Effects of β-hydroxybutyric acid and ghrelin on the motility and inflammation of gastric antral smooth muscle cells involving the regulation of growth hormone secretagogue receptor

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Received January 9, 2019; Accepted July 3, 2019

DOI: 10.3892/mmr.2019.10739

Abstract. Ghrelin is an orexigenic hormone that is produced by gastric cells. Ghrelin stimulates food intake and increases gastric movement. In rat model, injected β-hydroxybutyric acid (β-HB) leads to a decrease in body weight. It has been reported that patients with gastric erosions are slower to evacuate the stomach. The aim of the present study was to investigate the effects of ghrelin and β-HB on motility and inflammation in rat gastric antral smooth muscle cells (GASMCs). GASMCs were extracted from rat gastric antrum. Cell viability was determined using the Cell Counting Kit-8 assay. A reactive oxygen species (ROS) assay kit was used to analyze the levels of ROS using flow cytometry. Protein levels were determined using western blotting, and the expression levels of mRNAs were evaluated using reverse transcription-quantitative PCR. β-HB inhibited the expression of myosin regulatory light polypeptide 9 (MYL9), myosin light chain kinase (MLCK), transforming protein RhoA (RhoA), Rho-associated protein kinase-1 (ROCK-1) and growth hormone secretagogue receptor (GHS-R). By contrast, ghrelin increased the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R in β-HB-treated GASMCs. β-HB increased the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and ROS, and decreased the levels of manganese (Mn) superoxide dismutase (SOD), copper/zinc (Cu/Zn)SOD and catalase. Ghrelin decreased the expression of TNF-α, IL-6, ROS and catalase, whereas ghrelin promoted the expression of MnSOD and Cu/ZnSOD in β-HB-treated GASMCs. Short interfering RNA targeting GHS-R inhibited the expression of MYL9, MLCK, RhoA and ROCK-1, and increased the levels of TNF-α, IL-6 and ROS in β-HB-treated or ghrelin-treated GASMCs. The present study provided preliminary evidence that β-HB inhibits the motility of GASMCs and promotes inflammation in GASMCs, whereas ghrelin decreases these effects. GHS-R acted as a primary regulator of motility and inflammation in GASMCs treated with β-HB and ghrelin.

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Key words: β-hydroxybutyric acid, ghrelin, motility and inflammation, gastric antral smooth muscle cells, growth hormone secretagogue receptor

Introduction

Patients with gastric erosions have slower evacuation of the stomach and hypotonus of the stomach (1), as well as gastric inflammation and decreased gastric motility (2,3). Gastric diseases, such as achlorhydria, are well known to affect nutrient absorption of factors, including iron (4). Nutrient digestion causes movement of the stomach, including gastric secretions and gastric shaking (5). Hormones regulate the physiological functions of the stomach, such as gastric secretions and motility (6).

Ghrelin is one of the hormones that is produced by gastric cells, and is an orexigenic hormone as it increases appetite (7). Endocrine cells of the stomach regulate appetite through the hypothalamus and vagal afferent nerve fibers (8). Ghrelin has an important role in the regulation of food intake, stimulating food administration in humans and regulating energy metabolites (6,7). As such, ghrelin has been investigated as a target to treat obesity (9). The release of ghrelin may increase stomach movement according to the aforementioned studies.

β-hydroxybutyric acid/β-hydroxybutyrate (β-HB) regulates hormone synthesis and release, including of growth hormone-releasing hormone in the hypothalamus (10). Sun *et al* (11) demonstrated that the intracerebroventricular infusion of β-HB for 28 days significantly decreased the body weight in high-fat fed rats, although β-HB is similar to glucose as it provides energy for the brain in suckling rats (12). Nowroozi-Asl *et al* (13) reported that ghrelin and β-HB are sensitive indicators of energy balance. Poggioli *et al* (14) found that γ-HB increased gastric motility in a rat model. However, to the best of our knowledge, it is not known whether β-HB has an effect on gastric motility, which would affect food intake and digestion. The relationship among β-HB, ghrelin and gastric inflammation remains unclear. In the present study, the effect of β-HB and ghrelin on the motility of GASMCs, and inflammation in GASMCs, was investigated.

Materials and methods

GASMC separation and identification. In total, two Sprague Dawley rats (one male and one female), aged 6 weeks (200±10 g), were purchased from Guangdong Medical Laboratory Animal Center. Rats were kept in cages at 22˚C±3˚C with a stable humidity (50±10%) and a 12 h; light/dark cycle. The rats had free access to food and water. The gastric antrum was removed and D-Hanks medium (Beijing Solarbio Science and Technology Co., Ltd.) was used to wash the gastric antrum three times. The gastric antrum was cut into pieces; these pieces were digested using type II collagenase (Gibco; Thermo Fisher Scientific, Inc.) dissolved in M199 basic medium (Gibco; Thermo Fisher Scientific, Inc.) for 30 min in a 37˚C water bath. D-Hanks was added to resuspend the precipitate after removal of the type II collagenase and was agitated for 10 min. The mixed solution was centrifuged at 2,000 x g for 5 min at room temperature. The supernatant was discarded and M199, supplemented with 20% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% 10,000 U/ml penicillin-10,000 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), was used to resuspend and cultured the cells. The cell solution was passed through a size 200 mesh screen (Sigma-Aldrich; Merck KGaA). Animal experiments were approved by the Institutional Animal Care and Use Committee of Southwest University Hospital (no. 2017110853n).

Immunofluorescence was used to identify GASMCs. Cells ($5x10^4$ cells/ml) were seeded into 35 mm plates and 4% paraformaldehyde was used to fix the cells at 4˚C for 10 min. An antibody against α-smooth muscle actin (1:100; cat. no. 19245; Cell Signaling Technology, Inc.) was incubated with cells for 2 h at the room temperature. TBST was used to wash cells three times. A secondary antibody conjugated with Alexa Fluor® 594 (1:1,000; cat. no. 8889; Cell Signaling Technology, Inc) was incubated with the cells for 2 h at the room temperature. PBS was used to wash the cells. DAPI (5 µg/ml dissolved in PBS; Sigma-Aldrich; Merck KGaA) was used to stain the cells for 4 min at 25˚C and were then washed with PBS. The cells were observed using a fluorescence microscope (Olympus Corporation).

Treatment with reagents. Ghrelin (10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol/l; Sigma-Aldrich; Merck KGaA) was dissolved in PBS and diluted in culture medium. β-HB (0.5, 1, 5 and 10 mmol/l; MedChemExpress, LLC) was dissolved in DMSO and diluted in culture medium. In some experiments 10^{-8} mol/Ghrelin and 5 mmol/l β-HB were combined to treat cells.

Cell viability and transfection. GASMCs ($4x10^3$ cells/well) were seeded into 96-well plates. After cells were treated different concentrations of β-HB and Ghrelin as aforementioned for 24, 48 and 72 h, the medium was discarded. Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) was diluted with M199 basic medium (1:9). In total, 10 µl CCK-8 solution was added to each well and incubated with the cells for 1 h in a 37˚C incubator. Absorbance was determined at a wavelength of 450 nm using a Multiskan microplate reader (Thermo Fisher Scientific, Inc.).

For transfections, 50 nM small interfering (si)RNA against GHS-R (5'-CTGAAGGCATCTTTCACTACG-3') or a negative control siRNA (5'-CAGUACUUUUGUGUAGUACAA-3') were mixed with Lipofectamine® (Invitrogen; Thermo Fisher Scientific, Inc.) and diluted in M199 basic medium. This solution was incubated with cells for 3 h; the culture medium was then replaced, and cells were cultured for a further 48 h.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from GASMCs using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions and centrifuged at 15,000 x g for 15 min at 4˚C. RT was carried out using an Arcturus™ RiboAmp™ HS PLUS cDNA Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT was conducted at 42˚C for 15 min and 95˚C for 3 min.

The primers used are listed in Table I and were synthesized by Sangon Biotech Co., Ltd. qPCR was carried out using the PCR Taq Master Mix (MedChemExpress, LLC) using the following conditions: 95˚C for 2 min, followed by 40 cycles of 95˚C for 20 sec, 52˚C for 50 sec and 72˚C for 25 sec. GAPDH was used as an internal reference for qPCR. The relative mRNA expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (15).

Reactive oxygen species (ROS) assay. GASMCs ($4x10^6$ cells/ml) were seeded into 75 mm plates. After the cells were treated with reagents as aforementioned for 48 h, the cells were digested using 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) for 15 min. Cells were resuspended in PBS following centrifugation at 1,000 x g at room temperature for 3 min. A Total Reactive Oxygen Species (ROS) Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used, according to the manufacturer's protocol. Cells were analyzed using a flow cytometer (Invitrogen; Thermo Fisher Scientific, Inc.) and analysis software (FlowJo version 10.0; BD Biosciences) to determine the level of fluorescence.

Western blot analysis. Proteins were extracted from GASMCs using cell lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) at 4˚C for 2 h and centrifuged at 13,000 x g at 4˚C for 15 min. The bicinchoninic acid method was used to determine the protein concentration. Protein were separated by 10% SDS-PAGE. Proteins (30 µg/lane) were then transferred to PVDF membranes. Membranes were blocked using 5% milk solution for 2-3 h at room temperature. Primary antibodies (Table II) were obtained from Abcam and incubated with membranes at 4˚C for 12 h. A horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. ab7090; Abcam) was incubated with membranes for 2-3 h at room temperature. An ECL™ western blotting reagents kit (Sigma-Aldrich; Merck KGaA) was used to visualize protein bands and films were used to detect the signal in a dark room. Densitometry analysis was performed using ImageJ (Version 5.0; National Institutes of Health).

Table I. Primers used in reverse transcription-quantitative PCR.

Name	Forward (5'-3')	Reverse (5'-3')
MYL9	CACCAGAAGCCAGATGTCC	TTGAAAGCCTCCTTAAACTCC
MLCK	GGAATTCCATATGAAGACCCCTGTGCCTGAGAAG	CAGCCTCTAAGATCCCTGCC
RhoA	GTAGAGTTGGCTTTAT	CACTCCGTCTTGGTCTT
ROCK-1	AGTCTGTGGCAATGTGTGAG	CTTCAAGCCGACTAACAGTG
GHS-R	CCTGCTTCACCACCTTCTTG	CCAAAAGGGTCATCATCTCT
TNF-α	GCGACGTGGAACTGGCAGAAG	GGTACAACCCATCGGCTGGCA
IL-6	ACGCTAGTCCTCCACGAT	GGTTGTTTAACATTGCCTTT
MnSOD	GGCCAAGGGCGCTGTTACAA	CTGACCGAGCGTGGCTAC
Cu/ZnSOD	GTTCCGAGGCCGCGCGT	GTCCCCATATTGATGGAC
Catalase	AGTGAGAGAAGTTAGAAAAAAGAA	CAACTAACACAAATACCAAACT
GAPDH	AATGTGTCCGTCGTGGATCTGA	GATGCCTGCTTCACCACCTTCT

MYL9, myosin regulatory light polypeptide 9; MLCK, myosin light chain kinase; RhoA, transforming protein RhoA; ROCK-1, Rho-associated protein kinase-1; GHS-R, growth hormone secretagogue receptor; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc superoxide dismutase.

Table II. Primary antibodies used in western blotting.

Name	Item number	Weight	Dilution
MYL9	ab191393	20 kDa	1:1,000
MLCK	ab232949	211 kDa	1:1,000
RhoA	ab187027	22 kDa	1:5,000
ROCK-1	ab156284	158 kDa	1:1,000
GHS-R	ab85104	40 kDa	1:500
MnSOD	ab13533	25 kDa	1:5,000
Cu/ZnSOD	ab13498	19 kDa	1:1,000
Catalase	ab16731	60 kDa	1:2,000
GAPDH	ab181602	37 kDa	1:10,000

MYL9, myosin regulatory light polypeptide 9; MLCK, myosin light chain kinase; RhoA, transforming protein RhoA; ROCK-1, Rho-associated protein kinase-1; GHS-R, growth hormone secretagogue receptor; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc superoxide dismutase.

Statistical analysis. All experiments were independently performed at least three times and all values are presented as the mean ± SD. The data were analyzed using one-way ANOVA and significant differences were analyzed using Tukey's post hoc test using SPSS 19.0 (IBM, Corp). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of β-HB on cell viability and ROS levels in GASMCs. GASMCs isolated from rat gastric antrum had a normal shape (Fig. 1A). Treatment with 0-10 mmol/l β-HB had a mild inhibitory effect on the viability of GASMCs (Fig. 1B). β-HB stimulated the production of ROS in GASMCs (Fig. 1D and E). Therefore, β-HB effected cell viability and ROS levels in GASMCs in a dose-dependent manner.

Effects of β-HB on the expression of myosin regulatory light polypeptide (MYL) 9, myosin light chain kinase (MLCK), transforming protein RhoA (RhoA), Rho-associated protein kinase-1 (ROCK-1), growth hormone secretagogue receptor (GHS-R), tumor necrosis factor (TNF)-α and interleukin (IL)-6 in GASMCs. β-HB inhibited the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R. Higher concentrations of β-HB were found to have stronger inhibitory effects on the mRNA expression of these factors (Fig. 1C). By contrast, β-HB stimulated the expression of TNF-α and IL-6 in GASMCs (Fig. 1F), suggesting that β-HB could induce inflammation in GASMCs.

Effects of ghrelin on cell viability and the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R in GASMCs. Low concentrations of ghrelin were found to have no effect on the viability of GASMCs after 24 and 48 h (Fig. 2A). However, these low concentrations of ghrelin promoted the expression of MYL9, RhoA and ROCK-1 at 48 h (Fig. 2B).Ghrelin (10^{-8} mol/l) promoted the expression of MLCK and 10^{-9} mol/l ghrelin increased the mRNA expression of GHS-R (Fig. 2B). There was no significant effect on the expression of MYL9, MLCK, RhoA, ROCK 1 and GHS R in GASMCs with 10^{-10} mol/l of ghrelin.

Effects of ghrelin on cell viability and ROS levels in GASMCs treated with β-HB. Ghrelin (10^{-8} mol/l) improved the viability of GASMCs following β-HB treatment at 24, 48 and 72 h (Fig. 3A). Ghrelin ($1x10^{-8}$ mol/l) decreased the level of ROS following treatment with or without β-HB in GASMCs at 48 h (Fig. 3E and F).

Effects of ghrelin on the expression of MYL9, MLCK, RhoA, ROCK-1, GHS-R, catalase, manganese (Mn) superoxide dismutase (SOD), copper/zinc (Cu/Zn)SOD, TNF-α and IL-6 in GASMCs treated with β-HB. After 48 h, a low concentration

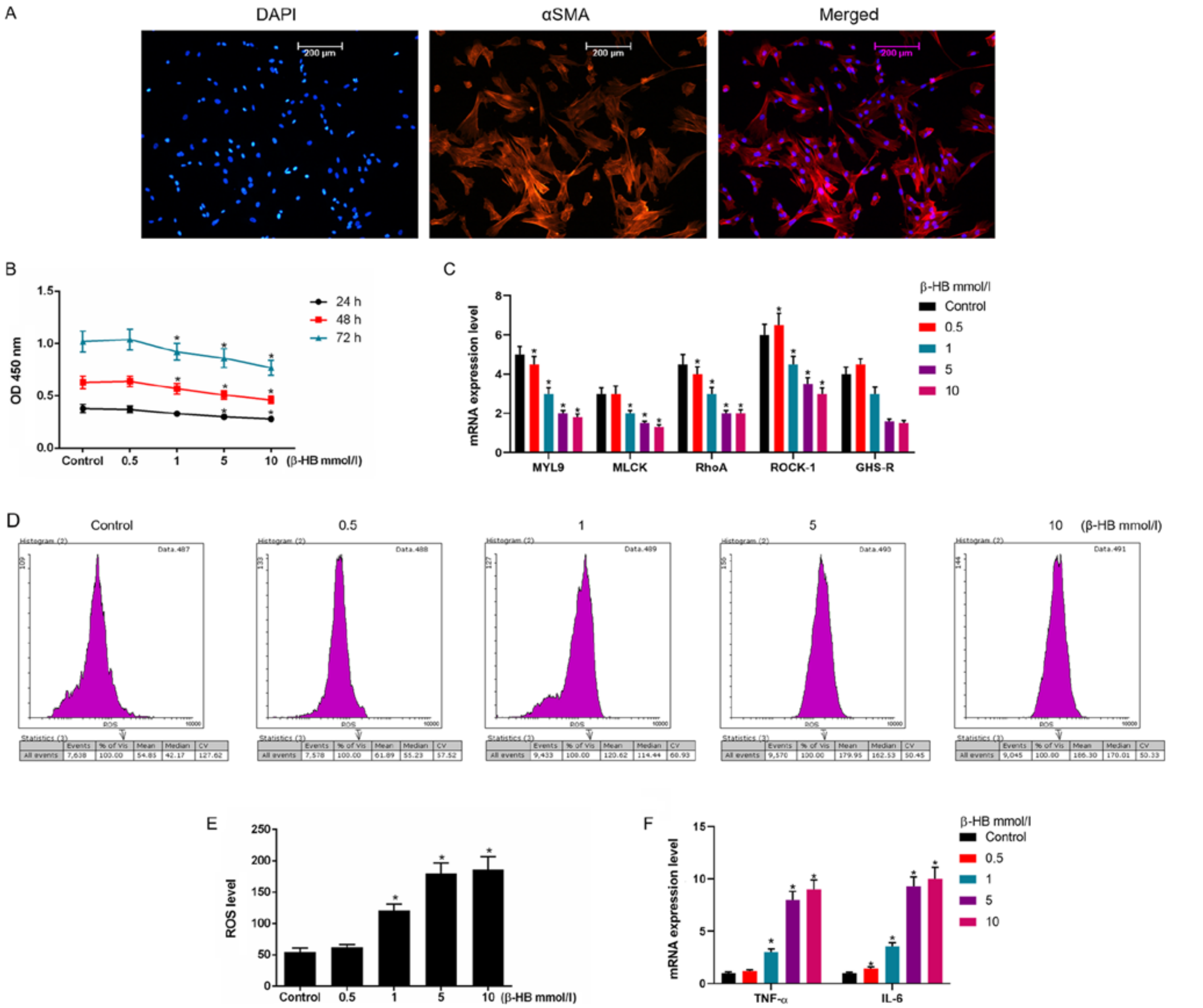

Figure 1. Effects of β-HB on the expression of MYL9, MLCK, RhoA, ROCK-1, GHS-R, ROS, TNF-α and IL-6 in rat GASMCs. (A) GASMCs were extracted from rat gastric antrum. GASMCs were stained with DAPI and an α-smooth muscle actin antibody. (B) GASMCs were treated with 0.5-10 mmol/l β-HB for 24, 48 and 72 h. The Cell counting Kit-8 assay was used to determine cell viability. GASMCs were treated with 0.5-10 mmol/l β-HB for 48 h. (C) RT-qPCR was used to determine the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R. (D) GASMCs were treated with 0.5-10 mmol/l β-HB for 48 h. An ROS assay kit was used to determine the ROS level using flow cytometry. (E) Quantification of ROS levels. (F) GASMCs were treated with 0.5-10 mmol/l β-HB for 48 h. The expression levels of TNF-α and IL-6 were determined using RT-qPCR. ANOVA was used to analyze the differences. $^{*}P<0.05$ vs. respective control. B-HB, β-hydroxybutyric acid; GASMCs, gastric antral smooth muscle cells; MYL9, myosin regulatory light polypeptide 9; MLCK, myosin light chain kinase; RhoA, transforming protein RhoA; ROCK-1, Rho-associated protein kinase-1; GHS-R, growth hormone secretagogue receptor; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; RT-qPCR, reverse transcription-quantitative PCR; OD, optical density.

of ghrelin ($1x10^{-8}$ mol/l) led to an increase in the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R following the treatment of GASMCs with β-HB (Fig. 3B-D). Treatment of GASMCs with β-HB or ghrelin inhibited the expression of catalase. β-HB decreased the expression of Cu/ZnSOD and MnSOD, whereas ghrelin increased the levels of Cu/ZnSOD and MnSOD after 48 h cultured (Fig. 4A-C). Ghrelin and β-HB co-treatment reduced the expression of Cu/ZnSOD and MnSOD and increased the expression of catalase compared to ghrelin treatment alone at 48 h. A low concentration of ghrelin ($1x10^{-8}$ mol/l) had a modest inhibitory effect on the expression of TNF-α and IL-6 (Fig. 4D), whereas a low concentration of ghrelin ($1x10^{-8}$ mol/l) significantly decreased the β-HB-induced expression of TNF-α and IL-6 (Fig. 4D).

Effects of siGHS-R on the levels of ROS and the expression of MYL9, MLCK, RhoA, ROCK-1, GHS-R, TNF-α and IL-6 in GASMCs. As shown in Fig. 5, transfection of cells with siGHS-R led to the significant depletion of GHS-R in GASMCs compared with the control (Fig. 5). β-HB inhibited the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R in GASMCs. By contrast, β-HB promoted the expression of TNF-α and IL-6 after 48 h of culture (Fig. 6A-C). β-HB promoted the production of ROS (Fig. 6D and E). siGHS-R significantly inhibited the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R following treatment with β-HB, ghrelin or their combination (Fig. 6A and B). siGHS-R significantly increased the levels of ROS, TNF-α and IL-6 following treatment with β-HB, ghrelin or their combination in GASMCs (Fig. 6C-E).

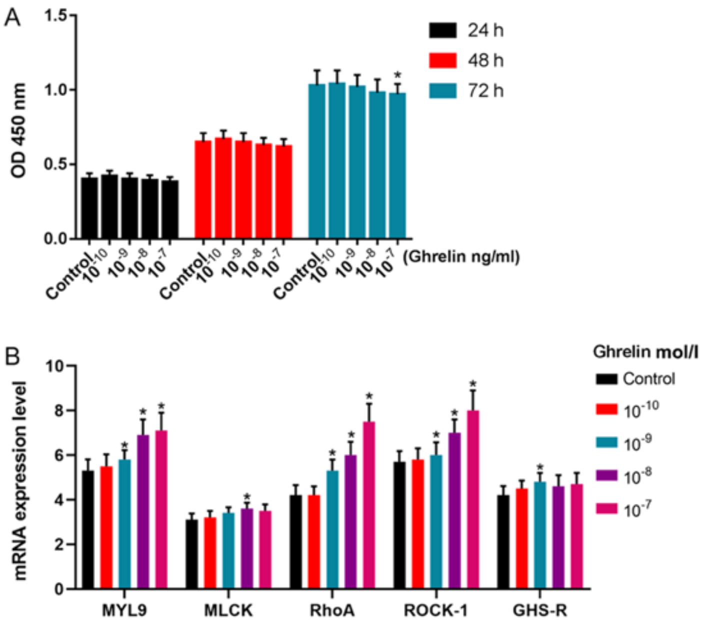

Figure 2. Effects of ghrelin on the expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R in rat GASMCs. (A) GASMCs were treated with 10^{-10}-10^{-7} mol/l ghrelin for 24, 48 and 72 h. The Cell Counting Kit-8 assay was used to determine cell viability. GASMCs were treated with 10^{-10}-10^{-7} mol/l ghrelin for 48 h. (B) The mRNA expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R were determined using reverse transcription-quantitative PCR. ANOVA was used to analyze the differences. *P<0.05 vs. Control. B-HB, β-hydroxybutyric acid; GASMCs, gastric antral smooth muscle cells; MYL9, myosin regulatory light polypeptide 9; MLCK, myosin light chain kinase; RhoA, transforming protein RhoA; ROCK-1, Rho-associated protein kinase-1; GHS-R, growth hormone secretagogue receptor; OD, optical density.

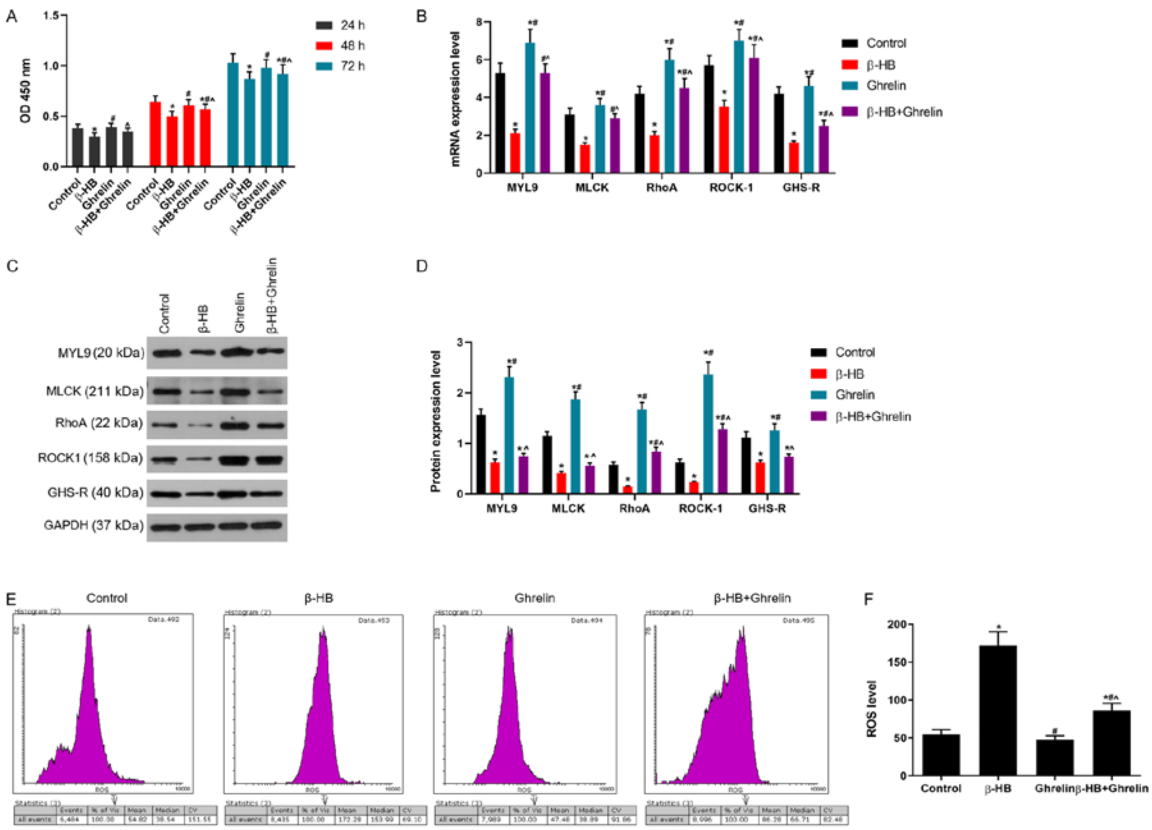

Figure 3. Effects of ghrelin in combination with β-HB on cell viability and the expression of MYL9, MLCK, RhoA, ROCK-1, GHS-R and ROS in rat GASMCs. (A) GASMCs were treated with 10^{-8} mol/l ghrelin and 5 mmol/l β-HB for 24, 48 and 72 h. Cell viability was determined using the Cell Counting Kit-8 assay. (B) Following treatment of GASMCs for 48 h, the mRNA expression of MYL9, MLCK, RhoA, ROCK-1 and GHS-R were evaluated using reverse transcription-quantitative PCR. (C) Western blot analysis was used to determine the protein levels of MYL9, MLCK, RhoA, ROCK-1 and GHS-R. (D) Quantification of the levels of MYL9, MLCK, RhoA, ROCK-1 and GHS-R. (E) ROS levels were analyzed using a ROS assay and flow cytometry. (F) Quantification of ROS levels. ANOVA was used to analyze the differences. *P<0.05 vs. Control group; $^{\#}$P<0.05 vs. β-HB; $^{\wedge}$P<0.05 vs. 10^{-8}. β-HB, β-hydroxybutyric acid; GASMCs, gastric antral smooth muscle cells; MYL9, myosin regulatory light polypeptide 9; MLCK, myosin light chain kinase; RhoA, transforming protein RhoA; ROCK-1, Rho-associated protein kinase-1; GHS-R, growth hormone secretagogue receptor; ROS, reactive oxygen species; OD, optical density.

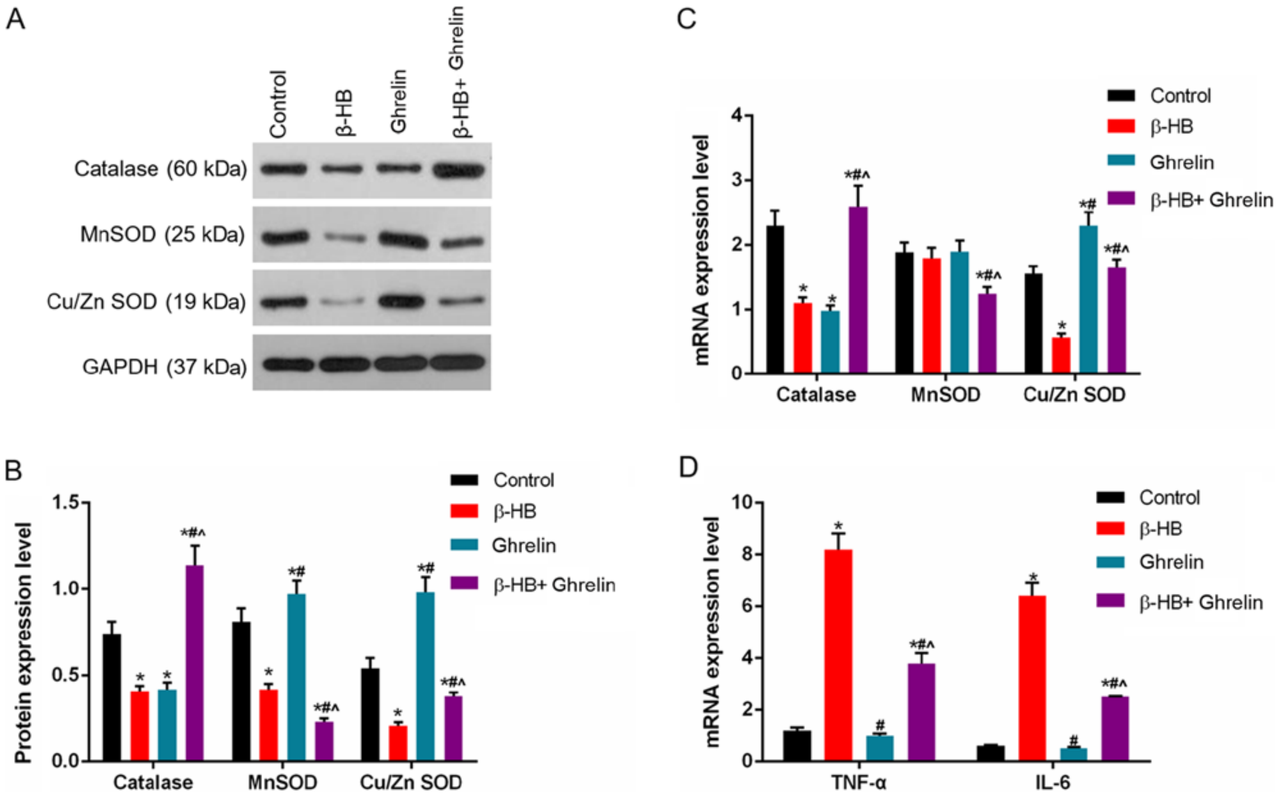

Figure 4. Effects of ghrelin and β-HB on the expression of catalase, MnSOD, Cu/ZnSOD, TNF-α and IL-6 in rat GASMCs. GASMCs were treated with 10^{-8} mol/l ghrelin and 5 mmol/l β-HB for 48 h. (A) The protein levels of catalase, MnSOD and Cu/ZnSOD were determined using western blot analysis and (B) quantified. Reverse transcription-quantitative PCR was used to determine the mRNA levels of (C) catalase, MnSOD, Cu/ZnSOD, (D) TNF-α and IL-6. ANOVA was used to analyze the differences. *P<0.05 vs. Control; $^{\#}$P<0.05 vs. β-HB; $^{\wedge}$P<0.05 vs. 10^{-8}. β-HB, β-hydroxybutyric acid; GASMCs, gastric antral smooth muscle cells; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc superoxide dismutase.

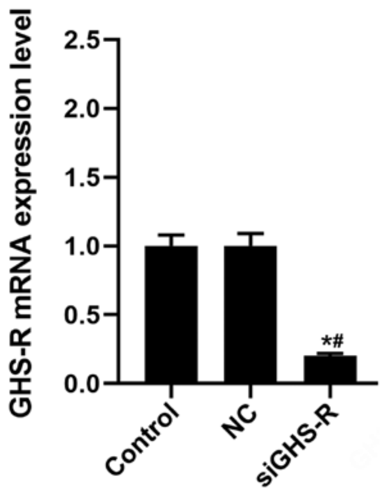

Figure 5. siGHS-R reduces the expression of GHS-R in GASMCs. The expression of GHS-R was determined in GASMCs following transfection with siGHS-R using reverse transcription-quantitative PCR. *P<0.05 vs. Control; $^{\#}$P<0.05 vs. NC. siGHS-R, small interfering RNA targeting GHS-R; GHS-R, growth hormone secretagogue receptor; GASMCs, gastric antral smooth muscle cells; NC, negative control.

Discussion

MLCK is a skeletal and smooth muscle enzyme that is encoded by two different genes in higher organisms: The mylk-1 and mylk-2 genes (16,17). The mylk-2 gene encodes an MLCK isoform that is only expressed in skeletal muscle cells. The mylk-1 gene encodes a 220 kDa MLCK, a 130 kDa MLCK and telokin, and is widely expressed in a diverse range of tissues and cells (18,19). MLCK is involved in adhesion and migration, which are basic characteristics of cells (20,21). A previous pharmacological study revealed that the inhibition of MLCK changed cell motility and wound contraction (22). There are three types of myosin regulatory light chains: MYL12B, MYL12A and MYL9 (23). The MYL9 gene is highly expressed in vascular smooth muscle cells (23). It has been reported that MYL9 impacts cell motility and contractility, and that is an important component of the contractile apparatus of cells (24). In the present study, β-HB inhibited the expression of MLCK and MYL9, while 10^{-8} mol/l ghrelin promoted the expression of MLCK and MYL9 in GASMCs.

There are three Ras homolog gene family members: RhoA, RhoB and RhoC in human and rat (25). Rho isoforms have GTPase activity and impact the levels of GDP and GTP. The sequences of RhoA, RhoB and RhoC share ~85% homology (26). Rho plays an important role in the regulation of cell shape and locomotion through actin and Rho is required for lamellipodia (27). RhoA directly promotes the polymerization of actin, and RhoA is considered to regulate actomyosin contractility, which is important for the ability of migrating cells to detach from the rear of the cell (26). ROCK and its two isoforms (ROCK-1 and ROCK-2) have an important role in cell motility and migration (28). ROCK-1 and

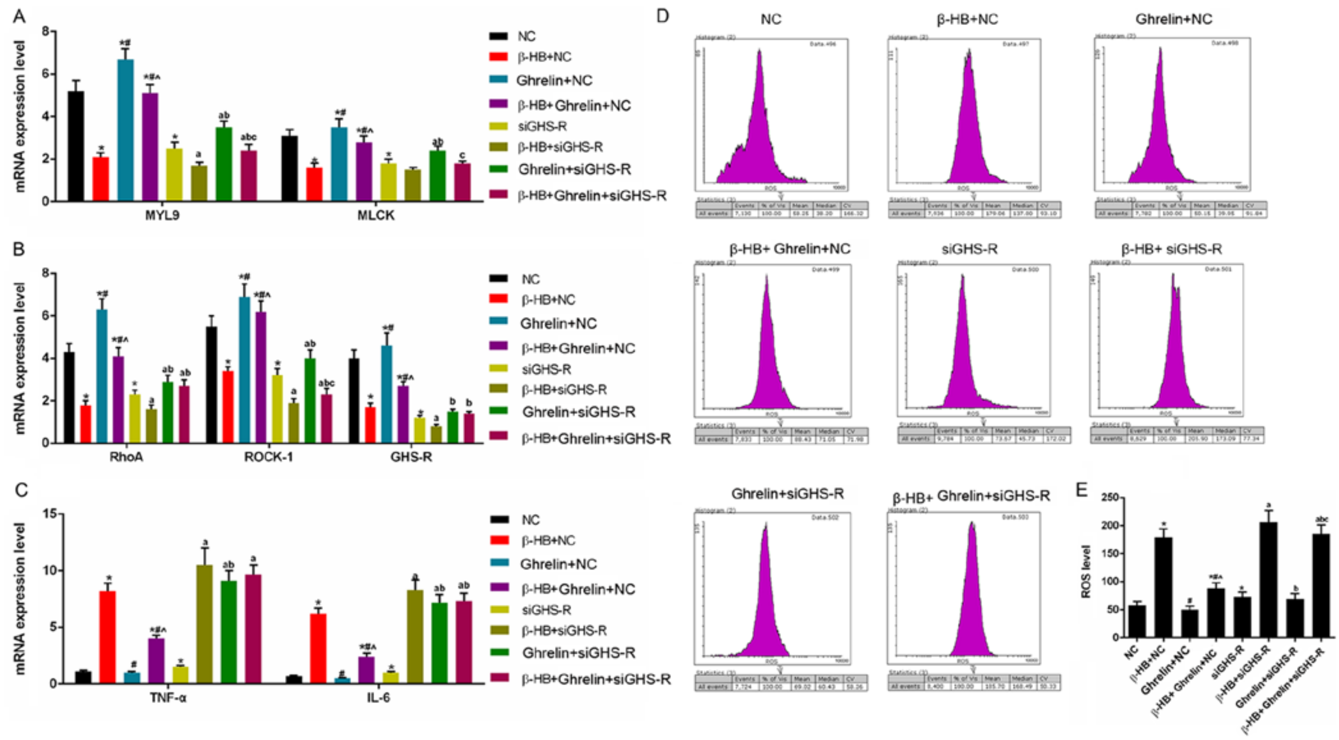

Figure 6. Effects of siGHS-R on the expression of MYL9, MLCK, RhoA, ROCK-1, GHS-R, TNF-α and IL-6, and ROS levels in rat GASMCs. GASMCs were transfected with siGHS-R and treated for 48 h, as indicated. The mRNA expression of (A) MYL9, MLCK, (B) RhoA, ROCK-1, GHS-R, (C) TNF-α and IL-6 were determined using reverse transcription-quantitative PCR. (D) ROS levels were analyzed using a ROS assay kit and flow cytometry. (E) Quantification of the ROS levels. ANOVA was used to analyze the differences. $^{*}P<0.05$ vs. NC; $^{\#}P<0.05$ vs. β-HB + NC; $^{\wedge}P<0.05$ vs. 10^{-8} + NC; $^{a}P<0.05$ vs. siGHS-R; $^{b}P<0.05$ vs. β-HB + siGHS-R; $^{c}P<0.05$ vs. 10^{-8} + siGHS-R. β-HB, β-hydroxybutyric acid; si, short interfering RNA; GASMCs, gastric antral smooth muscle cells; MYL9, myosin regulatory light polypeptide 9; MLCK, myosin light chain kinase; RhoA, transforming protein RhoA; ROCK-1, Rho-associated protein kinase-1; GHS-R, growth hormone secretagogue receptor; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; NC, negative control.

ROCK-2 contain different Rho-binding regions, and ROCK has a higher affinity with RhoC, which is important for cell locomotion (26). Previous studies reported that the inhibition of ROCK-1 decreased cell migration and motility (29,30). The present study identified that β-HB inhibited the expression of RhoA and ROCK1, whereas ghrelin increased the levels of RhoA and ROCK1 in GASMCs.

MnSOD is encoded by the SOD2 gene and the protein is located in the mitochondrial matrix. Cu/ZnSOD is encoded by the SOD1 gene, and the protein is located in the cytoplasm and the mitochondrial intermembrane space of cells (31). MnSOD and Cu/ZnSOD protect cells from oxidative damage (31); inflammation changes the antioxidative system, and increases inflammatory cytokines, which decreases the activities of the SOD proteins (32). Hydrogen peroxide can be neutralized by catalase during the process of antioxidation (33), and the overexpression of catalase reduces levels of DNA damage (34). ROS, TNF-α and IL-6 are involved in inflammation and immune responses; the inhibition of TNF-α, ROS and IL-6 expression reduces inflammation (35-37). A limitation of the present study may be the fact that the protein levels of TNF-α and IL-6 in GASMCs were not analyzed. However, changes in the mRNA expression level may reflect changes in the protein level. In the present study, β-HB promoted the expression of TNF-α and IL-6 in GASMCs, while ghrelin inhibited the β-HB-induced expression of TNF-α and IL-6. In addition, β-HB promoted inflammation in GASMCs, whereas ghrelin inhibited β-HB-induced inflammation in the GASMCs.

Ghrelin is a 28-amino-acid peptide, and is the endogenous ligand for GHS-R (38). In the present study, it was found that siGHS-R significantly inhibited the expression of MYL9, MLCK, RhoA and ROCK-1. By contrast, siGHS-R significantly increased the levels of ROS, TNF-α and IL-6 following treatment with β-HB, ghrelin or their combination in GASMCs. This indicated that silencing of GHS-R inhibited the motility of GASMCs, and promoted inflammation in GASMCs. The silencing GHS-R increased inflammation and the inhibition of GASMCs motility induced by the β-HB, ghrelin or their combination, which suggested that GHS-R may be a regulator of motility and inflammation in GASMCs. A limitation of the present study was that Transwell or wound healing assays were not used to determine the motility of GASMCs.

In conclusion, the present study has provided preliminary data to suggest that β-HB inhibits the motility of GASMCs and promotes inflammation, whereas ghrelin decreases these effects. GHS-R acted as a regulator of motility and inflammation in GASMCs treated with β-HB and ghrelin. Not analyzing the expression of classical markers of smooth muscle cells, such as osteopontin and calponin, may be a limitation of the present study, which should be addressed in future studies. Further research *in vivo* is also required.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Fundamental Research Funds for the Central University for the Southwest University (grant no. XDJK2018C083).

Availability of data and materials

The datasets used and/or analyzed during the study are available from the corresponding author on reasonable request.

Authors' contributions

XHu and LY provided substantial contributions to the concept and design of the study. CH, MA, JW, WH, XHe and ZW were involved in data acquisition, data analysis and interpretation. XHu and LY were involved in drafting the article or critically revising it for important intellectual content. All authors gave final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Animal experiments were approved by the Institutional Animal Care and Use Committee of Southwest University Hospital (no. 2017110853n).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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