Central effects of Humanin on hepatic triglyceride secretion

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ZG and RHM designed the experiments. ZG, KS, TZ, ET and LC performed the studies. ZG, ET,
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Abstract:

Humanin (HN) is an endogenous mitochondria-associated peptide that has been shown to protect against various Alzheimer disease-associated insults, myocardial ischemia-reperfusion injury, and reactive oxygen species induced cell death. We have previously shown that HN improves whole body glucose homeostasis by improving insulin sensitivity and increasing glucose-stimulated insulin secretion (GSIS) from the β cells. Here, we report that intraperitoneal treatment with one of HN analogues, HNG, decreases body weight gain, visceral fat and hepatic triglyceride (TG) accumulation in high fat diet fed mice. The decrease in hepatic TG accumulation is due to increased activity of hepatic microsomal triglyceride transfer protein (MTTP) and increased hepatic TG secretion. Both intravenous (IV) and intracerebroventricular (ICV) infusion of HNG acutely increase TG secretion from the liver. Vagotomy blocks the effect on both IV and ICV HNG on TG secretion, suggesting that effects of HNG on hepatic TG flux are centrally mediated. Our data suggest that HN is a new player in central regulation of peripheral lipid metabolism.
Introduction:

Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) are burgeoning health problems that affect one-third of adults and an increasing number of children in developed countries (1, 6). NAFLD is associated with obesity, insulin resistance, diabetes mellitus and metabolic syndrome (25). It is estimated that NAFLD affects approximately 70-80% of the obese people (3), sixty-seventy percent of people with diabetes (52) and virtually 100% of obese type 2 diabetic patients (53). Excess lipid accumulation in the liver could elicit an inflammatory response, thereby progressing to steatohepatitis, cirrhosis and liver cancer. In addition, fatty liver contributes to insulin resistance (7) and diabetes (48), which in turn increases the risks for many diseases including cardiovascular diseases (51), cancer (45) and Alzheimer’s disease (AD) (11). Thus, reducing lipid accumulation in the liver could have favorable hepatic as well as systemic consequences.

NAFLD arises from the imbalance of triglycerides (TG) storage and clearance in the liver. TG is generated in the liver by coupling free fatty acids (FFA), derived from adipose tissue lipolysis, diet or de novo lipogenesis, to a glycerol via ester bonds. TG in the liver can undergo hydrolysis to release FFA that can be used to generate ATP through β-oxidation in the mitochondria, or secreted from the liver in the form of very low-density lipoprotein (VLDL), thereby preventing hepatic TG accumulation. Microsomal triglyceride transfer protein (MTTP) is the rate-limiting enzyme in the assembly and secretion of hepatic VLDL. Mice lacking hepatic mttp gene show hepatic steatosis and a dramatic decrease in plasma lipid levels (39); while hepatic over-expression of mttp increases VLDL-TG secretion in vivo (4). In humans, mutations in MTTP cause abetalipoproteinemia, an autosomal recessive disorder characterized by the virtual absence of apolipoprotein B (apoB)-containing lipoproteins in plasma (34, 37). ApoB is an obligatory structural component of VLDL, and individuals heterozygous for inactivating mutations in ApoB produce fewer VLDL particles and have a three-fold increase in hepatic TG content relative to healthy individuals (50).

Humanin (HN) is a 24-amino acid mitochondria-associated peptide that was identified from a cDNA library from the surviving neurons of human Alzheimer's disease brain (15). Endogenous HN is both an
intracellular and secreted protein and has been detected in heart, liver, muscle, ovary, pancreas, kidney, brain, colon, testis plasma, cerebral spinal fluid (CSF) and seminal fluid (31, 49). HN has a well-described role in neuro-protection against AD-associated cell death (15), prion induced apoptosis (46) and chemically-induced neuronal damage (12, 24). Analogs of HN, created by a single amino acid substitution, are reported to increase the stability and biological potency; one such analog is HNG (with replacement of serine with glycine at position 14) (14). We have shown that in addition to the neuroprotective effects, HNG also displays a cardio-protective effect in ischemia and reperfusion mouse model (32). It was recently demonstrated that neuro-protective effects of HN are mediated through a trimeric cell surface receptor complex of CNTFR, WSX-1 and gp130 (13) and is associated with activation of STAT3 (26). Indeed, anti-apoptotic action and neuronal protection by HN were lost in the presence of dominant negative STAT3 (16). We have shown that HN increases overall insulin sensitivity in rodents through the activation of central STAT3 pathway (31). Very recently, our studies showed that HNGF6A, another analog of HN, increases insulin secretion in cultured βTC3 pancreatic β cell line, islets from wild type and db/db mice, and rats in vivo, and these effects were mediated through increased glucose oxidation and ATP generation (22).

Since insulin resistance is associated with NAFLD, and HN increases insulin sensitivity, we postulated that HN will decrease hepatic TG accumulation, the pathological hallmark of NAFLD. We tested our hypothesis in wild type and diet-induced obesity animal models where HNG was administered either centrally or peripherally.
MATERIALS AND METHODS

Animals

Male, 12-week-old C57BL/6J mice (Jackson Laboratory) and Sprague Dawley rats (Harlan Laboratory) were used in the present study. Mice were used for both acute and chronic studies, while rats were used for the acute studies evaluating potential signaling pathways. We used rats to confirm that the results are reproducible in a different species. Animals were housed under a standard 12-hour light–dark cycle (lights on at 7:00 AM) with access to food and water ad libitum.

Chronic studies: For the chronic treatment study, mice were fed with high fat diet (60% fat, Research Diets Inc., New Brunswick, NJ) and received once daily IP injection of 2 mg/kg scrambled peptide (SP, FRGGETRARAMPLIDLSPLCLLKV) or HNG (MAPRGFSCLLLLTGEIDLPVKRA, GenScript Piscataway, NJ) for 4 weeks.

Acute studies: For the acute studies, depending on the study group, the animals were prepared with survival surgeries. For the acute IV studies, catheters were placed in the right internal jugular vein and left carotid artery as previously described (28-31, 33). For the intracerebroventricular (ICV) studies, two weeks before the in vivo experiments, cannulas were implanted in the third cerebral ventricle, and after complete recovery, usually a week, catheters were placed in the right internal jugular vein and left carotid artery as previously described (28-31, 33). In the vagotomy group, hepatic vagotomy was performed as previously described (38) along with the catheter replacement. Briefly, mice were anesthetized and laparotomy was performed. The stomach was exposed and hepatic branch of the abdominal vagus were identified. The hepatic nerve bundles of mice were isolated on a fine wire loop and rapidly heated until the loop cut through the nerve. Based on the study group, the animals received ICV, IV or intraperitoneal (IP) administration of SP or HNG.

IV studies: A total of 2mg/kg of SP and HNG were infused through the jugular vein from -60 minute time point. One-third of the total amount (0.67mg/kg) was given as a bolus in first 3 minutes, and the remainder at the rate of 5.5 µg/kg/min over the duration of the study. The above dose was chosen based on our previous experience in vivo (31). A single dose injection of 600 mg/kg tyloxapol IV, an inhibitor
ICV studies: On the day of the experiments, SP and HNG (bolus of 36 µg/kg/min for 3 min followed by an infusion at 0.9 µg/kg/min) were started at -60 minutes and infused ICV throughout the duration of the study. PI3 kinase inhibitor LY294002 (3ul bolus of 1 mM and thereafter 2.5 ul/h for 5 hrs (40)) or Melanocortin-3/4 receptor antagonist SHU9119 (0.66nmole/3ul of bolus and thereafter 1.33nmole/5hrs (30)) (EMD Millipore, Darmstadt, Germany) or STAT3 inhibitor (31) (Genemed Synthesis Inc., San Antonio, TX), were infused to the 3rd ventricle along with HNG or SP depending on the study group. A single dose injection of 600 mg/kg tyloxapol IV, an inhibitor of lipoprotein lipase (LPL) that blocks degradation of TG in the plasma, was administered at the 0 minute time point.

Blood samples were collected for the assessment of serum TG levels at 0, 60, 120, 150 and 180 min after the IV injection of tyloxapol. TG secretion rate was calculated by the slope of the rise in serum TG over time. At the end of the study, the animals were sacrificed, the abdomen was quickly opened and visceral fat pads were carefully dissected, weighed and snap frozen in liquid nitrogen. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committees of the Albert Einstein College of Medicine and University of Pittsburgh.

Indirect calorimetry

Twelve-week-old weight-matched male mice were singly housed in metabolic chambers. Oxygen consumption, locomotor activity, and respiration exchange ratio were measured continuously for 2 weeks during 12:12 light-dark cycles, using a CLAMS (Columbus Instruments, Columbus, OH) open-circuit indirect calorimetry system. SP and HNG were given by IP injection daily.

Isolation and culture of primary hepatocytes

Primary hepatocytes were isolated from 12-week-old adult male C57/B6 mice by collagenase perfusion method as described previously (44) with modifications. In brief, livers were washed with Hanks’ calcium- and magnesium-free buffer for 3 min followed by a collagenase buffer (0.5 mg/ml) for 10 min at a perfusion rate of 7 ml/min. After the perfusion, livers were rapidly excised and transferred to a sterile
Petri dish. Hepatocytes were released by disrupting the liver capsule. The cells were separated from undigested tissue with a sterile 50µm mesh nylon filter and cultured in DMEM supplied with 10% FBS and grown on type I collagen coated plates. Primary cells were incubated with 150µM of oleic acid or palmitic acid overnight and then treated with SP or HNG for 4 hours. Intracellular TG and secreted TG levels were assessed by TG quantification kit (Abcam, Cambridge, MA) and normalized to cell number (per million cells). For MTTP activity assay, cells were treated with SP or HNG for 0.5, 1 or 3 hours and then harvested for the assay.

**Biochemical measurements**

Insulin was detected using ELISA kit (Crystal Chem, Downers Grove, IL). Blood glucose levels were measured by OneTouch glucometer (LifeScan Inc. Milpitas, CA). Free fatty acids were measured by a colorimetric assay (Wako Chemicals, Richmond, VA). Tissue and serum TG levels were determined using a colorimetric assay (Thermo-Fisher scientific, Middletown, VA). Liver TG was extracted using Folch method. Briefly, liver tissue was homogenized with chloroform/methanol at a ratio of 2/1. The homogenate was centrifuged and the liquid phase was recovered and washed twice with saline. After centrifugation and siphoning of the upper phase, the chloroform phase containing lipids was evaporated under a nitrogen stream, and lipids were dissolved in isopropanol alcohol. Due to the high concentration of TG in the serum in acute *in vivo* studies, samples were diluted by serum dilution buffer (25mM HEPES, 150mM NaCl and 1mM CaCl₂, pH 7.4). All other chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

**Lipoprotein fractions**

Serum samples from 5 mice in each group were pooled and fast protein liquid chromatography (FPLC) was performed to separate the lipoprotein fractions. TG levels in each fraction were determined using the TG kit (Thermo-Fisher scientific, Middletown, VA).

**RNA extraction and Real Time PCR**

Liver RNA was extracted using RNeasy purification kit (Qiagen, Valencia, CA). First strand cDNA was synthesized from 1 µg RNA (AMV First Strand cDNA Synthesis Kit, New England Biolab Inc., Ipswich,
Real time PCR was carried out in a 10 µl reaction mixture (LightCycler DNA master SYBR green, Roche) containing 1 µl of the diluted (1:10) first-strand cDNA, and the results were normalized to GAPDH using 'delta delta Ct' (ΔΔCt). The sequences of the primers used for amplification of mouse Cpt-1a, Srebp-1c, Fas, Dgat-2, mttp, ApoB and GAPDH are listed in table 1.

Immunoblotting

Livers were homogenized in RIPA buffer (50mM Tris (pH7.4), 150mM NaCl, 1% Triton X-100, 0.1% SDS) containing proteinase inhibitors (Roche). Twenty micrograms of protein were resolved on precast gradient 4-12% SDS-PAGE, and then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h at room temperature. Antibodies (Akt, pAkt ser473 and GAPDH from Cell Signaling Technologies Cambridge, MA diluted in TBS-T containing 5% of BSA were added to the membranes and incubated overnight at 4 °C. The membranes were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Signals detected using the SuperSignal West Dura extended duration substrate (Thermo Scientific, Rockford, IL).

MTTP Activity Assay

MTTP activity was measured using an MTTP assay Kit according to the manufacturer’s instructions (Roar Biomedical, New York, NY).

Oil Red O Staining

Liver sections were fixed in 10% formalin and then stained with oil red O solution at 60 ºC for 10 min. Images were taken by Olympus Light station 2.

Alanine Aminotransferase (ALT) activity Assay

Liver ALT activity assay was done using a colorimetric assay kit from Biovision (Milpitas, CA) according to the manufacturer’s instructions.

Statistical Analysis
All values shown are expressed as means ± SE. When comparing two groups between SP and HNG, independent two-tailed t test was used. When dynamic comparisons for variables such as TG secretion were made, two-way ANOVA (group×time) was used. For each statistically significant F value observed for the main effect or interaction, a two-tailed post hoc test (Tukey's) was applied to determine individual differences between means. Differences were considered to be statistically significant when $p \leq 0.05$ for primary outcomes and $p \leq 0.01$ for secondary outcomes.
RESULTS

Phenotype and basic metabolic profile in HNG treated HFD induced obese mice. Twelve-week-old C57BL/6J mice were fed 60% high fat diet and received once daily IP administration of either SP or HNG (2mg/kg) for 4 weeks. While no difference was observed on the food intake between the groups (Fig. 1A), there was a ~20% less body weight gain in HNG treated mice (Fig. 1B, p<0.05). HNG treated mice had less visceral fat (Fig. 1C, p<0.05). Increased energy expenditure was noted in HNG treated group on CLAMS (Fig. 1D and E, p<0.01). There was no preferential substrate oxidation between the groups (RQ-0.91 ± 0.01 vs. 0.92 ± 0.01 in SP vs. HNG, N.S). Fasting glucose was significantly decreased and insulin levels tended to increase (p=0.058) in the HNG treated group (Fig 1F and G). No significant differences were found in serum FFA (Fig. 1H) and total TG levels (Fig. 1I) in HNG treated mice. On Fast Protein Liquid Chromatography (FPLC) analysis of lipoprotein fractions from pooled samples, VLDL fraction was increased 2-fold in the HNG treated group (Fig 1J). There were no differences in LDL or HDL fractions.

HNG decreases hepatic lipid accumulation in HFD induced obese mice. Oil red O staining of liver sections demonstrates a significant decrease in liver steatosis in HNG treated mice (Fig. 2A). This was confirmed by liver TG levels (Fig. 2B); HNG treated mice showed decreased hepatic TG accumulation (Fig. 2B, p=0.011). There were no differences in liver ALT activity between SP and HNG treated mice (Fig. 2C).

HNG increases gene expression of ApoB and mttp, and increases the activity of MTTP. To further analyze the role of HNG in attenuation of hepatic TG accumulation, we analyzed the expression level of genes involved in TG synthesis (Diglyceride acyltransferase 2, DGAT2), free fatty acid synthesis (Sterol regulatory element-binding protein, Srebp-1c; Fatty Acid Synthase, Fasn), fatty acid β-oxidation (Carnitine palmitoyltransferase, Cpt-1a), gluconeogenesis (Phosphoenolpyruvate carboxykinase, Pepck), and VLDL packaging and secretion (ApoB and MTTP) in the liver. HNG significantly increased the mRNA levels of ApoB and mttp, genes involved in VLDL packaging and secretion (Fig. 3A). HNG also increased the mRNA level of hepatic lipase (HL, Fig. 2A), which hydrolyzes TG and phospholipids in
chylomicron remnants, IDL, and HDL (42). There were no changes in the mRNA levels of Dgat 2, Srebp-1c, Fasn, Cpt-1a and Pepck (Fig. 3A). Along with the increase in expression, there was a ~20% increase of the MTTP enzyme activity in the liver in HNG treated group (Fig. 3B), and an up-trend in the intestinal MTTP activity (Fig. 3C, p=0.15).

**Acute effects of HNG on hepatic TG flux.** To test if HNG increases hepatic TG secretion *in vivo*, we infused SP or HNG IV, along with a single dose of tyloxapol through the right internal jugular vein at 0 minute, to block the degradation of VLDL-TG in the circulation (Experimental design shown in fig 4A). Our results show that TG levels steadily increased in the serum after the administration of tyloxapol in both groups; and serum TG levels were significantly higher in the group that received IV infusion of HNG compared to SP (Fig. 4B). Liver TG secretion rate was increased in HNG treated mice (Fig. 4C).

To test if the effect of HNG on hepatic TG accumulation is mediated via the hypothalamus, we infused HNG or SP directly into the 3rd ventricle (ICV), and examined the acute TG secretion from the liver in mice. Similar to the IV infusion study, we found that ICV HNG increases TG accumulation in the circulation (Fig. 4D) and the calculated liver TG secretion rate was increased in the ICV HNG group (Fig. 4E). Consistent with the increased TG secretion rate, liver MTTP activity was increased by almost 50% in ICV HNG treated mice compared to those treated with SP (Fig. 4F).

**HNG does not directly affect TG flux in primary hepatocytes.** We then challenged the isolated primary hepatocytes overnight with FFAs (oleic acid or palmitic acid) and treated them with either SP or HNG for four hours to examine whether the HNG directly affects hepatic TG accumulation. All cells had significantly higher intracellular TG concentration after the treatment of FFAs, but there were no difference in the intracellular TG concentration (Fig. 5A) or the level of secreted TG in the media (Fig. 5B) between SP and HNG treated groups. We also measured the activity of MTTP, key regulator of VLDL assembly, and found no difference during 3 hour HNG treatment (Fig. 5C). Although overnight incubation with FFAs increased the expression levels of MTTP and apoB, there were no differences between the SP and HNG treated groups (Supporting data S1A, S1B). These studies indicate that the effects of HNG on TG secretion are not mediated through direct actions on the liver.
Effects of HNG on hepatic TG secretion are lost after vagotomy: To study the role of vagus nerve as the efferent signal from the hypothalamus in mediating the effects of HNG on hepatic TG secretion, we infused SP and HNG IV to animals that had sham surgery or vagotomy. IV HNG markedly increased serum TG levels compared to the SP treatment group in the sham surgery mice, whereas vagotomy completely abolished this effect (Fig. 6A and B). We also infused HNG ICV and measured the hepatocyte TG secretion and MTTP activity after vagotomy. Again, vagotomy completely abolished the effects of ICV HNG on hepatic TG secretion (Fig. 6C), TG secretion rate (Fig. 6D) and MTTP activity (Fig. 6E). Taken together with direct studies on the isolated primary hepatocytes, these data suggest that HNG affects hepatocyte lipid metabolism through central pathway(s).

Effects of HN on TG flux are independent of hypothalamic STAT-3 and insulin signaling pathways: We have previously shown that HN regulates whole body glucose homeostasis through activation of the central STAT-3 signaling pathway (31). Blockage of STAT-3 pathway in the hypothalamus by infusion of STAT-3 inhibitors into the 3rd ventricle abolished HN’s effects on glucose homeostasis. To examine if the effects of HNG on hepatic TG secretion is also through the hypothalamic STAT3 signaling pathway, cell-permeable STAT3 inhibitor was infused ICV an hour before the infusion of HNG in rats. Our results showed that HNG increased TG secretion from the liver in rats, replicating our results seen in mice. Furthermore, infusion of STAT-3 inhibitor did not block the effects of HNG on hepatic TG secretion (Supporting data Fig. S2). We found that ICV HNG increases the phosphorylation of Akt2 in the hypothalamus (Fig. 6B and C), but not in the liver (Fig 7C and D). However, we failed to block the effects of HNG on hepatic TG secretion by co-infusion of PI3K inhibitor, LY-294002 into the 3rd ventricle (Fig. 7E), suggesting that the activation of hypothalamic insulin signaling is not involved in the effects of HNG on TG flux.

It has been reported that central melanocortin system is critical for the control of lipid metabolism in the periphery including liver and fat tissues (35). In order to investigate whether the effects of HN on hepatic TG flux is mediated through the central melanocortin system we infused SHU9119, a melanocortin-3/4 receptor antagonist into the 3rd ventricle one hour prior to the infusion of HNG. While
SHU9119 alone did not induce any changes in TG levels in the serum, the accumulation of serum TG in response to HNG is greatly inhibited in the presence of SHU9119, suggesting that the effect of HNG on hepatic TG secretion could involve the central melanocortin system (Fig. 7F).
In studies presented here, we show that HNG, a potent analog of HN, decreases hepatic TG levels in HFD-induced obesity, increases hepatic MTTP activity and TG secretion, and these effects are mediated through the hypothalamus.

We have previously shown a role for HN and its analogs in glucose homeostasis (31). We have demonstrated that HN and analogs improve hepatic and skeletal muscle insulin sensitivity (31), and that HN increases insulin secretion through direct effects on β cell (21). Consistent with those observations, here we show that a 4-week IP injection of HNG in an obese mouse model leads to decrease in fasting glucose levels and a trend of increase in insulin levels (p=0.058) (Fig. 1F and G) compared to controls.

Importantly, we report here that HNG treatment reduces hepatic TG accumulation in obese mice (Fig. 2B). The demonstration of two-fold increase in VLDL by FPLC analysis (Fig. 1J) suggests increased hepatic TG efflux. However, the decrease in hepatic TG accumulation in the chronic study model could be multifactorial and attributable to a variety of factors including improved insulin sensitivity from decreased visceral fat, an increase in energy expenditure, as well as increased hepatic TG efflux as a result of increased MTTP activity, key regulator of the VLDL-TG secretion from the liver. Overall, the end result of decreased hepatic lipid accumulation, especially in the setting of high fat diet, could have significant hepatic and systemic consequences such as improvement in insulin sensitivity. Lack of changes in the expression level of genes involved in de novo lipogenesis by HNG (Fig. 2A), and observation that HNG does not affect phosphorylation of ACC (data not shown) suggest that HNG does not directly affect lipid deposition and utilization in the liver. Overproduction of TG and hypersecretion of VLDL-TG from the liver increases the risk for dyslipidemia and cardiovascular disease \textit{in vivo} (23, 54). The significant reductions in liver fat content (Fig. 2B), increase in serum VLDL (Fig. 1J) along with a reduction in adipose tissue LPL (data not shown) and decrease in visceral fat (Fig. 1C), suggest that HNG may influence lipid metabolism in other tissues, and requires further investigation.

One of the regulators of VLDL-TG secretion is insulin (19). Insulin controls the expression level of \textit{mttp} and the degradation of ApoB (5, 19). However, phosphorylation of Akt2 was unchanged in the
liver in the HNG treated mice (Fig. 7C and 7D), suggesting that the effects noted are not secondary to increased hepatic insulin signaling. Furthermore, our findings that HNG did not lower the intracellular or TG levels in the media or increase MTTP activity or mRNA levels in primary hepatocytes (Fig. 5 and S1), exclude the possibility of direct actions of HNG on the liver.

The hypothalamus plays central roles in energy and glucose homeostasis (8). We and others have demonstrated that the neurons in the arcuate nucleus (ARC) of hypothalamus are able to sense the nutrient levels and integrate hormonal signals such as insulin, IGF-1 and leptin, and ultimately control food intake and hepatic glucose production (HGP) (41). Indeed, hypothalamic insulin receptor disruption is sufficient to reduce the insulin-induced suppression of HGP (36), and adenovirus delivery of IRS-2 in the ARC slowed the rise in blood glucose levels in a mouse model of type 1 diabetes (9). Hypothalamic leptin signaling is also involved in regulating hepatic insulin sensitivity, HGP and whole body glucose homeostasis through both JAK-STAT and PI3K pathways (10, 17, 20, 27). Beyond HGP, hypothalamus also plays a role in adipose tissue metabolism. Mediobasal hypothalamus (MBH) infusion of insulin in SD rats increase WAT lipogenic protein expression, inactivates hormone-sensitive lipase (Hsl) and adipose tissue triglyceride lipase (Atgl), and suppresses lipolysis (43). On the other hand, mice that lack the neuronal insulin receptor exhibit unrestrained lipolysis and decreased \textit{de novo} lipogenesis in WAT (43). These indicate that hypothalamus is a critical organ for regulating whole body fat metabolism.

Our data in \textit{in vivo} models demonstrating that the effects of both IV and ICV HNG on hepatic TG secretion were lost in the mice after vagotomy (Fig. 6), suggest a central role for hypothalamus and vagus nerve in mediating the effects of HNG on hepatic TG secretion. Our studies point to the vagus nerve as the efferent carrying the signals from the hypothalamus to the liver, as effects of ICV and IV HNG on hepatic TG flux were lost in the mice post-vagotomy. Also, the significant increase in hepatic MTTP activity in response to hypothalamic HNG was lost post-vagotomy, providing evidence for the first time that HNG regulates hepatic MTTP activity through the hypothalamus. Previous studies have shown that hypothalamic leptin signaling controls intestinal MTTP activity and lipid absorption (18). However, MTTP activity in the intestine in the HFD mice were slightly but not significantly increased with HNG
treatment (Fig. 3C), suggesting that HNG increases hepatic TG flux without affecting lipid absorption \textit{in vivo}. We acknowledge that in our experimental model, vagotomy would have resulted in transection of both sympathetic and parasympathetic nerves and further studies are necessary to delineate the components of autonomic nervous system that are involved in the effects of HNG on VLDL-TG secretion.

Neuropeptide Y (NPY) (2) and glycine (54) have been shown to control hepatic VLDL-TG secretion through the hypothalamus. Our observation that inhibition of melanocortin-3/4 receptor by infusion of SHU9119 into the 3\textsuperscript{rd} ventricle reversed the effects of HNG on hepatic TG secretion (Fig. 7F) is inconsistent with observations that acute activation or inhibition of melanocortin pathway did not affect TG secretion from the liver (47). This could be due to the species and strains differences in the study (SD rats instead of Long Evans rats were used in our study) and merits further investigation using genetic models of altered melanocortin signaling.

In summary, we demonstrate that HNG, an analog of HN, increases TG flux from the liver through activation of hepatic MTTP and these effects are mediated through the hypothalamus, with the vagus nerve serving as the efferent from hypothalamus to the liver. In addition to the described role in glucose metabolism, these studies provide new evidence that HNG, a potent analogue of the endogenous mitochondrial peptide humanin, is a novel central regulator for hepatic TG secretion.
Acknowledgments:

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Disclosures: The authors have nothing to disclose.
References


Figure legends:

Fig. 1: Effects of HNG on phenotype and basic metabolic profile in HFD-induced obese mice. Food intake (A) and Body weight gain (B) of mice fed HFD and treated with SP (white) or HNG (grey) for 4 weeks (n=15 for SP and 17 for HNG); (C): Visceral fat and; (D and E): 24-hour and average energy expenditure levels (n=3 in each group). Fasting glucose (F); fasting insulin (G); serum FFA (H); serum TG (I) levels; and (J) lipoprotein fractions in HFD mice treated with SP or HNG. *p<0.05 and **p<0.01

Fig. 2: Effects of HNG on hepatic steatosis. (A): Representative images for Oil-red O staining of liver from mice fed with HFD and treated with SP or HNG. Arrows point to the lipid droplets in black puncta. Liver TG (B) and liver ALT (C) activity of HFD mice treated with SP (white) or HNG (grey) for 4 weeks (n=6 in each group).

Fig. 3: Effects of HNG on hepatic gene expression and activity of MTTP in HFD mice. (A): Relative gene expression levels for Srebp-1c, Fasn, Cpt-1a, Dgat2, Pepck, ApoB, mttp and HL in SP (white bar) or HNG (grey bar) treated HFD mice (n=6 each group); (B and C): activity of hepatic (B) and intestinal (C) MTTP in HFD mice treated with SP or HNG for 4 weeks. *p<0.05 and **p<0.01

Fig. 4: Acute effects of HNG on TG secretion in WT mice. (A): Timeline for the acute experiments. Dynamic TG secretion (B) and TG secretion rate over 3 hours (C) in mice that received acute IV infusion of SP (white) or HNG (grey) for 3 hours; Dynamic TG secretion (D) and TG secretion rate over 3 hours (E) in mice that received acute ICV infusion of SP (white) or HNG (grey) for 3 hours; (F): Relative hepatic MTTP activity in ICV SP or HNG mice. *p<0.05, **p<0.01

Fig. 5: Effects of HNG on TG secretion in primary hepatocytes. Intracellular TG (A) and TG secreted into the media (B) from the hepatocytes that were challenged with vehicle, Palmitic acid (PA) or oleic acid (OA) for overnight and then treated with SP (white) or HNG (grey) for 4 hours; (C): MTTP activity in primary hepatocytes treated with SP or HNG for 0.5, 1 and 3 hours. *p<0.05, **p<0.01

Fig. 6: Acute effects of HNG on hepatic TG secretion in vagotomized mice. Dynamic TG secretion following SHAM surgery or vagotomy (A) and TG secretion rate over 3 hours (B) in vagotomized mice that received acute IV infusion of SP (white) or HNG (grey) for 3 hours; Dynamic TG secretion following
SHAM surgery or vagotomy (C) and TG secretion rate over 3 hours (D) in vagotomized mice that received acute ICV infusion of SP (white) or HNG (grey) for 3 hours; (E): Relative hepatic MTTP activity in vagotomized mice following ICV SP or HNG. *p<0.05 and **p<0.01

Fig. 7: **Effects of HNG on hepatic TG secretion are independent of central insulin signaling**: Levels of GAPDH, total Akt and phosphor-Akt (Ser 473) in the hypothalamus by immunoblot (A) and quantification (B); Levels of GAPDH, total Akt and phosphor-Akt (Ser 473) in the liver by immunoblot (C) and quantification (D); (E): Serum TG levels in mice treated with SP (solid line), HNG (dotted line with open squares) and HNG+LY294002 (dotted line with open triangles). (F): Serum TG levels in mice treated with SP (solid line), HNG (dotted line with open squares), SP+SHU9119 (solid line with solid rounds) and HNG+SHU-9119 (dotted line with open triangles). *p<0.05
Table 1: Real Time PCR primers

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<th>Gene</th>
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</tr>
<tr>
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</tr>
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<td>ttcctgaagagctctcc</td>
<td>ttttggggatgggcac</td>
</tr>
<tr>
<td>HL</td>
<td>atgggaatcccccactcaatct</td>
<td>gtgcctaggctcgagacga</td>
</tr>
</tbody>
</table>
Fig. 1

**Energy expenditure (Kcal/kg/hr)**

- **A**: Food intake (g/day)
- **B**: Weight gain (g)
- **C**: Visceral Fat (g)
- **D**: Energy expenditure (Kcal/kg/hr)
- **E**: Energy expenditure (Kcal/kg/hr) by condition

**Food intake (g/day)**

- **A**: SP vs HNG

**Weight gain (g)**

- **B**: SP vs HNG

**Visceral Fat (g)**

- **C**: SP vs HNG

**Energy expenditure (Kcal/kg/hr)**

- **D**: Comparison of SP and HNG

**Food intake (g/day)**

- **A**: Comparison of SP and HNG

**Weight gain (g)**

- **B**: Comparison of SP and HNG

**Serum FFA (mM)**

- **D**: Comparison of SP and HNG

**Blood Glucose (mg/dl)**

- **F**: Comparison of SP and HNG

**Serum Insulin (ng/ml)**

- **G**: Comparison of SP and HNG

**Serum FFA (mM)**

- **H**: Comparison of SP and HNG

**Serum TG (mM)**

- **I**: Comparison of SP and HNG

**TG level (mM)**

- **J**: TG levels across different fractions

**VLDL**, **LDL+IDL**, **HDL**

- **J**: Fractional distribution of lipids
Fig. 2

A

Liver TG (ug/mg)

B

Liver TG activity (mU/ml)

C

Liver ALT activity (mU/ml)

SP  HNG

SP  HNG

*
**Fig. 3**

### A

- **Srebp-1**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

- **FASN**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

- **Cpt-1a**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

- **Dgat2**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

- **APEB**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

- **Srebp-1**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

- **PEPCK**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

- **MTPp**
  - Relative Gene expression
  - SP: Lower, HNG: Higher

### B

- **Liver**
  - Relative MTTP activity (%)
  - SP: Lower, HNG: Higher

- **Intestine**
  - Relative MTTP activity (%)
  - SP: Lower, HNG: Higher
Fig. 4

A

ICV surgery (ICV group only) IV catheter
Day -14 Day -7

IV/ICV-HNG/Control
TYLOXAPOL (600mg/kg)

TG
TG
TG
TG

Day of the study

B

Serum TG (mg/ml)

0 5 10 15 20

0 60 120 150 180

Time (Min)

SP HNG

**

C

TG Secretion Rate (mg/h)

0 1 2 3 4 5 6

SP HNG

**

D

Serum TG (mg/ml)

0 2 4 6 8 10 12 14 16

Time (Min)

SP HNG

*

E

TG Secretion Rate (mg/h)

0 1 2 3 4 5

SP HNG

*

F

Relative Mtp activity (%)

0 20 40 60 80 100 120 140 160

SP HNG

*
Fig. 5

(A) Intracellular TG (µM) levels in cells treated with Vec, OA, and PA, showing a comparison between con and HNG conditions.

(B) TG secretion (µM) levels in cells treated with Vec, OA, and PA.

(C) Relative MTTP activity over time (h) at 0.5, 1, and 3 hours for Vec and OA conditions.
Fig. 7

A) Hypothalamus

B) pAkt/Akt

C) Liver

D) pAkt/Akt

E) Serum TG (mg/ml)

F) Serum TG (mg/ml)