Ghrelin Relieves Obesity-Induced Myocardial Injury by Regulating the Epigenetic Suppression of miR-196b Mediated by IncRNA HOTAIR

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Keywords
Ghrelin · Obesity · miR-196b · Homeobox transcript antisense RNA

Abstract
Introduction: Obesity has been believed to be closely linked with many kinds of diseases including atherosclerosis, hypertension, cerebrovascular thrombosis, and diabetes. Ghrelin and Homeobox transcript antisense RNA (HOTAIR) were believed to be involved in the regulation of myocardial injury. Methods: The obesity mice model was established through feeding mice (C57BL/6J, male, eight-week-old) with high-fat diet and palmitate (PA)-induced cardiomyocyte injury. RNA and protein levels were detected with Quantitative real-time PCR and Western blotting. The levels of TG, TCH, LDL, CK-MB, cTnl, and BNP in the serum or cell medium supernatant were measured using ELISA kits. The ROS level was detected with the DCFH-DA method. Binding sites between different targets were identified using detection of dual luciferase reporter assay. Cell apoptosis was analyzed by flow cytometry. RNA-binding protein immunoprecipitation and chromatin immunoprecipitation were used to detect the binding of DNMT3B with HOTAIR or miR-196b promoter. Results: The expression of HOTAIR was downregulated, and miR-196b was upregulated in the obese myocardial injury. Ghrelin attenuated PA-induced cardiomyocyte injury by increasing HOTAIR. HOTAIR regulated the expression of miR-196b by recruiting DNMT3B to induce methylation of the miR-196b gene promoter. The binding site between miR-196b and IGF-1 was identified. Discussion/Conclusion: We demonstrated that ghrelin attenuated PA-induced cardiomyocyte injury by regulating the HOTAIR/miR-196b/IGF-1 signaling pathway. Our findings might provide novel thought for the prevention and treatment of obesity-induced myocardial injury by targeting HOTAIR/miR-196b.

Introduction

Obesity is characterized by a body mass index equal or more than 30 kg/m², and it is becoming common in the Western world. The incidence of obesity in China is increasing greatly in the past decades with the changes of...
Ghrelin Relieves Myocardial Injury

Ghrelin, a 28-amino acid peptide, has been proved to be closely linked with the regulation of cardiovascular disease. Ghrelin exerts cardioprotective function through growth hormone secretagogue receptor. Ghrelin and its receptor GHS-R 1A have been reported to be constitutively expressed in cardiomyocytes and other myocardial cells [4, 5], suggesting an autocrine/paracrine role of ghrelin in the heart. Meanwhile, total ghrelin levels were proved to be decreased in several obesity-associated heart disorders, such as left ventricular hypertrophy [6], coronary heart disease [7], and myocardial infarction [8]. The inhibition of endothelial dysfunction, sympathetic nerve activation, apoptosis, and inflammation by ghrelin accounts for the cardiovascular protection [9]. We previously demonstrated that ghrelin reduces the cardiac injury induced by obesity by affecting the IncRNA H19/miR-29a/IGH1 signaling pathway [10]. However, whether ghrelin could protect against cardiac injury through other signaling pathway needs to be further explored.

Long noncoding RNAs have been proved to play an important role in many types of life activities [11]. Some reports indicated that long noncoding RNA acted a regulatory role in myocardial cell injury [12]. Homeobox transcript antisense RNA (HOTAIR) is located at 12q12.13, and it is known as a custom tilling array of HOXC locus. It is reported that HOTAIR protected cardiomyocytes from oxidative stress-induced injury [13, 14]. However, its role and regulatory mechanism in obese myocardial injury are still unclear.

It was reported that DNMT3B regulated the Rictor levels through miR-196b promoter methylation [15]. We speculate that ghrelin upregulates the expression of HOTAIR, which promotes hypermethylation of the miR-196b gene by recruiting DNMT3B, and then increase the expression of IGF-1, thus alleviating the progress of obesity-induced myocardial injury. This report might provide novel thought for the prevention and treatment of obesity-induced myocardial injury by targeting HOTAIR/miR-196b.

Materials and Methods

Establishment of the Obesity Mice Model
C57BL/6 mice (Male, 8-week-old, 20–24 g) were purchased from Charles River (Beijing, China). All experiments were approved by the Second Affiliated Hospital of Nanchang University Medical Research Ethics Committee (the Examination and Approval No. Review [2020] No. [A901]). The animals were raised with free access to water and food. Mice in the group high-fat diet (HFD) were fed with HFD (carbohydrate-protein-fat ratio: 20%-20%-60%, 25 kJ/g) for 8 weeks. The mice in the control group were fed with normal diet (carbohydrate-protein-fat ratio: 20%-70%-10%, 14 kJ/g) for 8 weeks. After sacrifice, the blood and heart samples were collected for further experiments.

Hematoxylin-Eosin Staining
Tissue was isolated and fixed using 4% formaldehyde for 24 h. The optimal cutting temperature compound (OCT, Sigma, Ronkonkoma, NY, USA) was used for tissue embedding, and a cryostat was used to make 10-micron thick sections. Hematoxylin was used to stain tissues for 5 min. Tap water was used to wash 3 min, and 1% acetic acid was used for differentiation. After washing with water for 30 s, 0.1% ammonia water (20 s) was used for bluing. Tap water was used to wash 3 min, and tissues were rinsed using 95% alcohol. Then, the slides were stained using eosin for 15 s. Then, the slides were captured using Zeiss AxioVision (Jena).

Cell Culture and Treatment by Palmitate
Palmitate (PA) was purchased from Sigma (Ronkonkoma, NY, USA). PA (5 mM) was prepared using 5% BSA. Cardiac myocytes were cultured using DMEM medium (Gibco, Germany) containing 5% FBS on the condition of 5% CO2 at 37°C. After treatment with PA (5 mM) for 24 h, the cells were used for different experiments.

Cell Transfection
si HOTAIR, pcDNA-HOTAIR, and miR-196b mimics were purchased from RiboBio (Guangzhou, China). Cells (1 × 106 cells/well) were seeded into 6-well plates. Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) was used to package the plasmids into cells according to the instructions of manufacturer.

Detection of the GSH/GSSG Ratio
The measurement of GSH/GSSG ratio was performed as described previously [16]. Cells were plated into 6-well plates and cultured on the condition of 5% CO2 and 37°C. After treatment with transfection, cells were digested. GSSG and GSH were detected in cell extracts using HPLC method. Then, the GSH/GSSG ratio was calculated.

Flow Cytometry
Cells were plated into 6-well plates and cultured on the condition of 5% CO2 and 37°C. After treatment with transfection, cells were digested. After removing supernatant, the pellet was resuspended using 300 μL PBS buffer containing PI (5 μL) and Annexin V-FITC (5 μL). After incubation for 20 min, apoptosis was measured using flow cytometry.
RNA Isolation and Quantitative Real-Time PCR

TRizol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract RNA. Reverse transcription was performed with the Taqman miRNA Reverse Transcript Kit (Applied Biosystems, Waltham, MA, USA). The Prism 7500 Fast sequence detection system of Applied Biosystems was used to perform PCR reaction. Primers were listed as follows: miR-196b (forward: 5'-TTTTATTTGTTGTTGATTAGGTGGAG-3', reverse: 5'-AACCTTAACCTTGCCCCCTCTTAAC-3'), HOTAIR (forward: 5'-GGGGAAGTGCAAGTTAATACACGAGT-3', reverse: 5'-TAGGCGCTGAGTGGTCACGAGG-3'), IGF-1 (forward: 5'-CACATCACATCCTTCTTG-3', reverse: 5'-CTGGAACCGTACCCCTGTG-3'), U6 (forward: 5'-CTCGCTTCGGACACCACTCACAATTTGTC-3', reverse: 5'-AACCGCTCTCAGATTTGTCCT-3'), GAPDH (forward: 5'-ACAACAGCCTCAATTCGGCCAGCACA-3', reverse: 5'-GGTCCACACCTGACACGTGTG-3').

Western Blotting

Proteins were isolated with RIPA lysine buffer and measured using the BCA method. The proteins were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were cultivated with related primary antibodies (Abcam, UK) overnight at 4°C. After washing with PBS, membranes were cultivated with the secondary antibody (Abcam, UK) for 2 h at room temperature. Then, an ECL chemiluminescent kit (Advansta, San Jose, CA, USA) was applied to expose protein bands.

Measurement of TG, TCH, LDL, CK-MB, cTnI, and BNP

Blood samples were collected and centrifuged for 10 min at 1,000 g. Then, the concentrations of TG, TCH, LDL, CK-MB, cTnI, and BNP in the sera were measured through related commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Measurement of MDA and SOD

Blood samples were collected and centrifuged for 10 min at 1,000 g. The pyrogallol method (420 nm) was used to measure SOD activity. The SOD measurement kit was purchased from Shanghai Westang (Shanghai, China). The MDA level was measured using a commercial kit purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Measurement of ROS with the DCFH-DA Method

After treatments with transfection and PA, the cells were incubated with DCFH-DA (2 μM, Sigma, Ronkonkoma, NY, USA) for 30 min at 37°C. After washing 2 times with PBS, the ROS intensity was detected using a fluorescence microscope (488 nm).

Methylation-Specific PCR Assay

The miR-196b methylation was measured using methylation-specific PCR. The primer used for miR-196b was: forward: 5'-TTTTATTTGTTGTTGATTAGGTGGAG-3', reverse: 5'-AACCTTAACCTTGCCCCCTCTTAAC-3'. The PCR reaction was set as follows: initial denaturation (95°C, 4 min), 35 cycles of denaturation (95°C, 30 s), annealing (55°C, 40 s), extension (72°C, 55 s), and final extension (72°C, 5 min). Finally, electrophoresis was performed to separate PCR products.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation was performed according to the previous report [17]. The tissue was incubated with formaldehyde (2%) for 10 min. Then, glycine (2 mol/L) was added to incubate tissues for 15 min. After washing with PBS 3 times, the tissues were incubated with protease inhibitors and lysis buffer. After treatment with a homogenizer, 300–400 bp DNA fragments were achieved. After centrifugation (12,500 g, 5 min), chromatin was diluted using buffer and immunoprecipitated with protein A magnetic beads and nonspecific IgG or DNMT3B antibody. Then, the solutions were incubated for 12 h at 4°C. Immunoprecipitated complex-magnetic beads were collected using a magnetic separator. The primer used for miR-196b was: forward: 5'-TTTTATTTGTTGTTGATTAGGTGGAG-3', reverse: 5'-AACCTTAACCTTGCCCCCTCTTAAC-3'.

Detection of Dual Luciferase Reporter Assay

Cells were plated into 24-well plates first. Igf1-WT plasmid, Igf1-MUT plasmid, NC mimics, and miR-196b mimics were mixed using medium. The mixed solution was incubated with a transfection reagent for 24 h. Signals were identified using a Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

Statistical Analysis

Data are presented as means ± SD. Student’s t test was used to analysis between two groups. ANOVA was used to analyze among more than two groups. p < 0.05 was considered to be statistical significance. Experiments were conducted at least for three times.

Results

The Expression of HOTAIR Was Downregulated, and miR-196b Was Upregulated in the Obese Myocardial Injury

First, the HFD animal model was established. Compared with the group control, myocardial hypertrophy and fibrous disorder were observed in the HFD group through hematoxylin-eosin staining (Fig. 1a). In addition, remarkable higher levels of TG, TCH, LDL, CK-MB, cTnI, and BNP in the HFD group were found compared to the group control (Fig. 1b, c). Meanwhile, the expression of HOTAIR was significantly downregulated, but the level of miR-196b was upregulated in the HFD group (Fig. 1d). In the control group, the methylation of miR-
196b gene was obvious, while in the HFD group, the methylation of miR-196b gene promoter was not detected (Fig. 1e). Compared with the control group, the expression of HOTAI R was significantly downregulated, and miR-196b was significantly upregulated in the PA group (Fig. 1f). In the control group, the methylation of miR-196b gene was obvious, while in the PA-induced HCM cells, no methylation of miR-196b gene promoter was observed (Fig. 1g). Therefore, obesity caused myocardial injury, and HOTAI R and miR-196b might be related to this process.

**Fig. 1.** Expression of HOTAI R was downregulated, and miR-196b was upregulated in the obese myocardial injury. a Histological changes of myocardial tissues were measured using HE staining. b, c Levels of TG, TCH, LDL, CK-MB, cTnl, and BNP in the serum were detected by the ELISA kit. d, f Expression of HOTAI R and miR-196b was measured using qRT-PCR. e, g Methylation of the miR-196b gene was detected by MSP. HE, hematoxylin-eosin; qRT, quantitative real-time; MSP, methylation-specific PCR.

**Fig. 2.** Ghrelin attenuated PA-induced cardiomyocyte injury by increasing HOTAI R. a HOTAI R expression was measured using qRT-PCR. b Knockdown of HOTAI R was established using si HOTAI R transfection. c, d Levels of ROS, MDA and SOD were detected by using a commercial kit. e Ratio of GSH/GSSG was detected by using a commercial kit. f Cell apoptosis was analyzed by flow cytometry. g Apoptosis-related proteins were measured by Western blot. qRT, quantitative real-time.

(For figure see next page.)
a) Relative HOTAIR expression

b) Relative HOTAIR expression

c) Control vs PA

PA+Ghrelin vs HOTAIR

PA+Ghrelin+ si HOTAIR

b) MDA (mmol protein)

SOD (U/mg protein)

GSH/GSSG ratio

e) Control vs PA

PA+Ghrelin vs HOTAIR

PA+Ghrelin+ si HOTAIR

f) AnnexinV

Control vs PA

PA+Ghrelin vs HOTAIR

PA+Ghrelin+ si HOTAIR

g) Bcl-2

Bax

Cleaved caspase3

Total caspase3

β-actin

PA - + + +

Ghrelin - - + +

si HOTAIR - - + +

Control vs PA

PA+Ghrelin vs HOTAIR

PA+Ghrelin+ si HOTAIR

Relative protein level
Ghrelin Relieves Myocardial Injury

Compared with the control group, the expression of HOTAIR was significantly downregulated by PA, while the overexpression of ghrelin promoted the expression of HOTAIR (Fig. 2a). The expression of HOTAIR was significantly suppressed by si HOTAIR (Fig. 2b). The production of ROS induced by PA was significantly increased compared with the control group. Overexpression of ghrelin significantly inhibited the level of ROS induced by PA, but knockdown of HOTAIR significantly reversed the content of ROS (Fig. 2c). Meanwhile, PA induced a significant increase of MDA production and a remarkable decrease of SOD. However, overexpression of ghrelin significantly inhibited PA-induced MDA increase and promoted SOD levels. The changes of MDA and SOD by ghrelin induced were remarkably reversed by HOTAIR knockdown (Fig. 2d). Meanwhile, the PA-induced GSH/GSSG ratio was significantly reduced, and overexpression of ghrelin significantly reversed the inhibition of GSH/GSSG ratio by PA. While, HOTAIR knockdown significantly reversed the effect of ghrelin overexpression (Fig. 2e). In addition, compared with the control group, PA significantly increased the cell apoptosis rate, and ghrelin overexpression significantly inhibited PA-induced apoptosis. However, knockdown of HOTAIR reversed the influence of ghrelin on cell apoptosis (Fig. 2f). The expression level of Bcl-2 was suppressed, and the levels of Bax and cleaved caspase 3 were increased by PA. Overexpression of ghrelin significantly promoted the expression of Bcl-2 and inhibited the expression of Bax and cleaved caspase 3, while the expression of these apoptosis-

**Fig. 3.** HOTAIR regulated the expression of miR-196b by recruiting DNMT3B to induce methylation of the miR-196b gene promoter. a Methylation of the miR-196b gene promoter after overexpression of HOTAIR was measured by MSP. b Expression of miR-196b was detected by qRT-PCR. c Protein expression of DNMT3B was measured by Western blot. d Binding between HOTAIR and DNMT3B was measured by RIP. e Methylation of the miR-196b gene promoter after overexpression of DNMT3B was measured by MSP. f Expression of miR-196b was detected after DNMT3B knockdown by qRT-PCR. g Ability of DNMT3B to bind to the miR-196b promoter region was measured by ChIP. qRT, quantitative real-time; MSP, methylation-specific PCR; RIP, RNA-binding protein immunoprecipitation; ChIP, chromatin immunoprecipitation.
related proteins was significantly reversed after knockdown of HOTAIR (Fig. 2g). Therefore, ghrelin alleviated PA-induced cardiomyocyte injury by upregulating HOTAIR.

**HOTAIR Regulated the Expression of miR-196b by Recruiting DNMT3B to Induce Methylation of the miR-196b Gene Promoter**

Overexpression of HOTAIR promoted the methylation of miR-196b gene promoter significantly compared with group pcDNA3.1 (Fig. 3a). Meanwhile, overexpression of HOTAIR remarkably decreased the expression of miR-196b (Fig. 3b) and induced the upregulation of DNMT3B (Fig. 3c). In addition, we demonstrated that HOTAIR could bind with DNMT3B (Fig. 3d). In addition, overexpression of DNMT3B increased the methylation of the miR-196b gene promoter remarkably (Fig. 3e), but knockdown of DNMT3B markedly increased the expression of miR-196b (Fig. 3f). However, the ability of DNMT3B to bind with the miR-196b promoter region was significantly decreased after HOTAIR knockdown (Fig. 3g).

**Igf1 Was a Target Gene of miR-196b**

The relationship between miR-196b and Igf1 was predicted by StarBase (http://starbase.sysu.edu.cn/) in this study. The potential binding sites between miR-196b and Igf1 were found (Fig. 4a). Co-transfection of miR-196b mimics and the wild-type Igf1 vector significantly decreased the luciferase activity, but no significant change was found after treatment with the mutant-type Igf1 (Fig. 4b). miR-196b mimics significantly suppressed the mRNA and protein expression of IGF-1. Conversely, miR-196b inhibitor significantly promoted the mRNA and protein expression of IGF-1 (Fig. 4c, d).

**Ghrelin Attenuated PA-Induced Cardiomyocyte Injury by Regulating the HOTAIR/miR-196b/IGF-1 Signaling Pathway**

Overexpression of ghrelin significantly inhibited the level of ROS induced by PA. miR-196b mimics significantly promoted the level of ROS. Meanwhile, overexpression of IGF-1 significantly reversed the increased level of ROS caused by miR-196b mimics (Fig. 5a). Overexpression of ghrelin significantly inhibited the level of MDA induced by PA and promoted the level of SOD. miR-196b mimics significantly promoted the level of MDA and inhibited the
content of SOD. Overexpression of IGF-1 reversed the changes of MDA and SOD induced by miR-196b mimics (Fig. 5b). The decreased GSH/GSSG ratio induced by PA was significantly promoted by ghrelin overexpression. In addition, miR-196b mimics significantly inhibited the GSH/GSSG ratio, and overexpression of IGF-1 reversed the GSH/GSSG change caused by miR-196b mimics (Fig. 5c). The PA-induced cell apoptosis was inhibited by ghrelin overexpression, and miR-196b mimics remarkably promoted cell apoptosis. However, simultaneous overexpression of IGF-1 reversed the increase apoptosis rate induced by miR-196b mimics (Fig. 5d). In addition, the expression of Bcl-2 was increased and Bax and total caspase 3 were inhibited by overexpression of ghrelin. However, miR-196b mimics exerted the opposite effect on the levels of these apoptosis-related proteins. Overexpression of IGF-1 remarkably reversed the influence of miR-196b mimics on apoptosis-related proteins (Fig. 5e).

Fig. 5. Ghrelin attenuated PA-induced cardiomyocyte injury by regulating the HOTAIR/miR-196b/IGF-1 signaling pathway. **a**, **b** Level of ROS MDA and SOD were measured by a commercial kit. **c** GSH/GSSG ratio was measured by a commercial kit. **d** Cell apoptosis was analyzed by flow cytometry. **e** Apoptosis-related proteins were measured by Western blot.
Discussion

It was reported that more than 650 million adults were obese in the world, and the obese condition is closely associated with several types of diseases including cancers, type 2 diabetes, stroke, ischemic heart disease, and high blood pressure. Previous reports indicated that obesity might lead to cardiac injury through inducing apoptosis, lipotoxicity, mitochondrial dysfunction, and oxidative stress. However, the further mechanism how obesity affects cardiac injury has not been fully clarified.

Previous report suggested that the expression of HO-TAIR was promoted in the plasma and cardiac tissues of congenital heart diseases patients, and HO-TAIR might be a potential marker for the congenital heart diseases patients [18]. HO-TAIR could also act as the ceRNA through sponging miR-613 in regulating atrial fibrillation [19]. HO-TAIR presented protective function against hypoxia-induced injury and myocardial infarction via sponging miR-519d [20]. It was reported that miR-196b was linked negatively with cardiomyocytes differentiation [21]. In this study, the expression of HO-TAIR was significant lower, and miR-196b was increased in the myocardial injury induced by obesity. HO-TAIR and miR-196b played an important role regulating the obesity-induced myocardial injury.

Ghrelin has been believed to be an important regulator for vasculature and heart diseases. The myocardial injury caused by obesity was relieved by ghrelin by targeting the H19/miR-29a/IGF-1 signaling pathway [10] and TLR4/NLRP3 inflammasome [22]. Ghrelin also suppressed the hypoxia injury of cardiomyocyte via Akt-mTOR [23]. In this study, we demonstrated that ghrelin attenuated PA-induced cardiomyocyte injury by increasing HO-TAIR, which is in line with previous research.

DNA methylation in mammals acts an important role in many different kinds of life activities, and it mainly is established by de novo DNMT3B, DNMT3A, and DNMTs [15]. Telomerase reverse transcriptase affects DNA methylation in hepatocellular carcinoma through influencing DNMT3B expression [24]. In addition, increase of DNMT3B-mediated DNA methylation accelerates the leukemia development [25]. We demonstrated that HO-TAIR regulated the expression of miR-196b by recruiting DNMT3B to induce methylation of the miR-196b gene promoter, which might be the potential mechanism how HO-TAIR protects cardiomyocyte.

IGF-1 is believed to be closely linked with the regulation process of myocardial injury. It was reported that miR-150 protected myocardial infarction by targeting TP53/IGF-1 [26]. IGF-1 protects against myocardial infarction through activating PI3K/Akt [27]. Meanwhile, IGF-1 also suppresses myocardial injury via strengthening the function of BMSC [28]. In this study, the direct binding site between IGF-1 and miR-196b was identified. Meanwhile, we proved that ghrelin inhibited PA-induced cardiomyocyte injury by regulating the HO-TAIR/miR-196b/IGF-1 signaling pathway.

In this study, the low expression of HO-TAIR and high expression of miR-196b in the obese myocardial injury tissues were observed. We demonstrated that ghrelin might suppress PA-induced cardiomyocyte injury by targeting the HO-TAIR/miR-196b/IGF-1 signaling pathway. This study might provide a new thought for the treatment and prevention of cardiomyocyte injury induced by obesity.

Statement of Ethics

All experiments were approved by the Second Affiliated Hospital of Nanchang University Medical Research Ethics Committee; the Examination and Approval No. Review (2020) No. (A901).

Conflict of Interest Statement

The authors report no conflict of interest.

Funding Sources

This work was supported by the National Natural Science Foundation of China (82060162) and Natural Science Foundation of Jiang Xi (20202BABL206040).

Author Contributions

Guarantor of integrity of the entire study: Yang Liu; study concepts: Yuan-Yuan Lang; study design: Yang Liu; definition of intellectual content: Yan-Ling Liu; literature research: Chun-Feng Ye; clinical studies: Na Hu; experimental studies: Xin-Yue Xu; data acquisition: Qing Yao; data analysis: Wen-Shu Cheng; statistical analysis: Zu-Gen Cheng; manuscript preparation: Xin-Yue Xu; manuscript editing: Yuan-Yuan Lang; manuscript review: Yang Liu.

Data Availability Statement

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Ghrelin Relieves Myocardial Injury

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