Alleviation of Diet-Induced Fat Accumulation by a Small Molecule CMKLR1 Antagonist in Mice

Li Xue¹, Yan Yu², Fa Zeng³, Hui-Ru Tang⁴, Liang Xiang¹, Jie Chen¹, Tian-xia, Xiao¹, Pei-Gen Ren¹ and Jian V. Zhang^{1*}

¹Research Laboratory for Reproductive Health, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China

²Shenzhen Key Laboratory of Birth Defects, Shenzhen Baoan Maternal and Child Health Hospital, Shenzhen, Guangdong 518133, China.

³Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University, Shenzhen 518028, China

⁴Peking University Shenzhen Hospital, Shenzhen 518035, China

*Corresponding author: Jian V Zhang, 1Research Laboratory for Reproductive Health, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China, Tel: 86 755 9951 8743; E-mail: jian.zhang@siat.ac.cn

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Abstract

Objective: Non-alcoholic Fatty Liver Disease (NAFLD) is believed to be correlated with chemerin and its receptor, Chemokine-like Receptor 1 (CMKLR1). We analyse the role of CMKLR1 in NAFLD by using a novel small molecule CMKLR1 antagonist-NETA (2-naphthoyl ethyl trimethyl ammonium) in vitro and in vivo.

Methods: We assessed the effects of -NETA on a mouse model of high-fat-diet-induced fat accumulation in liver and adipose tissue and an analogous cell model established by culturing Hepa 1-6 and 3T3-L1 cells.

Results: We found that chemerin and CMKLR1 mRNA were significantly increased in the livers and fat tissue of the mice fed the high fat diet relative to those in mice fed the normal diet. α -NETA administration suppressed serum TC, TG, AST and ALT levels and hepatic TG content as well as inhibited lipid metabolism-associated factors in the livers and fat of high fat diet mice. Furthermore, in the cell model, α -NETA suppressed oleic acid induction of Hepa 1-6 cell steatosis, 3T3-L1 adipogenesis and the expression of mRNAs for related lipid metabolism-associated factors.

Conclusions: Chemerin/CMKLR1 signaling plays an important role in the progression of NAFLD.

Keywords: Chemerin; Chemokine-like receptor 1; α -NETA; Non-alcoholic fatty liver disease

Introduction

Obesity is a worldwide health problem. The concern over this metabolic syndrome is not obesity itself, but rather its various complications [1,2]. Non-alcoholic Fatty Liver Disease (NAFLD) encompasses a wide spectrum of liver diseases, ranging from simple steatosis to Non-alcoholic Steatohepatitis (NASH) [3,4], with the “two-hit theory” increasingly being adopted to explain the pathogenesis of both NAFLD generally and NASH specifically [5,6]. Progression to inflammation and fibrosis appear to result from oxidative stress [1,4,7], which ultimately causes liver damage [8]. Recent reports support the theory that obesity is a major risk factor for NAFLD. Most of the adipose tissue-derived proteins, such as adipokines, are elevated in obesity and may contribute to systemic inflammation and liver damage.

Adipose tissue can secrete more than 600 bioactives called adipokines [9], which participate in energy metabolism through autocrine and paracrine mechanisms. The main functions of these adipokines include the regulation of adipogenesis, appetite and satiety

among others [10]. Recently, a great deal of evidences have been found to support the idea that adipokines play pivotal roles in NAFLD [11,12,13]. The interplay of various adipokines may be very important in the progression from fatty infiltration to inflammation and fibrosis.

Chemerin is an adipokine originally identified in the skin in 1997 [14] and initially produced as an 163 amino acid pro-form [15,16]. In subsequent research, it has been isolated from ascitic fluid [17] (ovarian carcinoma), normal serum [1], liver, pancreas, kidney and adipose tissue [18], and that is why Chemerin as a novel chemokine is classified into the big family of adipokines. Chemokine-like Receptor-1 (CMKLR1) [19], one of three G-protein-Coupled Receptors (GPCR) of chemerin, was found earlier than its ligand. Recent studies have found that CMKLR1 is expressed in the liver and reduced in NAFLD. However, other researches have suggested that this receptor has nothing to do with NAFLD and that hepatic CMKLR1 abundance is related to adiponectin activity [20,21,22]. Thus, whether CMKLR1 contributes to the pathogenesis of NAFLD is still not clear.

2-naphthoyl ethyl trimethyl ammonium (α -NETA), an inhibitor of CMKLR1, was originally identified as an inhibitor of Choline Acetyltransferase (ChAT) [23], an enzyme that catalyzes the synthesis of acetylcholine from choline and acetyl-CoA [24]. In this study, our

objective was to determine (i) The effect of α -NETA on hepatic steatosis in Hepa 1-6 cells induced by oleic acid (OA). (ii) The effect of α -NETA on adipogenesis in 3T3-L1 cells and in the fat and liver of mice on high-fat diet. (iii) The role of chemerin/CMKLR1 signaling in the progression of NAFLD in both in vitro cell and in vivo mouse models.

Materials and Methods

Animal model

Male BALB/C mice (5-6w) were purchased from the Animal Experimental Center, Guangdong Academy of Medical Sciences, China, and were individually housed and fed either a normal chow diet (NCD, n=12) or a high-fat diet (HFD, n=12) with 45% of calories derived from fat for 2-12 weeks. All procedures related to animal usage were approved by the Committee on the Use of Live Animals for Teaching and Research, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. For the α -NETA treatment group (HFD + α -NETA, n=12), mice were also given intraperitoneal (i.p.) injections of 1 mg/kg α -NETA (dissolved in 1% DMSO in saline) twice a week. The control groups were given vehicle control injections. Mice were sacrificed and serum and livers were immediately collected and stored at -80°C for further analysis.

Materials

Dulbecco's Modified Eagle's Medium (DMEM), 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone sodium phosphate (DEX) were obtained from Invitrogen (USA); oleic acid (OA) and insulin were purchased from Sigma (USA); α -NETA was from Santa Cruz Biotechnology (USA); the 45% fat diet (HFD) was from Shanghai SLAC Laboratory Animal Company (China); Oil Red O was from Sigma (USA); Total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) ELISA kits were from the Nanjing Jiancheng Bioengineering Institute (China); the RNAiso Plus was from TaKaRa (Japan); PrimeScript RT reagent kit with gDNA Eraser was from TaKaRa (Japan); SYBR[®] Premix Ex TaqTM II was from TaKaRa (Japan).

Cell culture

3T3-L1 preadipocytes (obtained from Wuhan Boster, China) were cultured, maintained and differentiated. Cells were plated and grown in DMEM with 10% FBS, 1% Penicillin-Streptomycin(P/S), and 1% gluta-max. For differentiation, post-confluent cells were treated with induction medium (IM) containing 10% FBS, 0.5 M IBMX, 1.0 μ M DEX, and 5 mg/L insulin for two days. The culture medium was then replaced with DMEM supplemented only with 5 mg/L insulin and 10% FBS. This medium was changed every other day. For treatment, 300 nM, 1000 nM or 3000 nM α -NETA was added to 3T3-L1 cells with differentiation medium.

Hepa 1-6 cells (a mouse hepatocellular carcinoma cell line) were obtained from Wuhan Boster, China, and were maintained in culture

in 48-well plates with DMEM containing 10% FBS under an atmosphere of 5% CO₂ at 37°C with 95% humidity. Once approximately 70% confluence was reached, Hepa 1-6 cells were treated with different concentrations of OA (0.2–3.0 mM) for 1–3 days. For the α -NETA treatment group, confluent Hepa 1-6 cells were given 300nM, 1000nM or 3000nM α -NETA 24h before OA induction.

Oil Red O staining

For Oil Red O staining, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 10 min, and stained for 15 min with 0.1% (w/v) Oil Red O solution in 60% (v/v) isopropanol. Cells were then washed several times with water, and excess water was evaporated by placing the stained cultures at approximately 32°C. Following image acquisition, the cellular incorporation of Oil Red O was quantified using the following procedure: 200 μ l of isopropanol was added to each well; plates were shaken at reverse transcription for 3 minutes to dissolve the Oil Red O; and 100 μ l of each sample and 100 μ l of isopropanol (blank) were transferred into separate wells in a 96-well plate. Absorbance was measured using a spectrophotometer at 500 nm. These steps were performed very quickly to prevent significant evaporation of isopropanol.

Biochemical analysis

Serum TC, TG, HDL, LDL, ALT and AST levels were measured using ELISA kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Histological analysis

Formalin-fixed, paraffin-embedded adipose tissue was sectioned and stained with hematoxylin and eosin. An area of 30 adipocytes per animal, totaling 180 adipocytes, were randomly measured at high magnification (200X) using Image Pro-Plus 6.0.

Formalin-fixed and paraffin-embedded livers were sectioned and stained with hematoxylin and eosin. Steatosis was quantified as the percentage of hepatocytes containing macrovesicular fat (a fat droplet equal to or larger than the size of the nucleus, often displacing the nucleus) or microvesicular fat (numerous small fat droplets surrounding a centrally located nucleus). The degree of steatosis was graded as S0 (<5%), S1 (5-33%), S2 (34-66%), or S3 (>66%) [6].

Monitoring of gene expression by quantitative RT-PCR (QPCR)

Total RNA was extracted using TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were reverse-transcribed into cDNA, and relative transcript abundances were estimated by quantitative PCR using a SYBR RT-PCR kit (Takara, Shiga, Japan) according to the manufacturer's instructions. The relative gene expression levels normalized to beta-actin were calculated using the DDCT method, where CT was the cycle threshold. PCR primers for reference genes are listed in Table 1.

Gene	Forward primer(5'-3')	Reverse primer(5'-3')
Beta-Actin	GTATCCATGAAATAAGTGGTTACAGG	GCAGTACATAATTTACAGAGAAGCAAT

CMKLR1	CGAGTTCTCAAACCTGAAGTCGC	CAAGTCCACAAAGTAGCCAAAGCC
Chemerin	TACAGGTGGCTCTGGAGGAGTTC	CTTCTCCCGTTTGGTTTGATTG
LDLR	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT
ADRP	AAGAGGCCAAACAAAAGGCCAGGAGACCA	ACCCTGAATTTTCTGGTTGGCACTGTGCAT
TNFα	CCGATGGGTTGTACCTTGTC	CGGACTCCGCAAAGCTAAG
PPARδ	ATGAGAAGTGCGATCGGATC	TCAGCCCCGCCACCAGCTT
IL-6	TTCCATCCAGTTGCCTTCTTG	GAAGGCCGTGGTTGTCAACC
HMGCR	CTTGTGGAATGCCTTGATTG	AGCCGAAGCAGCACATGAT
PPARα	TCTGTGGGCTCACTGTTCT	AGGGCTCATCCTGTCTTTG
PPARγ	TCTGGGAGATTCTCTGTTGA	GGTGGGCCAGAATGGCATCT
HSL	ACAGTGCAGGTGGGAATCTC	GCCTAGTGCCTTCTGGTCT

Table 1: Primers were synthesized by Invitrogen. Mouse primer sequences were as follows.

Measurement of Hepatic TG

Liver tissues (100 mg wet tissue) were homogenized in an ice-cold 0.05% butylhydroxytoluene. After lipids were extracted with a slight modification, TG content in each sample was measured with a commercial assay kit (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

Data are presented as means ± SEM. The Student’s t-test was used to analyze all parametric data. The Fisher’s exact test was used to compare disease incidence. Values of p less than 0.05 were considered statistically significant.

Results

a- NETA suppresses fat deposition under high fat feeding

To determine the potential role of CMKLR1 in fat accumulation, BALB/C mice were fed a normal chow diet (NCD), a high fat diet (HFD), or a HFD in conjunction with injection of a- NETA twice a week (a-NETA+HFD, n=12). Adipose tissue was stained to measure the sizes of adipocytes by microscopy. As shown in Figure 1A, the adipocytes of epididymal fat pads were larger in mice fed the HFD for 8 weeks than in mice fed the NCD. This effect was reduced by a-NETA treatment, and there was a significant decrease in the areas of adipocytes between the HFD and HFD+a-NETA groups, as determined by morphometric analysis of adipose tissue (Figure 1B). Our results show that a-NETA treatment significantly reduces fat accumulation in adipose tissue. To test whether the histological differences in adipose tissue are associated with altered gene expression, we performed quantitative analysis (QPCR) on epididymal fat pads from NCD, HFD, and HFD+a-NETA mice (n=12) at 2 and 8 weeks of feeding. Chemerin and its receptor were more highly expressed in mice fed the HFD for 8 weeks, but no significant difference in chemerin and CMKLR1 expression was found between NCD and HFD mice fed for 2 weeks (Figure 1C). Expression of adipose accumulation marker genes, such as PPARγ, HSL and ATGL, was significantly increased in the HFD group relative to the NCD group at 2 weeks. The HFD effect at both 2 and 8 weeks was mitigated

by α-NETA treatment and these was more pronounced in the adipose tissue from mice fed the HFD for 8 weeks (Figure 1D). Expression of inflammation-related genes (TNFα and IL-6) was not different between the groups at 2 weeks, but there was a remarkable difference between the two groups after 8 weeks of feeding (Figure 1E). These results of QPCR further indicated that α-NETA affects fat accumulation by suppressing genes involved in adipogenesis and inflammation.

Figure 1: Effect of α-NETA on fat accumulation. **A:** H and E staining analysis of adipose tissue from male BALB/C mice fed a normal chow diet, a high fat diet, or a high fat diet in conjunction with 1 mg/kg α-NETA twice a week. **B:** Adipocyte areas in the fat of NCD, HFD and α-NETA-treated HFD BALB/C mice were measured (n=5-7). **C:** Chemerin and CMKLR1 mRNA expression in adipose tissue was determined by qRT-PCR. **D and E:** QRT-PCR analysis of metabolic and inflammatory gene expression in adipose tissue. Original magnification, 40x. The mRNA expression levels are presented as mean ± sem. aP<0.05 versus mice fed the NCD. bP<0.05 versus mice fed the HFD.

α-NETA suppresses adipose accumulation in liver

Macroscopic analysis revealed hypertrophy and the color became shallow in the livers from mice fed the HFD for 8 weeks relative to those from mice fed the NCD for 8 weeks (Figure 2A), while livers from HFD+α-NETA mice were less hypertrophic than those from HFD mice (Figure 2A). There was an excess of fat in the livers of mice fed the HFD at both 2 and 8 weeks, in which at least 5% of hepatocytes contained lipid droplets, a symptom of NAFLD, whereas livers were normal in the NCD group (Figure 2D). Livers from the NCD group showed no signs of lipid accumulation, with normal hepatic lobule structure (Figure 2A), whereas there was obvious lipid accumulation in the livers from the HFD group in H&E-stained sections (Figure 2B) that was reduced in the α-NETA treated HFD group. Figure 2C presents statistical analysis of lipid accumulation for the three groups. For each liver, lipid accumulation was assigned one of 4 grades according to the percentage of hepatocytes exhibiting significant lipid accumulation (Materials and Methods). At week 2, the frequency of steatotic hepatocytes seen in liver tissue sections was greater than 5% (grade S0). At week 8, the frequency of steatotic cells was greater than 66% (grade S3), and many cells contained macrovesicular fat. By contrast, the HFD+α-NETA group had far fewer steatotic cells (grade S1), especially at week 8, indicating that α-NETA treatment significantly inhibited the development of steatosis. To determine whether the observed differences in lipid accumulation between the groups was associated with changes in the expression of chemerin, CMKLR1 (Figure 2D), and lipid metabolism-associated genes (Figure 2E), we examined mRNA expression levels in the livers of all three groups (Figure 2E), which contrasts with the adipose tissue of these mice, in which both CMKLR1 and chemerin mRNA expression increased (Figure 1C). Expression of mRNAs for the lipid accumulation marker genes ADRP, HMGCR, C/EBPα, PPARγ, PPARδ, HSL, LDLR, and AdipoR1, and the inflammation-related genes IL-6 and TNFα was measured by qRT-PCR. In contrast to the NCD, the HFD taken for 2 weeks increased the levels of HSL, LDLR, C/EBP α, PPARγ, PPARδ, IL-6 and TNF α in the liver (Figure 2E). α-

NETA treatment significantly alleviated HFD induction of these genes. Furthermore, the HFD taken for 8 weeks increased the levels of HSL, PPARα, PPARγ, IL-6 and TNFα in the liver, while α-NETA treatment inhibited the upregulation of PPARα, IL-6 and TNFα in these livers.

Figure 2: Effect of α-NETA on hepatic fat deposition. Mice were fed NCD, HFD or HFD+α-NETA for 2 or 8 weeks. **A and B:** Representative images and morphological analysis of livers from different groups of BALB/C mice. **C:** Statistics for degree of hepatic steatosis in HFD and α-NETA treated HFD mice. Bar=50 um. **D:** Expression of chemerin and CMKLR1 mRNA in livers from the three groups, as determined by quantitative RT-PCR. **E:** QRT-PCR analysis of metabolic and inflammatory gene expression in liver for each group. Original magnification, 40X. Quantification of mRNA expression is presented as mean ± sem; aP<0.05 versus mice fed the NCD. bP<0.05 versus mice fed the HFD.

	Body weight gain	TC	TG	ALT	AST	HDL	LDL
	(g)	(mM)	(mM)	(U/L)	(U/L)	(mM)	(mM)
2 weeks of feeding							
NCD	3.77 ± 0.31	3.72 ± 0.27	1.13 ± 0.11	25.69 ± 2.1	21.22 ± 1.7	0.29 ± 0.02	3.43 ± 0.25
HFD	4.99 ± 0.38a	4.42 ± 0.26a	1.43 ± 0.14a	36.16 ± 3.8	23.09 ± 1.6	0.42 ± 0.02a	4.11 ± 0.28a
HFD+NETA	4.97 ± 0.23	3.81 ± 0.21b	0.78 ± 0.06b	33.96 ± 3.4	22.39 ± 1.9	0.34 ± 0.03	3.46 ± 0.27b
8 weeks of feeding							
NCD	7.29 ± 0.67	2.33 ± 0.19	1.12 ± 0.09	36.8 ± 2.9	19.07 ± 1.2	0.55 ± 0.04	1.78 ± 0.11
HFD	9.46 ± 0.56a	3.55 ± 0.29a	1.53 ± 0.12a	87.04 ± 7.3a	26.03 ± 1.9a	1.03 ± 0.06a	2.72 ± 0.16a
HFD+NETA	7.83 ± 0.61b	3.38 ± 0.27	0.89 ± 0.08b	51.93 ± 4.7b	21.57 ± 1.1b	1.12 ± 0.09	2.47 ± 0.17

Mice fed NCD, or HFD co-treatment with α-NETA for 2 or 8 weeks were fasted and subjected to each measurement (n=6-8). aP < 0.05 versus mice fed NCD. bP < 0.05 versus mice fed HFD.

Table 2: Lipids and liver enzymes in mice.

Lipids and liver enzymes in mice

To determine the effect of α -NETA on lipolysis in mice, we measured serum levels of TC, TG, ALT, AST, HDL and LDL, markers of fat synthesis and catabolism. As shown in Table 1, body weights increased more in mice fed the HFD for 2 or 8 weeks than in the mice fed the normal diets. No significant difference in weight gain was observed between mice fed the HFD and mice fed the HFD with α -NETA administration for 2 weeks, while weight gain in the 8-week α -NETA treated HFD mice was significantly lower than that in the 8-week HFD mice. The histological results shown in Figure 3 suggest that α -NETA suppresses HFD-induced fat deposition in the liver. We therefore measured hepatic TG content under the high fat dietary regimen. Consistent with the observed body weight gain, hepatic TG content was greater in mice fed the HFD for 8 weeks than in mice fed the NCD for 8 weeks. In the HFD+ α -NETA group, hepatic TG content was notably lower than that in mice fed the HFD.

In mice fed the HFD for 2 weeks, there was significant fat deposition in the liver and adipose tissue and increases in serum TG and TC; these changes were all markedly suppressed by α -NETA treatment. Similar results were observed after 8 weeks of feeding. However, α -NETA treatment did not suppress the increase in serum TC levels after 8 weeks on the HFD (Table 2).

The lipoprotein profiles shown in Table 1 reveal that mice fed the HFD for 2 and 8 weeks had higher serum levels of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) than mice fed normal diets. α -NETA administration significantly suppressed the increase in serum levels of LDL after 2 weeks of HF feeding but had no effect on mice after 8 weeks of high fat feeding. Elevation of serum aminotransferase level, a marker of liver damage, is correlated with NAFLD. In agreement with this, we show here that the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly higher in mice fed the HFD for 8 weeks than in mice fed the normal diet, but the serum levels of both ALT and AST were not significantly changed after 2 weeks of high fat feeding (Table 2). α -NETA treatment significantly suppressed the elevation in serum ALT and AST after 8 weeks of high fat feeding.

α -NETA treatment of 3T3-L1 cells

To further test our overall concept, we performed in vitro experiments to determine the effects of α -NETA on the differentiation of 3T3-L1 cells, an adipocyte cell line. Adipogenesis was verified by documenting an increase in intracellular lipid accumulation, as measured by Oil Red O staining (Figure 3A). As evidenced by Oil Red O staining, after differentiation for 10 d, α -NETA treatment suppressed lipid accumulation in 3T3-L1 cells when compared to untreated control cells (Figure 3A). Moreover, α -NETA treatment produced a dose-dependent reduction in lipid accumulation in the 3T3-L1 adipocyte differentiation model, which was verified by a quantitative test (Figure 3B). To quantify lipid accumulation, cellular incorporation of Oil Red O was measured using a spectrophotometer at 500 nm. The optical density (OD) was significantly decreased by 3 μ M α -NETA treatment over that in untreated control cells (Figure 3B). To verify the effects of α -NETA treatment on adipocyte differentiation in 3T3-L1 cells, qPCR analysis was used to measure the levels of mRNAs for the adipocyte marker genes C/EBP α , FABP4, PPAR γ , HSL, and ATGL and the inflammatory factors IL-6 and TNF α before differentiation and at 1 and 2 days post-differentiation. As shown in Figure 3C, the expression levels of both chemerin and CMKLR1 mRNAs were significantly increased in 3T3-L1 cells at 1 and 2 days post-differentiation relative to

the expression levels before differentiation. In parallel, C/EBP α , FABP4, PPAR γ , HSL, ATGL, IL-6 and TNF α mRNA levels increased with increasing time of exposure to the adipogenic medium (Figure 3D). To determine the effect of α -NETA on adipogenesis, different doses of α -NETA (300 nM, 1000 nM, and 3000 nM) were added to the differentiation medium. By two days post-differentiation, the expression levels of C/EBP α , HSL, ATGL, and TNF α were reduced by α -NETA treatment. Interestingly, α -NETA treatment produced a dose-dependent reduction in the levels of adipocyte target gene mRNAs. Significant reductions in C/EBP α , HSL, ATGL and TNF α expression were observed at 3000 nM α -NETA when compared with the untreated controls (Figure 3D).

Figure 3: Effect of α -NETA on lipid accumulation in the 3T3-L1 adipocyte differentiation model. **A:** 3T3-L1 cells were cultured with or without Induction Medium (IM). After differentiation for 10 d, lipid droplets in the cells were stained with Oil Red O. **B:** Quantification by spectrophotometry on uninduced and induced 3T3-L1 cells at different concentrations of α -NETA. **C:** Expression of chemerin and CMKLR1 was measured before differentiation and at 1 and 2 days post-differentiation. **D:** Gene expression of metabolic and inflammatory was measured after treatment with different dosages of α -NETA before differentiation and at 1 and 2 days post-differentiation. Quantification of Oil Red O staining and mRNA expression is presented as mean $\pm$ sem. aP<0.05 versus w/o IM group. bP<0.05 versus IM group

α -NETA treatment of Hepa 1-6 cells

An vitro model of steatosis was established in Hepa 1-6 cells through oleic acid (OA) treatment to explore the effects of α -NETA on hepatic steatosis. Hepa 1-6 cells were cultured at OA concentrations of 0.2, 0.5, 0.8, 1.0 and 3.0 mM for 3 days (data not shown), and Oil Red O staining was used to examine the accumulation of lipid droplets (Figure 4A). Oil Red O staining revealed an almost complete absence of intracellular lipids without OA treatment. After treatment with different doses of OA, however, large lipid droplets accumulated in the cytoplasm, and OA at a concentration of 0.8 mM reliably induced steatosis in the Hepa 1-6 cells.

Chemerin and CMKLR1 mRNAs were measured by quantitative real-time PCR in Hepa 1-6 cells after 0.8 mM OA induction for 1, 2 and 3 days. Expression of both chemerin and CMKLR1 mRNAs was

significantly increased in Hepa 1-6 cells in the cells treated with OA for 2 and 3 days when compared to cells not treated with OA (Figure 4C).

In parallel, the levels of ADRP, C/EBP α , PPAR α , PPAR γ , PPAR δ , HSL, HMGCR, IL-6 and TNF α mRNAs were significantly increased by 0.8 mM OA treatment, especially at 2 and 3 days (Figure 4D). When Hepa 1-6 cells were pre-treated with α -NETA (300 nM, 1000 nM or 3000 nM) before treatment with 0.8 mM OA for 3 days, expression of PPAR γ , IL-6 and TNF α mRNAs was decreased (Figure 4D), and PPAR δ was downregulated at 3000 nM α -NETA (Figure 1).

Figure 4: Oleic acid-induced steatosis in Hepa 1-6 cells. **A:** Different doses of α -NETA were added to Hepa 1-6 cultures 24 h before OA induction, then cells were incubated for 3 days with oleic acid (OA), and lipid droplets in cells were stained with Oil Red O and quantified by measuring OD. **B:** Results for untreated and OA-treated Hepa 1-6 cells with different doses of α -NETA. Hepa 1-6 cells were treated with OA or with OA followed by injection of α -NETA. **C, D and E:** Gene expression was measured in the different groups of cells. Quantification of Oil Red O staining and mRNA expression is presented as mean $\pm$ sem. aP<0.05 versus w/o OA group. bP<0.05 versus OA group.

Discussion

Here we describe the effect of α -NETA on fat accumulation in adipose tissue and liver. Extended periods of high fat feeding have been shown to lead to obesity and non-alcoholic fatty liver disease (NAFLD). Chemerin, as an adipokine, plays an important role in systemic inflammation, insulin sensitivity and fat accumulation [25].

α -NETA which reported by Kareem I, et al. [26] can inhibit the binding of CMKLR1 to its ligands. Morphology by H&E staining revealed a significant decrease in fat accumulation in α -NETA-treated adipose tissue and liver, demonstrating that CMKLR1 can indeed affect adipogenesis in these two organs. In our vitro study, we found that α -NETA inhibited OA-induced steatosis and delayed the progression of NAFLD. This indicated that CMKLR1 may participate in the development of NAFLD.

Our results show that both chemerin and its receptor, CMKLR1, are highly expressed in both the 3T3-L1 adipocyte differentiation model and the HFD mouse obesity model. Furthermore, we found that α -NETA strongly suppresses lipid accumulation and adipocyte gene

expression via the inhibition of binding of CMKLR1 with its ligands. Thus, this study indicates that binding of chemerin to CMKLR1 may participate in the process of adipogenesis. Both Oil Red O staining and measurement of adipocyte gene expression demonstrated differentiation of 3T3-L1 cells from preadipocytes to mature adipocytes. Interestingly, α -NETA treatment strongly suppressed the expression of C/EBP α , HSL, ATGL and TNF α in the 3T3-L1 adipocyte differentiation model. Moreover, the same effect was found in the HFD-induced obesity mouse model. Our results show that the upregulation of adipocyte genes in the WAT of obese mice was reduced by α -NETA administration. Thus, α -NETA could interfere with the lipid metabolism involved in both adipogenesis in the 3T3-L1 cell model and obesity in the HFD mouse model.

Otherwise both chemerin and CMKLR1 mRNAs are expressed in Hepa 1-6 cells and in the liver. Moreover, the expression of both chemerin and CMKLR1 increased in OA-induced Hepa 1-6 steatosis, and the expression of CMKLR1 increased in the livers of mice in the NAFLD model. Administration of α -NETA inhibited the hepatic steatosis induced by OA in the Hepa1-6 cells and attenuated the progression of hepatic steatosis in the liver. Furthermore, the expression of both PPAR γ and PPAR δ was suppressed by α -NETA. PPAR γ plays a major role in the regulation of adipogenesis, stimulates fatty acid storage in adipocytes and suppresses free fatty acid secretion, whereas PPAR α and PPAR δ stimulates the β -oxidation of fatty acids [20,27]. The fact that α -NETA suppressed the expression of PPAR γ and PPAR δ suggests that α -NETA may inhibit adipogenesis and the storage of fatty acids and cholesterol in the liver.

Low-density lipoprotein receptors (LDLRs) play a critical role in regulating the amount of cholesterol in the blood. They are particularly abundant in the liver [8]. Low-level expression of HSL was also observed at an early stage in the induction of steatosis by OA, consistent with the increase in serum triacylglycerol levels at 2 weeks in NAFLD mice, and both of these changes were suppressed by α -NETA treatment. In contrast, serum triacylglycerol levels decreased at 8 weeks in NAFLD mice, which indicates impaired liver function, and this impairment was not prevented by α -NETA. HSL is a triglyceride lipase that functions to hydrolyze the first fatty acid from a triacylglycerol molecule, freeing a fatty acid and diglyceride [8,28]. It has been reported that the expression of HSL is markedly decreased in the livers of obese mice [21,22,28]. Therefore, it suggests that α -NETA promotes triglyceride hydrolysis in the early stage of steatosis. Fatty acids are used for β -oxidation in mitochondria and peroxisomes under the regulation of PPAR α [23,24,29]. The impact of CMKLR1 deficiency on adipose development, glucose homeostasis, and inflammation in vivo was investigated. Regardless of diet, CMKLR1 $^{-/-}$ mice exhibited decreased hepatic TNF α and IL-6 mRNA levels [30]. In the current study, the increased expression of PPAR α induced the oxidation of fatty acids and resulted in NASH, which presents as high-level expression of IL-6 and TNF α in NAFLD mouse livers. Treatment with α -NETA suppressed the expression of PPAR α , IL-6 and TNF α . As summarized above, this indicates that α -NETA may inhibit the progression of NAFLD into NASH.

Our study describes the effect of α -NETA on fat accumulation in fat tissue and liver. High fat feeding leads to obesity accompanied by NAFLD, and we propose that α -NETA may inhibit steatosis and the progression of NAFLD through suppression of the accumulation of fatty acids/triglycerides/cholesterol in the liver. Our study suggests that CMKLR1 and its ligand chemerin could be targeted to treat NAFLD.

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