Original Paper

Effects of Ghrelin on the Intracellular Calcium Concentration in Rat Aorta Vascular Smooth Muscle Cells

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Key Words

Ghrelin • Vascular smooth muscle cells • Intracellular calcium • AngII • Sq22536

Abstract

Background/Aims: Ghrelin has been regarded as a cardioprotective factor with complicated mechanisms. Whether ghrelin is vasodilative or vasoconstrictive in nature is controversial, and the effects of ghrelin on intracellular calcium concentration are still unclear. To explore the mechanisms involved in the vasoactive regulation of ghrelin at the cellular level, we investigated the effects of ghrelin on calcium concentrations in rat aorta vascular smooth muscle cells (VSMCs). **Methods:** We obtained VSMCs via cell culture and stained the cells with Furo-2 AM. Western blotting was used to verify growth hormone secretagogue receptor (GHS-R1a) expression in VSMCs. The intracellular calcium variations affected by ghrelin and the interactions of ghrelin with angiotensin II (AngII), Sq22536, and potassium chloride (KCl) were observed using a calcium imaging and analysis system. **Results:** Western blotting revealed good GHS-R1a expression in VSMCs. The most prominent finding in the present study was that ghrelin inhibited the AngII-induced increase in the calcium concentration. This inhibition was reversed by the adenylate cyclase inhibitor Sq22536 and the GHS-R1a antagonist (D-Lys³)-GHRP-6. This finding revealed the potential vasodilative effects of ghrelin at the cellular level. We did not observe any effects of ghrelin on intracellular calcium concentrations in resting VSMCs or the increase of calcium concentration induced by KCl. **Conclusion:** Ghrelin inhibited the increase in the intracellular calcium concentration of rat aorta VSMCs induced by AngII, which may depend on the activation of the cAMP/PKA pathway.

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Introduction

Ghrelin, an acylated 28-amino acid gut-brain peptide, is predominantly produced by the stomach oxyntic mucosa. It is the endogenous ligand of the hormone secretagogue receptor (GHS-R) [1]. GHS-R is widely expressed in the central and peripheral tissues, but circulating ghrelin is mainly expressed in the gastric mucosal X/A-like cells (>90% of the body's ghrelin stores) [2-4]. In addition to the gastrointestinal tract, some expression was also identified in the endocrine organs (ovary, anterior pituitary, and adrenal gland) [5], pancreatic islets [6], hypothalamus [7], and cardiomyocytes [8]. In the cardiovascular system, ghrelin and GHS-R are distributed in the arteries, heart, veins, and vascular endothelial cells, and they affect the regulatory functions of the heart and vasculature [9-12]. Two main molecular forms of ghrelin are reported, namely acylated ghrelin and desacyl ghrelin. In acylated ghrelin, serine in position 3 is n-octanoylated by ghrelin-O-acyltransferase, and this n-octanoylation is crucial for ghrelin binding to its receptor (GHS-R1a) and exerting its biologic activity [13-14]. Both forms of ghrelin are predominantly derived from gastric X/A-like cells. The amount of desacyl ghrelin far exceeds that of acylated ghrelin, and the ratio of desacyl ghrelin and acylated ghrelin in the blood was reported to fluctuate from 15:1 to 55:1 [13-15]. The exact receptor for desacyl ghrelin is unclear. Most previous studies focused on the acylated form, usually referred to as "ghrelin" in the literature [16], we used acylated ghrelin in the present study.

Ghrelin is a cardioprotective factor that reduces the circulatory levels of catecholamines, rennin, angiotensin II (AngII), aldosterone, and endothelin-1 (ET-1) in rats with heart failure in addition to significantly decreasing arterial pressure in humans and rats [12, 17-19]. The effects of ghrelin on vascular smooth muscles can be observed by assessing the artery rings using a myograph or vascular smooth muscle cells (VSMCs) by the patch clamp technique [20-22]. Nevertheless, whether ghrelin is vasodilative or vasoconstrictive in nature is contradictory among studies depending on the vessels examined [23-24]. It is hypothesized that ghrelin may play a role VSMC adjustment by affecting intracellular calcium concentrations.

The known intracellular mechanisms involved in ghrelin signaling pathways are complicated. Ghrelin has been reported to activate several intracellular signaling pathways. First, ghrelin primarily binds to GHS-R1a, which preferentially activates Gq-protein and phospholipase C (PLC) and mobilizes calcium release from inositol 1, 4, 5-triphosphate (IP₃) stores [25]. This is a classic well-described pathway that is commonly used to explain the vasoconstrictive effect of ghrelin in rat coronaries, guinea pig femoral arteries, and other contexts [21, 23]. In addition, ghrelin activates two other targets: (1) adenylate cyclase (AC) and cAMP-dependent protein kinase (PKA), namely the AC/cAMP/PKA signaling pathway in the endothelial and smooth muscle cells of the human aorta [11, 22, 26] and bladder [27] and (2) pancreatic β -cells in rats [28]. It also modulates cGMP production and cGMP-dependent protein kinase (PKG) in the GH3 rat pituitary tumor cells [29]. Recent reports indicated that the extracellular signal-regulated kinases 1 and 2 were also enhanced by ghrelin in both mouse VSMCs [30] and human endothelial cells [26, 31].

However, the effects of ghrelin on the intracellular calcium concentrations of VSMCs are still unknown. To achieve a deeper understanding of the mechanisms involved in the vasoactive regulation of VSMCs, we investigated the effects of ghrelin on intracellular calcium concentrations in VSMCs using a calcium imaging and analysis system.

Materials and Methods

Animals and materials

Fifteen male Sprague-Dawley (SD) rats (200 $\pm$ 10 g) were obtained from the Experiment Animal Center, Shantou University Medical College. The animals were cared for and treated as per the guidelines of the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH

Publication No. 85-23, revised 1996). All experimental protocols were supervised by the Laboratory Animal Ethics Committee of Shantou University Medical College.

VSMCs culture

Rat aorta VSMCs were obtained and cultured as described previously [32]. In brief, rats were anesthetized with 10% chloral hydrate (0.4 mL/100 g; i.p.). The thoracic aortae were removed from the fat and connective tissues and cleaned. The endothelium was removed, and the smooth muscle layer was cut into segments of 1 mm². Next, the VSMC tissues were placed in M199 medium containing 20% FBS (only for primary culture), penicillin (1 × 10⁵ U/L), and streptomycin (100 mg/L) and cultured with 5% CO₂ at 37 °C. Cells in the third to fifth generations (cultured with 10% FBS) were placed in Petri dishes and incubated for 36 h continuously in preparation for staining.

Western blotting to verify GHS-R1a expression in VSMCs

Western blotting was performed as described previously [33-34]. In brief, VSMCs (5 × 10⁶) were lysed in lysis buffer (10 mM Tris-buffered saline, 1 mM EDTA, 2 mM sodium orthovanadate, 0.2 mM PMSF, 2 µg/mL leupeptin, 2 µg/mL aprotinin, and 1% Triton X100). Protein was loaded onto SDS-PAGE gradient gels, separated, and electrophoretically transferred onto polyvinylidene difluoride membranes. The blots were blocked by incubation in TBST [10 mM Tris-HCl (pH 7.5), 150 g NaCl, and 0.1% Tween 20] containing 5% skimmed dried milk for 1 h at room temperature. After washing, the membranes were probed with the primary antibody (rabbit anti-rat GHS-R1a, Phoenix Pharmaceuticals). The signal was revealed using an enhanced chemiluminescence detection kit (Pierce) after reacting with the second antibody.

Calcium fluorescence intensity (FI) measurement and analysis

Fura-2 AM was dissolved with cosolvent Pluronic F-127 (1 mmol/L). VSMCs were washed twice with cell physiological solution (134 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4) and incubated for 20 min with 200 µL of cell physiological solution containing 5 µmol/L Fura-2 AM. The cells were then washed twice with cell physiological solution, and 2 mL of the same solution was added to the Petri dishes.

The calcium imaging and analysis system, which consists of an Axiovert 2000 fluorescence microscope (Carl Zeiss, Germany), CCD cameras (Hamamatsu ORCA-ER, Japan), and an Ultra High Speed Wavelength Switcher (LAMBDA DG-4, Sutter, USA), was used for observing intracellular calcium variations. After the staining sample was prepared, we selected a ×40 oil object lens and switched the optical path to CCD when the cells were brought into focus. PCI 6.0 calcium imaging system software was used to capture and analyze the calcium images (the excitation wavelengths were 340 and 380 nm in the Fura-2 module). The parameters were optimized to maximize the image quality in prescanning accordingly. The relative calcium concentration was expressed as the FI ratio of FI at 340 and 380 nm as follows:

$$FI \text{ ratio} = \frac{\geq FI_{(340)}}{\geq FI_{(380)}}$$

Where $\geq FI$ is the average FI of the selected cytoplasm area minus the average background FI.

Drug administration

The stock solutions including 5 × 10⁻⁴ and 5 × 10⁻⁶ mol/L acylated ghrelin (Sigma, USA), 4 mol/L KCl, 2 × 10⁻⁵ mol/L AngII, and 1 × 10⁻³ mol/L Sq22536 were prepared, and 20 µL of each solution (which were diluted 100-fold) were added to 2 mL of cell physiological solution in the respective Petri dishes. To validate the specificity of the observed acylated ghrelin-induced inhibition of AngII-stimulated intracellular calcium elevation, we prepared 5 × 10⁻⁴ mol/L (1:1 with the concentration of ghrelin after being diluted, namely 5 × 10⁻⁶ mol/L) GHS-R1a antagonist (D-Lys³)-GHRP-6 solutions (Mocell, Shanghai, P.R of China) according to previous studies [22, 35], and 20 µL of each solution was added to 2 mL of cell physiological solution (which were diluted 100-fold) in the sequence of acylated ghrelin, (D-Lys³)-GHRP-6, and AngII.

Data analysis and statistics

All data were recorded as the mean ± SD. The rate of change of the intracellular calcium concentration was obtained by calculating the FI ratio after stimulation to the resting state FI. Statistical analysis of drug effects was performed using a self-control paired t-test. *P* < 0.05 was considered statistically significantly.

Fig. 1. Western blotting of GHS-R1a expression in rat aorta VSMCs. Rabbit anti-rat GHS-R1a antibody bound a component of the membrane with a molecular weight of 41 kDa, and good GHS-R1a expression in rat aorta VSMCs was observed.

Fig. 2. Results of the FI stability examination. (A) Images of calcium FI taken using the calcium imaging and analysis system ($\times 400$). The luminance of the fluorogram corresponds to calcium concentration; i.e., increased brightness of the fluorogram indicates a higher calcium FI ratio. (B) Time curve of the intracellular calcium FI ratio. Our data revealed that the FI ratio was stable for 15 min after initiation, after which it began to attenuate. The stability of the experimental system was confirmed.

Fig. 3. Influence of ghrelin on the intracellular calcium FI ratio in resting VSMCs. Administration of 5×10^{-8} and 5×10^{-6} mol/L ghrelin did not affect the calcium FI ratio of resting VSMCs.

Results

Examination of GHS-R1a in VSMCs

Figure 1 shows antibody binding to an approximately 41-kDa component of the membrane, which corresponds to the molecular weight of GHS-R1a. It was confirmed that GHS-R1a was well expressed in rat aorta VSMCs. This result was consistent with that of a previous study [33].

Examination of FI stability

Figure 2 shows that the FI ratio remained unchanged in the first 15 min after which it was attenuated. Fluorescence quenching by excitation therefore can be ignored because all observations were performed within 15 min.

Fig. 4. Effects of ghrelin on the calcium FI in VSMCs during the interactions of ghrelin with AngII, KCl, and Sq22536. (A) Calcium FI after AngII administration. AngII image nos. 4 and 5 are brighter than the control images, indicating that calcium FI was enhanced. In contrast, reduced luminance was observed in the no. 6 AngII image, indicating that calcium FI was attenuated. Such dynamic variation in the luminescence induced by AngII coincided with the FI ratio time curve shown in (B). (C) Calcium FI when VSMCs were preincubated with 5×10^{-6} mol/L ghrelin. Image nos. 3 and 4 were not different from the control images, indicating that the administration of ghrelin did not directly affect calcium FI. Moreover, AngII image nos. 5 and 6 did not exhibit the same increasing effects as Fig. 3A, indicating that preincubation with ghrelin inhibited the enhancing effect of AngII. Then, when 4×10^{-2} mol/L KCl was administered, as shown in image nos. 7, 8, and 9, the brightness of the images increased, indicating that calcium FI was increased by KCl. Such dynamic changes were also observed in the FI ratio time curve (D). (E) Calcium FI when VSMCs were preincubated with 1×10^{-5} mol/L Sq22536 and 5×10^{-6} mol/L ghrelin. AngII image nos. 4, 5, and 6 were brighter than the control image, indicating that Sq22536 inhibited the antagonizing effects of ghrelin on AngII. Such dynamic variations were also observed in the FI ratio time curve (F).

Fig. 5. Effects of ghrelin on the calcium FI ratio in VSMCs during the interactions between ghrelin and (D-Lys³)-GHRP-6. Time curve of the intracellular calcium FI changes induced by the successive administration of 5×10^{-6} mol/L ghrelin, 5×10^{-6} mol/L (D-Lys³)-GHRP-6, and AngII. Ghrelin partially inhibited the increase in the calcium FI ratio induced by AngII ($P < 0.05$).

Influence of ghrelin on the calcium FI ratio in resting VSMCs

Figure 3 shows that the administration of 5×10^{-8} and 5×10^{-6} mol/L ghrelin did not change the intracellular calcium FI ratio in resting VSMCs.

Effects of ghrelin on the calcium FI ratio in VSMCs during interactions of ghrelin with AngII, KCl, and Sq22536

Figure 4A and 4B show that 2×10^{-7} mol/L AngII induced a transient increase in the intracellular calcium FI ratio in VMSCs (FI ratio $155 \pm 40\%$, $P < 0.05$ compared with the resting state). Preincubation with 5×10^{-6} mol/L ghrelin inhibited the vasoconstrictive effect of AngII (FI ratio reduced to $103 \pm 13\%$, $P > 0.05$ compared with the resting state). However, when KCl was administered, the intracellular calcium FI ratio was increased in VMSCs (FI ratio $376 \pm 87\%$, $P < 0.01$ compared with the resting state; Fig. 4C and 4D). When VMSCs were preincubated with 1×10^{-5} mol/L Sq22536 and 5×10^{-6} mol/L ghrelin for 5 and 3 min, respectively, the effects of ghrelin were inhibited, and the vasoconstrictive effect of AngII was revealed (FI ratio enhanced to $163 \pm 23\%$, $P < 0.05$ compared with the resting state, Fig. 4E and 4F).

Effects of ghrelin on the calcium FI ratio in VSMCs during interactions of ghrelin with (D-Lys³)-GHRP-6 and AngII

Figure 5 reveals that 5×10^{-6} mol/L (D-Lys³)-GHRP-6 can partially block the inhibitory effect of 5×10^{-6} mol/L ghrelin on AngII (FI ratio increased to $146 \pm 27\%$, $P < 0.05$ compared with the resting state), which is consistent with previous findings [22]. The observed specificity of acylated ghrelin was validated by the administration of (D-Lys³)-GHRP-6.

Influence of ghrelin on KCl-induced increase in the calcium FI ratio in VMSCs

Figure 6A shows that the calcium FI ratio in resting VSMCs increased to $268 \pm 74\%$ ($P < 0.01$ compared with the resting state) by 4×10^{-2} mol/L KCl, but this increase could not be sustained. When VSMCs were preincubated with 5×10^{-6} mol/L ghrelin for 5 min, the increase in the calcium FI ratio was not inhibited (FI ratio $380 \pm 137\%$, $P < 0.01$ compared with the resting state). Acceleration of the decrease in the calcium FI ratio was also not obvious following the administration of 5×10^{-6} mol/L ghrelin (Fig. 6B).

Discussion

Effects of ghrelin on the cardiovascular system have been widely reported. Most studies confirmed that ghrelin inhibits proinflammatory cytokine expression as well as apoptosis of endothelial cells and cardiomyocytes in addition to alleviating ischemia/reperfusion injury in rat heart. Furthermore, it contributes to anti-atherosclerosis, protection against myocardial ischemia [31, 36-40], and reduction of blood pressure [17-19, 41-42]. However, other authors reported the contradictory effects of ghrelin on vascular smooth muscles.

Fig. 6. Effects of ghrelin on the calcium FI ratio in VSMCs during the interactions of ghrelin with KCl. (A) Time curve of the variations of the intracellular calcium FI ratio induced by KCl. Administration of 40 mmol/L KCl significantly increased the calcium FI ratio ($P < 0.05$). (B) Time curve of the intracellular calcium FI changes induced by the successive administration of ghrelin and KCl. Ghrelin did not inhibit the increase in the calcium FI ratio induced by KCl.

Initially, some studies concluded that ghrelin was a vasodilator [43], whereas Pemberton reported that ghrelin induces the contraction of rat coronary arteries [21]. Dimitrova found that ghrelin enhances the ET-1-induced contraction of guinea pig renal arteries including endothelium-denuded and intact endothelium vessels, which coincided with the results in guinea pig femoral arteries [20, 23]. Simultaneously, Wiley found that ghrelin significantly decreases the ET-1-induced contraction of human endothelium-denuded mammary arteries [44]. Moazed reported that ghrelin evokes endothelium-dependent vasodilatation of the rat mesenteric vascular bed [45]. Nevertheless, no clinical or bench study has investigated the effects of ghrelin on intracellular calcium concentrations. The roles of ghrelin in vasoactive mechanisms remain unknown.

The present study was the first to investigate the effects of ghrelin on intracellular calcium concentration using VSMCs. The most important finding in this study is that ghrelin inhibits the increase in the calcium concentration induced by AngII (Fig. 4C and 4D), although it does not affect the calcium concentration of resting VSMCs when administered directly (at concentrations of 5×10^{-8} mol/L and 5×10^{-6} mol/L, Fig. 3). This antagonistic effect of ghrelin on AngII could be reversed by the AC inhibitor Sq22536 (Figs. 4E and 4F). We also found that ghrelin had no effects on the increase in the calcium concentration induced by KCl (Fig. 5A and 5B). The interactions of ghrelin and the other vascular adjusting factors (including AngII and KCl) are complicated. It is helpful to clarify the pathophysiological mechanisms of ghrelin together with its pharmacological applications to achieve a deeper understanding of its interactions.

The detailed mechanisms of the interactions between ghrelin and AngII are unclear. AngII, which binds to the AT_1 receptor on the VSMC membrane, can activate PLC to produce IP_3 and stimulate intracellular calcium release from the sarcoplasmic reticulum (SR). Our findings revealed that preincubation with ghrelin can thoroughly inhibit the increase in the VSMC calcium concentration induced by AngII, whereas pretreating VSMCs with Sq22536 before the administration of ghrelin and AngII inhibits the effect of ghrelin. These data indicate that ghrelin inhibits intracellular calcium release by activating the cAMP/PKA signal pathway, which is consistent with the results reported by Rossi [22] and Tolekova [27], who proved that ghrelin inhibits SMC contraction in a concentration-dependent manner by activating the cAMP/PKA signaling pathway. Thus, we hypothesize that binding of ghrelin to GHS-R1a, increases cAMP production by activated AC, which further activates PKA or PKG and enhances calcium intake in SR. In contrast, the large conductance calcium-activated potassium channels and spontaneous transient outward currents activated by cAMP may also play a role in the mechanisms of ghrelin, which hyperpolarizes membranes and accelerates intracellular calcium discharge [46]. Furthermore, the inhibitory effects of ghrelin on AngII may enable us to understand whether ghrelin is vasodilative or vasoconstrictive in nature.

Because ghrelin inhibits the vasoconstrictive effects of AngII, our data support that ghrelin has vasodilative effects in rat aorta VSMCs.

To validate the specificity of acylated ghrelin-induced inhibition of AngII-stimulated intracellular calcium elevation, we used a known specific GHS-R1a receptor inhibitor, (D-Lys³)-GHRP-6. Our data indicate that 5×10^{-6} mol/L (D-Lys³)-GHRP-6 (1:1 with the concentration of ghrelin, Fig. 5) exerted strong inhibitory effects on ghrelin, and AngII-stimulated intracellular calcium elevation was observed. (D-Lys³)-GHRP-6 is a well-known selective GHS-R1a receptor inhibitor that only competes with the binding of acylated ghrelin [22, 35], therefore, the specificity of acylated ghrelin is validated. As to the administration sequence, in our preliminary experiment, we added the inhibitor before ghrelin, and found the (D-Lys³)-GHRP-6 administration would potentially make small intracellular calcium fluorescence. Since the aim of this experiment is only to confirm the specificity of acylated ghrelin-induced inhibition of AngII-stimulated intracellular calcium elevation, we changed the sequence of administration (inhibitor after ghrelin) to eliminate the potential noise.

In the present study, we found that ghrelin did not affect the increase in the calcium concentration induced by KCl. It is known that intracellular calcium concentrations can be enhanced by both intracellular calcium release and extracellular calcium influx, the latter of which is induced by opening of the voltage-dependent calcium channels (VDCCs). This finding indicated that ghrelin has no direct effect on VDCCs or extracellular calcium influx, as preincubation with ghrelin did not inhibit the KCl-induced increase in the calcium concentration in VSMCs.

We also found that ghrelin did not affect the calcium concentration in resting VSMCs when administered directly. This result was consistent with previous studies that reported the analogous conclusion that ghrelin did not induce any significant changes in the spontaneous tone of smooth muscles [20, 27, 33].

The results of the present study implied the ghrelin possess vasodilative effects. However, the detailed physiological effects of ghrelin remain controversial, as some researchers also reported the contractive mechanisms of ghrelin. Dimitrova reported that ghrelin exerted its contractile effects via Src kinase, MEK, cyclooxygenase 1, and T prostanoid receptor agonists in human mesenteric arteries [47]; Sax also validated the coronary vasoconstrictor effect of ghrelin, which is not mediated by GHS-R1a [33]. These results in different experimental conditions indicate that the mechanisms of ghrelin regarding intracellular calcium concentrations are complicated and affected by multiple factors.

In this study, we investigated the effects of the interaction of ghrelin with AngII and KCl in VSMCs. We did not find any direct effect of these interactions on intracellular calcium concentrations in resting rat aorta VSMCs or on KCl-induced increases in calcium concentrations. To validate these results, two experiments are needed. First, GHS-R1a expression in VSMCs should be examined. Western blotting confirmed good GHS-R1a expression in VSMCs, which was consistent with analogous previous studies [22, 34]. Second, a GHS-R1a agonist should be used to examine the function of GHS-R1a. Unfortunately, no GHS-R1a agonist (such as EP1572 and MK-0677) is commercially available, and we were unable to synthesize this compound because of which this profound experiment could not be completed. The limitation of this study was that we only used the GHS-R1a antagonist (D-Lys³)-GHRP-6 to examine the function of GHS-R1a. There is a possibility that a substantial quantity of acylated ghrelin was desacylated in the cell culture medium during the cell culture experiment between the addition of acylated ghrelin and that of AngII. The potential effects of desacyl ghrelin cannot be ruled out. In a previous study, a ghrelin-deacylating enzyme (acyl-protein thioesterase/lysophospholipase) was purified and identified [48]. This study validated the specificity of acylated ghrelin, and thus, we can hypothesize that desacylated ghrelin does not affect the inhibition of AngII. To verify this hypothesis, acyl-protein thioesterase 1/lysophospholipase I should be employed to investigate the effects of desacylated ghrelin [48]. This important experiment could not be performed because this enzyme was not available in our laboratory, which was another limitation of this study. These experiments will be conducted in the future if the reagents become available. We also found

that ghrelin antagonizes the vasomotor effects of AngII, which suggested the ghrelin has a potential vasodilative effect at the cellular level. In this regard, ghrelin can be considered a potential protective factor against cardiovascular disease. This study only begins to uncover the complicated mechanisms of ghrelin. Future studies should be conducted to clarify the detailed mechanisms of the interactions of ghrelin with vasoactive factors at the cellular level.

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