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Original Article

GDF-15 promotes proliferation of vascular smooth muscle cells through MAPK-activated protein kinase pathways

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Abstract: Growth differentiation factor 15 (GDF-15) is a multifunctional member of the TGF-b superfamily. Accumulating evidence indicates that GDF15 is an independent biomarker for the cardiovascular disease, and is closely involved in the development of atherosclerotic lesions induced by ox-LDL, such as inhibition of macrophage infiltration and increase of endothelial cell proliferation, while whether GDF-15 also participates into the VSMCs proliferation is still lack of systemic analysis. In the present study, ox-LDL was found to induce GDF15 expression and secretion in cultured rat VSMCs in a p38- and ERK1/2-dependent manner. Inhibition of ox-LDL-induced GDF-15 expression by siRNA transfection by lentivirus demonstrated that adaptively induced GDF15 played a proliferative role in VSMCs, and overexpression of GDF-15 alone was adequate to induce VSMCs proliferation. Further application of inhibitors of p38- and ERK1/2 attenuated the GDF-15 expression induced by ox-LDL. All data suggest that GDF15 is a proliferative molecule that gives rise to VSMCs proliferation secondary to ox-LDL stimulation *in vitro*.

Keywords: Atherosclerosis, GDF-15, MAPK kinases, VSMCs proliferation

Introduction

Atherosclerosis is a progressive disease in which vascular smooth muscle cells (VSMCs) mainly derived from the medial layer of the blood vessel play a pivotal role in atherogenesis. VSMCs proliferation is one of the important components of atherosclerosis [1]. The oxidized low-density lipoprotein (ox-LDL) as a major contributing factor promotes the atherogenic actions, in part by stimulating proliferation in a variety of cell types of cells within the vessel wall, including endothelial cells, macrophages, and fibroblasts [2, 3]. However, the proliferative activity of ox-LDL in VSMCs appear contradictory. Experimental studies have shown that a mildly ox-LDL might evoke a proliferative response to contribute to the formation of intimal plaque [4, 5]. Alternatively, high concentrations of ox-LDL cause LOX-1-mediated apoptosis of VSMCs to destabilize the atherosclerotic plaques [3, 6]. The mechanism whereby ox-LDL induces VSMCs proliferation and apoptosis might be dependent on the simultaneous induction of both cell cycle activators and suppressors [2]. In spite of the controversy, recent demonstration from our laboratory showed that ox-LDL evoked cell proliferation in a time- and concentration-dependent manner [7]. However, the mechanisms underlying the effect of ox-LDL on VSMCs proliferation are not fully understood.

Growth-differentiation factor-15 (GDF-15), a distant member of the transforming growth factor-β cytokine superfamily, is first found as macrophage inhibiting factor 1 (MIC-1) to inhibit tumor necrosis factor α (TNF-α) secreting from activated macrophages [8]. Accumulating evidence indicates that MIC-1/GDF-15 is induced in the myocardium following pathological stress associated with inflammation or tissue injury [9], and is closely related to the biomarkers of heart disease, such as B-type natriuretic peptide, N-terminal pro-BNP, cardiac troponin I and cardiac troponin T [10, 11]. Experimental studies have shown that, besides heart diseases, GDF-15 overexpression also occurs in other

cardiovascular cell types including macrophages, endothelial cells and vascular smooth muscle cells following pathological stresses [12, 13]. Consistent to correlation to common cardiac biomarkers, GDF-15 exhibits independent associations in those biomarkers of endothelial activation, both E- and P-selectin, VCAM-1, and ICAM-1. These adhesion molecules are involved in the recruitment of leukocytes into the vessel wall which is commonly regarded as a key step in the development and progression of atherosclerotic vascular disease [14]. These data suggest that GDF-15 may not only be a marker of cardiac abnormalities but also a marker of vascular pathologies, especially in atherogenesis.

In addition to overexpression of GDF-15 in activated endothelial cells, GDF-15 expression is also upregulated in macrophages activated by ox-LDL and is significantly correlated to MMP-9, probably reflecting the effects of macrophage activation on the progression of plaque development [15]. Given the prognostic value of GDF-15 in atherosclerotic plaques, a systematic analysis of the potential associations between GDF-15 and VSMCs proliferation is lacking so far. The aim of the current study was to illustrate the correlation of GDF-15 and VSMCs proliferation in the stimulation of ox-LDL and to explore the signaling pathways implicated in the expression of GDF-15.

Methods

Materials

Ox-LDL (YB-002) was purchased from the Yiyuan Biotechnologies Co. Ltd. Guangzhou, China. GDF-15 (1:1000, Abcam), ERK1/2 (1:1000, Abcam), p-ERK1/2 (1:1000, Abcam), GAPDH (1:2000, Santa Cruz) and detected using horseradish peroxidase-conjugated mouse IgG secondary antibodies (1:5000, Santa Cruz). ERK1/2 (ab17942) and p38 (ab31828) inhibitors were purchased from Abcam. Other chemicals were dissolved in double-distilled water or experimental solutions.

Cell culture

VSMCs were utilized and isolated from Wistar rats. Aortic strips were cut into about 2 mm small rings and placed in 6-well culture plates. These explants were grown in DMEM (Hyclone, Utah, USA) following the instruction [5]. VSMCs were maintained in DMEM supplemented with 10% FCS (Invitrogen, Carlsbad, CA, USA) containing 100 mg/mL penicillin and 100 mg/mL streptomycin (Invitrogen), and cultured in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The medium was changed after 24 hours and then every 3 days. These cells were identified as VSMCs by their characteristic hill-and-valley growth patterns and the presence of SMC-specific-actin.

ELISA assay

For quantitative measurement of GDF-15, the cell culture media was collected and diluted 20 times. Detailed procedures were conducted according to the protocol in the Mouse/Rat GDF-15 Quantikine ELISA Kit (MGD150, R&D system).

Plasmid construction and stable transfectants

The vector GV248 and helper plasmids pHelper were purchased from QIAGEN. The full-length CDS of rat GDF15 was cloned into the GV248 vector. GDF15-specific short hairpin RNAs were designed and cloned into GV248 (pFU-GW-007-hU6-Ubiquitin-EGFP-IRES-puromycin) vectors to inhibit GDF-15 expression. The viral particles were produced in 293T cells. A successful transfer of GDF-15 into VSMCs was achieved with a MOI (multiplicities of infection) of 100-200 viral particles/cell after 48 hour incubation in DMEM with 10% FBS. MOI 100 was the minimal concentration with the maximal transfer of the reporter gene into 100% of VSMCs. VSMCs were infected in DMEM with 10% FBS for 48 hour and cell numbers were determined by direct cell counting.

Western blot

The procedures were conducted according to our own publication [7]. Cultured VSMCs were lysed in lysis buffer (protease and phosphatase inhibitors), then lysates were centrifuged at 13, 500 g for 15 minutes at 4°C. Protein concentration was assessed using the BCA protein assay. The aliquots were then mixed with Laemmli sample buffer and boiled at 99°C for 5 min. The samples were resolved using 8-10% SDS/PAGE, followed by electrotransfer to polyvinylidene fluoride membranes. For visualization, blots were probed with antibodies against

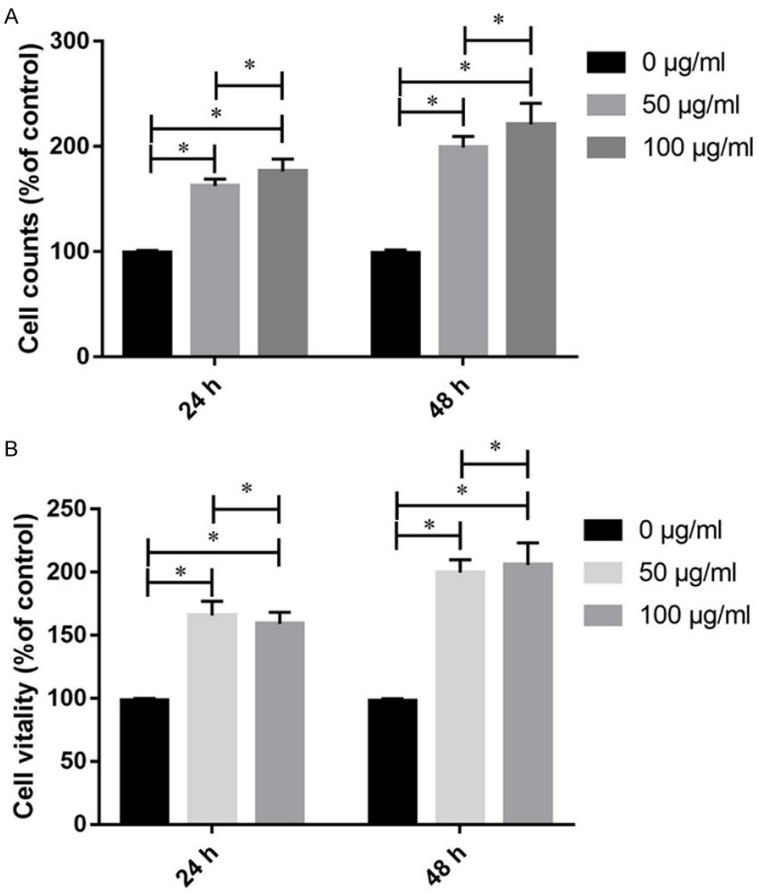

Figure 1. ox-LDL promotes smooth muscle cell proliferation *in vitro*. The proliferation of rat aortic smooth muscle cells incubated with ox-LDL (50 μg/ml and 100 μg/ml) was evaluated by the increment of cell number (A). The viability was detected by CCK-8 assay (B). *P<0.05; n=3 different experiments. Values are means ± SEM.

GDF-15 (1:1000), ERK1/2 (1:1000), p-ERK1/2 (1:1000), GAPDH (1:2000) and detected using horseradish peroxidase-conjugated mouse IgG secondary antibodies (1:5000).

Statistics

All data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to analyze the statistical differences among the groups. The *p*-values <0.05 were considered statistically significant. Statistical analyses and graphs were performed with SPSS 15.0 for Windows and GraphPad Prism 7.0 software.

Results

Ox-LDL induced VSMCs proliferation through MAPKs activation

Consistent with published data from our laboratory, ox-LDL promoted VSMCs proliferation in a time- and concentration-dependent way. Compared with the control group, VSMCs proliferation was robust under the treatments of ox-LDL at 50 and 100 μg/ml for 24 hours. In addition, VSMCs maintained the proliferation with the time increasing to 48 hours (**Figure 1A**). However, VSMCs proliferation was not a linear correlation with the concentrations of ox-LDL. The cell viability was tested using CCK-8 assay. After the exposure of ox-LDL for 24 hours, all cell suspensions were prepared in a 96-well plate to incubate with CCK-8 solution for 2 hours, and then the absorbance at 450 nm was measured using microplate reader (**Figure 1B**). ox-LDL enhanced VSMCs viability in a time- and concentration-dependent way.

MAPK activation, one of the major pathways involved in cell proliferation, was assessed through the protein expression level, including p38, ERK 1/2 and JNK (**Figure 2**). After 48 hour incubation with ox-LDL, VSMCs were collected and isolated the total proteins. The protein productions of p38 and ERK 1/2 followed a similar trend, significantly increasing with the treatment of ox-LDL. In contrast, the expression level of JNK phosphorylation remained unchanged to ox-LDL treatment.

GDF-15 was upregulated in VSMCs after the treatment of ox-LDL through an ERK 1/2 and p38-dependent mechanism

The supernatants of VSMCs in each group were collected, and the GDF-15 concentration was measured by ELISA assay. After exposure of 50 μg/ml ox-LDL for 24 hour, GDF-15 concentration in the supernatant of Control group was 0.375±0.023 ng/ml, and GDF-15 concentration in the supernatant of Control + ox-LDL group was 0.462±0.009 ng/ml; After 48 hours incubation without ox-LDL, GDF-15 concentration had no obvious change (0.349±0.048 ng/

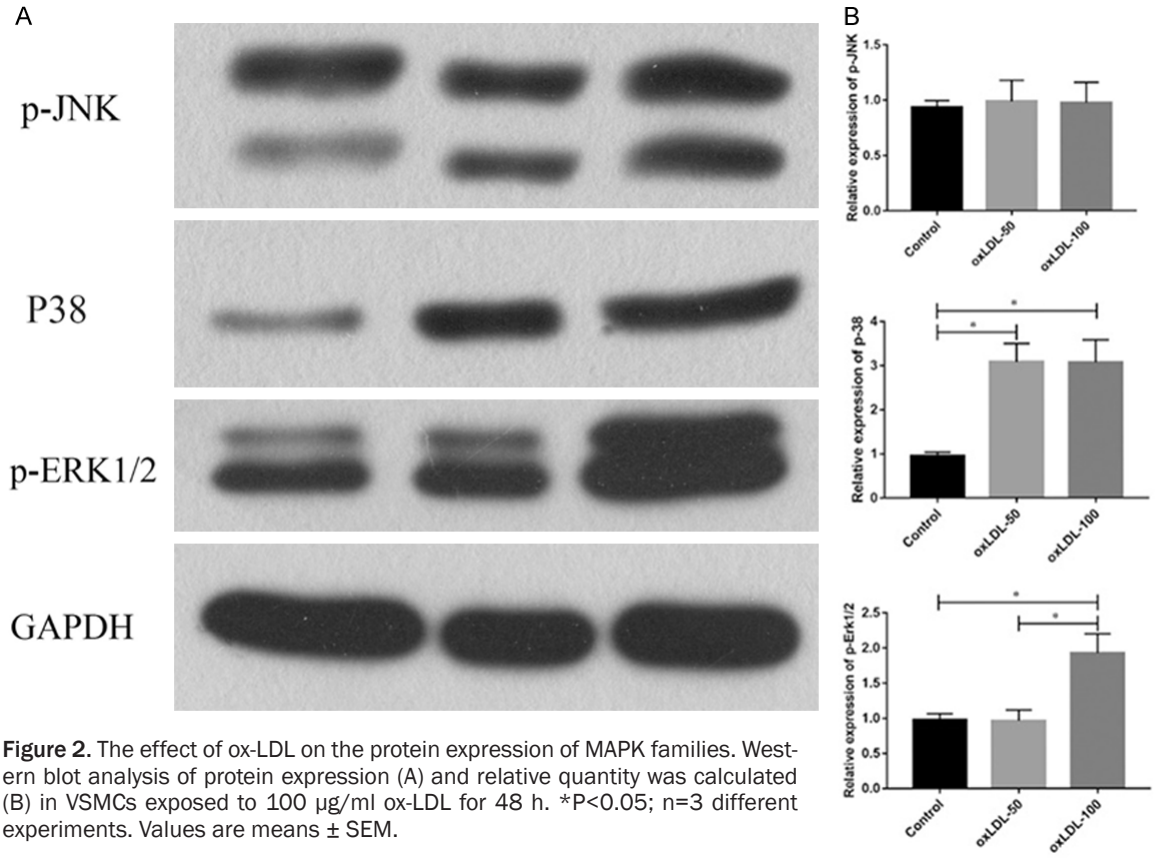

Figure 2. The effect of ox-LDL on the protein expression of MAPK families. Western blot analysis of protein expression (A) and relative quantity was calculated (B) in VSMCs exposed to 100 μg/ml ox-LDL for 48 h. *P<0.05; n=3 different experiments. Values are means ± SEM.

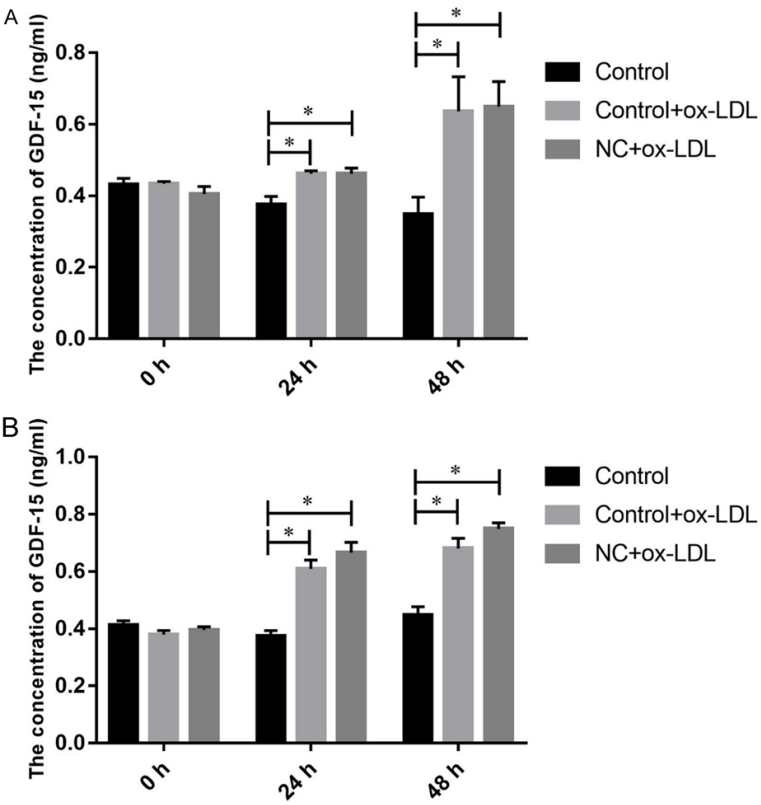

Figure 3. ox-LDL enhances GDF-15 protein expression in the smooth muscle cell. ELISA quantitative analysis of the GDF-15 in the supernatant of 50 μg/ml ox-LDL treated VSMC (A). ELISA quantitative analysis of the GDF-15 in the supernatant of 100 μg/ml ox-LDL treated VSMC (B). *P<0.05; n=3 different experiments. Values are means ± SEM.

ml), but ox-LDL dramatically increased the concentration level of GDF-15 to 0.637± 0.096 ng/ml. In this study, NC + ox-LDL group meant negative control of virus infection, a conclusion could be made that unpacked virus did not disturb the expression of GDF-15 stimulated by ox-LDL (**Figure 3A**). To further expose VSMCs to 100 μg/ml ox-LDL for 24 and 48 h, the concentration increased to 0.637±

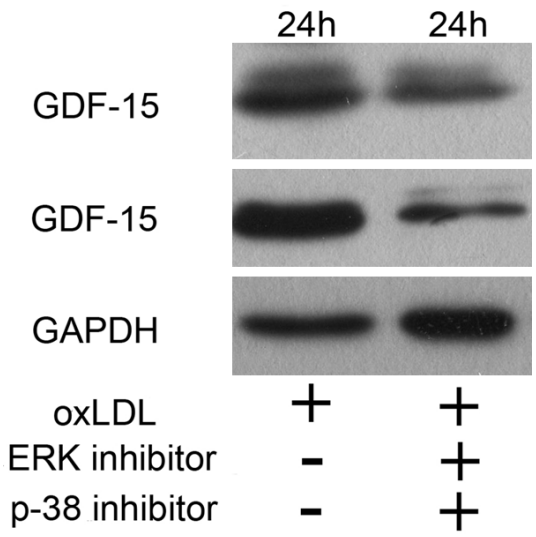

Figure 4. GDF-15 protein expression in ox-LDL-treated VSMCs was dependent on p-38 and ERK1/2 MAPK activation. The inhibitors of p-38 and ERK1/2 inhibited the GDF-15 protein expression in ox-LDL-treated cells. $P<0.05$; n=3 different experiments. Values are means ± SEM.

0.096 and 0.650±0.070 ng/ml in Control + ox-LDL and NC + ox-LDL groups respectively (**Figure 3B**).

The main objective of this study was to determine whether GDF-15 was involved in ox-LDL-induced proliferation. The GDF-15 expression in response to ox-LDL was stunted by ERK1/2 and p38 inhibition (**Figure 4**).

Gain- or loss-of-function of GDF-15 regulated the VSMCs proliferation in response to ox-LDL treatment.

Gain- or loss-of-function of GDF-15 was performed by the lentiviral infection of a full-length CDS of rat GDF-15 or GDF-15-specific short hairpin RNAs. Overexpression of GDF-15 significantly enhanced VSMCs proliferation. In contrast, knock-down GDF-15 followed a similar trend as utilization of ERK 1/2 and p38 inhibition significantly inhibited VSMCs proliferation with 100 μg/ml ox-LDL treatment for 48 hours (**Figure 5**).

Discussion

TGFβ has been intensively studied in atherosclerotic development [16], which is believed to be a critical factor in the formation of atherosclerotic plaque. GDF-15 is a member of the TGF-β cytokine superfamily and was first found as macrophage inhibiting factor 1, and it may inhibit tumor necrosis factor α (TNF-α) secreting from activated macrophages. TNF-α is important for recruiting macrophages into foam cells to form atherosclerotic plaque. Moreover, GDF-15 plays a pivotal role in ox-LDL-induced apoptosis of human macrophages in arteriosclerotic lesions [12, 17]. Thus, GDF-15 may orchestrate atherosclerotic lesion progression by regulating inflammatory responses to prevent atherosclerotic development [18, 19]. Overexpression of GDF-15 also protects the atherosclerotic process and reduces atherosclerotic lesion size [18]. Intriguing, it has also been found that deficiency of GDF-15 attenuates early atherosclerotic lesion formation and improves the stability of plaques by induction of collagen deposition [17, 20]. The diverse effects of GDF-15 may vary with the stage of the disease. GDF-15 mediates anti-atherosclerotic effects in mice by directly inhibiting myeloid cell recruitment, but indirectly promotes proinflammatory effects in atherosclerosis models [9, 17, 21-23].

In recent years, increasing investigation reveals that GDF-15 is an independent marker of cardiovascular dysfunction and disease [24-27]. Several lines of evidence reveal that GDF-15 is highly expressed in endothelial cells and VSMCs in normal and pathological conditions. In human endothelial cells, GDF-15 has independent associations with biomarkers of endothelial, such as E- and P-selectin, VCAM-1, and ICAM-1. These adhesion molecules are commonly regarded as an initial step in the formation of atherosclerosis by recruiting circulating leukocytes [14]. VSMCs proliferation is another crucial factor in developing atherosclerotic plaque [16, 28, 29]. ox-LDL has been thought to be mitogenic to VSMCs to induce VSMC proliferation through the activation of the ERK1/2 MAPK signaling pathway [5]. In addition, it has been reported that GDF-15 expression is upregulated by the treatment of ox-LDL, partially depending on the activation of ERK 1/2 [19]. However, a systematic analysis of the potential associations between GDF-15 levels and VSMCs proliferation is lacking so far. Our data indicate that ox-LDL elicits expression of GDF-15 in rat VSMCs, and overexpression of GDF-15 by lentiviral infection has the same effects on VSMCs proliferation induced by ox-LDL. Con-

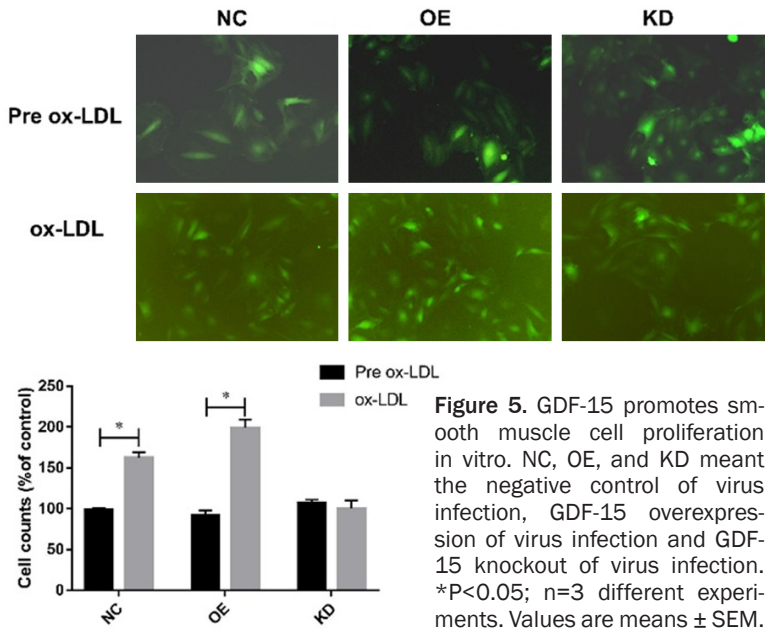

Figure 5. GDF-15 promotes smooth muscle cell proliferation in vitro. NC, OE, and KD meant the negative control of virus infection, GDF-15 overexpression of virus infection and GDF-15 knockout of virus infection. *P<0.05; n=3 different experiments. Values are means ± SEM.

sistent with macrophages treated with ox-LDL, GDF-15 expression is upregulated in VSMCs incubated with ox-LDL *in vitro*, and is dependent on the activation of MAPK pathways. Unfortunately, correlation of increased expression of GDF-15 was not validated with proliferative actions *in vivo*. Future research is needed to study the potential actions of GDF-15 on the arterial wall thickness and plaque stability in an animal model of atherosclerosis.

Conclusions

The results of the present study demonstrate that GDF-15 is a proliferative molecule that gives rise to VSMCs proliferation secondary to ox-LDL stimulation *in vitro*. VSMCs treated with ox-LDL increase the induction of GDF-15, which is mediated through the activation of the p38 and ERK 1/2 MAPK pathways. A better understanding of the relationship between GDF-15 and VSMCs proliferation will provide us with further insight into the mechanism of plaque formation. It also offers new evidence that GDF-15 is a suitable biomarker for cardiovascular disease, and enables us to focus on this specific molecular as a potential therapeutic target for atherosclerosis.

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Disclosure of conflict of interest

None.

Abbreviations

GDF-15, Growth differentiation factor 15; TGF-β, Transforming Growth Factor-Beta; ox-LDL, Oxidized low-density lipoprotein; VSMC, Vascular smooth muscle cell; SMC, Smooth muscle cell; MAPK, Mitogen-activated protein kinase; ERK1/2, Extracellular signal-regulated kinase; LOX-1, Lectin-type oxidized LDL receptor 1; FBS, Fetal bovine serum; MOI, Multiplicities of infection; TNF-α, Tumor necrosis factor alpha; VCAM-1, Vascular cell adhesion protein 1; ICAM-1, Intercellular adhesion molecule 1.

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