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Autoreferát disertační práce

UNIVERZITA KARLOVA
1. lékařská fakulta

Vzájemná interakce ghrelinu a jeho nového endogenního antagonisty LEAP2: možná úloha v patologii obezity

Interplay between ghrelin and its novel endogenous antagonist LEAP2: possible role in the pathology of obesity

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ABSTRAKT

Zvyšující se počet osob s nadváhou a obezitou se v naší společnosti stal závažným zdravotním problémem. Obezita je často způsobena nadměrnou hyperfagií, a proto je důležité komplexně rozumět regulaci příjmu potravy, abychom mohli toto chronické onemocnění úspěšně léčit. Ghrelin, periferní peptidový hormon zodpovědný za zvýšení příjmu potravy, přímo ovlivňuje hypotalamus prostřednictvím GHSR (zkratka anglického názvu growth hormone secretagogue receptor). Nedávno bylo zjištěno, že LEAP2 (zkratka anglického názvu liver expressed antimicrobial peptide 2) přirozeně inhibuje konstitutivní aktivitu GHSR jako inverzní agonista. Proto je LEAP2 potenciálně využitelný kandidát pro vývoj antiobezitního léčiva.

Tato disertační práce zkoumá interakci mezi ghrelinem a LEAP2 v kontextu regulace příjmu potravy a obezity. Nejprve se zaměřuje na modifikovaný zkrácený N-terminální peptid LEAP2(1-14) a jeho lipidizované analogy a zkoumá jejich afinitu ke GHSR a jeho aktivaci *in vitro* a *in vivo*. Výsledky ukazují, že palmitovaný LEAP2(1-14) (palm-LEAP2(1-14)) v porovnání s ostatními analogy vykazuje nejvýraznější afinitu ke GHSR, působí jako inverzní agonista GHSR, snižuje příjem potravy, inhibuje uvolňování růstového hormonu navozeného ghrelinem a vykazuje zvýšenou stabilitu v potkaní plasmě. Tato zjištění naznačují, že palm-LEAP2(1-14) by mohl být slibným lékem proti obezitě.

Studie dále zkoumá vliv potravy s vysokým obsahem tuků na obezitu a rozvoj rezistence vůči ghrelinu a LEAP2 u myší. Výsledky ukazují, že podávání vysokotukové diety snižuje aktivní a celkový ghrelin v plasmě, zvyšuje *LEAP2* mRNA v játrech a vede ke glukózové intoleranci. Přechod na standardní dietu normalizuje expresi mRNA *LEAP2* v játrech a hladinu aktivního ghrelinu, nikoli však celkového ghrelinu v plasmě. Studie dále prokazuje rezistenci vůči palm-LEAP2(1-14) vyvolanou vysokotukovou dietou a také rezistenci vůči stabilnímu GHSR agonistovi [Dpr^{3}]Ghrelinu která je reverzibilní po přechodu na standardní dietu.

Nakonec byl hodnocen potenciál palm-LEAP2(1-14) potlačit vliv vysokotukové diety na nárůst tělesné hmotnosti a normalizovat morfometrické a metabolické parametry spojené s obezitou. Palm-LEAP2(1-14) mírně snížil přírůstek tělesné hmotnosti vyvolaný podáváním vysokotukové diety a snížil hladinu leptinu v plasmě. Celkově však palm-LEAP2(1-14) nebyl schopen potlačit účinek vysokotukové diety pravděpodobně v důsledku rezistence k palm-LEAP2(1-14).

Tato zjištění přispívají k lepšímu pochopení patofyziologie obezity a naznačují nutnost dalšího zkoumání alternativních strategií ke zlepšení účinnosti léčby obezity zaměřené na dráhy ghrelinu a LEAP2.

ABSTRACT

The increasing number of overweight and obese individuals has become a major health issue in our society. The etiology of obesity often involves excessive hyperphagia, highlighting the importance of comprehensive understanding the regulation of food intake regulation in order to effectively treat this chronic condition. Ghrelin, a peripheral peptide hormone responsible for increasing food intake, directly affects the hypothalamus through the growth hormone secretagogue receptor (GHSR). Recently, it was found that liver expressed antimicrobial peptide 2 (LEAP2) naturally counteracts the effects of the GHSR as an inverse agonist. This makes LEAP2 a potential candidate for the development of anti-obesity treatment.

This thesis explores the interaction between ghrelin and LEAP2 in the context of food intake regulation and obesity. Firstly, it focuses on modified N-terminal peptide LEAP2(1-14) and its lipidized analogs, examining their affinity to and activation of GHSR *in vitro* and *in vivo*. The results demonstrate that palmitoylated LEAP2(1-14) (palm-LEAP2(1-14)) exhibits the most pronounced affinity for GHSR, acts as GHSR inverse agonist, reduces food intake, inhibits growth hormone release, and shows increased stability in rat plasma. These findings suggest that palm-LEAP2(1-14) holds promise as an anti-obesity treatment.

Furthermore, the study investigates the impact of a high-fat (HF) diet on obesity and the development of ghrelin and LEAP2 resistance in mice. The results reveal that HF diet feeding decreases active and total plasma ghrelin, increases liver *LEAP2* mRNA expression, and leads to glucose intolerance. The switch to a standard diet normalizes liver *LEAP2* mRNA expression and active ghrelin levels but not total ghrelin. Furthermore, the study demonstrates resistance to palm-LEAP2(1-14) induced by the HF diet and also resistance to GHSR stable agonist [Dpr^3]Ghrelin, which is reversible upon switching to a standard diet.

Lastly, the potential of palm-LEAP2(1-14) to counteract the effects of a HF diet on body weight gain and normalize morphometric and metabolic parameters associated with obesity was evaluated. Palm-LEAP2(1-14) slightly reduced the body weight gain induced by HF diet feeding and decreased plasma leptin level. But overall, palm-LEAP2(1-14) was not able to suppress the effect of HF diet due to palm-LEAP2(1-14) resistance.

These findings advance our comprehension of obesity pathophysiology and indicate the necessity for additional investigation into alternative approaches to improve the efficiency of anti-obesity treatments that target the ghrelin and LEAP2 pathways.

1. INTRODUCTION

The increase in overweight and obese individuals is one of the most serious health problems in our society (Muller et al., 2022). Currently, the mostly recommended approach to treat and manage obesity is to modify lifestyle by diet and exercise (Wadden et al., 2012). However, not everyone is able to achieve significant weight loss through lifestyle changes and therefore pharmaceutical treatment is recommended in individuals, who are at risk of obesity comorbidities (Krentz et al., 2016). Since obesity is frequently caused by hyperphagia, there is a need to fully understand food intake regulation to treat this chronic disease.

The only known peripherally released orexigenic peptide hormone is ghrelin. Ghrelin positively regulates food intake, adiposity, and body weight (Tschöp et al., 2000). Ghrelin consists of 28 amino acids, and its Ser^3 is n-octanoylated by non-stable ester bond. This modification is essential for ghrelin biological activity (Sato et al., 2012). The octanoyl residue is post-translationally attached to the peptide by ghrelin O-acyl-transferase (GOAT). Ghrelin without the acyl group (des-acyl ghrelin) is biologically inactive (Kojima et al., 1999).

Ghrelin is expressed in stomach, but it acts directly in the hypothalamus through the growth hormone secretagogue receptor (GHSR) (Andrews, 2011; Kojima et al., 1999). GHSR is known to be a constitutively active G-protein-coupled receptor with intrinsic activity in a non-active state (Holst et al., 2004). Therefore, attention has turned to reducing the high constitutive activity of GHSR by the use of inverse agonists. Although several GHSR inverse agonists and antagonists have been shown to function *in vitro* and *in vivo*, no drug that reduces body weight by targeting GHSR has been developed yet (Schalla and Stengel, 2019).

Recently, liver-expressed antimicrobial peptide 2 (LEAP2) was identified as an endogenous inverse agonist of GHSR (Ge et al., 2018), which makes it potential candidate for anti-obesity drug development.

LEAP2 is a 40-residue cationic peptide (Krause et al., 2003). In the organism, the peptide is first synthesized as a 77-amino acid precursor that is subsequently processed into the mature peptide consisting of 40 amino acids. NMR-based structure analysis indicates that mature LEAP2 consists of an unstructured, hydrophobic N-terminal region and a compact central region containing two disulfide bridges linked in an I-III, II-IV pattern (Henriques et al., 2010). The part of LEAP2 structure responsible for binding to GHSR is located in the first 8 amino acids of the N-terminus, which contains hydrophobic amino acids Met^1, Pro^3, Phe^4, and Trp^5. However, to achieve maximal potency and effectiveness of the binding, a longer N-terminal sequence containing at least the first 12 amino acids is necessary (M'Kadmi et al., 2019).

The plasma levels of ghrelin and LEAP2 display opposite patterns during fasting and feeding/refeeding. In both humans and mice, plasma LEAP2 rises with higher levels of body weight, body fat, blood sugar, food intake, serum triglycerides (TAG), visceral adiposity, and

intrahepatic lipid content (Mani et al., 2019). Plasma LEAP2 decreases after a 24-hour fast and weight loss brought on by a vertical sleeve gastrectomy or a Roux-en-Y gastric bypass (Mani et al., 2019). Ghrelin levels follow an inverse trajectory; thus, the LEAP2-to-ghrelin ratio could be considered a marker of obesity (Mani et al., 2019).

Furthermore, peripherally injected ghrelin has no effect after acute administration to agouti mice (Martin et al., 2004), or diet-induced obese (DIO) mice (Perreault et al., 2004). Resistance to ghrelin is observed also in DIO mice after its chronic administration (Gardiner et al., 2010). A low-calorie diet helps obese people to lose weight and restores ghrelin sensitivity, but a following increase of ghrelin plasma levels in the blood promotes weight gain after ending the diet (Briggs et al., 2013). It was suggested that ghrelin resistance is a mechanism created to maintain a greater body weight set point during periods of food availability, maximizing energy reserves during periods of food scarcity (Zigman et al., 2016). This could be the cause of limited efficacy of ghrelin antagonists in the treatment of obesity to date (Vodnik et al., 2016).

Although ghrelin resistance occurs in obese individuals, targeting GHSR could still be a way to develop an anti-obesity drug using LEAP2 analogs. LEAP2 analogs might be able to stop rebound weight gain after switching to a low-calorie diet (Andrews, 2019). In less severe states of obesity, LEAP2 does not show a compensatory effect, thus further increasing LEAP2 may decrease food intake and body weight gain (Mani et al., 2019). Additionally, people who have lost weight through lifestyle changes are vulnerable to regaining it due to an increase in plasma ghrelin, so LEAP2 therapies may be helpful (Gupta et al., 2021).

Peptides are increasingly being recognized as potential therapeutics due to their beneficial properties such as high affinity to natural receptors and low toxicity for the organism. However, their low stability and short half-life present significant challenges (Goodwin et al., 2012). To maximize their pharmacological potential, it is important to find effective ways to modify peptides to make their syntheses feasible and preserve their biological activity and increase their stability.

Lipidization of peptides is a potential tool to increase stability and overcome the inability to cross the blood-brain barrier (Zhang and Bulaj, 2012). Diverse fatty acids are covalently attached to the peptide/protein. The most commonly used fatty acids are: octanoic acid (C8), myristic acid (C14), palmitic acid (C16), and stearic acid (C18) (Maletinska et al., 2015).

The biological stability of ghrelin is highly limited, because of its ester bond in Ser^3-O-octanoyl, which is an easy target for hydrolysis (Holubova et al., 2018). Octanoyl-ghrelin hydrolysis can be overcome by replacing Ser^3 with diaminopropionic acid (Dpr). Octanoic acid, which is essential for the biological activity of the peptide, is anchored to the [Dpr^3]Ghrelin chain by stable amid bond (Bednarek et al., 2000; Maletinska et al., 2012).

Synthesis of natural LEAP2 is extremely difficult because of its two S-S bonds between cysteines. However, the entire sequence of LEAP2 is not required for its effects. Specifically, the N-terminal part 1-12 is responsible for binding to the receptor and exerting its activity. M'Kadmi et al. proved, that the N-terminal segment of LEAP2 acts as an inverse agonist and competitive antagonist of GHSR *in vitro* (M'Kadmi et al., 2019). Moreover, the N-terminal part of LEAP2 can effectively reduce ghrelin-induced food intake in mice (M'Kadmi et al., 2019), which makes it potential candidate for anti-obesity drug development.

2. AIMS OF THE THESIS

- **Investigation of *in vitro* characteristics of LEAP2(1-14) and its lipidized analogs compared to natural LEAP2.**

LEAP2 is a potent inverse agonist of GHSR. Its N-terminal LEAP2(1-14) analog was modified with various fatty acids because N-terminal fragment LEAP2 is crucial for LEAP2 biological activity. The first aim of the thesis was to select the most stable and bioavailable lipidized analog of LEAP2(1-14) by analyzing affinity and inverse agonist and antagonist activity at GHSR overexpressed in U2OS cell line.

- **Study of LEAP2(1-14) analogs in short-term *in vivo* experiments.**

The second aim was to study the ability of lipidized and non-lipidized LEAP2(1-14) analog to suppress the orexigenic actions of high endogenous ghrelin in fasted mice as well as of exogenous stable ghrelin analog [Dpr^3]Ghrelin in free-fed mice.

- **Research of the long-term effect of high-fat diet on progress of obesity regarding the development of ghrelin and LEAP2 resistance.**

The third aim was to study the long-term effects of high-fat diet on the interplay of ghrelin and LEAP2 in mice and the development of obesity and ghrelin and LEAP2 resistance.

- **Observation of palm-LEAP2(1-14) ability to suppress effect of high-fat diet.**

The fourth aim was to find out if palm-LEAP2(1-14) has the potential to minimize the effects of high-fat diet and normalize morphometric and metabolic parameters which are associated with obesity.

3. METHODS

Ghrelin and its stable analog [Dpr^3]Ghrelin were synthesized using solid-phase peptide synthesis at the Institute of Organic Chemistry and Biochemistry as previously described (Maixnerova et al., 2007). LEAP2(1-14) and its lipidized analogs were synthesized at the Institut des Boimolécules Max Mousseron, University of Montpellier by solid-phase peptide synthesis as previously described (Hola et al., 2022). LEAP-2(38-77) (#075-40) was purchased from Pheonix Pharmaceuticals (Burlingame, CA USA).

3.1. *In vitro* experiments

T-REx™ Tango™ GHSR-bla U2OS cells, which overexpress GHSR and have a β-lactamase reporter gene controlled by an upstream activation site response element, were provided by Thermo Fisher Scientific Inc. (Waltham, MA, USA). The cells were grown in a humidified incubator at 37°C with 5% CO_2 in media prepared according to manufacturer's protocol.

Competitive binding and saturation experiments were performed according to Motulsky and Neubig (Motulsky and Neubig, 2002). Cells were seeded in 24-well plates at a density of 20,000 cells/well and allowed to grow for 3 days. Doxycycline was added 16 hours before the experiment at a final dosage of 1.25 ng/mL. Saturation experiments were carried out with increasing concentration of ^{125}I-ghrelin from 0.05 to 2.5 nM and non-labeled ghrelin at concentration 10^{-5} M. Competitive binding experiments were conducted with 0.1 nM ^{125}I-ghrelin and peptide analogs at final concentrations from 10^{-12} to 10^{-5} M.

The agonist, inverse agonist and antagonist assays were performed according to Thermo Fisher's protocol and according to our previous study (Holubova et al., 2018). Agonists and inverse agonists of GHSR were tested at the final concentration from 10^{-12} to 10^{-5} M of peptides. Antagonist assay mode was performed with LEAP2 analogs at the final concentration from 10^{-8} to 10^{-6} M and ghrelin at final concentrations ranging from 10^{-12} to 10^{-5} M.

3.2. *In vivo* experiments

Male C57Bl/6J (for short-term experiments) or C57Bl/6N (for long term experiments) mice (Charles River, Sulzfeld, Germany) were housed in the animal facility under standard conditions at temperature of 23 °C and a daily cycle of 12 h light and dark (light from 6:00 AM). Mice had free access to water and either standard (St) diet (ssniff R/M-H cat. no. V1534; Spezialdiäten GmbH, Soest, Germany), which contained 8% kcal from fat, 21% kcal from protein, 71% kcal from carbohydrates or high fat (HF) diet, which contained 60% kcal from fats, 13% kcal from proteins and 27% kcal from carbohydrates (Maletinska et al., 2015).

3.2.1. Acute food intake after administration of LEAP2(1-14) analogs to mice

12-week-old mice (n = 5) were isolated in individual cages and provided with St diet and water *ad libitum*. The anorexigenic effect of LEAP2 analogs was tested in overnight fasted (17 h) mice. Mice were SC injected with either saline, which was used as a control, or LEAP2 analog, which was dissolved in saline to a final dose of 5 mg/kg of body weight. Mice were given pre-weighted pellets 30 minutes after the beginning of the experiment. Food intake was monitored every 30 min for at least 7 hours.

The ability of LEAP2 analogs to suppress the orexigenic effect of [Dpr^3]Ghrelin was tested in free-fed mice. Mice were SC injected with either saline or LEAP2 analog, which was dissolved in saline to a final dose of 5 mg/kg of body weight. 15 minutes after the first injection, mice were SC injected with saline or [Dpr^3]Ghrelin at a dose of 1 mg/kg of body

weight. Mice were given pre-weighted pellets 30 minutes after the beginning of the experiment. Food intake was monitored every 30 min for at least 7 hours.

3.2.2. GH release after administration of [Dpr^3]Ghrelin and LEAP2(1-14) analogs to mice

8-week-old mice (n = 5-8) were placed in individual cages and provided with St diet and water *ad libitum*. Mice were SC injected with either saline or LEAP2 analog which was dissolved in saline to a final dose of 10 mg/kg of body weight. 15 minutes after the first injection, mice were SC injected with saline or [Dpr^3]Ghrelin at a dose of 1 mg/kg of body weight. Mice were sacrificed by decapitation 30 minutes after the beginning of the experiment. The concentration of GH in plasma samples was measured in plasma using a kit for rat/mouse GH enzyme-linked immunosorbent assay (ELISA) (#EZRMGH-45K).

3.2.3. Experimental designs of long-term *in vivo* experiments

3.2.3.1. Long-term *in vivo* study 1: Progress of obesity at HF diet feeding

Mice were separated into 12 groups (n = 8) when they reached 8 weeks of age. Body weights were monitored every week, and mice were sacrificed at various time points (Figure 1). On the day of sacrificing, free-fed mice were anesthetized with pentobarbital. Blood from heart was collected in tubes with EDTA and plasma was separated and stored at -20°C. Mice were then perfused with heparinized saline (at a concentration of 20 U/ml) and dissected. Epididymal white adipose tissue (eWAT) was weighted. Levels of active (#EZRGRA-90K) and total (#EZRGRT-90K) ghrelin and leptin (#EZML-82K) were measured in plasma by ELISA according to manufacturer's protocol and mRNA analyses of tissues were performed as described previously (Maletinska et al., 2015).

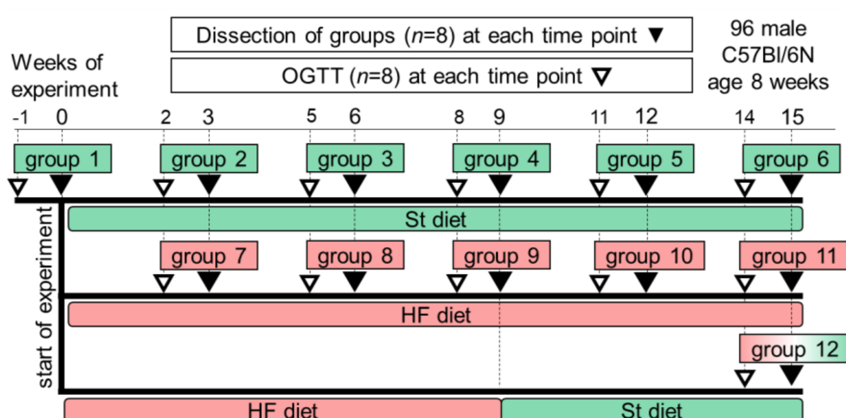

Figure 1: Long-term *in vivo* study 1: Mice were separated into 12 groups. Group 1 was sacrificed at the beginning of the experiment. Every 3 weeks, 1 group fed a St diet (2-6) and one group fed a HF diet (7-11) were sacrificed until week 15. The last group (12) was fed HF diet for the first 9 weeks, then switched to St diet for the following 6 weeks before being sacrificed at week 15.

3.2.3.2. Long-term *in vivo* study 2: Long-term effect of HF diet on development of [Dpr^3]Ghrelin and palm-LEAP2(1-14) resistance

Mice were separated into 5 groups (n = 8) (Figure 2) and housed in separate cages when they reached 8 weeks of age. Two groups were provided with a St diet, while three groups were given a HF diet from the age of 8 weeks. The sensitivity to [Dpr^3]Ghrelin and palm-LEAP2(1-

14) was evaluated in mice that were allowed to feed freely at weeks 0, 2, 4, 8, 10, and 12 of the experiment. At week 8, groups fed with HF diet were switched to a St diet. The food intake experiment was performed similarly to part 3.2.3.1.

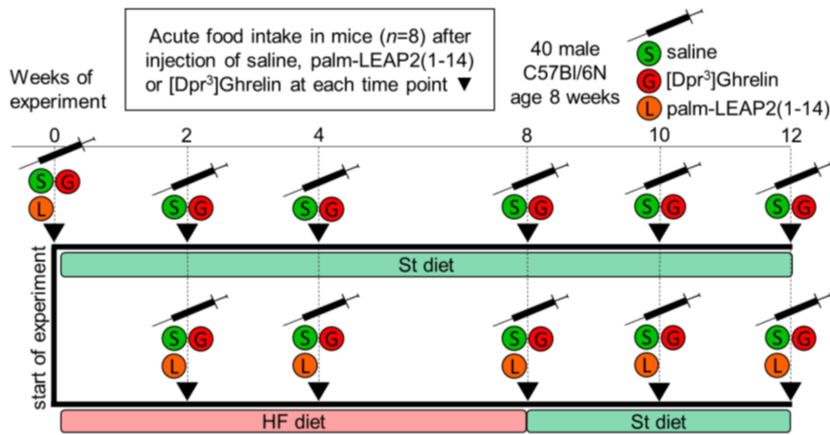

Figure 2: Long-term *in vivo* study 2: Mice were separated into 5 groups. 2 groups were given St diet, while the remaining 3 groups were given HF diet. In the 8th week of the experiment, groups fed with HF diet were switched to St diet. Amount of food consumed was recorded after SC administration of either a saline injection, palm-LEAP2(1-14), or [Dpr^{3}]Ghrelin injection.

3.2.3.3. Long-term *in vivo* study 3: Ability of palm-LEAP2(1-14) to suppress effect of HF diet

Mice were separated into 4 groups (n = 10) and housed in separate cages when they reached 8 weeks of age (Figure 3). One group was provided with St diet, while three groups were given HF diet from the age of 8 weeks. Mice were injected daily with saline, LEAP2(1-14) or palm-LEAP2(1-14), which were dissolved in saline to a final dose of 10 mg/kg of body weight. The experiment was ended similarly as in the part 3.3.1 after 6 weeks of HF diet feeding and interventions.

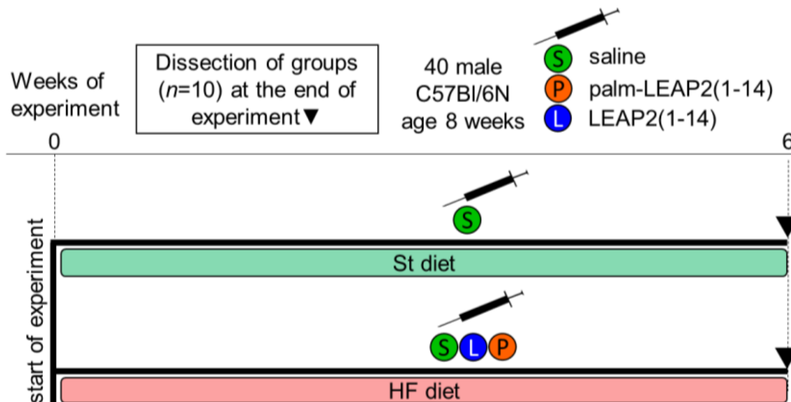

Figure 3: Long-term *in vivo* study 3: 40 male C57Bl/6N mice were separated into 4 groups, one group was given St diet, while the remaining three groups were given a HF diet. Mice were injected daily with saline, LEAP2(1-14) or palm-LEAP2(1-14), which were dissolved in saline to a final dose of 10 mg/kg of body weight. Mice were sacrificed at the week 6 of the experiment.

4. RESULTS

4.1. *In vitro* testing of LEAP2(1-14) analogs

We designed a series of new truncated LEAP2 analogs to choose the most stable and bioavailable inverse agonist of GHSR. Biologically active N-terminal LEAP2 analog, LEAP2(1-14), was used and lipidized with different fatty acid residues (myristoyl, palmitoyl or stearoyl) at its C-terminus. Here, we compare *in vitro* properties of LEAP2(1-14) and palmitoylated analog palm-LEAP2(1-14) together with ghrelin, stable ghrelin analog

[Dpr³]Ghrelin and natural LEAP2 (Figure 4). In a competitive binding experiment, ghrelin and LEAP2 analogs competed with ^{125}I-ghrelin for binding to GHSR on U2OS cells with K_i falling within the 10^{-8} M range. The affinity of LEAP2(1-14) for GHSR was increased by palmitoylation.

	Competitive binding assay K_i [nM]	GHSR agonist assay EC_{50}[nM]	GHSR inverse agonist assay EC_{50}[nM]
Ghrelin: G S S(octanoyl) F L S P E H Q R V Q Q R K E S K K P P A K L Q P R	3.35 ± 0.35	3.10 ± 0.47	-
[Dpr³]ghrelin: G S Dpr(octanoyl) F L S P E H Q R V Q Q R K E S K K P P A K L Q P R	6.63 ± 0.42	5.28 ± 0.63	-
LEAP2: M T P F W R G V S L R P I G A S C R D D S E C I T R L C R K R C S L S V A Q E	29.97 ± 1.59	-	46.46 ± 5.38
LEAP2 (1-14): Nle T P F W R G V S L R P I G βA K–NH₂	11.01 ± 0.96	-	152.54 ± 19.24
palm-LEAP2 (1-14): Nle T P F W R G V S L R P I G βA K(palmitoyl)–NH₂	1.91 ± 0.09	-	52.43 ± 7.95

Figure 4: Binding affinities and agonist and inverse agonist effect of ghrelin and LEAP2 analogs to GHSR. Data are presented as the mean ± SEM. K_i values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Data analyzed in Graph-Pad Software were performed in 3–5 independent experiments in duplicates.

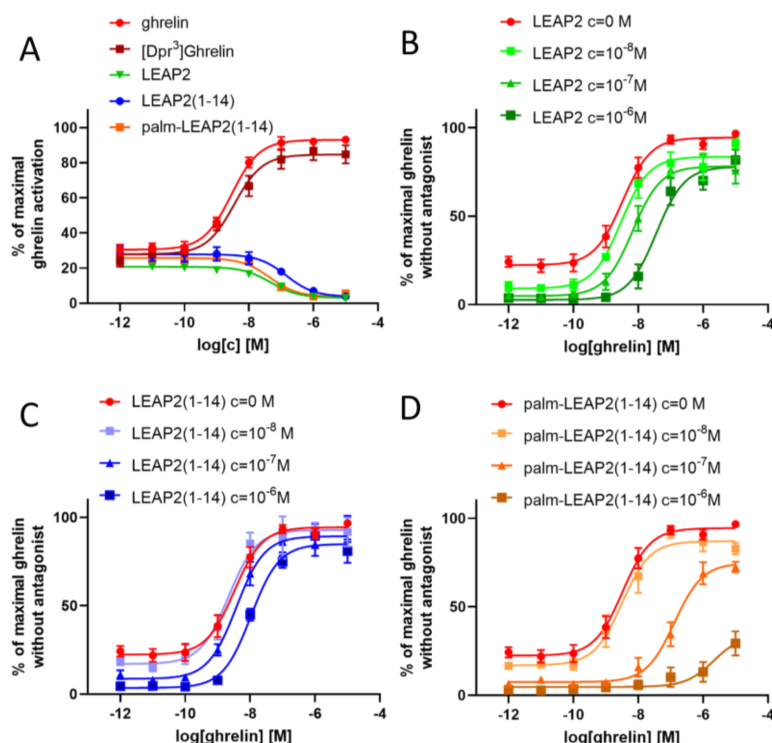

Figure 5: Inverse agonist and antagonist properties of LEAP2(1-14) analogs. Effect of LEAP2 compounds on GHSR activation in inverse agonist mode assay (A). Effect of LEAP2 (B), LEAP2(1-14) (C), and palm-LEAP2(1-14) (D) on GHSR activation in antagonist mode assay. The maximum ghrelin-induced GHSR activation in U2OS cells overexpressing GHSR was set as the standard value of 100%. Data are presented as the mean ± SEM. The experiments were performed in duplicates and repeated at least three times and analyzed using non-linear regression.

A T-REx™ Tango™ GHSR-bla U2OS Cell-based Assay was used to detect the activation of GHSR. Both ghrelin and [Dpr³]Ghrelin were strong GHSR agonists and activated GHSR at nanomolar EC_{50} levels (Figure 4 and 5). On the other hand, natural LEAP2 and palm-

LEAP2(1-14) analogs showed a strong inverse agonist and antagonist activity and suppressed the constitutive activity of GHSR with EC_{50} levels in the 10^{-8} M range. LEAP2(1-14) is a weaker GHSR inverse agonist and antagonist with an EC_{50} value, three times higher than the palm-LEAP2(1-14) or natural LEAP2.

4.2. Short-term *in vivo* testing of LEAP2(1-14) analogs

The impact of LEAP2(1-14) and its lipidized analogs on the orexigenic effect of [Dpr³]Ghrelin was evaluated in free-fed mice (Figure 6A-B). All the peptides caused a significant decrease in [Dpr³]Ghrelin-induced food intake. Moreover, palm-LEAP2(1-14) completely suppressed the orexigenic action of [Dpr³]Ghrelin and reduced food intake even below the food intake of saline-treated mice.

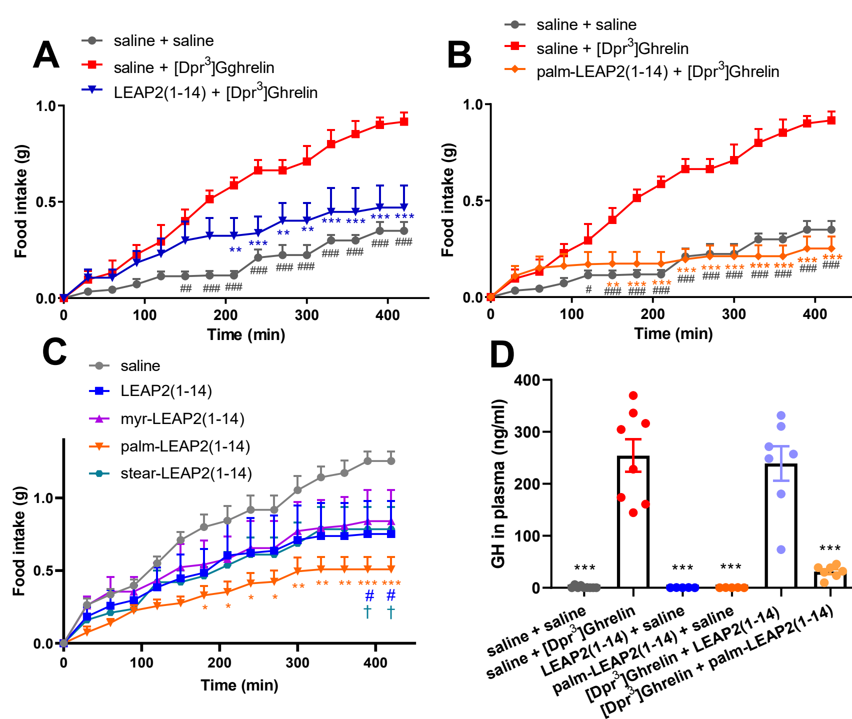

Figure 4: Effect of (A) LEAP2(1-14) and (B) palm-LEAP2(1-14) on [Dpr³]Ghrelin-induced cumulative food intake after SC administration to free fed mice; cumulative food intake after SC administration of LEAP2(1-14) analogs to fasted mice (C); and effect of LEAP2(1-14) analogs on [Dpr³]Ghrelin-induced GH release in 8-weeks-old mice. (**A, B, C**) LEAP2(1-14) and palm-LEAP2(1-14) were SC injected at a dose of 5 mg/kg, [Dpr³]Ghrelin at a dose of 1 mg/kg of body weight. The food intake was monitored every 30 minutes for 7 hours. The data were analyzed using 2-way ANOVA followed by Bonferroni post hoc test. (**A, B**) The significance levels are *P < 0.05; **P < 0.01; ***P < 0.001, LEAP2(1-14) analog vs [Dpr³]Ghrelin-treated group, #P<0.05, ##P<0.01, ###P<0.001 saline vs [Dpr³]Ghrelin-treated group. (**C**) *P<0.05; **P<0.01; ***P<0.001 palm-LEAP2(1-14) vs saline-treated group, #P<0.05, ##P<0.01, ###P<0.001 LEAP2(1-14) vs saline-treated group; †P<0.05, ††P<0.01, †††P<0.001 stear-LEAP2(1-14) vs saline-treated group. (**D**) Mice were SC injected with saline or LEAP2(1-14) analog at a dose of 10 mg/kg of body weight. 15 minutes after injection, mice were SC injected with saline or [Dpr³]Ghrelin at a dose of 1 mg/kg of body weight. Blood was collected 30 minutes after beginning of the experiment. The concentration of GH in plasma samples was measured using a commercially available ELISA kit. The data were analyzed using 1-way ANOVA followed by Bonferroni post hoc test. The results are expressed as means ± SEM. The significance levels are *P<0.05; **P<0.01; ***P<0.001, compared to the saline + [Dpr³]Ghrelin treated group, (n = 5-8).

The anorexigenic effect of LEAP2(1-14) analogs was tested in overnight fasted mice (Figure 6C). Palm-LEAP2(1-14) strongly reduced the cumulative food intake in fasted mice. However, non-lipidized LEAP2(1-14) showed only a weak anorexigenic effect.

The effect of LEAP2(1-14) and palm-LEAP2(1-14) on GH release was tested in young 8-week-old mice (Figure 6D). SC injection of neither LEAP2(1-14) nor palm-LEAP2(1-14) affected the plasma level of GH. Since the level of GH in plasma is naturally low, we tested the ability of LEAP2(1-14) analogs to inhibit GH release induced by [Dpr^3]Ghrelin. It was found that the palm-LEAP2(1-14) significantly reduced the release of GH induced by [Dpr^3]Ghrelin. However, this effect was not observed when the non-lipidized form of LEAP2(1-14) was administered.

4.3. Progress of obesity at HF diet feeding

Feeding mice with a HF diet resulted in a continuous increase in both body weight and eWAT weight (Figure 7) compared to a St diet. These differences became noticeable as early as 3 weeks after beginning of the HF diet feeding. Significant decrease in body weight was observed in mice that were switched to a St diet after 9 weeks on a HF diet already after 2 weeks of St diet feeding and their final body weight was comparable to the control group fed a St diet for 15 weeks. 6 weeks of St diet feeding after 9 weeks of HF diet feeding did not result in the complete reduction of eWAT, however the eWAT weight was significantly lower than in the group fed exclusively a HF diet for 15 weeks.

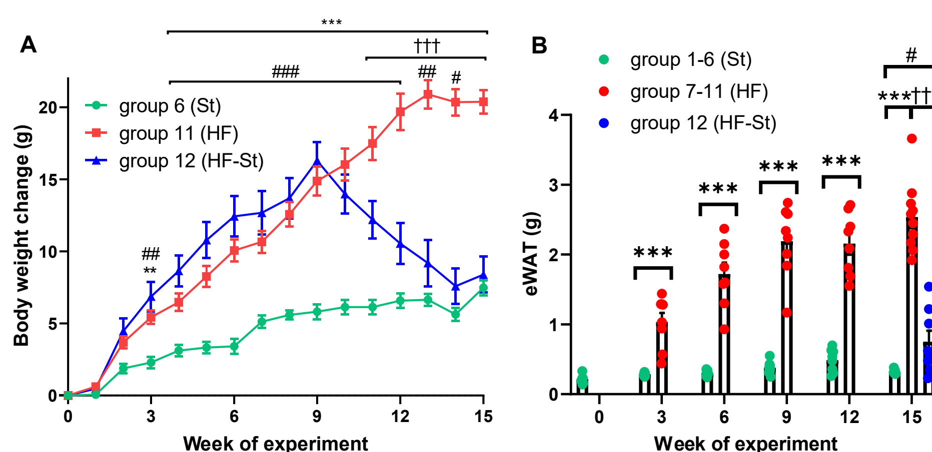

Figure 5: Effect of HF diet on body weight (A) and eWAT weight (B) in mice. Data are presented as mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with Bonferroni's post hoc test (A) and multiple t-tests with Bonferroni-Dunn's method for multiple comparisons. Significance is *P<0.05, **P<0.01, ***P<0.001 HF vs St; #P<0.05, ##P<0.01, ###P<0.001 HF-St vs St; †P<0.05, ††P<0.01, †††P<0.001 HF-St vs HF (n=8).

The levels of leptin in the blood (Figure 8A) had a similar trend in both the body weight and the amount of eWAT in all the groups. Similarly to leptin, *LEAP2* mRNA expressed in the liver (Figure 8B) increased with increasing adipose tissue in the body. The group that was given a HF diet for 9 weeks and then switched to a St diet for 6 weeks showed levels of leptin in their

blood and *LEAP2* mRNA in their liver that were similar to the group that was only given a St diet for 15 weeks. Active (Figure 8C) and total ghrelin (Figure 8D) levels in plasma had the opposite trend than leptin in plasma and *LEAP2* mRNA in the liver. Mice that were given a HF diet had lower levels of both active and total ghrelin compared to those that were given a St diet. Mice that were fed HF diet for 9 weeks and St diet for 6 weeks had higher active ghrelin level than those fed HF diet; their active ghrelin was similar to the level found in the group that was only given a St diet for 15 weeks. It is interesting that the mice that were given a HF diet for 9 weeks and then switched to a St diet for 6 weeks had the same total ghrelin levels as the mice that were only given the HF diet for 15 weeks.

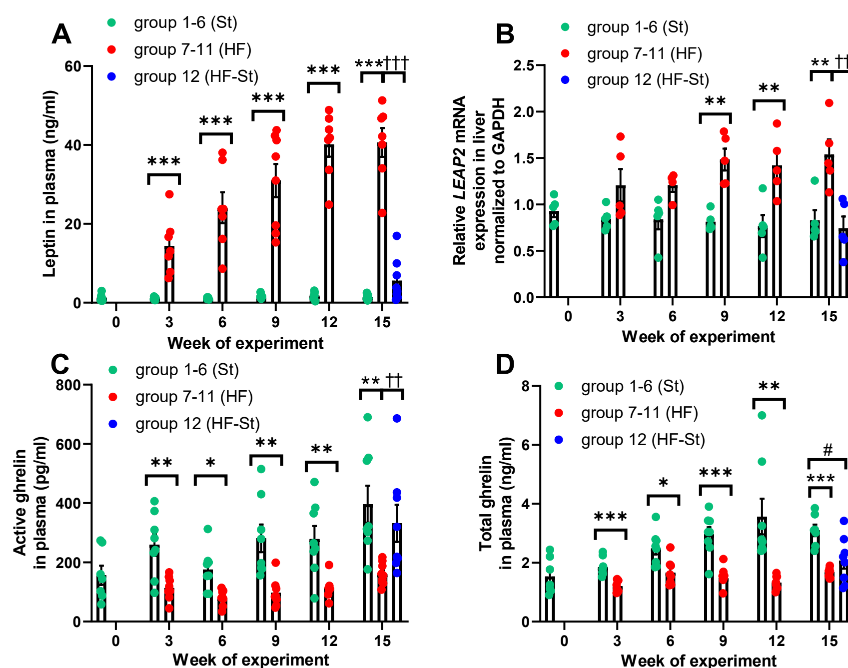

Figure 6: Effect of HF diet on the level of leptin in plasma (A), *LEAP2* mRNA in the liver (B), and active (C) and total (D) ghrelin in plasma. Data are presented as mean ± SEM. Statistical analysis was performed using the multiple *t*-test with Bonferroni-Dunn's method for multiple comparisons. Significance is **P*<0.05, ***P*<0.01, ****P*<0.001 HF vs St; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 HF-St vs St; †*P*<0.05, ††*P*<0.01, †††*P*<0.001 HF-St vs HF (*n*=8).

To observe the development of LEAP2 resistance, mice were fed HF diet and SC injected with either [Dpr^3]Ghrelin or palm-LEAP2(1-14) (Figure 9). Mice fed a St diet were used as the control group. Just two weeks after being fed a HF diet, mice had already developed a resistance to the acute effects of ghrelin, meaning that [Dpr^3]Ghrelin was no longer able to increase their food intake. Additionally, food intake had decreased below the baseline level due to the administration of palm-LEAP2(1-14). After being fed a HF diet for 4 weeks, the mice developed a resistance to the effects of palm-LEAP2(1-14), which was no longer able to decrease their basal level of food intake. However, after switching to a St diet for 4 weeks, their sensitivity to [Dpr^3]Ghrelin had been restored, while their sensitivity to palm-LEAP2(1-14) remained unchanged.

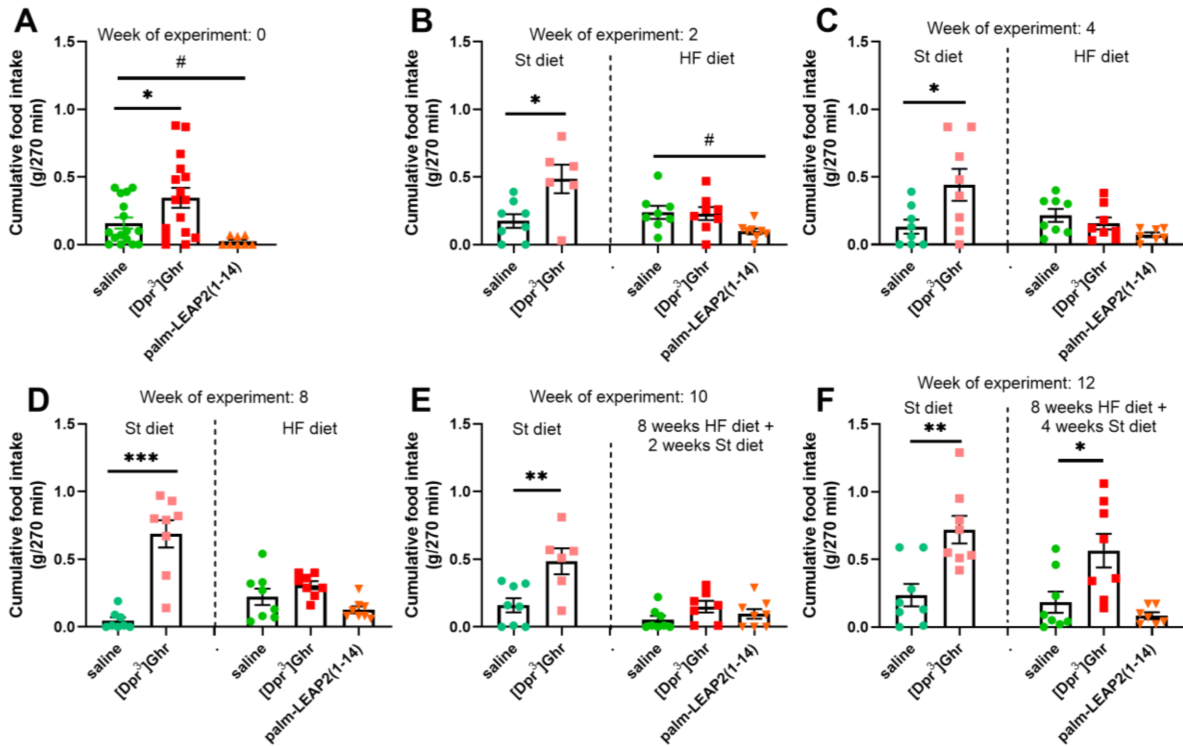

Figure 7: Cumulative food intake 270 minutes after SC [Dpr³]Ghrelin (1mg/kg) or palm-LEAP2(1-14) (5 mg/kg) administration in mice fed HF diet or St diet for 0 (A), 2 (B), 4 (C), and 8 (D) weeks followed by a St diet only for a further 2 (E) and 4 (F) weeks. Data are presented as mean ± SEM. Statistical analysis was performed by t-test. Significance is *P<0.05, **P<0.01, ***P<0.001 [Dpr³]Ghrelin vs saline; #P<0.05, ##P<0.01, ###P<0.001 palm-LEAP2(1-14) vs saline (n=6-8).

4.4. Ability of palm-LEAP2(1-14) to prevent development of HF diet-induced obesity

Feeding mice with a HF diet resulted in a continuous increase in both body weight and eWAT weight during the experiment (Figure 10) compared to a St diet. Non lipidized LEAP2(1-14) intervention did not have any effect on body weight nor eWAT weight, however long-term administration of palm-LEAP2(1-14) tended to slightly reduce body weight gain induced by a HF diet.

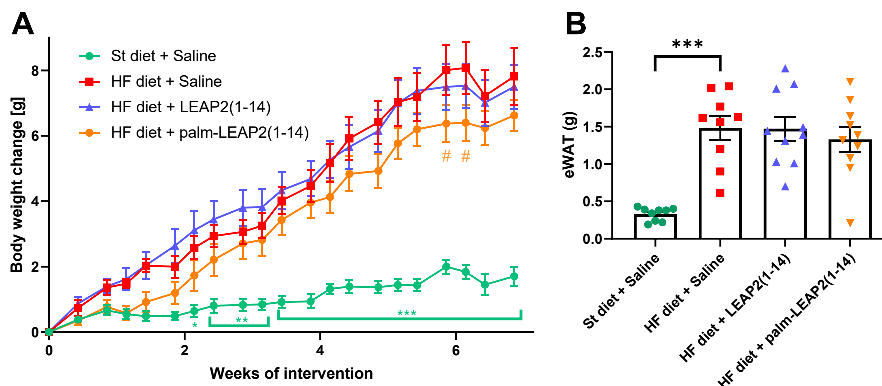

Figure 8: Effect of chronic palm-LEAP2(1-14) on body weight (A) and eWAT weight (B) in mice. Data are presented as mean ± SEM. Statistical analysis was conducted using 2-way ANOVA (A) and 1-way ANOVA (B) with Bonferroni's post hoc test. Significance is *P<0.05, **P<0.01, ***P<0.001 St diet + saline vs HF diet + saline; #P<0.05, ##P<0.01, ###P<0.001 HF diet + palm-LEAP2(1-14) vs HF diet + saline (n=10).

No differences were observed in either total or active ghrelin between groups fed the HF or St diet nor after the LEAP2 analogs interventions (Figure 11 A, B). Six weeks of HF diet induced an increase in leptin (Figure 11C) and LEAP2 (Figure 11D) levels in plasma. LEAP2(1-14) interventions did not have any effect on plasma levels of leptin nor LEAP2. Long-term administration of palm-LEAP2 significantly decreased plasma leptin in HF diet fed mice.

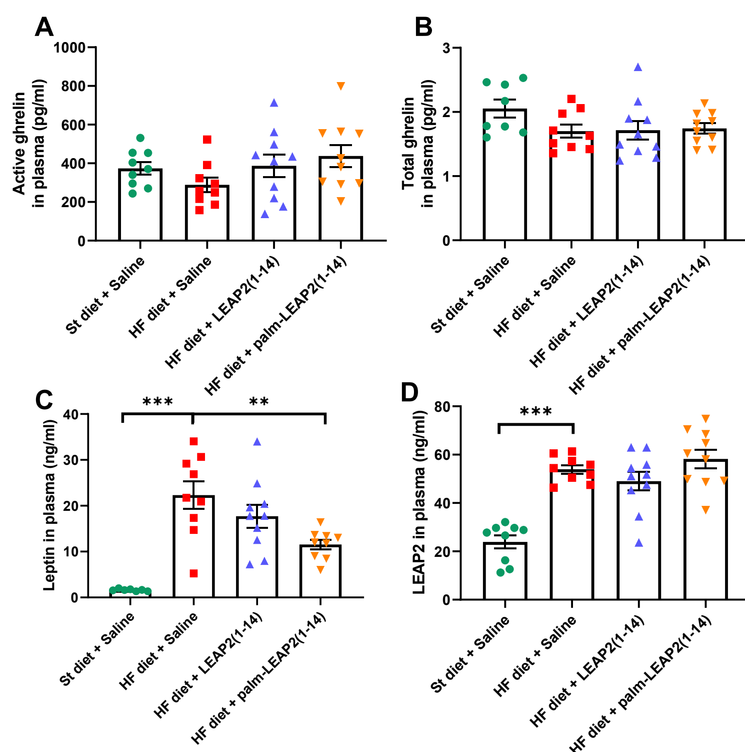

Figure 9: Effect of chronic palm-LEAP2(1-14) on active ghrelin (A), total ghrelin (B), leptin (C), and LEAP2 (D) in plasma. Data are presented as mean ± SEM. Statistical analysis was performed using the 1-way ANOVA with Bonferroni's post hoc test for multiple comparisons. Significance is *P<0.05, **P<0.01, ***P<0.001 vs HF diet + Saline (n=10).

5. DISCUSSION

5.1. *In vitro* testing of LEAP2(1-14) analogs

M'Kadmi recently proved that N-terminal sequence that contains at least the first 12 amino acids is necessary for full GHSR binding and activation (M'Kadmi et al., 2019). We verified that truncation of LEAP2 to its N-terminal fragment LEAP2(1-14) does not impair its ability to bind to GHSR compared to native LEAP2. We proved that the lipidization did not impair ability of LEAP2(1-14) to bind to the overexpressed GHSR in U2OS cells. LEAP2(1-14) and palm-LEAP2(1-14) displayed K_i values in the 10^{-9}-10^{-8} M range that were equivalent to those of natural LEAP2. The highest affinity for GHSR was obtained with the palm-LEAP2(1-14).

With an EC_{50} of roughly 10^{-8}-10^{-7} M, both tested analogs reduced GHSR constitutive activity, which proves that they are inverse agonists of GHSR. This finding is consistent with the work of M'Kadmi et al., who disproved Ge's claim that LEAP2 does not have inverse agonist activity (Ge et al., 2018). The activity of LEAP2(1-14) on the GHSR receptor was slightly lower than that of the natural LEAP2. However, lipidization increased the inverse agonist activity of LEAP2(1-14) analogs at GHSR with EC_{50} comparable to that of natural LEAP2.

In antagonist assay, EC_{50} of ghrelin increased together with higher concentration of LEAP2 analogs without changing the maximal effect induced by ghrelin. This indicates that LEAP2 acts as a competitive antagonist and shares a common ligand-binding pocket with ghrelin at GHSR which is in accordance with M'Kadmi's and Wang's studies (M'Kadmi et al., 2019; Wang et al., 2019), but contradicts the study of Ge (Ge et al., 2018). Similarly to the inverse agonist assay, LEAP2(1-14) exhibited diminished antagonist activity in comparison to natural LEAP2, but palm-LEAP2(1-14) demonstrated even higher levels of antagonist activity compared to natural LEAP2.

5.2. Short-term *in vivo* testing of LEAP2(1-14) analogs

The blood levels of ghrelin and LEAP2 have opposite trend in fasting and feeding in mice and humans. Ghrelin levels are higher and LEAP2 levels are lower in fasting individuals (Mani et al., 2019). Palm-LEAP2(1-14) had the highest anorexigenic activity in fasted mice. LEAP2(1-14) showed a slight reduction in food intake, which was already proved in previous studies, where N-terminal LEAP2 analog decreased food intake in fasted mice similarly to natural LEAP2 (M'Kadmi et al., 2019, Fernandez et al., 2022). LEAP2(1-14) partially attenuated [Dpr^3]Ghrelin-induced food intake in free-fed mice similarly as described by M'Kadmi et al. (M'Kadmi et al., 2019). Palm-LEAP2(1-14) was able to completely attenuate [Dpr^3]Ghrelin-induced food intake in free-fed mice.

The stimulation of the GHSR by ghrelin in pituitary cells was shown to trigger significant GH secretion (Kojima et al., 1999) whereas natural LEAP2 was found to suppress ghrelin-induced GH secretion in mice (Islam et al., 2020, Ge et al., 2018). While palm-LEAP2(1-14) suppressed [Dpr^3]Ghrelin-induced GH release, LEAP2(1-14) had no effect on it. Reduced ability of LEAP2(1-14) to inhibit [Dpr^3]Ghrelin-induced GH release could potentially be attributed to its lower stability or bioavailability in organisms compared to palm-LEAP2(1-14).

5.3. Progress of obesity at HF diet feeding

In order to be able to monitor the long-term effect of LEAP2 analogs on obese individuals, we first wanted to examine the interplay between endogenous LEAP2 and ghrelin at obesity progression. The increase in body weight resulting from the consumption of a HF diet in our study was accompanied by elevated plasma leptin and increased production of *LEAP2* mRNA in the liver and a decrease in active plasma ghrelin. Similarly, after the switch to a St diet, there was a corresponding rise in active ghrelin levels and a decrease in LEAP2 mRNA expression, accompanied by a restoration of body weight. This is consistent with numerous studies that have shown that increased body weight, adiposity, and plasma leptin after HF diet feeding of mice and rats (Enriori et al., 2007, Lin et al., 2000, Papathanassoglou et al., 2006, de Git et al., 2018, Williams et al., 2014, Varghese et al., 2020). Obesity decreases ghrelin levels in blood (Cummings et al., 2001, Tschop et al., 2001). Briggs et al. proved that switching the HF diet to

the control diet after 12 weeks of HF diet feeding re-increased level of active ghrelin but did not normalize the total ghrelin level (Briggs et al., 2014). In our experiment, we observed that already 3 weeks of HF diet resulted in decreased levels of active and total ghrelin. After switching the HF diet for St diet, only active ghrelin levels were restored, as in the previous research (Briggs et al., 2014). We believe that an increase in active ghrelin – rather than total ghrelin – is considerably more significant for ghrelin sensitivity.

The plasma LEAP2 is elevated in obese mice and humans (Andrews, 2019) and so is liver *LEAP2* mRNA expression in liver in DIO mice (Holm et al., 2022). On the other hand, plasma LEAP2 is decreased in diet-induced weight loss (Mani et al., 2019). After three weeks of consuming HF diet, we observed a slight rise in liver *LEAP2* mRNA levels in mice. *LEAP2* mRNA expression in liver significantly increased after 9 weeks of HF diet feeding and it decreased after switching from HF diet to a St diet to the level seen in animals fed a St diet exclusively. We are the first who compared a time course of *LEAP2* mRNA expression in the livers of mice fed a HF diet, a St diet, or a diet that alternates between the two.

In previous studies, it was shown that ghrelin resistance developed after 3-4 weeks of HF diet feeding (Briggs et al., 2014, Naznin et al., 2015) and that both peripheral and central administration of ghrelin to DIO mice did not induce food intake (Perreault et al., 2004, Briggs et al., 2010, Gardiner et al., 2010). However, the ghrelin sensitivity could be restored after the diet-induced weight loss (Briggs et al., 2013). To determine whether obese mice would respond to palm-LEAP2(1-14) by reducing food intake, we acutely SC administered palm-LEAP2(1-14) to mice fed a HF diet at several time points of HF diet feeding. Our findings indicate that mice develop [Dpr^3]Ghrelin resistance as early as after 2 weeks feeding a HF diet, while resistance to palm-LEAP2(1-14) develops after 4 weeks. Unlike palm-LEAP2(1-14) resistance, [Dpr^3]Ghrelin resistance was reversible and it was restored 4 weeks after the switching HF diet to St diet.

5.4. Ability of palm-LEAP2(1-14) to prevent development of HF diet-induced obesity

We decided to find out if the chronic administration of palm-LEAP2(1-14) can suppress the effect of the HF diet on mouse metabolism before the onset of palm-LEAP2(1-14) resistance. Palm-LEAP2(1-14) was SC administered to mice from the first day of HF diet feeding. Its effect was compared with non-lipidized analog LEAP2(1-14).

The non-lipidized analog had no effect on the metabolomic and morphometric parameters affected by the HF diet. Palm-LEAP2(1-14) tended to decrease body weight gain induced by HF diet feeding, but the difference was mostly not significant. A significant difference between the LEAP2-treated group and the control group was observed only in reduced plasma leptin levels. Other parameters, such as active and total ghrelin and LEAP2 in plasma did not change after chronic administration of palm-LEAP2(1-14).

The use of other GHSR inverse agonists than LEAP2 and its analogs could help to discover the mechanism of LEAP2 resistance. Small molecule GHSR inverse agonist GHSRIA2 induced decrease in body weight and visceral fat in DIO mice (Abegg et al., 2017). This would suggest that LEAP2 resistance occurs upstream of LEAP2 binding to the GHSR and is related to either elevated plasma LEAP2 levels or impaired LEAP2 transport to the hypothalamic GHSR. Therefore, the addition of another LEAP2 analog would not affect the metabolomic and morphometric parameters in DIO mice.

6. CONCLUSIONS

This study provides insights into the interaction between ghrelin and LEAP2 in the regulation of food intake and obesity. Series of novel N-terminal LEAP2(1-14) analogs, lipidized with different fatty acids was tested for its affinity to and activation of GHSR in vitro and in vivo.

Palm-LEAP2(1-14) had the highest affinity for GHSR and acted as inverse agonist as well as competitive antagonist of GHSR. In vivo experiments proved that palm-LEAP2(1-14) reduced food intake in both fasted mice with high endogenous ghrelin level and free fed mice with exogenous [Dpr^3]Ghrelin. Palm-LEAP2(1-14) inhibited [Dpr^3]Ghrelin-induced GH release and showed increased stability in rat plasma compared to non-lipidized LEAP2(1-14). In order to study long-term effect of palm-LEAP2(1-14) in obese mice, we studied HF diet-induced obesity and the development of ghrelin and LEAP2 resistance in mice. HF diet feeding decreased plasma active and total ghrelin and increased liver *LEAP2* mRNA expression. The switch to a St diet normalized liver *LEAP2* mRNA expression and active ghrelin levels but not total ghrelin. Palm-LEAP2(1-14) and [Dpr^3]Ghrelin resistance developed after HF diet feeding, [Dpr^3]Ghrelin sensitivity restored upon switching to a standard diet. Effect of palm-LEAP2(1-14) on HF diet feeding since the first day of HF diet feeding was studied. Palm-LEAP2(1-14) slightly reduced the body weight gain and decreased plasma leptin level. However, palm-LEAP2(1-14) was not able to suppress the effect of HF diet due to palm-LEAP2(1-14) resistance.

In conclusion, the findings contribute to the understanding of obesity and suggest that modified N-terminal peptide LEAP2(1-14) and its lipidized analogs, particularly palm-LEAP2(1-14) holds promise for anti-obesity treatment, but further investigations are necessary to overcome the challenges of long-term treatment and resistance development. Alternative approaches are needed to improve the effectiveness of anti-obesity therapies that target the ghrelin and LEAP2 pathways.

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Publications related to Ph.D. thesis:

1. Holá, L.; Železná, B.; Karnošová, A.; Kuneš, J.; Fehrentz, JA.; Denoyelle, S.; Cantel, S.; Blechová, M.; Sýkora, D.; Myšková, A.; Maletínská, L. A Novel Truncated Liver Enriched Antimicrobial Peptide-2 Palmitoylated at its N-Terminal Antagonizes Effects of Ghrelin. *J Pharmacol Exp Ther.* 2022, 383(2), 129-136. https://doi.org/ 10.1124/jpet.122.001322. **IF_{2022} = 4.40**

2. Holá, L.; Tureckiová, T.; Kuneš, J.; Železná, B.; A.; Maletínská, L. High-fat diet induces resistance to ghrelin and LEAP2 peptide analogs in mice. *J. Mol. Endocrinol.* 2023. Under major revesion. **IF_{2022} = 4.87**

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Publications not related to Ph.D. thesis:

1. Strnadová, V.; Karnošová, A.; Blechová, M.; Neprašová, B.; Holá, L.; Němcová, A.; Myšková, A.; Sýkora, D.; Železná, B.; Kuneš, J.; Maletínská, L. Search for lipidized PrRP analogs with strong anorexigenic effect: In vitro and in vivo studies. *Neuropeptides*. 2023, 98**,** 102319. https://doi.org/10.1016/j.npep.2022.102319. **IF$_{2023}$ = 3.29**

2. Mráziková, L.; Neprašová B.; Mengr A.; Popelová A.; Strnadová V.; Holá L.; Železná B.; Kuneš J.; Maletínská L. Lipidized Prolactin-Releasing Peptide as a New Potential Tool to Treat Obesity and Type 2 Diabetes Mellitus: Preclinical Studies in Rodent Models. *Front. Pharmacol*. 2021, 12, 779962. https://doi.org/10.3389/fphar.2021.779962. **IF$_{2021}$ = 5.55**

3. Kořínková, L.; Pražienková, V.; Černá, L.; Karnošová, A.; Železná, B.; Kuneš, J.; Maletínská, L. Pathophysiology of NAFLD and NASH in Experimental Models: The Role of Food Intake Regulating Peptides. *Front Endocrinol.* 2020, 11, 597583. https://doi.org/10.3389/fendo.2020.597583. **IF$_{2020}$ = 5.56**

4. Novotná, B.; Holá, L.; Staś, M.; Gutten, O.; Smola, M.; Zavřel, M.; Vavřina, Z.; Buděšínský, M.; Liboska, R.; Chevrier, F.; Dobiaš, J.; Boura, E.; Rulíšek, L.; Birkuš, G. Enzymatic Synthesis of 3′–5′, 3′–5′ Cyclic Dinucleotides, Their Binding Properties to the Stimulator of Interferon Genes Adaptor Protein, and Structure/Activity Correlations. *Biochemistry.* 2021, 60, 48, 3714-3727. https://doi.org/10.1021/acs.biochem.1c00692. **IF$_{2021}$ = 3.32**