Nesfatin-1 acts as an inhibitory factor in human gastrointestinal smooth muscle cells in diabetes mellitus-induced delayed gastric emptying

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Abstract: Diabetes mellitus (DM) brings about multiple gastrointestinal complications. Nesfatin-1 is an ingestion regulating peptide which possesses a considerable effect in delaying gastric emptying. We aimed to investigate effects and mechanism of nesfatin-1 application on human gastrointestinal smooth muscle cells (HGSMC). HGSMC were treated with different concentrations of nesfatin-1, and expression of endothelial nitric oxide synthases (eNOS) was confirmed. Besides, HGSMCs were administrated nesfatin-1 along or combination of nesfatin-1 and eNOS inhibitor NG-Nitro L-arginine Methyl Ester (L-NAME). Thereafter, the cell viability, apoptosis, and adhesion were assessed. Western blot analysis was performed to analyze expression of key proteins extracellular regulated protein kinas (ERK) 1/2, p38 mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) on the signaling pathway. The eNOS was significantly upregulated by nesfatin-1 at the optimal concentration of 100 nM (P < 0.05). Nesfatin-1 remarkably suppressed the HGSMC viability and adhesion (P < 0.05) and expedited apoptosis (P < 0.01). Additionally, nesfatin-1 significantly reduced expressions of ERK1/2, p38MAPK and mTOR in HGSMC compared with those in the control group (P < 0.05). However, administration of eNOS inhibitor L-NAME could relieve these effects on HGSMC. Nesfatin-1 inhibited cell viability and adhesion of HGSMC, and promoted cell apoptosis. These effects might be by regulating eNOS-mediated downstream ERK/MAPK/mTOR signaling pathway. It reveals that eNOS inhibitor is praised to be a novel therapeutic strategy and target for DM induced gastrointestinal complications.

Keywords: Nesfatin-1, human gastrointestinal smooth muscle cells, endothelial nitric oxide synthases, delayed gastric emptying

Introduction

Diabetes Mellitus (DM) is a metabolic disorder disease, characterized by hyperglycemia on account of insulin resistance (IR) and impaired insulin secretion, which has reached pandemic levels worldwide [1, 2]. Chronic DM brings about multiple gastrointestinal complications such as delayed gastric emptying (DGE) and gastroparesis, which diminish life quality in affected individuals [3-6]. Gastric emptying dysfunction occurs in 30-50% of DM patients and contributes to irregular blood glucose fluctuations and weakened oral antidiabetic drug emptying and absorption. As documented, DGE has been present in 25-55% of patients with type 1 and 30% of those with terminal type 2 diabetes mellitus (T2DM) [7-9]. Evidence points to a pivotal role of gastric motility disorder in DM patients, which markedly influences nutritional status and therapeutic effects. To our current knowledge, treatment options for DGE in DM patients remain limited.

Since first identified in 2006 [10], nesfatin-1 has been an anorexic hormone in the region of the precursor peptide nucleobindin-2 (NUCB2) [11]. As an ingestion regulating peptide, nesfatin-1 possesses a considerable effect in restraining food intake, losing weight, delaying gastric emptying and adjusting blood glucose [11-15]. Foregoing studies in rodents have concluded that nesfatin-1 was proposed to remedy obesity with the systemic or local admin-
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istration [11, 16]. Recently it has been revealed that gastrointestinal nesfatin-1 might be involved in the control of gastric mobility by adjusting the vagal activity [17, 18]. Ayada et al. found nesfatin-1 administration was related to endothelial nitric oxide synthases (eNOS) in balanced oxidative status [19]. Further, a novel site of action of nesfatin-1 on the Akt kinase (Akt)/AMP-dependent protein kinase (AMPK)/target of rapamycin complex 2 (TORC2) pathway [20] and mammalian target of rapamycin (mTOR)-signal transducer and activator of transcription 3 (STAT3) signaling pathway was detected in diet-induced IR [21].

However, the mechanism of nesfatin-1 on eNOS level and signaling pathways remains to be elucidated in DM-induced DGE. We aimed to investigate the effects and mechanism of nesfatin-1 application on human gastrointestinal smooth muscle cells (HGSMC), which is expected to address the unmet need and provide a theoretical basis for DM-induced gastrointestinal complications.

Materials and methods

Cell culture and treatment

HGSMC isolated from the human stomach were obtained from ScienCell Research Laboratories (San Diego, CA, USA). Cells were grown in Smooth Muscle Cell Medium (SMCM, Cat. #1101; Beijing Yuhengfeng Biotech Co., LTD, Beijing, China). HGSMC were maintained at 37°C in a humidified atmosphere containing 5% CO2. All cells were cultured to 80-90% confluency as judged under a phase contrast microscopy (Olympus, Tokyo, Japan) before the medium was replaced with Dulbecco's Modified Eagle's Medium (DMEM, Lonza, USA) for a further 15 h. Then HGSMC were assigned to three groups: control (C) group, nesfatin-1 (N) group and nesfatin-1 + NG-Nitro-L-arginine Methyl Ester (L-NAME) (NL) group (n = 2 × 10^4 in each group). Cells in C group were kept in DMEM as control. N group was treated by nesfatin-1 (R&D Systems, Minnesota, USA) of optimal concentration for 48 h selected from corresponding concentrations 10, 50, 100, 200 nM. NL was applied with 1.0 mmol/L eNOS inhibitor L-NAME (AMQUAR, Guangzhou, China) for 24 h after HGSMC were treated with nesfatin-1 of optimal concentration.

Cell viability assay

The cell viability of C, N and NL groups was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. HGSMC treated with 100 nM nesfatin-1 were cultured in DMEM with 10% fetal bovine serum (FBS, Invitrogen, CA, USA) containing 0.5 mg/mL MTT (Sigma, USA) for 4 h on days 1, 2, 3 and 4. Then 100 μl dimethylsulfoxide (DMSO, Lonza, USA) was added to dissolve the blue formazan (Sigma, USA) product. HGSMC viability was measured by absorbance at a wavelength of 550 nm.

Detection of apoptosis

Apoptotic cells were identified and quantified by flow cytometry (Beckman Coulter, USA) with Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). HGSMC of C, N and NL groups were seeded in 6-well culture plate respectively and washed twice with cold phosphate buffer saline (PBS). Then they were co-incubated with serum-free culture medium containing 10 μM dichloro fluorescein diacetate (20 min, 37°C, in dark). Subsequently, samples were collected by a trypsin digestion approach and centrifuged. Then they were resuspended in 100 μl annexin-binding buffer and measured according to the manufacturer’s protocol to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

Adhesion assay

Cell adhesion in C, N and NL groups was carried out with the Adhesion assay kit (Cell Biologs, CA, USA) based on the manufacturer’s introductions. After treatment in C, N and NL groups, HGSMC were trypsinized and added to Matrigel-coated inserts (BD Bioscience, CA, USA) in DMEM containing 2% FBS and 5 ng/ml transforming growth factor beta 1 (TGFb1) (Sino Biological Inc., Beijing, China) for 24 h. Then attached HGSMC were stained in 4% paraformaldehyde with 4, 6-diamidino-2-phenylindole (DAPI; Southernbiotech, Birmingham, USA) for 10 min. Inoculated HGSMC were detected by the optical density 560 nm in DigitScan Microplate Reader (Assys Hitech, Korneburg, Austria) and their percentages in extracted samples were calculated.
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Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA of HGSMC in C, N and NL groups was isolated respectively using Trizol reagent (Invitrogen, CA, USA) and treated with DNaseI (Promega, Madison, USA). A total of 2 μg RNA was used to synthesize poly-oligo (dT) primed complementary DNA (cDNA) with the RevertAid H Minus First strand Cdna Synthesis Kit (Thermo Fischer Scientific Inc., MA, USA). qRT-PCR reactions for eNOS, p53, factor associated suicide (Fas), extracellular regulated protein kinases (ERK) 1/2, p38 mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) were performed using RibomAX Large Scale RNA Production System T7 (Promega, Karlsruhe, Germany). The specific primer sequences were: eNOS, forward 5’TCTGCCGCGATGTATG’3, reverse 5’TATGGCGTCCTCCTGTGG’3; p53, forward 5’ACTGGTGCTCTGACTGCTTTTCA’3, reverse 5’CCAGCAT-TGAAGTCTCATGGAAAGC’3; Fas, forward 5’ATGCTGGGAATCGTTCAGGACC’3, reverse 5’CTCTTCGTCGTGTCTTGG’3; ERK1, forward 5’CCAGAGTGCTATCAAGAAG’3, reverse 5’TCCATGAGGC-CTGACCTCAGT’3; p38MAPK, forward 5’AGAGTTGGATGAGCTGGT’3, reverse 5’GAAGAGCCTGACCTACATG’3; mTOR, forward 5’GGGCTCATACATTGGT’3, reverse 5’GAAGAGCCTGACCTACATG’3; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, United Kingdom), forward 5’GTAAGAATATCTGTGACGT’3, reverse 5’GATGGGTGATGGCCACAGACT’3.

Western blot analysis

HGSMC in C, N and NL groups were lysed by means of 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membrane (Millipore, USA). The proteins used for western blotting were extracted using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Applngen Technologies Inc., Beijing, China). The total amount of proteins was quantified by Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Appleton, USA). A Bio-Rad Bis-Tris Gel system was employed to establish the western blot system, in which GAPDH was regarded as an internal control. Primary antibodies eNOS (ab66127), p53 (ab1101), Fas (ab82419), ERK1/2 (ab196883), p38MAPK (ab31828) and mTOR (an2732) were obtained from Abcam (Cambridge, United Kingdom). After incubation with the membrane at 4°C overnight, secondary antibodies were marked by horseradish peroxidase for 1 h at room temperature. Then Images were developed and photographed using Image Lab Software (Bio-Rad, Shanghai, China).

Figure 1. Nesfatin-1 upregulated eNOS in HGSMC. A. HGSMC were treated with nesfatin-1 for 48 h at corresponding concentrations 10, 50, 100, 200 nM; B. The eNOS proteins were measured by different concentrations of nesfatin-1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; eNOS, endothelial nitric oxide synthases; HGSMC, human gastrointestinal smooth muscle cells. *, P < 0.05.

Figure 2. Nesfatin-1 inhibited cell viability in HGSMC. C, control group; N, nesfatin-1 group; NL, nesfatin-1 + NG-Nitro-L-arginine Methyl Ester group. *, P < 0.05.
Statistical analysis

Each experiment was carried out in triplicate. All results were presented as mean ± standard deviation (SD). Values were performed by one-way analysis of variance (ANOVA) with SPSS 19.0 software (SPSS, IL, USA). Statistical significance was defined as $P < 0.05$.

Results

Impact on eNOS by nesfatin-1 treatment in HGSMC

In the N group, HGSMC were treated by nesfatin-1 with corresponding concentrations 10, 50, 100, 200 nM for 48 h. Figure 1A showed that the expression of eNOS mRNA was increased by nesfatin-1 with a concentration-dependent manner. However, no statistical difference was witnessed on expressions of eNOS mRNA between nesfatin-1 concentration of 0 nM, 10 nM, and 50 nM, respectively ($P > 0.05$). At concentration of 100 nM and 200 nM, the expressions of eNOS mRNA raised observably compared to that at concentration of 0 ($P < 0.05$). We detected that there was the highest expression of eNOS mRNA at concentration of 100 nM. The western blotting result declared as concentrations of nesfatin-1 increased, the expressions of eNOS protein were higher (Figure 1B). At nesfatin-1 concentration of 100 nM, there was the highest expression of eNOS protein. Thereby, 100 nM was selected as the optimal concentration of nesfatin-1 to treat HGSMC in the N group.

Nesfatin-1 inhibited cell viability in HGSMC

MTT colorimetric assay was applied to determine cell viability of C, N and NL groups. In the N group, the cell viability was statistically lower than that in the C group ($P < 0.05$; Figure 2) at day 4. The addition of eNOS inhibitor L-NAME made cell viability increased to the level of C group and there was no difference between C and NL group ($P > 0.05$). These results claimed that nesfatin-1 inhibited adhesion capacity in HGSMC by upregulating expressions of eNOS.

Nesfatin-1 promoted cell apoptosis in HGSMC

Apoptotic HGSMC was quantified by flow cytometry and pro-apoptotic factors were measured. Apoptotic HGSMC quantified by flow cytometry was 3.1% in the C group, 15.6% in the N group and 8.0% in the NL group (Figure 3A). Apoptosis cells went up significantly by nesfatin-1 compared to the C group ($P < 0.01$; Figure 3B). The addition of eNOS inhibitor L-NAME made apoptotic HGSMC reduce and there was no difference between C and NL group ($P > 0.05$). It revealed that nesfatin-1 promoted cell apoptosis in HGSMC by upregulating expressions of eNOS. Both pro-apoptotic factors p53 and Fas were markedly upregulated in the N group compared to those in the C group ($P < 0.05$; Figure 3C). The addition of L-NAME made mRNA expressions of p53 and Fas reduce to the level of C group and there was no difference between C and NL group ($P > 0.05$). In Figure 3D both p53 and Fas proteins were upregulated by nesfatin-1 and weakened by addition of L-NAME.

Nesfatin-1 suppressed adhesion in HGSMC

HGSMC adhesion capacity in C, N and NL groups was identified in Figure 4. In the N group, the percentage of relative adhesion was statistically lower than that in the C group ($P < 0.05$). The addition of eNOS inhibitor L-NAME made the percentage increased to the level of C group and there was no difference between C and NL group ($P > 0.05$). These results claimed that nesfatin-1 inhibited adhesion capacity in HGSMC by upregulating expressions of eNOS.

Nesfatin-1 inhibited ERK/MAPK/mTOR signaling pathway

To further understand the function and mechanism of nesfatin-1 in gastrointestinal complication of DM, we evaluated the impact of nesfatin-1 on expression of key proteins in ERK/MAPK/mTOR pathway. Figure 5A showed that nesfatin-1 significantly reduced mRNA expressions of ERK1/2, p38MAPK and mTOR in HGSMC compared with those in the C group ($P < 0.05$). The mRNA expressions of ERK1/2, p38MAPK and mTOR were observably higher in addition of L-NAME than those in the C group ($P < 0.05$ or $P < 0.01$). Similar outcomes were observed in western blot results (Figure 5B). The eNOS protein was upregulated in N group and downregulated in NL group. P-ERK1/2, p-p38MAPK and p-mTOR proteins were downregulated in N group and upregulated in NL group. It revealed that nesfatin-1 inhibited ERK/MAPK/mTOR pathway in HGSMC and the pathway was activated by restraining eNOS in addition of eNOS inhibitor.
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Discussion

To our knowledge, this study provided the first insight into the role and mechanism of nesfatin-1 in HGSMC. The eNOS was significantly upregulated by nesfatin-1 at the optimal concentration of 100 nM. Nesfatin-1 significantly suppressed the HGSMC viability and adhesion. Nesfatin-1 remarkably expedited apoptosis and upregulated pro-apoptotic factors p53 and Fas. The addition of eNOS inhibitor L-NAME could relieve these effects in HGSMC. Additionally, nesfatin-1 significantly reduced expressions of ERK1/2, p38MAPK and mTOR in HGSMC compared with those in the C group.

DM induced gastric complications, including DGE, diabetic gastroparesis (DGP) and gastroesophageal reflux disease (GERD), are chronic syndromes with complex pathogenesis and high morbidity [4]. Most of DGE patients undergo lower living quality and bear higher medical costs. Although improving gastric motility is proposed to be a pertinent therapeutic strategy for DGE, to our current knowledge of the pathophysiology, there remains no established therapy of DGE for clinical use. Thus, the application of gastrointestinal regulatory peptides is expected to be effective.

For nesfatin-1, the anorectic physiological action was a hotspot [22] and recently its function in gastrointestinal movement and motility has

Figure 3. Nesfatin-1 promoted cell apoptosis in HGSMC. A. Apoptosis cells were quantified by flow cytometry; B. Apoptosis cells were measured in C, N and NL groups; C. Expressions of pro-apoptotic factors p53 and Fas mRNA were measured in C, N and NL groups; D. Pro-apoptotic factors p53 and Fas proteins were measured in C, N and NL groups. C, control group; N, nesfatin-1 group; NL, nesfatin-1 + NG-Nitro-L-arginine Methyl Ester group; HGSMC, human gastrointestinal smooth muscle cells; Fas, factor associated suicide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *, $P < 0.05$; **, $P < 0.01$.

Figure 4. Nesfatin-1 suppressed adhesion in HGSMC. C, control group; N, nesfatin-1 group; NL, nesfatin-1 + NG-Nitro-L-arginine Methyl Ester group; HGSMC, human gastrointestinal smooth muscle cells. *, $P < 0.05$. 

For nesfatin-1, the anorectic physiological action was a hotspot [22] and recently its function in gastrointestinal movement and motility has
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Figure 5. Nesfatin-1 inhibited ERK/MAPK/mTOR signaling pathway in HGSMC. A. Expressions of ERK1/2, p38MAPK and mTOR mRNA were assessed in HGSMC; B. The eNOS, p-ERK1/2, p38MAPK and p-mTOR proteins were assessed in HGSMC; C, control group; N, nesfatin-1 group; NL, nesfatin-1 + NG-Nitro-L-arginine Methyl Ester group; HGSMC, human gastrointestinal smooth muscle cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; eNOS, endothelial nitric oxide synthase; ERK, extracellular regulated protein kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin. *, P < 0.05; **, P < 0.01.

To further understand the mechanism of nesfatin-1 inhibitory action in DGE, we evaluated the impact of nesfatin-1 on eNOS. It is well known that eNOS plays a potent vasodilator role in restraining the gastric secretion and protecting the gastric mucosa against various damaging agents and corrosive substances in upper gastrointestinal tract [29]. Huang et al. found that nitric oxide (NO) synthase acted as an inhibitory factor in modulating gastric motility [30]. However, to date, activation of eNOS has not been investigated as a mechanism for gastrointestinal movement at cell level. In the present study, eNOS was activated by nesfatin-1 and addition of eNOS inhibitor L-NAME could relieve effects of nesfatin-1 in HGSMC. Thus inhibitory implications of nesfatin-1 for HGSMC might be by activation of eNOS and eNOS inhibitor might alleviate DM induced DGE, which raised potential possibility to DM induced gastric emptying disorder treatment.

MAPKs, comprising c-Jun N-terminal kinase (JNK), ERK, ERK5 and p-38 pathways, have been involved in numerous aspects of key cellular processes and regulated mitochondrial pathway activation in cell apoptosis [31, 32]. Accumulating studies have shown that ERK1/2 signaling pathway mediates cell growth and differentiation [33, 34]. A growing body of literature implies mTOR signaling is of great importance in cell growth and metabolism, which is frequently upregulated in malignancies [35, 36]. Our results showed that nesfatin-1 decreased expressions of p-ERK1/2, p-p38MAPK and p-mTOR proteins, which suggested that nesfatin-1 inhibited phosphorylation of ERK1/2, p38MAPK and mTOR in HGSMC. eNOS inhibitor achieved aberrant activation of ERK/MAPK/mTOR signaling pathway. These results pointed to a critical role of nesfatin-1 in ERK/MAPK/mTOR signaling pathway by regulating eNOS. It was considered that the inhibitory effects of nesfatin-1 on HGSMC were by means of activation of eNOS and inhibition of ERK/MAPK/mTOR pathway.

In summary, the present study detects regulatory effect and mechanism of nesfatin-1 in HGSMC, which provides theoretical basis for further research on function of nesfatin-1. It reveals that eNOS inhibitor is praised to be a novel therapeutic strategy and target for DM induced gastrointestinal complications.

Disclosure of conflict of interest

None.

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