Resistin-like molecule beta augments phenotypic modulation of human aortic smooth muscle cell triggered by high glucose

Yi Wang¹⁾, Yawen Zhang¹⁾, Xiangyu Gao¹⁾, Jiali Qian¹⁾, Jia Yang¹⁾, Wanwan Sun¹⁾, Hao Wang²⁾ and Yehong Yang¹⁾

¹⁾ Department of Endocrinology, Huashan Hospital, Fudan University, Shanghai 200040, China

²⁾ Shanghai Medical College, Fudan University, Shanghai 200040, China

Abstract. Vascular muscle cells (VSMCs) participate in the pathophysiology of atherosclerosis. Resistin-like molecule beta (Relm β) contributes to atherosclerosis development by activating macrophage. This study aims to investigate whether Relm β regulates VSMC phenotypic modulation under high glucose environment. Human aortic vascular smooth muscle cells were cultured and treated with Relm β in the presence or absence of high glucose. VSMC phenotypic modulation was assessed by expression of related markers. The migration of VSMCs was detected by wound healing assay and transwell assay. The proliferation of VSMCs was measured using CCK-8 assay. In this study, we observed that Relm β modulated VSMC phenotypic modulation by down-regulating expression of smooth muscle α -actin (α -SMA), smooth muscle myosin heavy chain (SM-MHC), and calponin while up-regulating expression of osteopontin (OPN). Relm β increased the expression of inflammatory genes in VSMCs. Relm β also augmented VSMCs migration as well as proliferation. It is worth noting that all the effects of VSMCs were enhanced upon high glucose stimulation. The phosphorylation levels of p38MAPK and ERK1/2 were increased by co-treatment with Relm β and high glucose. The p38 MAPK pathway inhibitor RWJ64809 and pERK1/2 inhibitor PD98059 significantly inhibited the proliferation of VSMCs induced by Relm β and high glucose. Our results provide evidence that Relm β augments phenotypic modulation and migration of human aortic smooth muscle cell induced by high glucose. Relm β might be a potential target for treatment of atherosclerosis induced by hyperglycemia.

Key words: Resistin-like molecule beta, Vascular smooth muscle cell, Phenotypic modulation, High glucose

ATHEROSCLEROSIS is a key cause of various vascular diseases and has high morbidity and mortality worldwide. Hyperglycemic status activates multiple maladaptive signaling pathways and leads to the occurrence and progression of atherosclerotic diseases with distinct characteristics [1]. Macrophage accumulation in the vascular wall is a hallmark of atherosclerosis [2]. Recently, accumulating studies reveal that vascular smooth muscle cells

(VSMCs) and especially VSMC phenotypic modulation play an important role in the development of atherosclerosis. VSMCs possess splendid phenotypic plasticity, which allows rapid adaptation upon various environmental stimuli. Upon vascular damage, contractile VSMCs will dedifferentiate into a synthetic, proliferative and migratory phenotype (known as phenotypic modulation), which contributes to vascular remodeling during atherosclerosis [3, 4]. However, little is known about how the VSMC phenotypic modulation is regulated, especially under hyperglycemic status.

Resistin and the resistin-like molecules (Relms) comprise a novel class of cysteine-rich proteins, which include resistin, Relm α , Relm β and Relm γ . Resistin and Relm β , but not Relm α and Relm γ , are expressed in humans [5, 6]. Resistin is initially identified as an adipokine, which can reduce insulin action. Relm β is produced limitedly in the colon and the lung; and it is report to be involved in local immune response regulation in the gut and bronchial epithelial cells [7, 8]. Relm β produced by colon goblet cells is induced during intestinal

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Correspondence to: Yehong Yang, MD, Department of Endocrinology, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, China.
E-mail: yehongyang@fudan.edu.cn

Abbreviations: VSMCs, vascular smooth muscle cells; HASMC, Human aortic vascular smooth muscle cells; Relm β , resistin-like molecule beta; α -SMA, smooth muscle α -actin; MHC, smooth muscle myosin heavy chain; OPN, osteopontin; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; CDK, cyclin-dependent kinase; MAPKs, mitogen-activated protein kinases.

inflammation, including microbiota and high-fed diet [5, 9, 10]. Relm β also contributes to hypoxia-induced pulmonary vascular remodeling [6], pathogenesis of colon cancer [11] and type 2 diabetes [12].

Recently, Relm β is identified in activated macrophages and demonstrated to accelerate the development of atherosclerosis [13]. Relm β is found to be expressed in the foam cells of atherosclerotic lesion in both human samples and ApoE $^{-/-}$ mice. Relm β activates the classical M1 macrophage and contributes to the atherosclerosis development and plaque instability [13]. However, whether Relm β is involved in the regulation of VSMCs, which also play an important role in atherosclerosis development, is unknown.

In this study, we showed that Relm β modulated the behaviors of VSMCs, including phenotypic modulation, inflammation, migration and proliferation, after high glucose stimulation. We also found that mitogen-activated protein kinases (MAPKs) signaling pathway participated in VSMC phenotypic proliferation induced by co-stimulation of Relm β and high glucose.

Material and Methods

Cell culture and treatment

Human aortic vascular smooth muscle cells (HASMCs) were purchased from ScienCell. They were cultured in Smooth Muscle Cell Medium (SMCM) (ScienCell, US). The cells of the third to the eighth-generation were used for the following experiments. The 25 mM high glucose medium was used for high glucose intervention. The 20 ng/mL Relm β (ProSpec, Israel) was used for Relm β treatment. For Relm β and high glucose co-treatment, the cells were stimulated by 20 ng/mL RELM and 25 mM high glucose simultaneously.

Western blot analysis

Cells were lysed with Radio-Immunoprecipitation Assay solution (Thermo, US) containing the protease inhibitor on ice for 20 min with rotation. Lysate was collected and the supernatant was collected after centrifugation for 30 min at 4°C. Protein concentration was determined using BCA protein assay kit (Beyotime, China). Proteins (20 μ g) were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked by 5% milk for 1 hour, and then incubated with targeted protein primary antibody overnight. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Boster, China). The blots were developed with enhanced chemiluminescence detection reagents.

RT-PCR

Total RNA was extracted with TRIzol (Thermo, US) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using M-MLV (Takara, Japan). Real-time PCR (RT-PCR) was performed using SYBR Green Mix (Applied Biosystems, Woolston Warrington, UK). The total volume for RT-PCR was 20 μ L. The PCR procedures were as follows: denaturation at 50°C for 2 min, annealing at 60°C for 30 seconds and extension at 72°C for 60 seconds with a total of 40 cycles. The results were analyzed with $2^{-\Delta\Delta C_t}$ method.

Wound healing assay

HASMCs were seeded in the six-well plates and cultured with serum-free SMCM for 24 hours. A 200 μ L of pipettor was used to streak the bottom of each well and PBS was used to wash away the suspended cells. Then complete medium was added into the culture plate. Pictures were taken under the microscope and digital images were obtained every 24 hours.

Transwell assay

HASMCs were starved with serum-free culture medium for 24 hours. Then, five thousand HASMCs were seeded in each transwell chamber (Corning, US). The 200 μ L serum-free medium was added into the lower chamber and 500 μ L SMCM medium containing 10% FBS was added into the upper chamber. SMCM medium containing 10% FBS was removed from the upper chamber after 24 hours of culture. The cells were gently washed with PBS and fixed with 4% paraformaldehyde for 30 min. Image were observed and taken under a light microscope.

CCK-8 assay

HASMCs were seeded in 96-well plates as a density of 5,000 cells per well and treated according to the indicated treatments and timepoints. The 10 μ L of CCK8 solution was added to each well followed by incubation for 30 minutes at 37°C. Absorbance value at 450 nm was measured by a microplate reader.

Statistical analysis

Data are expressed as mean $\pm$ standard difference (SD). Statistical analysis was performed using SPSS 25.0 (IBM). The *t*-test was used for comparison between groups. Statistical significance was indicated at **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Graphs was plotted using GraphPad Prism 8.

Results

Relm β promotes the phenotypic modulation and inflammation of VSMCs induced by high glucose

To investigate how Relm β modulates the phenotype of VSMCs, we treated the cells with high glucose, Relm β and the combination of high glucose and Relm β and assessed specific markers of contractile VSMCs, including smooth muscle α -actin (α -SMA), smooth muscle myosin heavy chain (MHC), calponin, matricellular protein, and osteopontin (OPN). As shown in Fig. 1A, Relm β as well as high glucose significantly regulated the expression of phenotypic modulation-related genes. They suppressed the expression of α -SMA, SM-MHC and calponin, but elevated the expression of OPN. It is worthy to note that Relm β treatment could further downregulate the expression of α -SMA, SM-MHC and calponin and upregulate the expression of OPN upon high glucose stimulation. Consistent with gene expression results, α -SMA protein expression was also reduced by high glucose. Although Relm β alone didn't affect α -SMA protein content significantly, Relm β suppressed α -SMA protein level in the presence of high glucose (Fig. 1B). Meanwhile, high glucose or Relm β treatment induced the expression of OPN (Fig. 1A). Relm β treatment further enhanced the expression of OPN upon high glucose stimulation (Fig. 1B). Collectively, these data demonstrate that Relm β promotes the phenotypic modulation of VSMCs induced by high glucose.

It is well-known that atherosclerosis is an inflammatory disease and immune response is involved in atherosclerosis development [4, 14]. We therefore assessed whether Relm β treatment affects the inflammatory gene expression. we found that high glucose significantly upregulated the expression of IL-1 β , IL-8, TGF- β , TNF α , and MCP-1, which are classic inflammatory markers. Relm β treatment augmented the upregulation of these inflammatory markers induced by high glucose, while Relm β alone didn't increase the expression of inflammatory genes (except MCP-1) dramatically (Fig. 1C). These data suggest that Relm β increases inflammatory gene expression in VSMCs upon high glucose treatment.

Relm β induces VSMCs migration upon high glucose stimulation

Wound healing assay was used to demonstrate the effect of Relm β and high glucose on VSMCs migration. Compared with non-treated cells, 20 ng/mL Relm β treatment, as well as high glucose, remarkably enhanced cell migration (Fig. 2A). In line with the phenotypic modulation, Relm β and high glucose synergistically enhanced the cell migration. Next, the transwell Boyden chamber

experiment was performed in order to confirm the promoting effect of Relm β on VSMCs migration. As expected, the number of VSMCs that migrated through the transwell chamber was strikingly induced by treatment with high glucose for 24 h or Relm β for 24 h. Noteworthy, VSMCs migration was further enhanced when cells were co-treated with Relm β and high glucose (Fig. 2B). Furthermore, we investigated the regulatory effects of Relm β on the migration markers. As shown in Fig. 2C, migration markers, including matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), were significantly higher in high glucose or Relm β treated cells compared with control cells. Relm β treatment further elevated MMP-2 and MMP-9 gene expression upon high glucose treatment. Platelet-derived growth factor-platelet-derived growth factor receptor (PDGF-PDGFR) signaling represents one of the most prominent inducers of VSMC migration and proliferation. VSMCs can secrete PDGF in response to some stimulation and accelerate atherosclerosis [15]. In our study, consistent results were observed in expression of PDGFA and PDGFR-A after Relm β and high glucose treatment (Fig. 2C). These findings suggest that Relm β induces VSMCs migration upon high glucose stimulation.

Relm β stimulates proliferation of VSMCs upon high glucose treatment

To explore whether Relm β affects the proliferation of VSMCs with or without high glucose, we performed CCK-8 assay after treating VSMCs with Relm β with or without high glucose. As shown in Fig. 3A, the proliferation of VSMCs was significantly induced by Relm β or high glucose treatment, as well as Relm β and high glucose co-treatment. To further confirm the effect of Relm β on proliferation, we tested the expression of cell phase-specific cyclin-dependent kinase (CDK)-cyclins. The results showed that the protein levels of CDK2, cyclin D1, CDK4, and cyclin E were greatly increased by high glucose stimulation. Relm β slightly increased the expression of CDK2, cyclin D1, CDK4, and cyclin E. However, this effect was tremendously augmented by co-treatment with high glucose (Fig. 3B, 3C). This result suggests that Relm β stimulates proliferation of VSMCs upon high glucose treatment.

MAPKs signaling pathway maybe involved in proliferation of VSMC induced by Relm β and high glucose co-stimulation

It is reported that MAPKs signaling pathway, especially ERK1/2 signaling pathway, is a major driver of VSMC proliferation and inflammation [16]. Relm β also regulates proliferation of human diabetic nephropathy

Fig. 1 Relm β promotes the phenotypic modulation and inflammation of VSMCs induced by high glucose ($n = 3$). HASMCs were treated with 20 ng/mL Relm β with or without 25 mM high glucose for 24 hours. (A) RT-PCR analyses of smooth muscle α -actin (α -SMA), smooth muscle myosin heavy chain (MHC), calponin, matricellular protein, and osteopontin (OPN) in HASMC of four groups. (B) Western blot analyses of α -SMA and OPN in HASMC of four groups. Left graph was western blot bands. Right graphs were histogram of quantitative analysis of protein levels (***) $p < 0.001$ compared with control, (###) $p < 0.001$ compared with HG. (C) RT-PCR analyses of inflammation markers including IL-1 β , IL-8, TGF- β , TNF α , and MCP-1 in HASMCs of four groups. NC, normal control; HG, high glucose. Data are presented as mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$ (compared with NC). ## $p < 0.01$, ### $p < 0.001$ (compared with HG).

mesangial cells through p38 signaling [17]. We assessed whether those two pathways are involved in VSMC phenotypic proliferation induced by Relm β and high glucose co-stimulation. The expression of p-ERK1/2 and p-p38 was drastically increased by high glucose treatment and was further elevated by Relm β and high glucose co-stimulation (Fig. 4A). After inhibiting p38 and ERK1/2

phosphorylation using p-p38 inhibitor (RWJ64809) and p-ERK1/2 inhibitor (PD98059), the vitality of VSMCs were strikingly attenuated. Moreover, when Relm β or high glucose synergistic inhibitor acts on VSMC, the inhibitory effect on cell migration and proliferation are more significant (Fig. 4B, 4C), which suggests that ERK1/2 and p38MAPK signal pathways may be involved

Fig. 2 Relm β induces VSMCs migration upon high glucose stimulation ($n = 3$). HASMCs were treated with 20 ng/mL Relm β with or without 25 mM high glucose for 72 hours (A) or 24 hours (B and C). (A) Wound healing assay and (B) Transwell assay in HASMCs of four groups. Left panels were images of wound healing assay and transwell assay, respectively and right panels were histogram of quantitative analysis. (C) RT-PCR analyses of MMP-2, MMP-9, PDGF-A and PDGFR-A in HASMCs of four groups. NC, normal control; NG, high glucose. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared with NC). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (compared with HG).

in proliferation of VSMC induced by Relm β or high glucose co-stimulation.

Discussion

VSMCs are highly plastic cells characterized by striking plasticity in response to environmental stimuli [18]. Accumulating evidence shows that VSMC phenotypic plasticity plays an important role in various proliferative vessel disorders, such as essential hypertension and arteriosclerosis [19-21]. However, the details of the molecular mechanisms of VSMC phenotypic modulation are still not elucidated yet. The illustration of the mechanisms regulating the VSMC phenotypic modulation is critical for better understanding the occurrence and

development of vascular disorders, especially atherosclerosis. Atherosclerosis always occurs in patients with diabetes and is one of the major causes of diabetes related mortality [22]. High glucose environment may affect VSMC cell cycle and accelerate the development of atherosclerosis, which prompts us to further investigate the regulation of VSMC phenotypic modulation upon high glucose stimulation.

In this study, we investigated the effect of Relm β on VSMC in terms of phenotypic modulation, inflammation, migration and proliferation, which are critical steps during atherosclerosis development. Our results were consistent with previous studies that hyperglycemia condition could induce VSMC proliferation and migration [23, 24]. Additionally, we revealed that Relm β could

Fig. 3 Relmβ stimulates proliferation of VSMCs upon high glucose treatment ($n = 6$). HASMCs were treated with 20 ng/mL Relmβ with or without 25 mM high glucose. NC, normal control; HG, high glucose. (A) CCK-8 assay of HASMCs in four groups. *** $p < 0.001$ (compared with HG). (B) Cell phase-specific cyclin-dependent kinase (CDK)-cyclins protein expression of HASMCs in four groups. Data are presented as mean $\pm$ SD. *** $p < 0.001$ (compared with NC). (C) Histogram of quantitative analysis of protein levels (*** $p < 0.001$ compared with control, # $p < 0.05$, ### $p < 0.001$ compared with HG).

exacerbate the VSMC proliferation and migration induced by high glucose. Unexpectedly, even though comparable levels of absorbance between HG group and Relmβ plus HG group were observed when CCK-8 assay was conducted for cell proliferation assessment, the protein levels of CDK2, cyclin D1, CDK4, and cyclin E in HG group were greatly augmented by co-treatment with Relmβ treatment. This could be because the CCK-8 assay was so sensitive that high glucose maximized cell proliferation and it's difficult to further be enhanced by Relmβ treatment.

The mechanisms of the synergistic effect of Relmβ and high glucose on VSMC is not elucidated yet. Some pathways have been determined to be involved in hyperglycemia including diacylglycerol production, the protein kinase C pathway activation, MAPKs, advanced glycation accumulation and multiple cytokine production and secretion [25]. MAPKs signaling pathway modulates the expression of numerous transcription factors and participates the VSMC phenotypic modulation [26]. Growing evidence supports that various microRNAs, membrane receptors and ion channels are involved in VSMC phenotypic modulation, migration and proliferation [27]. Whether Relmβ could synergistically work with these molecules to regulate VSMC phenotype warrant further investigations. Our study also observed that MAPKs pathway, especially ERK1/2 and p38 phosphorylation levels, were significantly induced by Relmβ

and high glucose co-stimulation. More importantly, after suppression of ERK1/2 and p38 pathway by using inhibitors, the proliferation of VSMC was attenuated strikingly. Although the NC groups were also affected by the inhibitor, the more significant down-regulation occurs when high glucose or Relmβ synergistic inhibitors act in VSMCs. However, the inhibitors also down-regulated the proliferation ability of normal VSMCs to varying degrees, which may indicate that the proliferation effect of high glucose or Relmβ on cell proliferation was not necessarily caused by P38 or pERK. Whether Relmβ could synergistically work with these molecules to regulate VSMC phenotype warrant further investigations.

In conclusion, Relmβ regulates the VSMC phenotypic modulation upon high glucose environment, which could be mediated by MAPKs pathway. These results provide evidence that Relmβ could be a potential target for the therapeutic strategy of atherosclerosis induced by hyperglycemia.

Highlights

- Relmβ as well as high glucose stimulate VSMC phenotypic modulation, migration and proliferation
- Relmβ augments VSMC phenotypic modulation, migration and proliferation induced by high glucose
- MAPKs pathway are involved in proliferation of VSMC induced by Relmβ and high glucose co-

Fig. 4 MAPKs signaling pathway maybe involved in proliferation of VSMCs induced by Relmb β and high glucose co-stimulation ($n = 3$). (A) Phosphorylation level of ERK1/2 and p38 of in HASMCs treated with high glucose with or without 20 ng/mL Relmb β for 24 hours. (B) Representative cell density and (C) CCK-8 of HASMCs after Relmb β and/or high glucose co-treatment in the absence or presence with p-p38 inhibitor (RWJ64809) (20 ng/mL) or p-ERK1/2 inhibitor (PD98059) (20 ng/mL) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control, # $p < 0.01$, ### $p < 0.001$ compared with HG, $\Delta p < 0.05$ compared with Relmb β , $\star p < 0.05$, $\star\star p < 0.01$ compared with HG + Relmb β).

stimulation.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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