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RNAi-mediated Ghrelin affects gastric H^+–K^+-ATPase activity and expression of GOAT–Ghrelin system *in vitro*

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ABSTRACT

Ghrelin has been implicated in the regulation of gastric functional development, and its physiological functions are mediated by Ghrelin-*O*-acyltransferase (GOAT) which is capable of generating the active form of this polypeptide hormone. However, whether and how ghrelin gene silencing may modify gastric acid secretion and GOAT–Ghrelin system is yet to be explored. The study was performed in gastric mucosal cells from weanling piglets *in vitro*. We evaluated the effect of ghrelin on gastric acid secretion, gene expression of GOAT and ghrelin as well as ghrelin levels by RNA interference assay. shGhrelin triggered the down-regulation of ghrelin mRNA expression ($P < 0.05$) via an RNAi mechanism, as observed by real-time RT-PCR. In addition, shGhrelin showed reduced total ghrelin production and secretion ($P < 0.05$) using ELISA *in vitro*. We also detected that GOAT mRNA expression was reduced in shGhrelin group ($P < 0.05$), compared with control groups. In accordance with the GOAT expression, acylated ghrelin production and secretion were reduced in gastric mucosal cells and culture medium ($P < 0.05$). Silencing of ghrelin gene achieved by RNAi-mediation inhibited the activity of H^+–K^+-ATPase and pepsin ($P < 0.05$) in gastric mucosal cells. These results indicated that RNAi of Ghrelin gene inhibited the gastric acid secretion with decreased GOAT mRNA and acylated Ghrelin in gastric mucosal cells.

1. Introduction

Growth performance and health status of weanling piglets are issues of great concern to swine industry, because growth retardation and diarrhea reduce animal productivity and welfare. These problems are related, at least partly, to immature gastric function, especially gastric acid secretion, in nursing piglets (Hayashida et al., 2001). The proton pump, H^+–K^+-ATPase is typically located in the parietal cells, mediating the electroneutral exchange of intracellular H^+ and extracellular K^+ to achieve acid secretion when parietal cells are under the stimulation of secretagogues (Yao and Forte, 2003). Therefore, H^+–K^+-ATPase can serve as an accurate indicator for evaluating the ability of gastric acid secretion from parietal cells. The acidification of the stomach contents by H^+–K^+-ATPase is essential for the activation of the digestive enzyme pepsinogen, which is the main enzyme degrading food proteins into peptides.

Gastric acid secretion is subjected to an important hormonal regulation. The bulk of ghrelin in the body occurs in the A-like cells of the acid-producing part of the stomach, and plasma ghrelin-like immunoreactivity levels in totally gastrectomized patients were reduced to 35% of those in normal controls (Li et al., 2011), which suggested that ghrelin played the important role in regulating gastric acid secretion. Ghrelin is a 28-amino acid polypeptide produced and secreted from endocrine X/A-like cells in oxyntic glands of the stomach (Hayashida et al., 2001). Subsequently, researches demonstrated that ghrelin has a wide spectrum of biological activities, such as stimulation of gastric motility, gastric acid secretion (Date et al., 2001; Masuda et al., 2000; Yakabi et al., 2008) and stimulation of appetite (Kiewiet et al., 2009). Biological activities of ghrelin are mainly dependent on the attachment of octanoate to the third serine residue, a reaction catalyzed by Ghrelin *O*-acyltransferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008). Acylated ghrelin may then exert a stimulating effect and play a significant role in modulating gastric function development via a variety of mechanisms. Conflicting results are available about the effects of both ghrelin isoforms on gastric acid secretion (Sakurada et al., 2010). What is exactly the function

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of ghrelin in gastric acid secretion is still not clear. Furthermore, the exact mechanism of action and influence on gastric acid secretion remains unexplored. In the GOAT–Ghrelin system, GOAT plays a critical role through modulating the acyl ghrelin levels. This is important and the involvement of GOAT in ghrelin levels or its biological effects could represent an attractive therapeutic target (Kang et al., 2012).

RNA interference (RNAi) is a mechanism responsible for transcriptional (Janowski et al., 2006; Calero-Nieto et al., 2010) and post-transcriptional gene silencing (Elbashir et al., 2001). The silencing mechanisms can lead to the degradation of a target mRNA, as induced by small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs). shRNAs are introduced into the nuclei of target cells using either bacterial or viral vectors that, in some cases, can stably integrate into the genome. But siRNAs typically require perfect homology to induce degradation. Advantages of shRNA over siRNA include the ability to use viral vectors for delivery to overcome the difficulty of transfecting certain cell types, the option to control shRNA expression using inducible promoters, and the ability to co-express them with a reporter gene (Rao et al., 2009).

In this work, we evaluated the effects of ghrelin on gastric acid secretion *in vitro* using gastric mucosal cells models, by investigating the activity of H^+–K^+-ATPase and pepsin, as well as to investigate the biological role of GOAT involvement in regulation mechanisms by RNA interference assay.

2. Materials and methods

2.1. Design of shRNA and plasmid construction

The siRNA sequence targeting ghrelin gene (GenBank accession number: AF308930) was designed using Dharmacon siRNA Design tool (http://www.dharmacon.com). The siRNA sequences used in this study are as follows: siGhrelin: 5′-CAAGAAGCCAGCAGC CAAAU-3′. A siRNA with a scrambled sequence, 5′-CAGAACAGAGC CACACAGAU-3′, unable to induce degradation in any cellular mRNA, was used as negative control of specificity (shNC). All these DNA oligonucleotides were chemically synthesized by Invitrogen (Shanghai, China). A widely used siRNA expression vector, pGPU6/GFP/Neo (GenePharma, Shanghai, China) was selected to be the parental plasmid.

2.2. Cell culture and RNA transfection

Gastric mucosa was obtained from 21-week-old weanling Suzhong piglets with average body weight of 10 kg and cells were dispersed as described previously (Terano et al., 1982) with minor modifications. Briefly, the tissue was washed thoroughly in D-Hank's solution containing antibiotics (100 U penicillin/ml and 100 U streptomycin/ml) for about 1 h and digested with 0.15% trypsin at 37 °C for 30 min, filtered and centrifuged (1000 r/min, 5 min). Thereafter cells were cultured at a density of 2×10^6 per well (37 °C, 5% CO_2) in DMEM/F-12 supplemented with 10% fetal bovine serum, 5 mM HEPES buffer, and 100 U/ml antibiotics.

For transfection experiments, 1×10^5 gastric mucosal cells were cultured in six-well plates to 80–90% confluency. Cell transfection was performed using Lipofectamin 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Briefly, for each well, 3 μg of the respective shRNA plasmid was incubated with 250 μL serum-free medium for 5 min. Subsequently, a mixture of 9 μL Lipofectamine 2000 and 241 μL serum-free medium incubated for 5 min was added slowly to the plasmid mixture. After incubation for 20 min at room temperature, 1 mL serum-free medium was added to the dilution, mixed gently and then added to the well. After incubation at 37 °C for 5 h, the medium containing the transfection mix was replaced with growth medium. After 48 h of incubation, the plates were freeze/thawed three times and supernatants and cells were collected for real-time RT-PCR analysis and ELISA. At the same time, non-transfection normal cells were used as black group (BC). All experiments in this study were performed in triplicate.

2.3. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA from cells was extracted using a Trizol kit (TaKaRa, China). Total RNA concentration was then quantified by measuring the absorbance at 260 nm in a photometer (Eppendorf Biophotometer). Ratios of absorption (260:280 nm) of all preparations were between 1.85 and 2.01. Trace DNA contamination was removed by DNase digestion (TaKaRa, China). Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose–formaldehyde gel to verify their integrity. cDNA was synthesized from aliquots (1 μg) of DNAase-treated total RNA with the PrimeScript 1stStrand cDNA Synthesis kit (TaKaRa, China). It is a routine practice to confirm the absence of genomic DNA contamination in samples used for Real Time PCR analysis. Primers were designed to span an intron so any genomic DNA contamination can be easily reported by an extra PCR product in melting curve analysis.

The real-time RT-PCRs of ghrelin and GOAT cDNA and GAPDH as internal control were carried out using the SYBR-Green I chemistry (TaKaRa Co. Nanjing, China). Amplification primers of the three genes were synthesized by TaKaRa Co. (Nanjing, China) as follows: ghrelin F: 5′-GCTCCTCATGGCAGACTTGG-3′ and R: 5′-TGGACTCCTTTCTCTGCTGC-3′, GOAT F: 5′-TTCAGATGAGCTGGCAGACG-3′ and R: 5′-CGCTTCCTACAGCTTCCGAT-3′, and GAPDH F: 5′-CGTCCCTGAGACACGATGGT-3′ and R: 5′-CCCGATGCGGCCAAAT-3′. Two microliters of diluted cDNA (1:10) were used in each real-time PCR assay with the 7500 Fast Real-Time PCR System (Applied Biosystems). The cycle profile was programmed as follows: 30 s at 95 °C (initial PCR activation); and 40 cycles of 5 s at 95 °C (denaturation), 30 s at 63 °C (annealing); the final dissociation step was 15 s at 95 °C, 30 s at 63 °C, 15 s at 95 °C. Results were analyzed with the Ct ($2^{-\triangle\triangle Ct}$) method to compare the transcriptional levels of the target genes relative to BC group.

2.4. Biochemical analysis

Whole cells samples were prepared by homogenization and mild sonication in cold RIPA buffer with antiproteases (Sigma, Shanghai, China). Tissue lysates were centrifuged for 15 min at 12,000*g* in a microfuge at 4 °C. Culture medium and cells lysates supernatant were collected in a tube containing AEBSF (BEST, Chengdu, China) to a final concentration of 1 mg/mL, let at room temperature for 30 min, and immediately centrifuged at 4 °C. Cultural medium and supernatant were acidified with HCl to a final concentration of 0.05 M. Total and active ghrelin levels in the cells culture medium and cells were determined using a Sandwich ELISA kit (EMD Millipore, Hong Kong, China) according to the manufacturer's instructions. The limit of assay sensitivity was 40 pg/mL for total and 8 pg/mL for active ghrelin.

2.5. H^+–K^+-ATPase and pepsin activity

H^+–K^+-ATPase activity (Catalogue number A069) and pepsin (Catalogue number A080-1) in gastric mucosal cells were respectively measured with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activity of H^+–K^+-ATPase was analyzed by measured the inorganic phosphate release after hydrolysis of ATP. The lysates of gastric mucosal cells was made and centrifuged at 3500 rpm for 15 min. The amount of inorganic phosphate released per milligram of protein per hour (μmol Pi/