog decreased food intake in free-fed rats

The results presented in this chapter were published in Peptides (Mikulaskova et al., 2016).

Figure 8A shows food intake in free-fed Wistar rats after single SC administration of palm-PrRP31 at doses of 5 and 10 mg/kg, IP at doses 1, 5, and 10 mg/kg and IV administration of palm-PrRP31 at doses 0.01 and 0.1 mg/kg compared to administration of vehicle. Rats were injected two hours before lights off and food intake was monitored 14 hours later (next morning). As shown in Fig. 8A, after SC administration, the maximal food intake decreasing effect was obtained at a dose of 5 mg/kg compared to the vehicle-treated group. IP injections showed decreasing food intake for doses 1, 5, and 10 mg/kg and IV administration of palm-PrRP31 also significantly lowered food intake at both doses, but the dose 0.1 mg/kg did not futher lower food intake compared to the dose 0.01 mg/kg. Food intake after IP administration of non-lipidized human PrRP31 (5 mg/kg) was not changed compared to the vehicle-treated group (results not shown).

A/



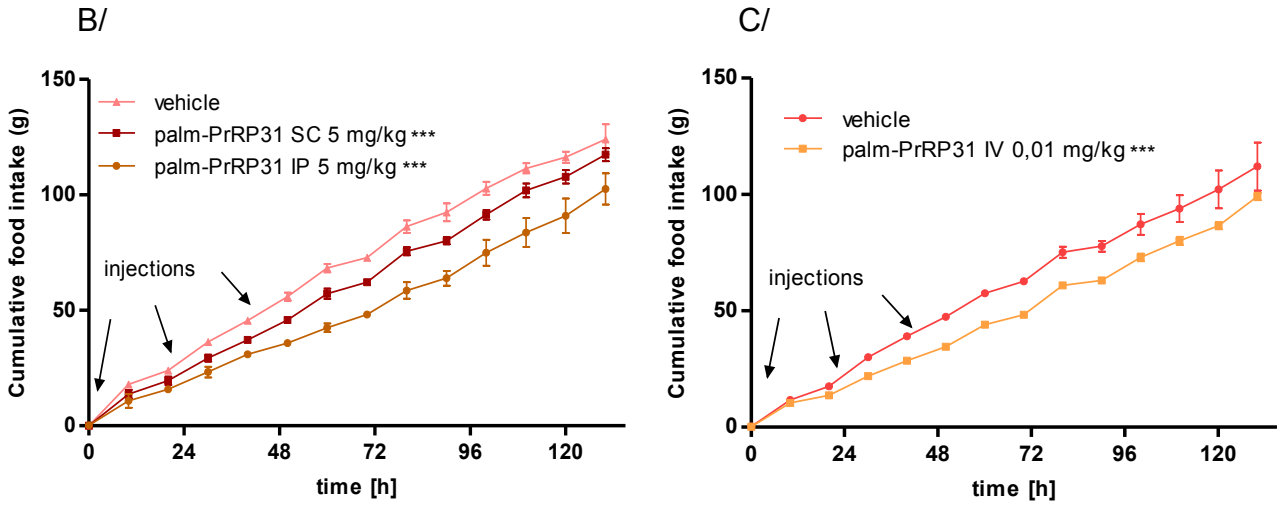


Fig. 8 Food intake after peripheral administration of palm-PrRP31 for one or three consecutive days by different routes of administration to Wistar rats

A/ Single injection of vehicle and palm-PrRP31 at doses and routes indicated in the figure (food intake monitored after 14 h)

B/ Repeated administration of vehicle and palm-PrRP31 at a dose of 5 mg/kg SC and IP

C/ Repeated administration of vehicle and palm-PrRP31 at a dose of 0.01 mg/kg IV into the jugular vein

*Cumulative food intake was monitored continuously for one week using an automatic feeding system. Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA followed by Dunnett´s post hoc test. The significance level was *P < 0.05, **P < 0.01 and***P < 0.001 vs the respective vehicle-treated group (n = 5–6).*

Figure 8B,C shows repeated SC and IP (dose 5 mg/kg) and IV (dose 0,01 mg/kg) administration of palm-PrRP31. The doses were selected based on the results from the single injection dose experiment. The cumulative food intake was monitored after three consecutive injections. Food intake was significantly decreased on the first day of the experiment after administration by all routes, SC, IP and IV, and this effect lasted after the second and third injection and on the following days of the experiment without injection. The most significant effect was observed after the IV injection because the dose of palm-PrRP31 was five hundred times lower than IP and SC doses, as shown in Fig. 8C.

4.2. Palm^{11}-PrRP31 analog decreased food intake, BW, and MAP in DIO mice

The results obtained in the following experiment have not yet been published.

Inbred C57BL/6 male mice were fed with HFD for four months to induce obesity – HFD groups. The STD group was fed a standard rodent chow diet.

Mice were SC injected twice a day with saline (HFD and STD vehicle) or palm^{11}-PrRP31 (5 mg/kg) (HFD palm^{11}-PrRP31) for 21 days. Food intake after the 21-day treatment was significantly decreased, as shown in Fig. 9A. Consumption of HFD for four months significantly increased BW compared to the STD group; the average BW of the STD group was 27.6 ± 0.5 g, while the average BW of the HFD group was 44.9 ± 1.1 g (Fig. 9B). Palm^{11}-PrRP31 markedly decreased BW (38.3 ± 1 g) in the HFD group in comparison with the HFD group treated with vehicle (Fig. 9B). Arterial blood pressure was measured at the end of the experiment by direct puncture of the carotid artery; the results are shown in Fig. 9C. MAP was significantly increased in the HFD group in comparison to the STD group and treatment with palm^{11}-PrRP31 returned MAP in the HFD group to normal levels compared to the STD group.

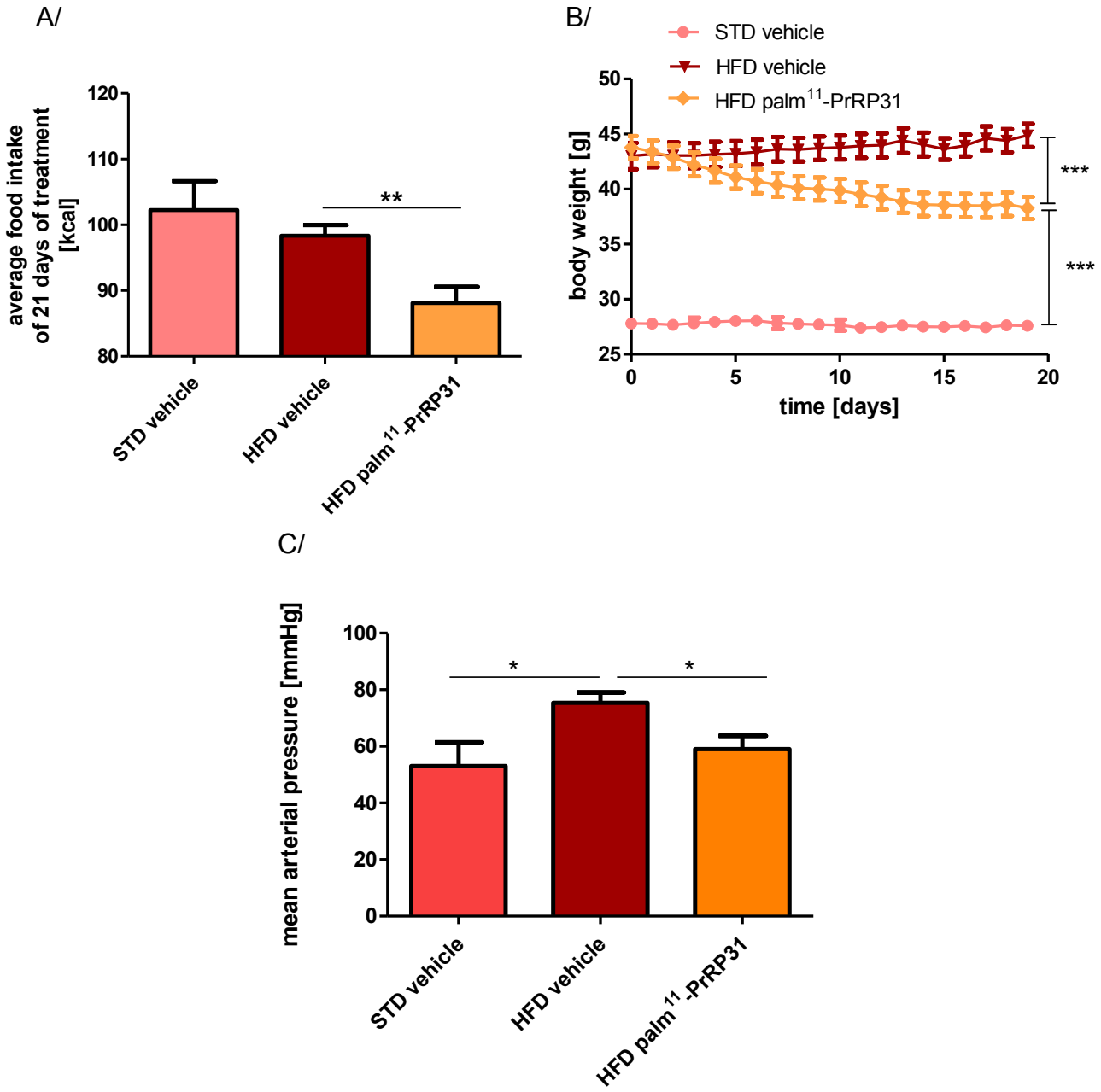


Fig. 9 Chronic effect of palm^{11}-PrRP31 on the food intake (A), body weight (B) and mean arterial pressure (C) in DIO mice

*Palm^{11}-PrPR31 was administered SC at a dose of 5 mg/kg twice a day for 21 days. Food intake and BW were monitored every day. Food intake is expressed as the average value of the 21 days of treatment. Arterial blood pressure was measured at the end of the experiment by direct puncture of the carotid artery under pentobarbital anesthesia. Data are presented as means ± S.E.M. Statistical analysis was performed by unpaired t-test (A, C) or repeated measures ANOVA with Bonferroni post hoc test (B), significance is *P < 0.05, **P < 0.01, ***P < 0.001 vs the respective group (n = 15).*

4.3. Palm-PrRP31 analog decreased BW and improved glucose tolerance in Sprague-Dawley DIO rats but not in diabetic ZDF rats

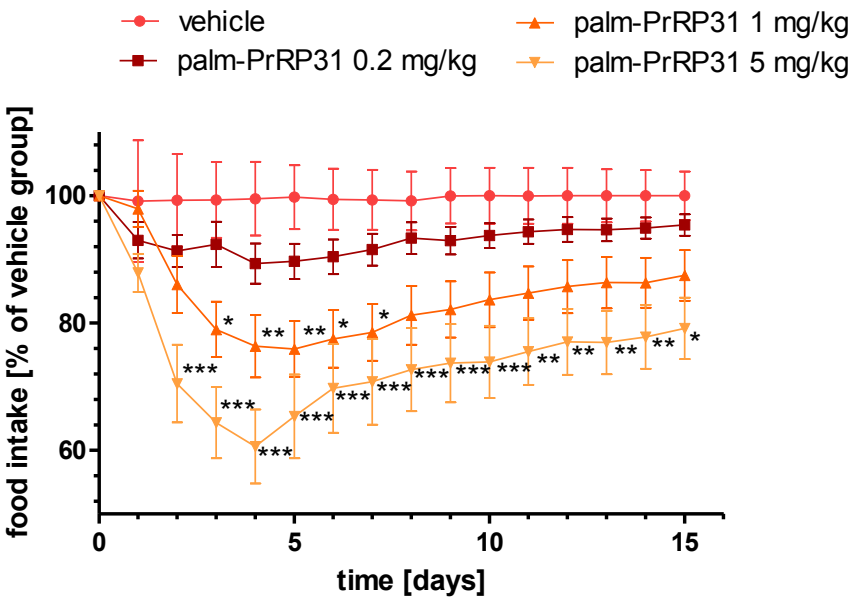
The results obtained in the experiments performed in DIO and ZDF rats presented in the following chapters were published in the Journal of Endocrinology (Holubová et al., 2016).

4.3.1. DIO Rats

The consumption of HFD for 25 weeks – start of the treatment (31-33 weeks of age) resulted in a significantly affected body weight gain; the average BW of the STD diet-fed control group was 581.4 ± 4.4 g, whereas the average BW of the HFD-fed control group was 638.6 ± 10.4 g ($P < 0.001$).

Rats on HFD were divided into four groups (n=8) and IP injected with vehicle or palm-PrRP31 at doses 0.2, 1, or 5 mg/kg for 17 days twice a day. Food intake and BW after the treatment was lowered in a dose-dependent manner, with the effect being more pronounced at week 1 and significant at 1 and 5 mg/kg doses (Fig. 10A,B). The highest tested dose of palm-PrRP31 lowered BW by 8 % (Fig. 10B). OGTT blood glucose levels were lowered in a dose-independent manner. This decrease was significant compared with the vehicle-treated obese control group only after treatment with the 1 mg/kg dose of palm-PrRP31 (Fig. 10C,D).

A/



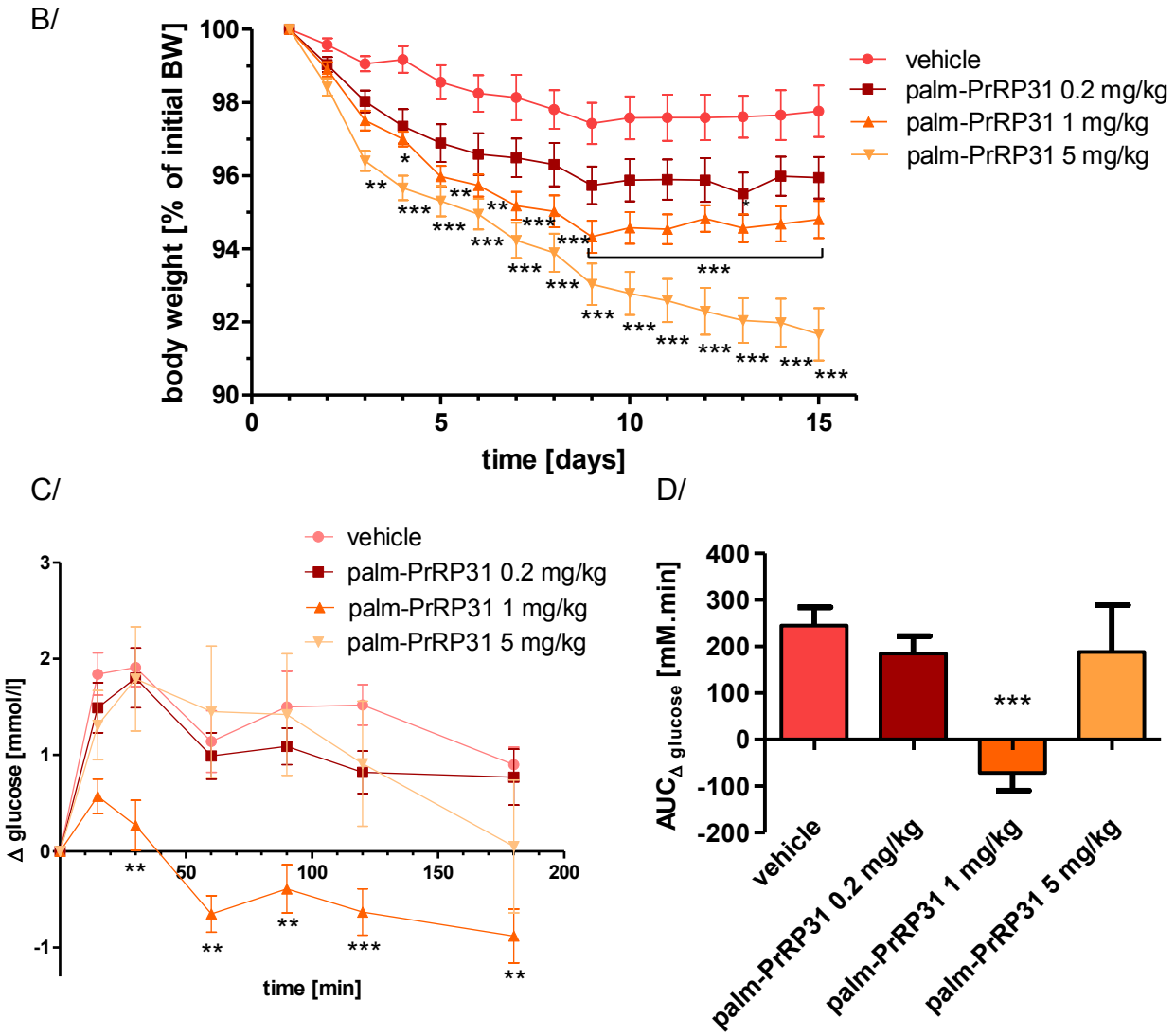


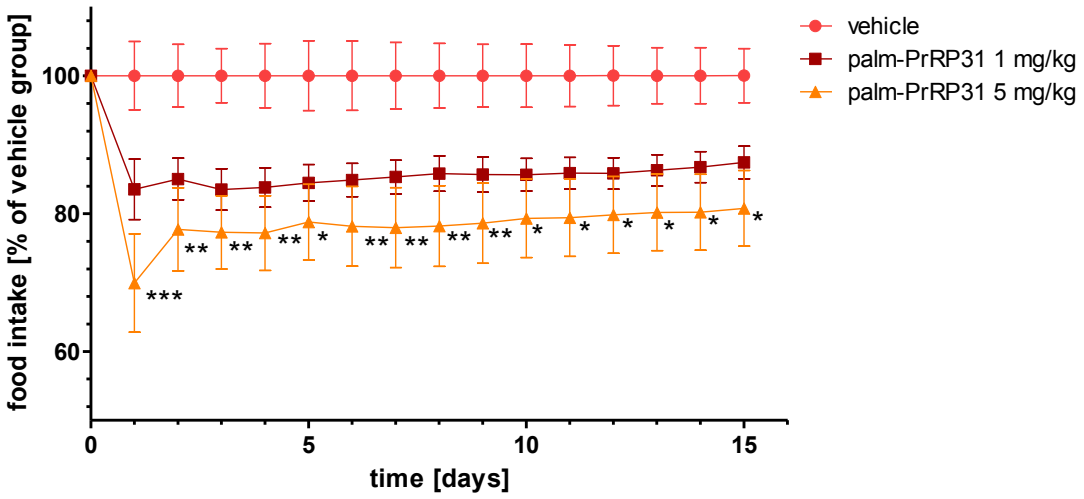
Fig. 10 Chronic effect of palm-PrRP31 on the food intake (A), body weight (B), and OGTT response (C,D) in DIO rats

*Palm-PrPR31 was administered IP at doses of 0.2, 1, and 5 mg/kg twice a day for 17 days. Food intake is expressed as a percentage of food intake in the vehicle-treated control group, BW is expressed as a percentage of the initial BW. OGTT was performed at the end of the experiment and its results are shown as Δ glucose and $AUC_{\Delta\ glucose}$. Data are presented as means ± S.E.M. Statistical analysis was performed by repeated measures ANOVA with Bonferroni post hoc test (A, B,C) or by one-way ANOVA followed by Dunnett´s post hoc test (D), significance is *P < 0.05, **P < 0.01, ***P < 0.001 vs the vehicle-treated obese control group (n = 7−8) (Holubová et al., 2016).*

4.3.2. Diabetic ZDF rats

The IP treatment of diabetic ZDF rats with palm-PrPR31 significantly lowered food intake at a dose of 5 mg/kg (Fig. 11A). All rats were gaining weight during the dosing period and the body weight gain was not significantly lowered after the palm-PrRP31 treatment (Fig. 11B). The treatment with palm-PrRP31 resulted in a nonsignificant dose-dependent decrease in blood glycemia during OGTT (Fig. 11C,D).

A/



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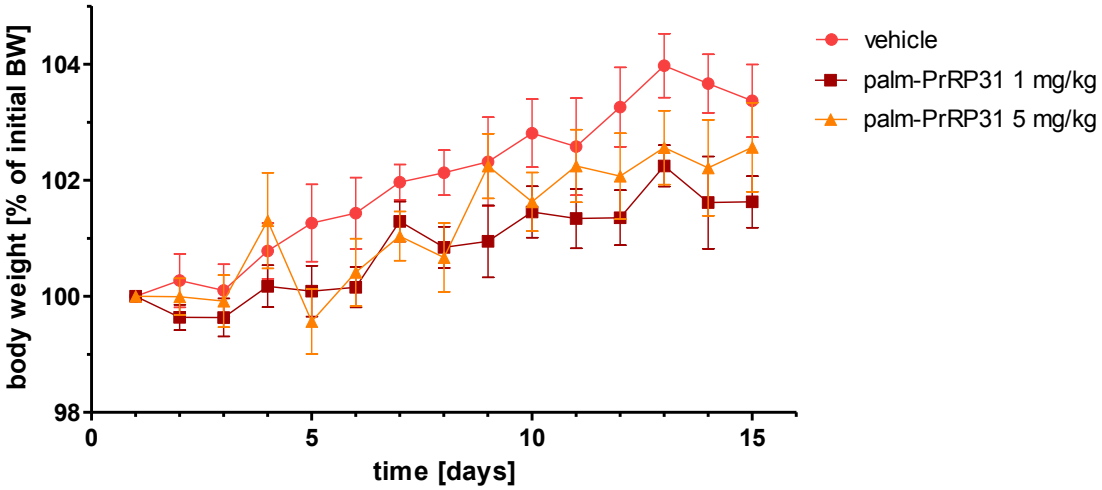


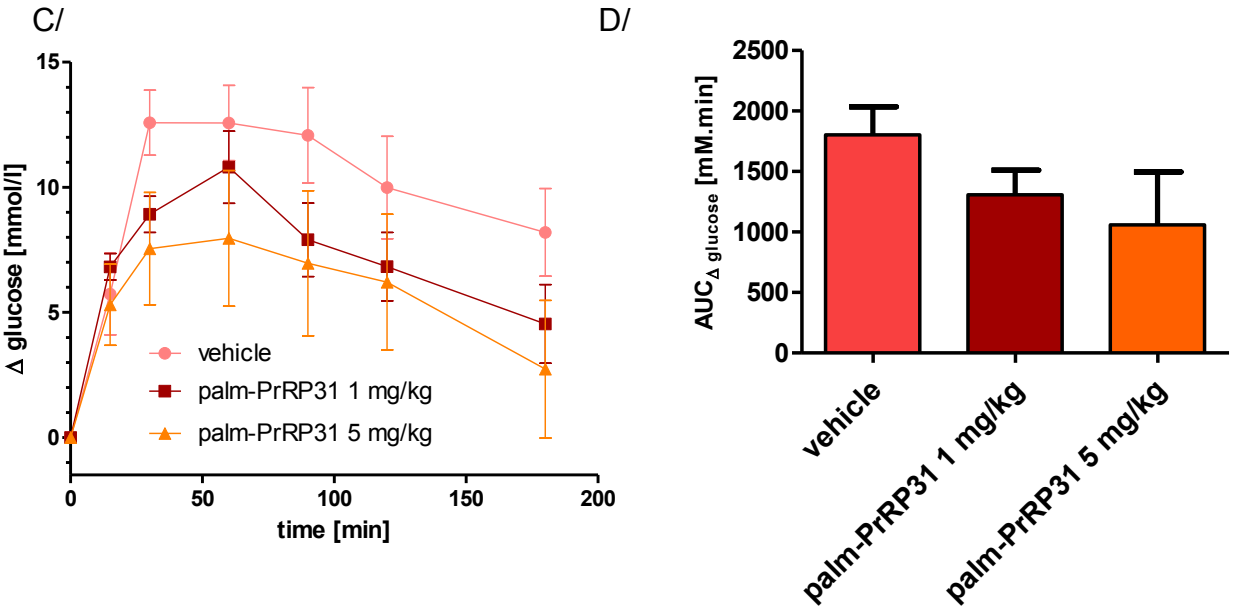


Fig. 11 Chronic effect of palm-PrRP31 on the food intake (A), body weight (B), and OGTT response (C,D) in ZDF rats

*Palm-PrPR31 was administered IP at doses of 1 and 5 mg/kg twice a day for 17 days. Food intake is expressed as a percentage of food intake in the vehicle-treated control group, BW is expressed as a percentage of the initial BW. OGTT was performed at the end of the experiment and its results are shown as Δ glucose and $AUC_{\Delta\ glucose}$. Data are presented as means ± S.E.M. Statistical analysis was performed by repeated measures ANOVA with Bonferroni post hoc test (A, B,C) or by one-way ANOVA followed by Dunnett´s post hoc test (D), significance is *P < 0.05, **P < 0.01, ***P < 0.001 vs the vehicle- treated obese control group (n = 7−8) (Holubová et al., 2016).*

4.4. Palm^{11}-PrRP31 improved glucose tolerance and metabolic parameters in SHROB and SHR rats

The results obtained in the experiment performed in SHROB and SHR rats presented in this chapter were published in Nutrition & Diabetes (Mikulášková et al., 2018).

Treatment with palm^{11}-PrRP31 decreased food intake in both, SHROB and SHR rats. However, the effect was more pronounced in the SHR rats compared to the SHROB rats (Fig. 12A). Similarly, BW was lowered significantly in the SHR rats (−13%, P < 0.001), but not in the SHROB rats after palm^{11}-PrRP31 treatment

(Fig. 12B). However, palm^{11}-PrRP31 administration improved tolerance to glucose measured by OGTT in both genotypes (Fig. 12C,D) according to the significantly decreased AUC in the palm^{11}-PrRP31-treated groups compared to the vehicle-treated groups.

At the end of the experiment at the age of 19 weeks, the metabolic parameters were measured in fasted plasma (Table 1). The higher BW in the vehicle-treated SHROB rats was accompanied by significantly higher plasma leptin, triglycerides, total cholesterol, and insulin levels as well as the HOMA index and insulin/glucagon ratio compared to vehicle-treated SHR rats. Furthermore, higher triglyceride (TAG) contents in the liver, but lower free fatty acids (FFA) and glucagon levels in the plasma, were observed in the SHROB model compared to SHR. The treatment insignificantly decreased leptin levels in the SHROB model and significantly decreased them in the SHR model. The levels of FFA were significantly increased in both, SHR and SHROB models after three weeks of treatment. The administration of palm^{11}-PrRP31 significantly decreased liver TAG in the SHR rats. Increased plasma glucagon levels but lowered insulin levels resulted in a decreased insulin/glucagon ratio in the palm^{11}-PrRP31-treated SHROB rats. On the other hand, no significant changes in the insulin and glucagon plasma levels were found in the palm^{11}-PrRP31-treated SHR rats. The treatment decreased the HOMA index in the SHROB but not in the SHR rats. The systolic blood pressure (SBP) did not change after the treatment in either rat model (Table 1).

Individual components of the insulin signaling cascade were explored in the hypothalami by immunochemistry. The representative samples of western blots are shown in Fig. 13A. Significantly lower hypothalamic IRβ was detected in the vehicle-treated SHROB rats compared to the vehicle-treated SHR rats; in addition, significant enhancement of IRβ was observed in the SHROB rats after the treatment (Fig. 13B). PI3K was significantly increased in both genotypes (Fig. 13C). A significant increase in MAPK/ERK1/2 phosphorylation was observed after the treatment in both SHR and SHROB rats (Fig. 13D).

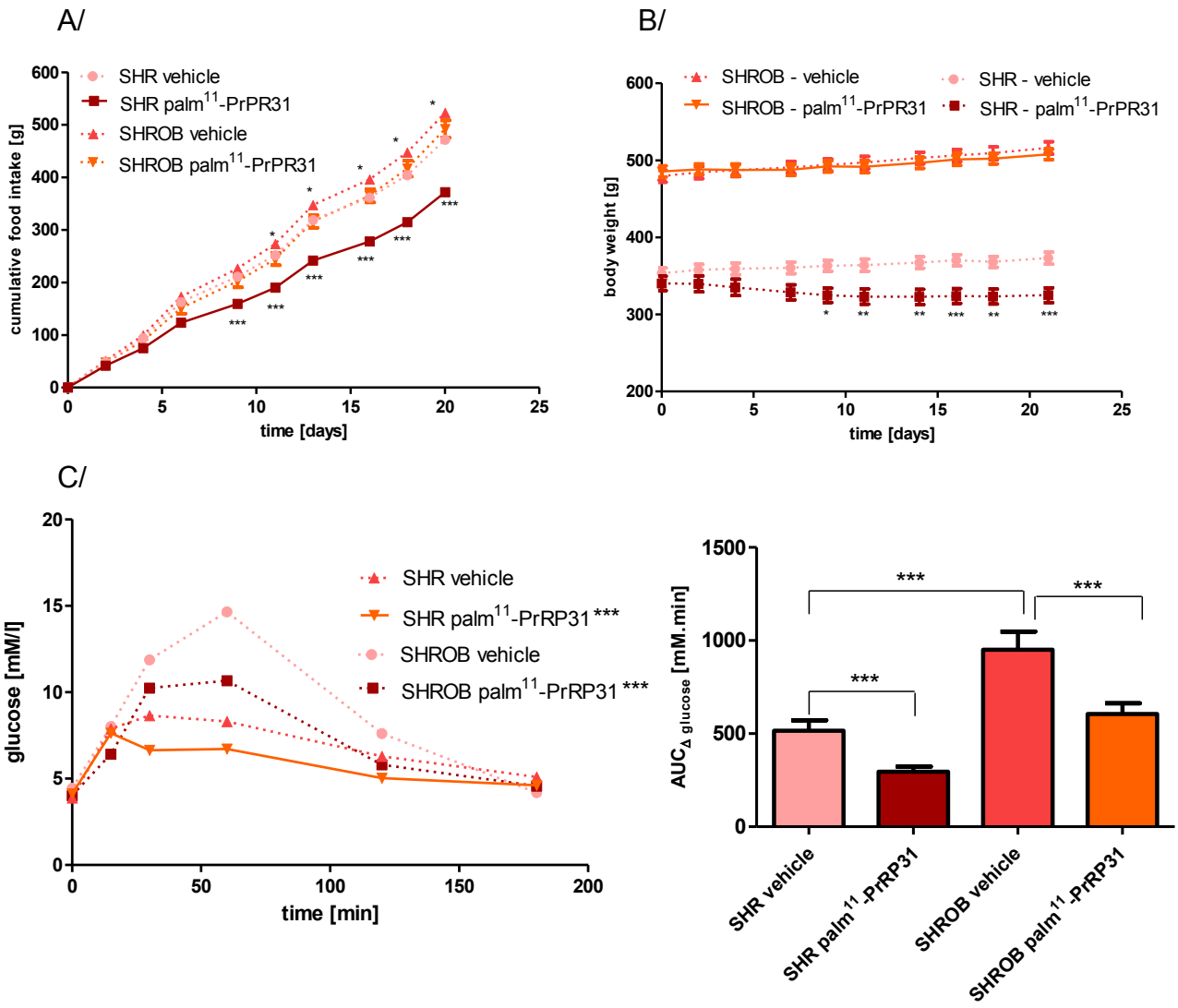


Fig. 12 Chronic effect of palm^{11}-PrRP31 on the food intake (A), body weight (B), and OGTT response (C) in SHR and SHROB rats

*Palm^{11}-PrPR31 was administered IP at a dose of 5 mg/kg once a day for 21 days. Food intake and body weight were monitored every two days during the drug application. OGTT was performed at the end of the experiment; results are shown as a glucose profile and delta AUC. Data are presented as means ± S.E.M. Statistical analysis was performed by repeated measures ANOVA with Bonferroni post hoc test, significance is *P < 0.05, **P < 0.01, ***P < 0.001 vs the respective control group (n = 8) (Mikulášková et al., 2018).*

Table 1: Metabolic parameters analyzed in the fasted blood plasma, triglycerides in the liver, and systolic blood pressure of SHR and SHROB rats after the treatment (19 weeks of age) with palm^{11}-PrRP31.

Genotype **Treatment**	**SHR** **vehicle**	**SHR** **palm^{11}PrRP31**	**SHROB** **vehicle**	**SHROB** **palm^{11}PrRP31**
body weight (g)	373 ± 8	325 ± 10$^{\#}$	516 ± 8***	508 ± 8
free fatty acids (mmol/l)	0.82 ± 0.04	0.97 ± 0.05$^{\#}$	0.60 ± 0.07*	0.80 ± 0.05$^{\#}$
leptin (ng/ml)	3.97 ± 0.46	2.37 ± 0.39$^{\#}$	179.10 ± 8.78***	166.20 ± 17.76
insulin (ng/ml)	0.72 ± 0.08	0.60 ± 0.06	19.73 ± 2.16***	13.08 ± 1.71$^{\#}$
HOMA index	21.89 ± 3.17	19.32 ± 2.26	734.70 ± 172.20**	411.80 ± 74.37
glucagon (ng/ml)	34.01 ± 2.12	29.71 ± 2.63	25.65 ± 2.70*	36.32 ± 1.42$^{\#\#\#}$
insulin/glucagon ratio	2.20 ± 0.29	2.11 ± 0.26	85.16 ± 14.25***	35.53 ± 3.39$^{\#\#}$
TAG in the liver (mmol/g of protein)	1.23 ± 0.18	0.47 ± 0.08$^{\#\#}$	7.02 ± 0.64***	4.47 ± 1.30
SBP (mmHg)	201.50 ± 8.74	205.30 ± 4.78	177.40 ± 9.41	184.80 ± 8.47

*Data are presented as means ± S.E.M. Statistical analysis was performed by unpaired t-test. Significance is *P<0.05, **P<0.01, ***P<0.001 vs the lean control SHR group (n=8), $^{\#}$P<0.05, $^{\# \#}$P<0.01, $^{\# \# \#}$P<0.001 vs the respective vehicle-treated control group (n = 8). SBP – systolic blood pressure, TAG – triglycerides*

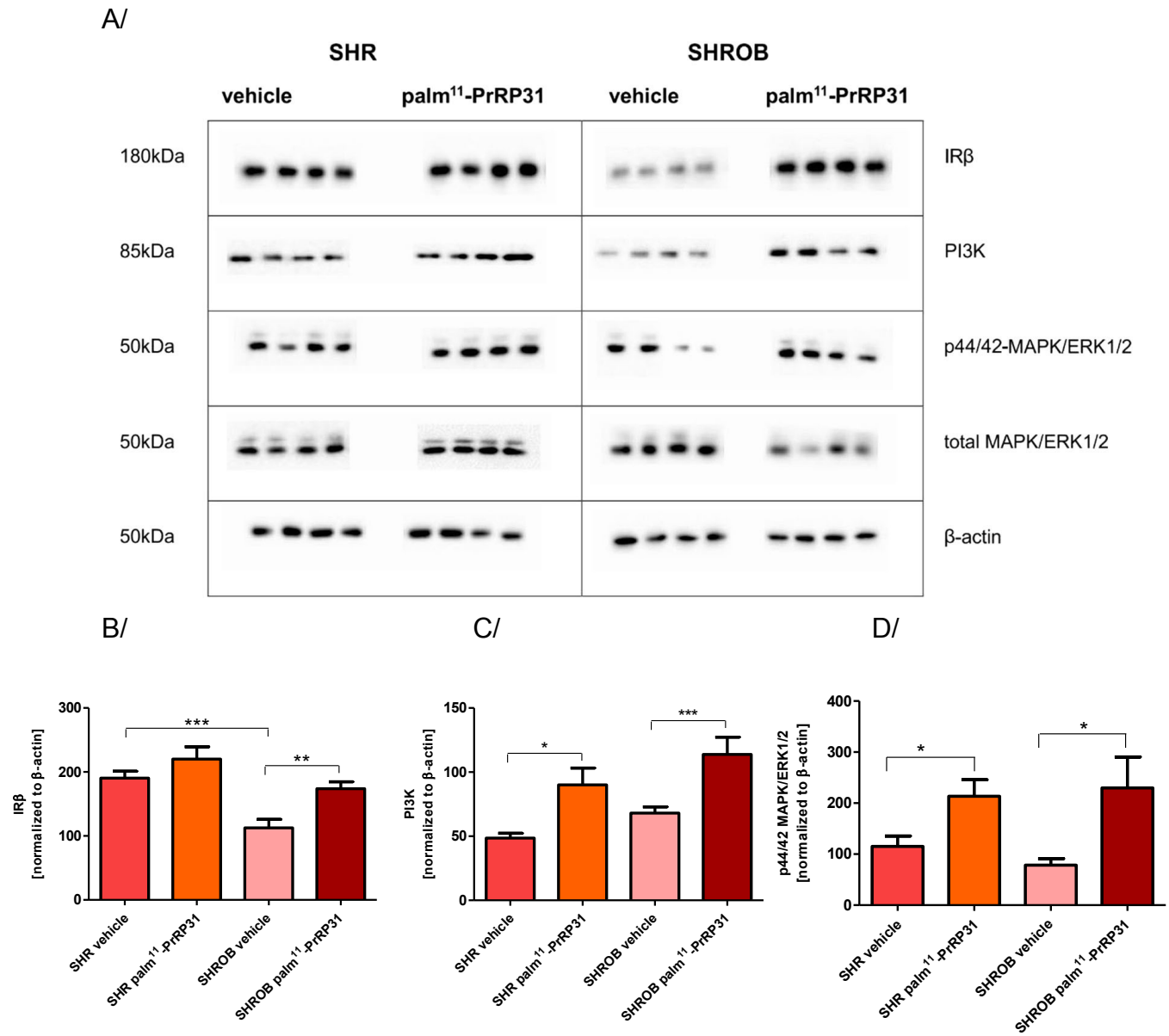


Fig. 13 Signaling pathways in the hypothalamus

A/ Western blots - levels of IRβ, PI3K, and MAPK/ERK1/2.

Densitometric quantification of western blots normalized to β-actin, B/ IRβ, C/ PI3K, D/ MAPK/ERK1/2/total MAPK/ERK1/2

*Data are presented as means ± S.E.M. Statistical analysis was performed by unpaired t-test. Significance is *P < 0.05, **P < 0.01, ***P < 0.001 vs respective control group (n = 8) (Mikulášková et al., 2018).*

4.5. Blood pressure experiments

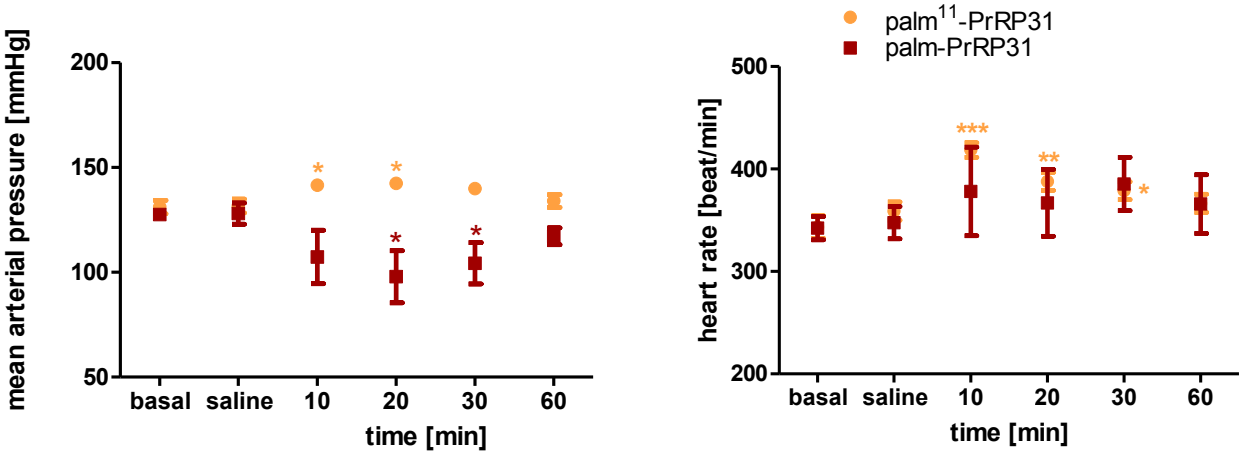
The results obtained in the following experiments have not yet been published.

4.5.1. The effect of acute injections of palm^{11}-PrRP31 and palm-PrRP31 on the blood pressure and heart rate in Wistar rats

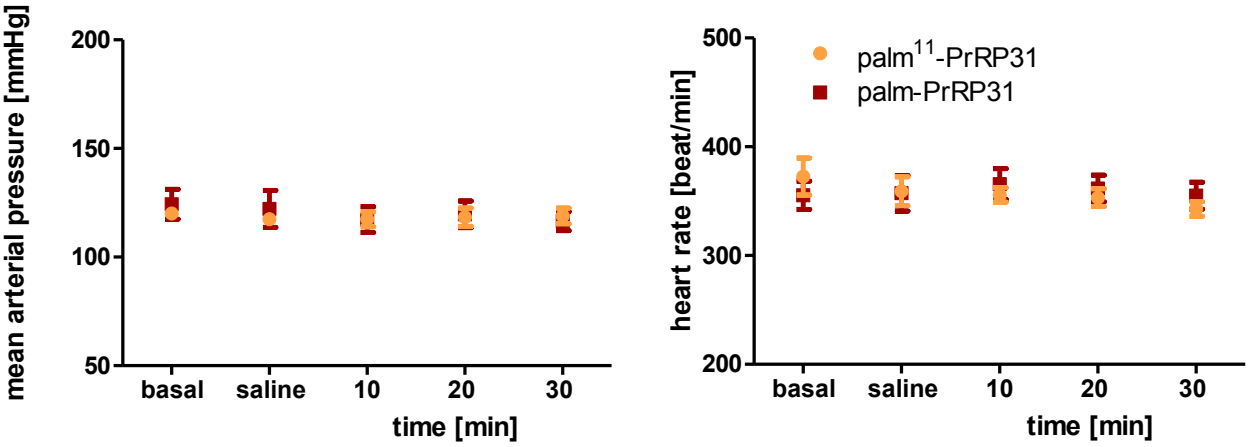
Acute measurement of BP and HR was performed in conscious Wistar male rats. Basal MAP was measured at the begginig of the experiment for 10 minutes. Thereafter, rats were injected IP (Fig. 14A) or SC (Fig.14B) with saline, to receive control MAP. Then palm^{11}-PrRP31 or palm-PrRP31 at a dose of 5 mg/kg was injected IP (Fig. 14A) or SC (Fig. 14B). There were no changes after the injection of saline (control MAP) in both, SC and IP injections. A temporary significant increase of MAP after IP treatment was observed 10 and 20 minutes after injection of palm^{11}-PrRP31 and a temporary significant decrease of MAP after IP treatment was observed 20, and 30 minutes after injection of palm-PrRP31. HR was temporarily increased 10, 20, and 30 minutes after IP injection of palm^{11}-PrRP31 (Fig. 14A). After SC treatment, there were no significant changes in MAP and HR after injections of both analogs (Fig. 14B).

In the IV administration experiment, the basal MAP and HR were measured for the first 10 minutes and then the rats were injected with palm-PrRP31 or palm^{11}-PrRP31 at a dose of 0.1 mg/kg. We did not obsereve any significant changes in MAP and HR after IV administration of both compounds (Fig. 14C).

A/ IP administration



B/ SC administration



C/ IV administration

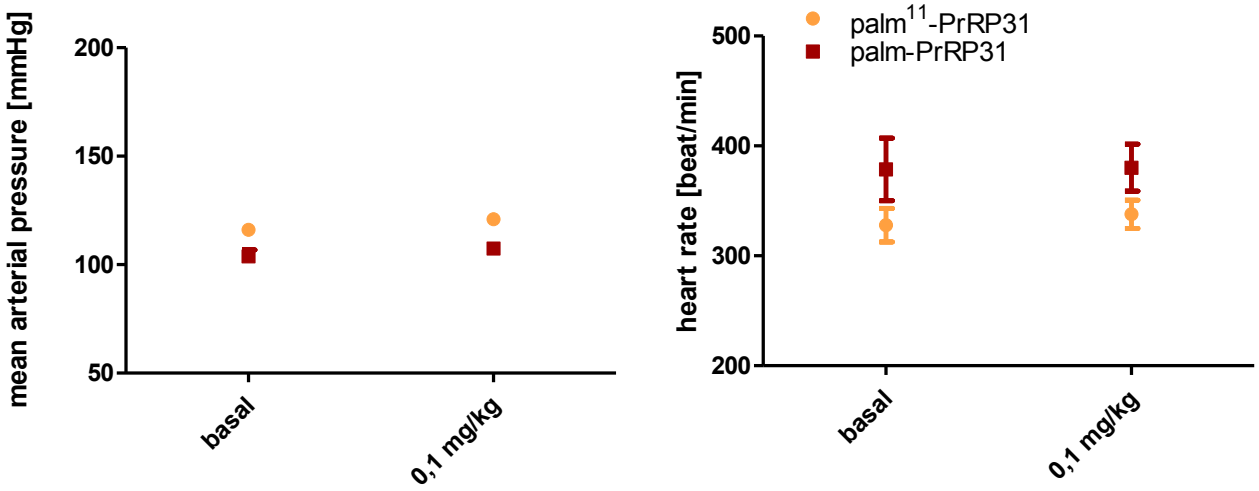


Fig. 14 Acute effect of palm-PrRP31 and palm^{11}-PrRP31 on the blood pressure and heart rate in Wistar rats after intraperitoneal administration (A), subcutaneous administration (B), or intravenous administration (C)

(A,B) Rats were injected with saline to receive control MAP. After saline injection, palm-PrRP31 or palm^{11}-PrPR31 was administered IP (A) or SC (B) at a dose of 5 mg/kg.

(C) Rats were injected IV at a dose of 0.1 mg/kg.

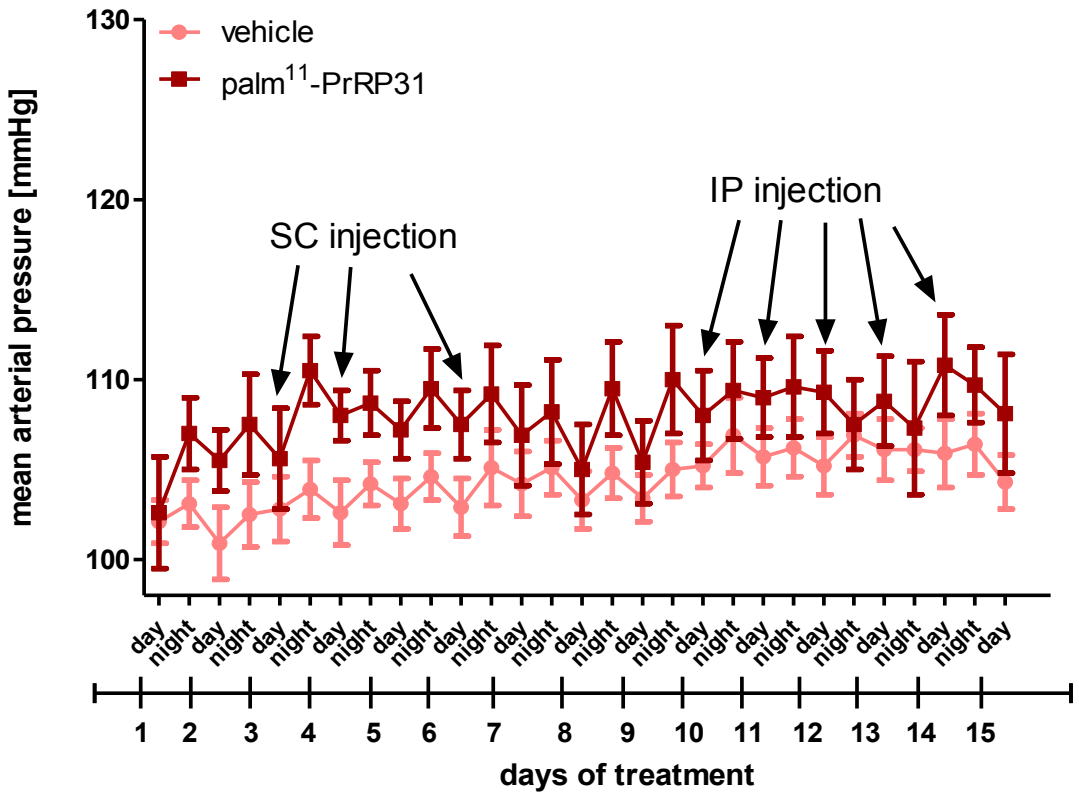
*Data are presented as means ± S.E.M. Statistical analysis was performed by unpaired t-test. Significance is *P < 0.05, **P < 0.01, ***P < 0.001 vs basal mean arterial pressure or basal heart rate (n=3-9).*

4.5.2. Telemetric measurement of BP

4.5.2.1. Palm^{11}-PrRP31 had no effect either on BP or on the heart rate after SC and IP administration to Wistar rats

Blood pressure and heart rate measurement by telemetry was perfomed in Wistar male rats. Continous measurement started one week after implantation of the telemetric probe, and BP and HR was monitored for 15 consecutive days. For the first two days, basal MAP was recorded. As shown in Fig. 15, on the 3rd, 4th and 6th day rats were injected SC with palm^{11}-PrRP31 at a dose of 5 mg/kg. From the 10th to the 14th day palm^{11}-PrRP31 was injected IP at a dose 5 mg/kg. Neither way of treatment (SC and IP) with palm^{11}-PrRP31 did significantly change the mean arterial pressure or the heart rate (Fig. 15A,B).

A/



B/

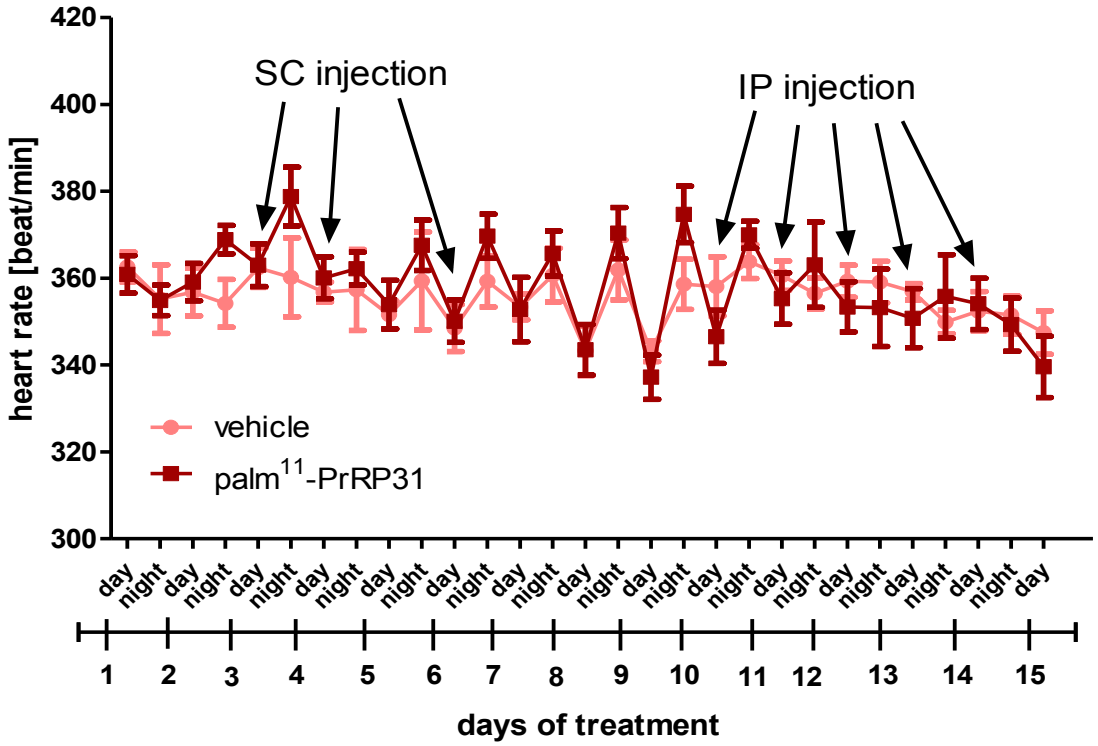


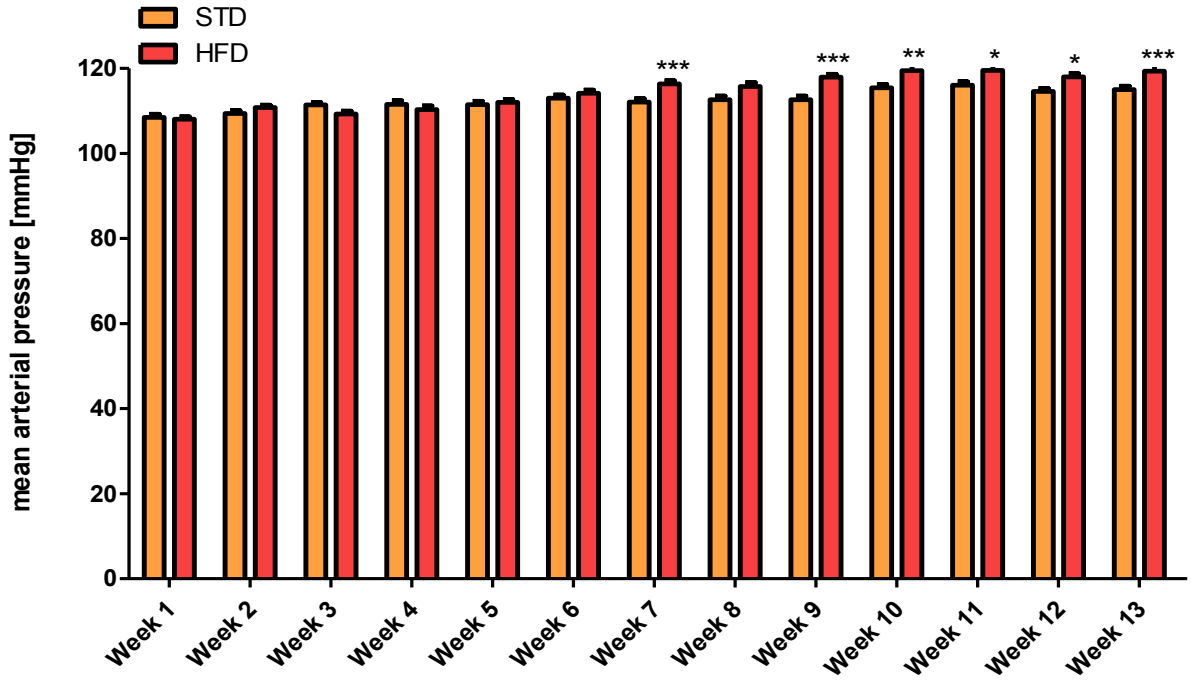
Fig. 15 Effect of palm^{11}-PrRP31 on telemetry-measured MAP (A) and HR (B) in Wistar rats

Rats were injected SC or IP with palm^{11}-PrPR31 at a dose of 5 mg/kg. Blood pressure was measured continuously for 15 days. Data are presented as means ± S.E.M. Each point represents the average value of daily or nightly values of BP/HR. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test (n=5-6).

4.5.2.2. HFD increased BP and HR in Wistar Kyoto rats

One week after implantation of the telemetric probe Wistar Kyoto rats were fed either the HFD or STD diet for 13 weeks. BP and HR measurement started at the beginning of HFD feeding. BP and HR was monitored every week on Thursday for 24 hours for a period of 13 weeks. As demonstrated in Fig. 16 A,B, at the beginning of the experiment both groups, STD and HFD have comparable MAP and HR. The consumption of HFD significantly increased BP and HR compared to the STD diet- fed group. A significant increase in BP was already observed in 7th week and HR increased even already in 2nd week after HFD feeding.

A/



B/

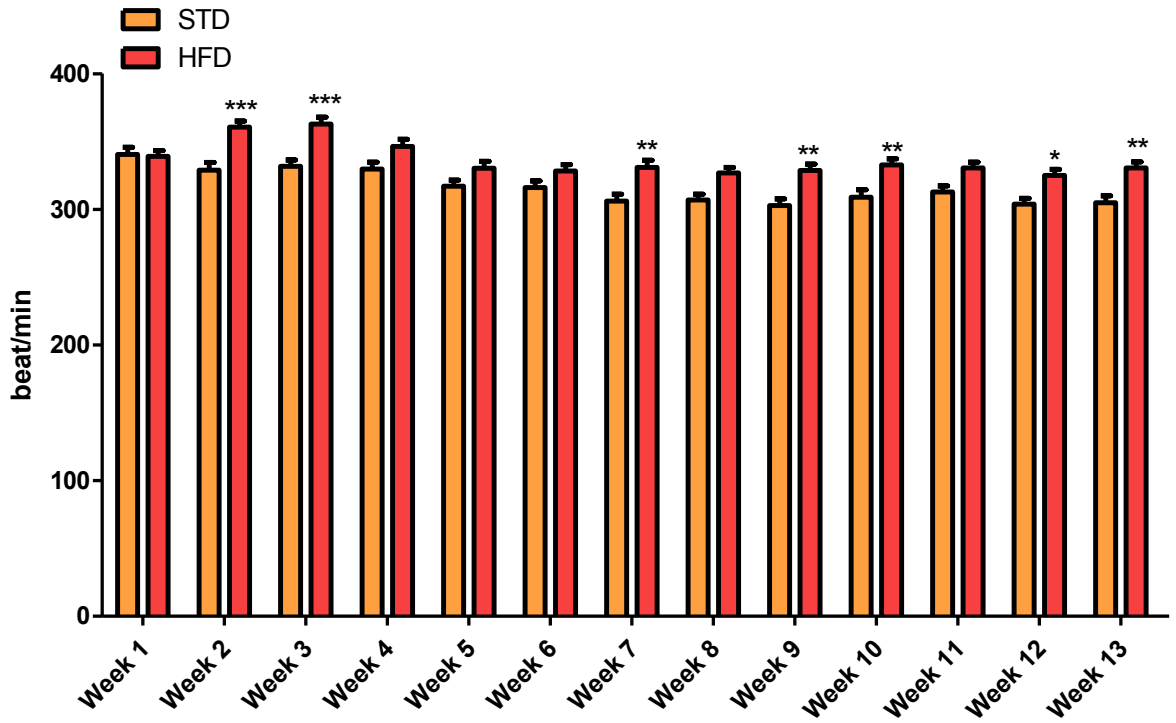


Fig. 16 Effect of HFD on telemetry-measured BP (A) and HR (B) in WKY rats

*Rats were fed with HFD for 13 weeks. Data are presented as means ± S.E.M. Each point represents the average value of the 24 hour values. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test. Significance is *P < 0.05, **P < 0.01, ***P < 0.001 vs STD diet control group (n = 6).*

5. DISCUSSION

Obesity is a serious health problem reaching epidemic proportions; more patients are therefore at risk of TD2M and cardiovascular diseases. Obesity as a particular symptom of MetS can play an important role in cardiovascular morbidity and mortality. Since mortality of cardiovascular diseases is one of the biggest problems in the world, the individual risk factors of MetS, including obesity and hypertension, need to be treated in order to prevent T2DM and cardiovascular diseases.

The treatment of obesity has proven to be challenging. Despite that pharmacotherapy for weight loss has progressed extensively over the past decade (for review see (Jackson et al., 2015)), a large medical need exists for new weight loss drugs, with higher efficacy and improved side-effect profile compared to the current antiobesity therapeutics.

Native anorexigenic neuropeptides have the potential to decrease food intake and ameliorate obesity. However, they are not able to cross the blood-brain barrier after peripheral application. Recently, we have designed and synthetized lipidized analogs of prolactin-releasing peptide, which resulted in stabilization of the molecule and allowed us to apply the peptide to the periphery to achieve its central biological effects (Kuneš et al., 2016; Maletínská et al., 2015).

In my PhD thesis, two potent analogs of prolactin-releasing peptide palmitoylated either at the N-terminus (palm-PrRP31) or at position 11 (palm^{11}-PrRP31) were used for metabolic studies in different animal models.

5.1. Palmitoylated PrRP31 analog decreased food intake in free-fed rats

Many neuropeptides affect food intake and body weight when injected centrally (Kalra et al., 1999), including PrRP, which was shown to decrease food intake and BW gain after central administration to rats without causing taste aversion (Lawrence et al., 2000). In our previous study, we have shown that modification of PrRP by lipidization not only led to its increased stability in the blood, but also enabled exertion of the PrRP central effect after its peripheral administration (Maletínská et al., 2015). In this study, we investigated the central effect of human PrRP31 analog palmitoylated at the N-terminus after administration by three different routes (IV, SC and IP). We expect that this modification of PrRP31 allowed its entrance to the brain and we have several indirect proofs supporting this hypothesis. Firstly, only the lipidized PrRP analogs had the anorexigenic effect after peripheral administration; natural PrRP had no effect (Maletínská et al., 2015). Secondly, the anorexigenic effect of lipidized PrRP molecules was associated with the presence of c-Fos immunostaining, marker of neuronal activation in specific brain nuclei and areas involved in the food intake regulation such as PVN, DMN, ARC, LHA, NTS, which contain GPR10 and NPFF2 receptors (Maletínská et al., 2015; Pražienková et al., 2017). Moreover, the central neuronal activation after peripheral palm-PrRP31 administration was associated with selective activation of specific hypothalamic oxytocin and hypocretin neuronal subpopulations (Pirnik et al., 2015), both involved in the food intake inhibition and energy expenditure.

This study demonstrated that three repeated injections (IV, SC or IP) of palm-PrRP31 decreased food intake even in free-fed rats independently of the route of delivery; however, the degree of food intake reduction was partially dependent on the route of administration and on the dose of the peptide. Our study clearly shows that SC absorption could be different even from intraperitoneal absorption, which is in good agreement with the study of Hayes et al. (Hayes et al., 2011). Hayes and coworkers found that food intake and BW suppression by liraglutide, palmitoylated GLP-1 agonist, were of greater magnitude and shorter latency following IP compared to SC delivery. Surprisingly, the effect of non-lipidized GLP-1 agonist exendin-4 was similar after IP and SC administration in

rats. Therefore, more studies are needed in the future to understand the exact mechanisms of palm-PrRP31 absorption after SC administration. In our study, the dose of 1 mg/kg of palm-PrRP31 injected SC did not have any significant effect on food intake (data not shown), but IP application of this analog in respective dose decreased the food intake significantly. This may relate to the speed at which peptides are released from the subcutis, optionally with other unknown factors. The maximal food intake-decreasing effect was achieved after the IV administration of palm-PrRP31 because the dose of palm-PrRP31 was five hundred times lower than IP and SC doses.

These findings are supported by the pharmacokinetics data. We have shown that palm-PrRP31 concentration in the rat plasma is dependent on the way of administration (IV, SC or IP) (Mikulaskova et al., 2016). The absorption of the peptide after SC and IP administration was similar, but at different levels in the blood (Mikulaskova et al., 2016). After IV application of 0.1 mg/kg, a fast and sharp peak appeared, which dropped to the basal level after one hour. We conclude that the pharmacokinetic parameters after IV application correspond to a one/two compartmental model, while several compartmental models correspond to SC and IP administration. It is evident that specifically for SC administration, the transfer of the tested peptide from the subcutis into the blood could have some limitations.

5.2. Palm^{11}-PrRP31 analog decreased food intake, BW, and MAP in DIO mice

In this study, C57BL/6J mice were fed with HF diet to induce obesity and then treated with palm^{11}-PrRP31. After 21-day SC administration of palm^{11}-PrRP31 we observed a significant decrease of food intake and body weight, as was previously seen in our other studies (Holubová et al., 2018; Pražienková et al., 2017). The mean arterial pressure at the end of the experiment was significantly increased after the HF diet feeding compared to the LF control group. A significant increase of MAP following the HF diet feeding was also observed by Rao and coworkers in their study in C57BL/6J mice (Rao et al., 2007). The treatment with palm^{11}-PrRP31 returned MAP to the normal levels. Thus, the palm^{11}-PrRP31 analog may represent an important therapeutic target for obesity and its complications such as increased blood pressure.

5.3. Palm-PrRP31 analog decreased BW and improved glucose tolerance in Sprague-Dawley DIO rats but not in diabetic ZDF rats

This study was aimed to characterize the subchronic effect of palm-PrRP31 in two rat models of obesity and diabetes: DIO Sprague-Dawley rats and ZDF rats (Holubová et al., 2016). Both these genotypes are hyperleptinemic; however, the origin of hyperleptinemia is different. In DIO rats, the leptin resistance developed because of dysregulation of the energy homeostasis in consequence of growing adipose tissue mass that secreted increasing amounts of leptin. On the other hand, in ZDF rats, a high level of circulating leptin results from the absence of a functional leptin receptor (Phillips et al., 1996), and leptin is thus not able to regulate food intake properly in ZDF rats.

In DIO rats, the two-week IP treatment with palm-PrRP31 resulted in a significantly decreased food intake and BW in a dose-dependent manner and in reduced leptin levels and adipose tissue masses. Palm-PrRP31 already caused a significant effect at the 1 mg/kg dose and the most significant effect was achieved at the dose of 5 mg/kg, when food intake was decreased for several days by 40 % and by 24 % at the end of the experiment compared to the vehicle-treated group. The BW was decreased by 8 % at the end of the palm-PrRP31 treatment. Similar effects were shown previously after chronic treatment in the DIO rat model with the GLP-1 analogs liraglutide (Madsen et al., 2010; Raun et al., 2007) and exenatide (Reidelberger et al., 2011) or with a lipidized α-MSH analog (Fosgerau et al., 2014). In our study, the palm-PrRP31 treatment significantly improved glucose tolerance, and this effect was caused at least partially by an attenuating effect on lipogenesis. However, at the moment we are not able to satisfactorily explain the observed nonlinear relationship in OGTT (Fig. 10D), and the possible antidiabetic effect should be further investigated.

In contrast, in ZDF rats, the same treatment lowered food intake but did not significantly affect body weight, fat mass, leptin levels, or glucose tolerance, probably in consequence of severe leptin resistance due to a non-functional leptin receptor. Food intake was decreased from the first day of the treatment with a 5 mg/kg dose of palm-PrRP31 and dropped by 15–20 % compared with the control vehicle-treated group. The most probable reason for the unchanged BW is that

ZDF rats at the age of 11 weeks had still increasing BW, but starting the treatment at this age was necessary for a proper modeling of type 2 diabetes. In this study, palm-PrRP31 tended to lower the OGTT curves in ZDF diabetic rats, but the results did not reach significance. The primary effect of palm-PrRP31 was anorexigenic and occurred both in DIO and ZDF diabetic rats. However, in ZDF rats, a deficiency in the functional leptin receptor could cause diminished PrRP efficacy, as the synergism of leptin and the PrRP anorexigenic effect is well known (Ellacott et al., 2002).

5.4. Chronic administration of palm^{11}-PrRP31 improved glucose tolerance in SHROB and SHR rats

Palm^{11}-PrRP31 was tested in spontaneously hypertensive obese rats, model of metabolic syndrome very suitable for exploring the interactions of all symptoms of MetS (e.g., obesity, hypertension, hyperlipidemia, and salt sensitivity) (Koletsky et al., 1995). Furthermore, SHROB rats are extremely insulin-resistant because of their impaired leptin receptor signaling. As a control group, spontaneously hypertensive rats were used.

In agreement with literature data, we have demonstrated in our study (Mikulášková et al., 2018) higher fat and BW, normal fasting glucose, but impaired glucose tolerance after an oral load and higher plasma levels of insulin and leptin in the SHROB rats compared to the lean SHR controls (Friedman et al., 1997; Chen et al., 2011; Velliquette et al., 2002). Moreover, SHROB rats had higher content of TAG in the liver compared to SHR animals. In contrast, we have observed lower fasting plasma FFA and glucagon levels in the SHROB rats compared with the SHR controls. In the hypothalamus of SHROB animals we observed a decreased insulin receptor level compared to the SHR rats. The reduced expression of the insulin receptor was previously linked to attenuated insulin signaling in fat (Friedman et al., 1997; Velliquette et al., 2002). Furthermore, the expression of several genes related to lipogenesis in the adipose tissue and liver were significantly higher in the SHROB rats. In the BAT of SHROB rats, negligible UCP-1 mRNA expression was detected, which points to a possible distorted/attenuated energy expenditure in this strain (Keen-Rhinehart et al., 2005; Keen Rhinehart et al., 2004; Mikulášková et al., 2018).

Compared to normotensive Wistar-Kyoto rats, the SHROB rats are hypertensive (results not shown), although SBP tended to be lower in comparison with lean SHR rats, in agreement with the study by Friedman et al. (Friedman et al., 1997).

The main goal of this study was to find out whether palm^{11}-PrRP31 could accomplish its antiobesity and antidiabetic effects in SHROB rats lacking leptin signaling and whether its effect depends on functional leptin. The three-week treatment significantly lowered the food intake in both SHROB and SHR rats. However, the BW-lowering effect was seen only in the SHR control group, probably because of impaired leptin receptor signaling in the SHROB rats. Similar results were seen in our previous study of ZDF rats with nonfunctional leptin receptor (Holubová et al., 2016). Therefore, we may speculate that intact leptin signaling is an important prerequisite for the PrRP body weight-lowering effect. The most important result from this experiment is a significant improvement of glucose tolerance observed in both SHROB and SHR rats (Mikulášková et al., 2018). This result was similar to the observations in diet-induced obese Sprague Dawley rats (Holubová et al., 2016). Taken together, these data suggest that the improvement of glucose tolerance is mostly independent of antiobesity effects in the SHROB rats, which makes palm^{11}-PrRP31 an interesting candidate for direct targeting of prediabetes/diabetes along with obesity.

A decrease in the plasma insulin and glucagon levels and a subsequent decrease in the HOMA index and insulin/glucagon ratio were observed after the treatment in SHROB rats. The increase in FFA observed in this study could be the result of a decreased insulin/glucagon ratio, as insulin blocks and glucagon stimulates the release of FFAs from adipocytes. We may speculate that the treatment with palm^{11}-PrRP31 markedly improved the liver insulin sensitivity with regard to decreased ectopic lipid storage in spite of the enhanced FFA levels. In a previous study, liver TAG production in type-2 diabetes was shown to be dependent on FFA levels rather than circulating insulin levels (Vatner et al., 2015).

Finally, increased insulin receptor and PI3K levels and MAPK/ERK1/2 phosphorylation were detected in the hypothalami of both genotypes. The increased MAPK/ERK1/2 phosphorylation in the hypothalamus could be a result of either insulin or PrRP effects. For PrRP, ERK1/2 is its main activation pathway through its GPR10 receptor (Maixnerová et al., 2011). It is known that leptin and

insulin act together in the hypothalamus to target energy homeostasis (Thon et al., 2016). The anorexigenic effect is mediated by STAT3 through the leptin receptor LepRb activation and by PI3K through the insulin receptor IR activation. In this study, palm^{11}-PrRP31 increased PI3K levels and additionally ERK activation in the hypothalamus, both pathways that are known to be activated by insulin.

Thus, the three-week treatment with our novel palmitoylated PrRP31 analog ameliorated glucose tolerance and attenuated hyperinsulinemia and the insulin/glucagon ratio in SHROB rats similarly to previously described treatments with angiotensin-converting enzyme inhibitors, angiotensin receptor 1 blockers (Ernsberger et al., 2007; Rong et al., 2010; Zhao et al., 2011), a PPARγ agonist (Velliquette et al., 2002), and the dipeptidyl peptidase IV inhibitor sitagliptin (Chen et al., 2011), although most likely via a different mechanism of action and by targeting different receptor(s). In spite of a modest effect of palm^{11}-PrRP31 on BW in SHROB rats, the glucose tolerance was significantly improved in both genotypes. We suggest that functional leptin is required for the anorexigenic but not for the antidiabetic effects of lipidized PrRP.

5.5. Blood pressure experiments

5.5.1. The effect of single injection of palm^{11}-PrRP31 and palm-PrRP31 on the blood pressure and heart rate in Wistar rats

Several neuropeptides produced in the brain that are involved in the food intake regulation and energy expenditure also have influence on the cardiovascular system. These effects could be mediated through the activation or inactivation of the sympathetic nervous system (Mikulášková et al., 2016). However, the relationship between the food intake regulation and BP regulation is complicated and not fully understood. Although one should expect that if there is a link between obesity and hypertension, then anorexigenic peptides should decrease BP and orexigenic peptides should increase BP, recent studies indicated the opposite effect. It was demonstrated that anorexigenic neuropeptides predominantly cause an increase in sympathetic nerve activity and thus an increase in BP. On the other hand, orexigenic neuropeptides decrease sympathetic nerve activity, and thus decrease BP (for review see (Mikulášková et al., 2016)).

Most studies about the effect of food intake-regulating peptides on BP (Mikulášková et al., 2016) show results after administration of a particular neuropeptide. Samson and coworkers found that ICV administration of native PrRP increased the arterial blood pressure in conscious and unrestrained rats (Samson et al., 2000). Moreover, microinjection of native PrRP into the most caudal ventrolateral medulla oblongata, recognized as the caudal pressor area, elicited dose-dependent increases in MAP, HR, and SNA (Horiuchi et al., 2002). In contrast, in our previous study we found that peripheral administration of native PrRP had no effect on BP and HR (unpublished results).

In this study, we tested the effect of palmitoylated analogs of PrRP31 on BP and HR after their peripheral administration (IP, SC and IV) into conscious rats in doses that significantly decreased the food intake. A temporary significant increase of MAP after IP treatment with palm^{11}-PrRP31 (5 mg/kg) and a temporary significant decrease of MAP after IP treatment with palm-PrRP31 (5 mg/kg) were found in our study. HR was temporarily increased only after IP injection of palm^{11}-PrRP31. After SC treatment (5 mg/kg), there were no significant changes in MAP and HR after injections of both analogs. We did not observe any significant changes in MAP and HR after IV administration (0.1 mg/kg) of both compounds. We assume that the effect of PrRP and its lipidized analogs on BP is dependent on the way of administration, similarly as the effect on food intake (chapter 4.1.); however, these changes were very modest at doses decreasing the food intake.

Food intake-regulating peptides might be considered as therapeutic tools for obesity treatment and possibly even for influencing blood pressure as well, but further research is needed to better understand the link between the pathways involved in the food intake regulation and BP regulation.

5.5.2. Telemetric measurement of BP

5.5.2.1. Palm^{11}-PrRP31 had no effect either on BP or heart rate after SC and IP administration to Wistar rats

In acute blood pressure experiments, palmitoylated analogs had no effect on BP and HR after SC and IP administration.

This study investigated the chronic effect of palm^{11}-PrRP31 on BP and HR on free-fed unrestrained Wistar rats. The dose of palm^{11}-PrRP31 (5 mg/kg) was a food intake-lowering dose and was chosen based on the previous food intake experiments. We did not observe any significant changes either after three SC injections or after five consecutive IP injections of palm^{11}-PrRP31. Thus, the food intake-lowering effect of palm^{11}-PrRP31 (Mikulášková et al., 2018; Pražienková et al., 2017) is not followed by changes in BP and HR after the chronic administration to rats.

5.5.2.2. HFD increased BP and HR in DIO Wistar Kyoto rats

Obesity causes or exacerbates many health problems including hypertension (Baltatzi et al., 2008). Our goal in this study was to investigate the effect of high-fat diet on BP. The BP was significantly increased from the seventh week of HF diet feeding to the end of the experiment and HR significantly increased already after the second week of HF diet feeding. In a previous study, Roza and coworkers observed significantly higher systolic BP measured 5 and 8 weeks post treatment with HF diet when compared to BP values in an age-matched group of female Wistar HanUnib rats (Roza et al., 2016).

These findings are in agreement with our study in DIO mice, where the BP increased after the consumption of the HF diet (chapter 4.2.).

6. SUMMARY

To sum up, lipidized analogs of PrRP31 designed in our laboratory showed strong anorexigenic, body weight-reducing and glucose tolerance-improving effects. However, the mechanism of their action is still unclear. In this work we used various animal models with different metabolic disorders to clarify the mechanisms how lipidized PrRP analogs act *in vivo*. The models with impaired leptin receptor signaling, the ZDF and SHROB rats, and two models of rodents fed high-fat diet, DIO mice and DIO rats, were used in this study. All these models are frequently used for studying obesity and its complications.

Firstly, we investigated the effect on food intake in free-fed rats, and based on these data we selected the dose for chronic experiments (Mikulaskova et al., 2016). Palm-PrRP31 analog influenced food intake even in free-fed rats; however, the degree of food intake reduction was partially dependent on the route of administration and on the dose of the peptide. The most significant food intake-decreasing effect was achieved after IV injections and a less pronounced significant effect was shown after SC administration. In spite of the fact that subcutaneous drug administration should provide an important route for its delivery, the exact mechanism underlying SC absorption is not completely understood. Future study towards a better formulation of the drug could help to decrease the effective dose and increase the release of the compound both after intraperitoneal and subcutaneous administrations.

Secondly, the long-term treatment with these analogs decreased food intake, body weight and improved metabolic parameters in DIO mice and DIO rats with metabolic complications. In ZDF rats with severe leptin resistance due to a nonfunctional leptin receptor (Ishizuka et al., 1998), two-week treatment with palm-PrRP31 decreased only food intake and failed to decrease BW, and only tended to improve glucose tolerance (Holubová et al., 2016). Similarly, three-week treatment with palm^{11}-PrRP31 decreased food intake and did not decrease BW in SHROB rats with impaired leptin receptor signaling. Surprisingly, glucose tolerance in SHROB rats was significantly improved after the treatment (Mikulášková et al., 2018). Taken together, these data suggest that improving glucose tolerance could be mostly independent of antiobesity effects. Moreover, as the effects of palm^{11}-PrRP31 were observed in the leptin receptor-deficient

SHROB rats, improvements in the glucose metabolism appear to be completely independent of the leptin signaling. Our data suggest that functional leptin is required for the anorexigenic but not for the antidiabetic effects of lipidized PrRP, which make palmitoylated analogs of PrRP31 interesting candidates for direct targeting prediabetes/diabetes along with obesity.

In the study with SHROB rats (Mikulášková et al., 2018), palm^{11}-PrRP31 increased PI3K levels and additionally ERK activation in the hypothalamus of SHR and SHROB rats, both pathways that are known to be activated by insulin. Based on these data, we can hypothesize that the anorexigenic effect of the PrRP31 analog was mediated by PI3K through the insulin receptor IR activation. Increased MAPK/ERK1/2 phosphorylation in the hypothalamus could also be the result of PrRP effects, because ERK1/2 is the main activation pathway through its GPR10 receptor (Maixnerová et al., 2011).

It is known that peptides that regulate food intake also play an important role in cardiovascular regulation. Therefore, in my PhD. thesis the blood pressure was measured after acute and chronic administration of PrRP31 analogs to normotensive Wistar rats. Acute IP administration of palm^{11}-PrRP31 at a dose that was used in metabolic studies (food decreasing dose – 5 mg/kg) temporarily increased blood pressure, and the same administration of palm-PrRP31 temporarily decreased blood pressure. On the other hand, SC and IV administration of both analogs at respective food intake-decreasing doses did not cause any effect on the blood pressure. Chronic SC and IP administration of palm^{11}-PrRP31 had no effect on the blood pressure in Wistar rats.

Last but not least, as the high blood pressure is one of the obesity-related complications, the effect of PrRP31 palmitoylated analog (palm^{11}-PrRP31) on BP was investigated. High-fat diet significantly increased blood pressure in DIO rats and DIO mice, and chronic administration of palm^{11}-PrRP31 (5 mg/kg) decreased the blood pressure in DIO mice to the normal level. Therefore, lipidized PrRP analogs may be important tools for the treatment of obesity complications such as increased blood pressure.

7. CONCLUSION

In general, peptides are key regulators of physiological processes with low risk of toxicity and side effects. Despite their clinical potential, natural peptides have several limitations such as poor bioavailability, low stability in the organism, and difficulties to cross the blood-brain barrier after peripheral application. One of the recently used strategies for peptide drug development is lipidization of peptides. These lipidized peptides are more stable, with a high effectiveness and potential to act centrally after peripheral application.

In our laboratory we designed palmitoylated PrRP31 analogs that exhibited strong anorexigenic and antidiabetic effects after peripheral administration to several rat and mouse models of obesity and diabetes. Moreover, palm^{11}-PrRP31 was able to return increased blood pressure in DIO mice to the normal levels.

Based on the results from my PhD thesis, we would like to hypothesize that the full effect of the lipidized PrRP analogs on food intake and hence on body weight but not improvement in the glucose metabolism might be related to intact leptin signalization.

To conclude, our data suggest a good efficacy of lipidized PrRP in rat and mouse models of obesity and diabetes. Thus, the strong anorexigenic, body weight-reducing, and blood glucose-improving effects make palmitoylated PrRP analogs attractive candidates for antiobesity and glucose-lowering treatment.

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LIST OF MY PUBLICATIONS

Publications related to PhD thesis:

1. **Mikulášková, B.**, Maletínská, L., Zicha, J., & Kuneš, J. 2016. The role of food intake regulating peptides in cardiovascular regulation. *Mol Cell Endocrinol*, 436: 78-92. **IF = 3.754**

2. Kuneš, J., Pražienková, V., Popelová, A., **Mikulášková, B.**, Zemenová, J., & Maletínská, L. 2016. Prolactin-releasing peptide: a new tool for obesity treatment. *J Endocrinol*, 230(2): R51-58. **IF = 4.706**

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Publications not related to PhD thesis:

1. Špolcová, A., **Mikulášková, B.**, Kršková, K., Gajdošechová, L., Zórad, Š., Olszanecki, R., Suski, M., Bujak-Giżycka, B., Železná, B., & Maletínská, L. 2014. Deficient hippocampal insulin signaling and augmented Tau phosphorylation is related to obesity- and age-induced peripheral insulin resistance: a study in Zucker rats. *BMC Neurosci*, 15: 111. **IF = 2.312**

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Granted patents involved in PrRP project:

AU2015266464. Lipidized peptides as neuroprotective agents. Maletínská, L., Zělezná, B., Blechová, M., Popelová, A., Neprašová, B., Kuneš, J. (granted 2017)

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Commercial activities:

Based on the results of this PhD. thesis the research collaboration and license agreement between IOCB AS CR and IP AS CR and Novo Nordisk A/S was signed in August 2017.

SUPPLEMENT

1. Mikulášková, B., Maletínská, L., Zicha, J., & Kuneš, J. 2016. The role of food intake regulating peptides in cardiovascular regulation. *Mol Cell Endocrinol*, 436: 78-92.

2. Kuneš, J., Pražienková, V., Popelová, A., Mikulášková, B., Zemenová, J., & Maletínská, L. 2016. Prolactin-releasing peptide: a new tool for obesity treatment. *J Endocrinol*, 230(2): R51-58.

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