Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

HIF-1α mediates visfatin-induced CTGF expression in vascular endothelial cells

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Received October 18, 2016; **Accepted** February 3, 2017; **Published** April 29, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.4.5

Abstract: Visfatin is an adipokine that functions as a mediator of endothelial dysfunction and cardiovascular diseases. Connective tissue growth factor (CTGF) is a key factor in vascular remodeling and atherosclerosis. However, the association between visfatin and CTGF is unclear. Therefore the study was to test the hypothesis that visfatin could modulate the expression of CTGF in vascular endothelial cells. In our study, cultured endothelial cell line EA.Hy926 cells were treated with different concentrations of visfatin for different times. The CTGF gene expression was analyzed by real-time PCR, and the protein expression of CTGF was assessed by Western Blot. The results showed that 100ng/mL concentration of visfatin could induce CTGF mRNA expression after 6 hours treatment, which peaked at 24 hours. And 100ng/mL concentration of visfatin also increased CTGF protein production after 12 hours treatment in EA.Hy926 cells. The expression of transforming growth factor-β1 (TGF-β1) mRNA was almost unaffected in cells treated with visfatin, whereas the expression of hypoxia inducible factor-1α (HIF-1α) was increased significantly. Moreover, knockdown of HIF-1α by its specific shRNA inhibits the effect of visfatin on CTGF expression. In conclusion, the up-regulation of CTGF expression by visfatin might be mediated via HIF-1α -dependent pathway, but not the TGF-β1 pathway in EA.Hy926 cells.

Key words: Visfatin; Connective tissue growth factor (CTGF); Vascular endothelial cells; Hypoxia inducible factor-1α (HIF-1α).

Introduction

The epidemic studies have provided evidence that obesity and overweight lead to many diseases including cardiovascular diseases (1,2). Obesity is now considered as a low inflammation state and is an independent risk factor for cardiovascular diseases such as atherosclerosis (3,4). Adipose tissue acts as an endocrine organ, generating some secreted factors (namely adipokines or adipocytokines) such as leptin, adiponectin, tumor necrosis factor-α (TNF-α), resistin and visfatin. Increased adipokines production from truncal adipose tissue could cause vascular endothelial dysfunction and insulin resistance (5,6). Inflammatory adipokines may even have influence on blood vessels endothelial function without their increase in plasma concentrations (3). Recent studies have indicated that adipokines play important roles in the pathogenesis of atherosclerosis (6,7).

Visfatin has recently been recognized as a visceral adipokine, which may be involved in obesity-related vascular disorders (8,9). Previous studies have identified the potential effects of visfatin in the progression of atherosclerosis. It could induce the expression of intercellular cell adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) through reactive oxygen species (ROS)-dependent nuclear transcription factor-κB (NF-κB) activation in vascular endothelial cells (VECs) (10-12). Visfatin is also thought to be a pro-inflammatory mediator localized in macrophages within the unstable atherosclerotic lesions, and potentially plays a role in plaque destabilization (13,14). Plasma visfatin levels are associated with coronary artery disease (CAD), particularly acute coronary syndrome (ACS), independent of other CAD risk factors (15). It is recently reported that, of all the adipokines/cytokines analysed (including adiponectin, visfatin, lipocalin-2, resistin and IL-6), only visfatin levels are significantly elevated in unstable carotid atherosclerotic plaque secretomes compared with that in non-atherosclerotic mammary artery secretomes (16). However, the mechanism of visfatin in endothelial dysfunction and atherosclerosis remains to be elucidated.

Connective tissue growth factor (CTGF) is a member of the CCN (including Cyr61, CTGF and Nov) family of early immediate genes (17), and is a potent profibrotic factor implicated in fibroblast proliferation, angiogenesis and extracellular matrix (ECM) syntheses (18,19). CTGF is expressed abundantly in atherosclerotic blood vessels, but only marginally in normal vascular tissues (20,21). It is regarded as a key factor in vascular remodeling and atherosclerosis (21,22). The CTGF gene contains a transforming growth factor-β1 (TGF-β1) response element in its promoter region and is a downstream mediator of the profibrotic effect of TGF-β1 (22,23). But the expression of CTGF can also be regulated by other factors, including angiotensin-II (22), high glucose (23,24), endothelin-1 (25) and hypoxia inducible factor-1α(HIF-1α) (26,27). More importantly, some adipokines with potent proinflammatory properties, could induce CTGF expression in different cell types (28-30). For example, visfatin may be involved in liver fibrogenesis and activation of hepatic stellate cells via increasing the CTGF levels (28). However, whether visfatin plays a role in the regulation of CTGF gene expression in the VECs, or more generally in atherogenesis, remains largely unknown. And so far, the connection between visfatin and CTGF expression in endothelial cells is still unclear.

In the view of the increased expression of CTGF in atherosclerosis, we hypothesized that CTGF might be upregulated by visfatin in VECs. Therefore, this study aimed to test the hypothesis that visfatin could modulate the expression of CTGF in VECs.

Materials and Methods

Cell culture and treatments

The human vascular endothelial cell line (EA. Hy926) was purchased from Institute of Hematology of Jiangsu Province. The characters of this cell line have been identified in our lab before. EA.Hy926 cells were cultured in 1640 medium containing 10% fetal bovine serum (FBS, Hyclone, USA) in the incubator at 37℃ under an environment of 5% CO_2 and starved in serum free medium for 6h before exposure to 10, 50, 100ng/mL of visfatin (Bio Vision, USA) for 6h, 12h or 24h.

RNA interference

Hypoxia inducible factor-1α (HIF-1α) specific targeting small inhibitory RNA (shRNA) plasmid (HIF-1α-shRNA) was constructed before, and was transient transfected into EA.Hy926 cells using Lipofectamine2000™ (Invitrogen, USA) according to the manufacturer's protocol. Expressing non-targeting (scrambled) shRNA plasmid was used as a negative control. After transfection for 24 h, the cells were treated with visfatin at the concentration of 100ng/mL.

RNA extraction and Real-time polymerase chain reaction(Real time PCR)

Total RNA was extracted from the cells using TRIzol (Invitrogen, USA) reagent. The RNA concentration was measured using Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using a reverse transcription (RT) kit (Toyobo, Japan). RT-PCR was carried out on CFX96™ Real-Time PCR Detection System (BIO-RAD, USA) with fluorescence dye SYBR Premix ExTaqII kit (Takara, China). Relative expression values were obtained by normalizing CT values of the tested genes in comparison with CT values of β-actin using the $^{\Delta\Delta}$CT method as previously described. Primers were used as follows: TGF-β1 F: 5'- TGAGTGGCTGTCTTTTGACG-3' and R: 5'- ACTGAAGCGAAAGCCCTGTA-3'; CTGF F: 5'- ACTGAAGCGAAAGCCCTGTA-3' and R: 5'-ACTGAAGCGAAAGCCCTGTA-3'; HIF-1α F: 5' CCAGCAGACCCAGTTACAGA-3' and R: 5'-TTCC-TGCTCTGTCTGGTGAG-3'; β-actin F: 5'-ACTATCG-GCAATGAGCGGTTC-3'and R: 5'- ATGC- CACAG-GATTCCATACCC-3'.

Western Blot Analysis

Cells were lysed in RIPA lysis buffer after stimulation. The protein concentrations were subsequently determined using the BCA Protein Assay Kit (ThermoFisher, USA). Then 25ug of cell lysates were subjected to 10% SDS-PAGE, transferred to a PVDF membrane (Millipore, Bedford, MA), blocked with 5% skim milk and probed with corresponding primary antibodies(CTGF,1:1000 or β-actin,1:2000). Finally, enhanced chemiluminescence detection kit (Pierce, USA) was applied to visualize the brands and protein expression was analyzed by Quantity One software. β-actin was regarded as the equal-loading control.

Immunofluorescent staining and determination of HIF-1α protein expression

The cells cultured on 12-well plates were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 after stimulated by visfatin. Mouse anti-HIF-1α (1:200, Santa Cruz, USA) were incubated overnight at 4°C after blocking with 1% BSA. Then the cells were incubated with an anti-mouse Alexa Fluo 488-conjugated secondary antibody (1:500, invitrogen). Finally, Images were captured on an Eclipse TE2000-U fluorescent microscope system (Nikon, Japan) and analyzed with the ImageJ software to semi-quantitatively determine the HIF-1α protein expression.

Statistical Analysis

Data are presented as mean ± SEM and analyzed with SPSS17.0 software. Differences between groups were evaluated for significance using single tailed Student's t-test of unpaired data or one-way analysis of variance (ANOVA). P value <0.05 was considered statistically significant in all cases.

Results

CTGF expression is up-regulated by visfatin stimulation in EA.Hy926 cells

To detect whether visfatin could induce CTGF exprssion, different concentrations of visfatin at 10ng/mL, 50ng/mL, and 100ng/mL were used to treat the EA.Hy926 cells separately. The mRNA level of CTGF was determined by quantitative RT-PCR. Compared with controls, CTGF expression was markedly increased by about 30% and 46% after treated for 12h or 24h by visfatin at 50ng/mL. Correspondingly, 100ng/mL of visfatin increased CTGF expression by about 65%, 116% and 190% after treated for 6h, 12h and 24h (Fig. 1A). These results suggested that visfatin induced CTGF mRNA expression in a time-dependent and concentration-dependent manner. And the concentration of 100ng/mL of visfatin was selected to treat the EA.Hy926 cells for 24h in the further studies.

Next, the protein expression of CTGF in visfatin treated EA.Hy926 cells was elevated by Western Blot.

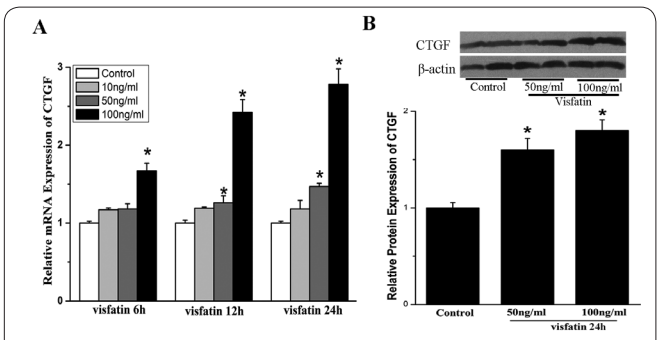

Figure 1. Visfatin significantly induced the expression of CTGF in EA.Hy926 cells. A. The mRNA expression of CTGF in EA.Hy926 cells was induced by 50ng/mL and 100ng/ml visfatin treated for 6h, 12h, and 24 h. **B**. Protein expression of CTGF in EA.Hy926 cells was increased after treated by visfatin for 24h at concentration of 50ng/mL and 100ng/mL * P<0.05 vs Control (n=3).

The results were consistent with the changes of CTGF's mRNA level (Fig. 1B), suggesting that CTGF was triggered by visfatin.

The expression of HIF-1α, but not TGF-β1, is increased in visfatin-treated EA.Hy926 cells

Previous studies have demonstrated that both TGF-β1 and HIF-1α are important stimulators of CTGF expression, therefore quantitative RT-PCR was performed to determine the expression of TGF-β1 and HIF-1α in visfatin treated EA.Hy926 cells. The data showed that the expression of TGF-β1 kept unchanged upon the stimulation of visfatin, while the expression of HIF-1α was induced about 90% by visfatin treatment for 24h at 50ng/mL, and about 163% at 100ng/mL in EA.Hy926 cells, compared with those in the controls without treatment (Fig.2A).

In addition, we also detected HIF-1α expression in EA.Hy926 cells treated with visfatin by Immunofluorescence staining. As shown in Fig. 2B, visfatin could significantly increase the protein level of HIF-1α in the cells by about 2.5-fold compared with that in the untreated controls.

Knockdown of HIF-1α partially blocks visfatin-induced CTGF expression

To evaluate the possible action of HIF-1α in visfatin-treated EA.Hy926 cells, specific inhibition of HIF-1α by HIF-1α-shRNA expressing plasmid was performed to investigate whether HIF-1α could regulate CTGF expression. As presented in Fig. 3A, knockdown efficiency of the HIF-1α shRNA expressing plasmid in endothelial cells was only approximate 30%. However, after down-regulation of the HIF-1α expression by HIF-1α-shRNA, the increasement level of CTGF was remarkably suppressed by 34% in cells treated with visfatin for 24h, compared with the cells transfected with the scrambled shRNA plasmid (Fig. 3B).

Taken together, these observations suggest that the up-regulation of CTGF expression induced by visfatin in EA.Hy926 endothelial cells might be mediated by HIF-1α, but not TGF-β1.

Discussion

In the present study, we used visfatin to induce CTGF expression and gained three findings: (1) CTGF was up-regulated in endothelial cells with visfatin treatment;

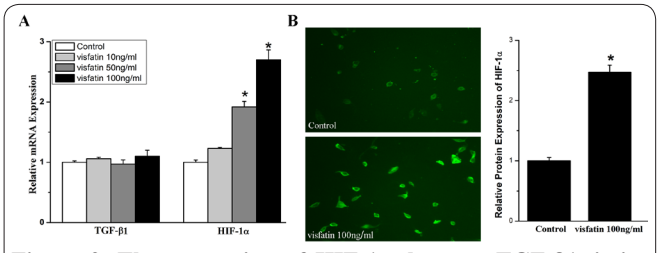

Figure 2. The expression of HIF-1α, but not TGF-β1, is increased in visfatin-treated VECs. **A**. The expression of TGF-β1 kept unchanged upon the stimulation of visfatin. But the level of HIF-1α was increased about 90% in EA.Hy926 cells after stimulated by visfatin at 50ng/ml and about 163% at 100ng/ml. **B**. The protein expression of HIF-1α in VECs treated by visfatin was elevated 2.5-fold compared with untreated control. * P<0.05 vs Control (n=3).

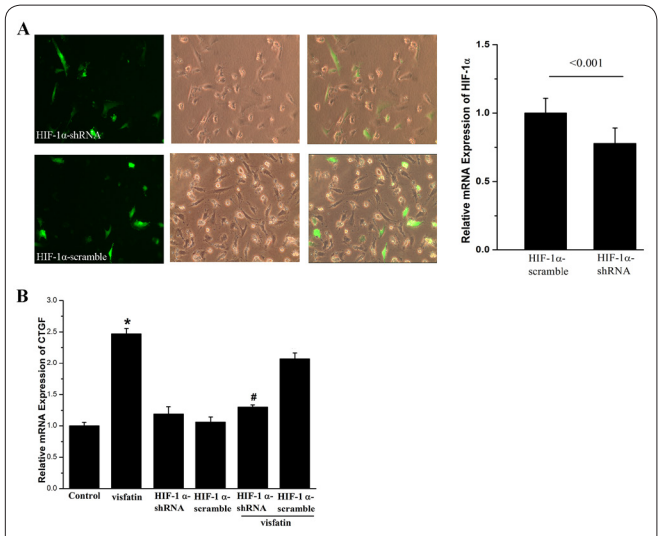

Figure 3. Knockdown of HIF-1α blocks visfatin-induced CTGF expression. A. The morphology of cells transfeted with HIF-1α-shRNA (the green color indicated cells transfected by HIF-1α-shRNA) and the knockdown efficiency of HIF-1α-shRNA validated by real time RT-PCR. B. Transfection of HIF-1α-shRNA inhibited 34% CTGF expression when treated with 100ng/ml visfatin at 24h (Fig 3). * P<0.05 vs control, # P<0.05 vs Scramble (n=3).

(2) TGF-β1 expression was unchanged whereas HIF-1α expression was increased after visfatin stimulation; (3) Down-regulation of HIF-1α expression attenuated CTGF induction by visfatin in the endothelial cells.

Vascular endothelial dysfunction is critical in the initial pathogenesis of atherosclerotic lesion formation (31,32). Adipokines, such as resistin, leptin and visfatin, are proinflammatory mediators that directly contribute to endothelial dysfunction and atherogenesis (2,10,11,33). The present study demonstrated that visfatin increased the production of CTGF in EA.Hy926 endothelial cells. This finding is in agreement with previous study, which showed that visfatin induced the CTGF production in the process of hepatic stellate cell activation (28). CTGF is a kind of secretion polypeptide rich in cysteine, which only keeps low expression in human vessels, but it is increased about 50-100 folds in atherosclerosis endothelial cells and smooth muscle cells (21). It could promote the adhesion, proliferation and migration of endothelial cells and accelerate the angiogenesis process, suggesting that CTGF plays an important role in atherosclerosis and other cardiovascular diseases (22). Moreover, CTGF is also involved in the pathogenesis of obesity-induced insulin resistance and atherosclerosis (34). Our results showed that the production of CTGF in EA.Hy926 cells was increased following exposure the cells to visfatin, implying that CTGF mediated the visfatin-induced injury to endothelial cells.

TGF-β1 has been identified as a potent inducer of CTGF expression, however, the TGF-β1 expression was almost unaffected upon visfatin stimulation, revealing that up-regulation of CTGF was independent of TGF-β pathway. There are two functional hypoxia response elements in the visfatin promoter, and visfatin gene expression is upregulated in the fat tissue of obesity via the activation of HIF-1α (35). Therefore, in present study, whether HIF-1α was involved in visfatin-induced CTGF upregulation was investigated. HIF-1α, the ac-

tive subunit of HIF-1, is undetectable under normoxia conditions because of the rapid proteasomal degradation. But it is stabilized under the hypoxia conditions (26). Evidences suggest that HIF-1α pathway is associated with the progression and angiogenesis of human atherosclerosis (36).

Of particular note, HIF-1α regulation by non-hypoxic stimuli has gained considerable interests (37). Studies have shown that in normal oxygen conditions, G-protein-coupled receptor agonists including angiotensin II and thrombin could potently induce and activate HIF-1α in vascular smooth muscle cells (38). In the current study, in order to block HIF-1α actions, we used a HIF-1α-specific small inhibitory RNA construct (HIF-1α-siRNA) to knock-down HIF-1α expression. The specific HIF-1α-siRNA expressing plasmid was constructed and used to knock-down HIF-1α expression in the lung cancer cell line A549 cells and in human vascular smooth muscle cells in our previous reports (39,40). Our present data demonstrated that, upregulation of CTGF induced by visfatin was suppressed in EA.Hy926 endothelial cells transfected with HIF-1α-shRNA plasmid. More importantly, visfatin could induce the expression of HIF-1α under the normal oxygen condition. Based on the observation that HIF-1α can be induced by G-protein-coupled receptor agonists, we postulate that visfatin may cause activation of G-protein-coupled receptor. However, further studies are necessary to elucidate the detailed mechanism by which visfatin activates HIF-1α in vascular endothelial cells under normal oxygen conditions.

In conclusion, our study discovered a novel mechanism of HIF-1α mediated the visfatin- induced CTGF expression in endothelial cells. These results suggest a more general role of this transcription factor in the vascular response to injury.

Acknowledgements
This work is supported by the National Natural Science Foundation of China (Grant No. 11372204).

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