Gastrin-releasing peptide promotes the migration of vascular smooth muscle cells through upregulation of matrix metalloproteinase-2 and -9

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Gastrin-releasing peptide (GRP) has been reported to be implicated in the pathogenesis of inflammatory disorders. The migration and proliferation of vascular smooth muscle cells (VSMCs) are key components of vascular inflammation that leads to the development of atherosclerosis. The present study aimed to investigate the molecular effect of GRP on VSMC proliferation and migration. We report that GRP significantly enhanced the proliferation and migration of rat VSMCs. GRP increased mRNA and protein expression of matrix metalloproteinase-2 and -9 (MMP-2/9) in VSMCs. The induction of MMP-2/9 by GRP was regulated by the activation of the signal transducer and activator of transcription-3 (STAT3). In addition, STAT3-knockdown of VSMCs by siRNA or blockade of the GRP receptor inhibited GRP-induced migration of VSMCs. Taken together, our findings indicate that GRP promotes the migration of VSMCs through upregulation of MMP-2/9 via STAT3 activation. [BMB Reports 2017; 50(12): 628-633]

INTRODUCTION

Gastrin-releasing peptide (GRP), a primary member of mammalian bombesin-like peptides, is a small regulatory peptide that has been implicated in various cellular responses including cell growth, proliferation, survival, inflammation, and angiogenesis under physiological and pathological situations (1). GRP binds with high affinity to the GRP receptor in an autocrine, paracrine, or neuroendocrine manner to regulate diverse biological functions (2). The GRP receptor is a specific member of the G-protein-coupled receptor family and is found in different types of normal and cancerous tissues (3).

The vascular inflammatory response involves the adherence and recruitment of inflammatory cells (lymphocytes, neutrophils, and monocytes) to vascular cells (endothelial cells and vascular smooth muscle cells (VSMCs) (4). Upon exposure to pro-inflammatory stimuli, vascular endothelial cells and VSMCs regulate the expression of cell adhesion molecules, growth factors, cytokines, and matrix metalloproteinases (MMPs), which can lead to the progression of cardiovascular diseases such as atherosclerosis (5). GRP has been shown to mediate the migration and recruitment of neutrophils, which acts as inflammatory mediators (6). We have previously demonstrated that GRP directly induces endothelial dysfunction through the upregulation of cell adhesion molecules in endothelial cells (7). However, little attention has been devoted to the potential role of GRP on inflammatory responses in VSMCs.

In this study, we examined the effects of GRP on VSMC migration and proliferation, which have been linked to the development and progression of atherosclerosis (8). We also demonstrated that GRP-induced migration of VSMCs is mediated by the upregulation of MMP-2/9 and have explored the underlying mechanism(s).

RESULTS

GRP induces proliferation and migration in VSMCs

VSMCs were treated with different concentrations of GRP for up to 72 h, and cell proliferation was measured by the MTT assay. As shown in Fig. 1A, the proliferation rate increased in a dose-dependent manner with increasing GRP concentrations at 48 h and 72 h, respectively, but no such effects were observed at 24 h. The maximal increase in proliferation, a 1.7-fold increase over control, was induced by GRP. BrdU incorporation assay was employed to further test VSMC proliferation. The results showed that treatment of VSMCs with GRP significantly induced cell proliferation in a dose- and time-dependent manner (Fig. 1B). The migration of VSMCs is known to be a
Effect of GRP on VSMC migration
Hyun-Joo Park, et al.

Fig. 1. Effect of GRP on proliferation and migration in vascular smooth muscle cells. (A) VSMCs were exposed to different concentrations of GRP for the indicated times. After treatment, cell proliferation was determined by MTT assay. The data are expressed as percentage ± SE from at least three different experiments performed in triplicate. *P < 0.05; **P < 0.01 vs. control. (B) VSMCs were incubated with the indicated concentrations of GRP for 48 and 72 h, and the proliferating cells were assayed using BrdU incorporation. *P < 0.1 vs. control. (C) VSMCs were seeded on gelatin-coated filters for the migration assay. VSMCs in the absence or presence of GRP (100 nM) were incubated in Transwell chambers for 24 h. The migrated cells were stained with hematoxylin-eosin and photographed. The number of migrated cells was counted. *P < 0.1 vs. control. (D) The migration capability of VSMCs was analyzed by scratch wound migration assays. The number of cells that migrated beyond the reference line was counted.

Fig. 2. Effect of GRP on the expression and activation of MMP-2/9 in vascular smooth muscle cells. (A) VSMCs were incubated with GRP (100 nM) for the indicated times. Total RNA was isolated and then analyzed by real-time PCR using primers specific for rat MMP-2 and MMP-9. The expression level of the control (untreated) was set to 1, and the values are normalized to the β-actin mRNA levels. *P < 0.05; **P < 0.01 vs. control. (B) MMP-2 and MMP-9 protein levels were examined by western blotting using anti-MMP-2 and anti-MMP-9 antibodies. β-actin served as the loading control. (C) VSMCs were exposed to GRP for the indicated times. The conditioned medium was concentrated and subjected to gelatin zymography. (D) Protein and mRNA expression levels of GRP-R were observed by western blotting and RT-PCR, respectively. β-actin and GAPDH served as the loading control. (E) VSMCs were incubated with GRP (100 nM) alone or in combination with RC-3095 (100 nM) for 8 h. Using real-time PCR, the expression levels of MMP-2 and MMP-9 mRNA were also quantified. The expression level of the control (untreated) was set to 1, and the values were normalized to the β-actin mRNA levels. *P < 0.05 vs. control; #P < 0.05 vs. GRP alone.

key event in the arterial response to injury, vessel development and atherosclerosis (9). In this study, the effect of GRP on VSMC migration was examined using a modified Boyden's chamber assay. We found that GRP significantly enhanced the migration of VSMCs, by approximately 3.8-fold (Fig. 1C). We also performed a scratch wound migration assay to qualitatively observe the effect of GRP-induced motility of VSMCs. As shown in Fig. 1D, GRP treatment increased the number of cells that migrated into the scratch wound compared to that observed with control VSMCs.

**GRP promotes the expression and activity of MMP-2 and MMP-9 in VSMCs**

MMP-2 and MMP-9 are known to be major proteinases that contribute to the migration ability of VSMCs (10). Thus, we investigated the role of GRP on the expression of MMP-2 and MMP-9 in VSMCs. Cells were treated with GRP for the indicated times, and levels of MMP-2 and MMP-9 mRNA were measured real-time quantitative PCR analysis. As shown in Fig. 2A, GRP significantly induced the expression of MMP-2 and MMP-9 mRNA in a time-dependent manner. However, GRP had little effect on the mRNA expressions of MMP-1, MMP-3, MMP-7, MMP-12, MMP-13, and MMP-14 (data not shown). The protein levels of MMP-2 and MMP-9 in GRP-treated VSMCs were further analyzed by western blot analysis. Consistent with results of the real-time quantitative PCR analysis, GRP significantly induced the protein levels of MMP-2 and MMP-9 in VSMCs (Fig. 2B). Gelatin zymography showed that GRP
Effect of GRP on VSMC migration
Hyun-Joo Park, et al.

significantly increased levels of mature and active MMP-2 and MMP-9 proteins (Fig. 2C). Recent studies showed that the effects of GRP are known to be mediated by the GRP receptor in many cell types (11). The presence of GRP-R mRNA and protein in VSMCs was confirmed by RT-PCR and western blot analysis, respectively (Fig. 2D). Next, we tested the effect of pretreatment with RC-3095, a selective GRP-R antagonist (12), on the increase in expression of MMP-2 and MMP-9 mRNA caused by GRP. As expected, GRP induced the expression of MMP-2 and MMP-9 mRNA compared with that in the untreated control, which was suppressed by pretreatment with RC-3095 (Fig. 2E).

**GRP-induced MMP-2/9 expression is mediated by JAK2/STAT3 signaling pathways**

JAK2/STAT3 signaling is required for expression of MMP-2 and MMP-9 in VSMCs (13). We determined whether GRP stimulates the activation of JAK2 and STAT3 in VSMCs. As shown in Fig. 3A, treatment of VSMCs with GRP increased the phosphorylation of JAK2 and STAT3 in a time-dependent manner, and the maximal activation was observed after 30 min of GRP treatment. Next, to elucidate the involvement of JAK2/STAT3 signaling in GRP-induced MMP-2/9 expressions, we performed real-time PCR analysis. As shown in Fig. 3B, pretreatment with the JAK2/STAT3 inhibitors, AG490 or WP1066 (14, 15), significantly decreased the mRNA levels of MMP-2 and MMP-9 otherwise induced by GRP. To confirm that the expression of MMP-2 and MMP-9 was directly regulated by STAT3, we used a STAT3-targeted siRNA or a control siRNA. We demonstrated that the protein and mRNA levels of MMP-2 and MMP-9 were decreased by STAT3-knockdown in VSMCs with siRNA (Fig. 3C and D). In the presence of STAT3-targeted siRNA, the mRNA expression of GRP-induced MMP-2 and MMP-9 was markedly reduced compared with that in control...
siRNA-transfected VSMCs (Fig. 3E).

Involvement of JAK2/STAT3 signaling pathway in GRP-induced VSMC migration

To examine whether JAK2/STAT3 signaling is involved in GRP-induced migration in VSMCs, we adopted Boyden’s chamber migration assay. As shown in Fig. 4A, pretreatment with WP1066 significantly decreased GRP-induced migration of VSMCs. We also performed a scratch wound migration assay to qualitatively observe the effect of AG490 on GRP-induced motility of VSMCs. As expected, AG490 treatment decreased the number of cells that migrated into the scratch wound compared to that observed in GRP-treated VSMCs (Fig. 4B). Next, to elucidate the role of STAT3 in migration of VSMCs, we knocked down endogenous STAT3 in VSMCs and assessed the rate of GRP-induced migration. As shown in Fig. 4C and D, depletion of STAT3 markedly reduced the ability of GRP to induce VSMC migration.

DISCUSSION

The proliferation, migration, and accumulation of VSMCs are critical steps in the pathogenesis of intimal hyperplasia after arterial injury and atherosclerosis (16). During the atherosclerotic process, several mediators, such as growth factors and pro-inflammatory cytokines, induce the phenotypic switch of VSMCs from the quiescent, contractile state to the active, synthetic phenotype as well as VSMC proliferation and migration from the media to the subendothelial region (17, 18). Activation of STAT3 by JAK2 regulates a variety of biological responses, including proliferation, survival, differentiation, and inflammation (19). It has been reported that STAT3 regulates the phenotypic change of VSMCs by association with myocardin (20). The activation of STAT3 by JAK2 in VSMCs is induced in response to various growth factors, cytokines and hormones, such as platelet-derived growth factor (PDGF)-BB, angiotensin II, vascular endothelial growth factor (VEGF) and interleukin-6 (21). In this study, we showed that GRP markedly stimulates the activation of STAT3 in VSMCs. Further investigations will be needed to examine whether GRP could reduce the contractile phenotype or/and induce the synthetic phenotype of VSMCs through the JAK-STAT3 signaling pathway.

The major function of MMPs, which form a family of proteolytic enzymes, is to degrade various components of the extracellular matrix (ECM) and essential in physiological and pathological processes including wound healing, angiogenesis, tumorigenesis, and inflammation (10). Among the MMPs, the gelatinases MMP-2 and -9 have been found to be predominantly upregulated in VSMCs within atherosclerotic plaques and of the mouse atherosclerosis model (22, 23). Many studies have identified increased MMP-2 and MMP-9 expression has been coincided with VSMC migration from the media to the intima (10). MMP-9 overexpression increases the migration of VSMCs and alters vascular remodeling in the injured carotid artery (24). MMP-2 contributes to VSMC migration by the degradation of ECM and non ECM, or by inducing a phenotype switch (25).

We demonstrated in this report that GRP upregulates the expression of MMP-2/9 in VSMCs and in turn mediates the migration of VSMCs, which suggesting a pathogenic role of GRP as a MMP-2/9 regulator in the progression of atherosclerosis. The promoter region of MMPs contains binding sites for nuclear factor-κB (NF-κB), Activator Protein-1 (AP-1), PEA3, SP-1, and STAT-3 (26). It has been reported that salusin-β-induced MMP-9 expression is mediated by NF-κB in VSMCs (27). We previously found that GRP induces NF-κB activation in vascular endothelial cells (7). We also observed that the treatment of GRP slightly stimulated IkBα phosphorylation and degradation in VSMCs (data not shown). The activation of NF-κB has been reported to be positively or negatively associated with STAT3 activation in different types of human cancers (28). We are currently investigating whether NF-κB activation or the association of NF-κB and STAT3 contribute to GRP-induced MMP expression and VSMC migration.

In conclusion, our research shows for the first time that GRP increases the migration of VSMCs by promoting STAT3-mediated expression of MMP-2/9. Additional in vivo studies will be required to consider whether intervention targeting the GRP-STAT3 axis may be an efficient strategy for the prevention or treatment of vascular diseases.

MATERIALS AND METHODS

Reagents and antibodies

GRP and RC-3095 were purchased from Sigma. AG490 and WP1066 were supplied by Calbiochem and Sigma, respectively. The antibodies for GRP-R and MMP-9, were supplied by Santa Cruz Biotechnology. The antibodies for phospho-JAK2, JAK2, phospho-STAT3, and STAT3 were obtained from Cell Signaling. MMP-2 and β-actin antibodies were purchased from Abcam and Bioworld Technology, respectively. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Thermo Fisher Scientific.

Cell isolation and cell culture

To isolate VSMCs, Sprague-Dawley rats (3 weeks old) were euthanized via intraperitoneal injection of sodium pentobarbital (60 mg/kg) by a tissue explanting method. Thoracic aorta was isolated and surrounding fat and connective tissues were discarded. Vessels were cut longitudinally and the lumen side was scraped with a razor blade to remove the intima. Vessels were fragmented into 3-5 mm lengths and explanted lumen side down on collagen-coated culture dishes. After seven days of explanting, tissue fragments were discarded and sprouted VSMCs were collected (referred to as P0). The VSMCs were grown in DMEM (Gibco) with 10% FBS (Gibco) and 1% antibiotics, at 37°C in a humidified 95% air/5% CO₂ environment.
MTT assay
VSMCs (5 × 10^4 cells) were seeded into 24-well plates and incubated for 24, 48, and 72 h. After exposure to different doses of GRP, cells were treated with MTT solution (0.5 mg/ml) and analyzed by MTT assay as described (29).

BrdU incorporation assay
To evaluate cell proliferation, we used an FITC BrdU Flow kit purchased from BD Pharmingen according to the manufacturer’s instructions. VSMCs were treated with GRP for 48 and 72 h. VSMCs were then labeled with BrdU for 3 h, washed, fixed and permeabilized with BD Cytofix/Cytoperm buffer. After repeated incubation on ice, washing, and centrifugation, cells were treated with DNase to expose BrdU epitopes for 1 h at 37°C, washed, then stained with fluorescent anti-BrdU for 20 min at room temperature, washed again, and analyzed by FACS Calibur (BD Bioscience).

Boyden chamber migration assay
Transwells with 8 μm porosity polycarbonate membrane inserts were coated with 10 μg gelatin. VSMCs were suspended in DMEM as 1 × 10^5 cells/100 μl, and were added to the upper chamber. GRP (100 nM) or WP1066 (10 μM) in DMEM was added into the lower chamber. VSMCs migrating through the filter and appearing on the lower side were fixed by careful immersion of the filter into methanol for 1 minute, stained with H&E solution and cells counted in three random fields per well (30). Each experiment was performed in duplicate and three separate experiments were performed for each group.

Scratch wound migration assay
VSMCs were seeded in 6-well plates at a concentration of 1 × 10^5 cells/well in 1 ml of serum-free DMEM for 6 h, until an adherent monolayer was obtained. A 10 μl pipette tip was used to create a scratch in the monolayer and the cells were washed 3 times with serum-free medium. The cells were then placed in fresh serum-free medium and treated with either GRP or AG490. Samples were taken at the beginning and after 24 h of culture in 5% CO2 at 37°C. Images of the scratch wounds were taken and measured by Image-J software to calculate the mean and standard deviation. Each experimental group was compared with its respective control. The experiments were repeated three times.

Real-time RT-PCR analysis
Real-time PCR quantification was performed using a SYBR® Green method (Light Cycler; Roche Applied Science). Cycling parameters included 1 cycle at 95°C for 10 min, followed by amplification for 30 cycles at 95°C for 10 s, 57°C for 5 s, and 72°C for 7 s. The entire cycling process, including data analysis, took less than 1 h and was monitored using Light Cycler software (version 4.0). The oligonucleotide primers for real-time PCR were as follows: MMP-2, 5'-ACCGTCGCCCATCATG-3' and 5'-TTGCATGCTTTGGATCCA -3'; MMP-9, 5'-TCGAAGGCGACCTCAAGTG-3' and 5'-TTCGGTGTAGCTTTGGATCCA -3'.

Gelatin zymography assay
Confluent VSMCs were maintained in serum-free medium for 12 h prior to being treated with GRP for 24 h. The conditioned medium was subsequently collected, cleared by centrifugation and mixed with 2 × SDS sample buffer, followed by electrophoresis on polyacrylamide gels containing 0.1% (w/v) gelatin. After electrophoresis, the gels were incubated in renaturing buffer (2.5% Triton X-100) with gentle agitation to remove the SDS and were then incubated in developing buffer (50 mM Tris-HCl buffer, pH 7.4, and 10 mM CaCl2) overnight at 37°C to allow digestion of the gelatin. Finally, the gels were stained with 1% Coomassie brilliant blue R-250 and incubated in destain buffer (10% acetic acid and 30% methanol).

RNA interference experiment
Small interfering RNA (siRNA) duplexes for rat STAT3 and a negative control siRNA were obtained from Santa Cruz Biotechnology and Bioneer Co, respectively. Transfection of VSMCs was carried out using oligofectamine, according to the manufacturer’s instructions.

Statistical analysis
Data shown are the mean ± standard deviation (S.D.) obtained for at least three independent experiments. Statistical comparisons between groups were made by one-way analysis of variance (ANOVA) followed by Student’s t-test.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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