Galectin-3 exacerbates ox-LDL-mediated endothelial injury by inducing inflammation via integrin β1-RhoA-JNK signaling activation

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Abstract
Oxidized low-density lipoprotein (Ox-LDL)-induced endothelial cell injury plays a crucial role in the pathogenesis of atherosclerosis (AS). Plasma galectin-3 (Gal-3) is elevated inside and drives diverse systemic inflammatory disorders, including cardiovascular diseases. However, the exact role of Gal-3 in ox-LDL-mediated endothelial injury remains unclear. This study explores the effects of Gal-3 on ox-LDL-induced endothelial dysfunction and the underlying molecular mechanisms. In this study, Gal-3, integrin β1, and GTP-RhoA in the blood and plaques of AS patients were examined by ELISA and western blot respectively. Their levels were found to be obviously upregulated compared with non-AS control group. CCK8 assay and flow cytometry analysis showed that Gal-3 significantly decreased cell viability and promoted apoptosis in ox-LDL-treated human umbilical vascular endothelial cells (HUVECs). The upregulation of integrin β1, GTP-RhoA, p-JNK, p-p65, p-IKKα, and p-IKKβ induced by ox-LDL was further enhanced by treatment with Gal-3. Pretreatment with Gal-3 increased expression of inflammatory factors (interleukin [IL]-6, IL-8, and IL-1β), chemokines (CXCL-1 and CCL-2) and adhesion molecules (VCAM-1 and ICAM-1). Furthermore, the promotional effects of Gal-3 on NF-κB activation and inflammatory factors in ox-LDL-treated HUVECs were reversed by the treatments with integrin β1-siRNA or the JNK inhibitor. We also found that integrin β1-siRNA decreased the protein expression of GTP-RhoA and p-JNK, while RhoA inhibitor partially reduced the upregulated expression of p-JNK induced by Gal-3. In conclusion, our finding suggests that Gal-3 exacerbates ox-LDL-mediated endothelial injury by inducing inflammation via integrin β1-RhoA-JNK signaling activation.

KEYWORDS
atherosclerosis, endothelial injury, Galectin-3, inflammation, integrin β1, JNK

1 | INTRODUCTION

Endothelial cells (ECs) play a key role in maintaining vascular homeostasis in response to various stimuli. Endothelial dysfunction in...
arterial walls has been considered to be the basis and initial step of atherosclerosis (AS), the most common cause in cardiovascular diseases (Davignon & Ganz, 2004; Tabas, García-Cardena, & Owens, 2015). Increasing evidence has demonstrated that oxidized low-density lipoprotein (ox-LDL) induces apoptosis of endothelial cells and is considered as the major risk factor in the progression of AS (J. Li, Liang, Wang, Xu, & Li, 2017; Qin et al., 2017; Veas et al., 2016). To date, the underlying molecular mechanisms of AS progression are complicated and not completely understood.

Many studies have demonstrated the role of inflammation in endothelial dysfunction (Cho, Lee, Chang, Lee, & Kim, 2018; Chrysohoou, Kollia, & Tousoulis, 2018; Stolberg et al., 2018). Under conditions of chronic inflammation, sustained activation of ECs by inflammatory stimuli causes alterations in normal endothelial function, resulting in endothelial dysfunction. Accumulating evidence shows that ox-LDL induces endothelial cell injury by changing pro-inflammatory genes expression (J. B. Li et al., 2017; S. Zhang et al., 2017). In addition, several studies have demonstrated that ox-LDL stimulates vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) to exacerbate ECs dysfunction (Wang, Hao, Wang, Wang, & Li, 2011; H. Zhang, Zheng, Zhao, Guo, & Chen, 2013). However, the effective regulators and accurate mechanisms of inflammation in ox-LDL induced AS remain to be thoroughly investigated.

As a multifunctional protein, Galectin-3 (Gal-3) is broadly expressed in inflammatory cells to induce the functions of macrophage chemotaxis, angiogenesis, lipid loading, and inflammation responding to biochemical and biophysical stimuli. An elevated level of Gal-3 has been found to be significantly associated with higher risk of death in both acutely decompensated heart failure and chronic heart failure populations (Lok et al., 2010; van Kimmenade et al., 2006). Some studies have indicated that Gal-3 inhibition significantly reduces hypertension and fibrosis (Martínez-Martínez et al., 2018; Yu et al., 2013). The role of Gal-3 in promoting AS has recently received great interest. Gal-3 participates in different mechanisms involved in AS, such as inflammation, proliferation, or macrophage chemotaxis. However, the role and related mechanisms of Gal-3 in ox-LDL induced AS have not been reported.

A large number of basic and clinical studies have shown that Gal-3 is mainly involved in the formation and development of AS by exacerbating the inflammation of coronary arteries (Madrigal-matute et al., 2014). Integrins are transmembrane receptors that facilitate cell-extracellular matrix adhesion and signal transduction. Some studies have shown that Gal-3 can interact with integrin β1 (Hönig et al., 2018; Nachtigal, Ghaffar, & Mayer, 2008; Kianoush, F., 2017) and indicated that integrin β1 activates RhoA, thus upregulating JNK signaling, an important branch of the MAPK (mitogen-activated protein kinase), which plays an important role in many physiological and pathological processes, such as cell stress, inflammation and apoptosis (Wang et al., 2016; L. Zhang et al., 2017).

JNK, a common receptor tyrosine kinase, participates in wide ranges of physiological processes. Recent reports also indicate that JNK produces a molecular link between inflammation and AS (Kwok, K. H. M., 2016; Mammen et al., 2018). Hence, the activation of JNK can accelerate the formation of AS to some extent. In the present study, we hypothesize that the Gal-3-integrin β1 interaction may promote the expression of inflammatory factors and adhesion molecules by modulating the RhoA-JNK signaling pathway, thus resulting in the progression of AS.

2 | MATERIALS AND METHODS

2.1 | Collection of human specimens

Blood samples were obtained from people diagnosed with color Doppler ultrasound in Nanjing BenQ Hospital, affiliated to Nanjing Medical University. Specimens were collected from patients who underwent carotid endarterectomy (CEA) in The First Affiliated Hospital of Nanjing Medical University. The splenic arteries in the control group were from traumatic splenectomy. The study was approved by the ethics committee of Nanjing BenQ Hospital and The First Affiliated Hospital of Nanjing Medical University. The protein levels in blood and CEA specimens were analyzed by enzyme-linked immunosorbent assay (ELISA) and western blot (WB), respectively.

2.2 | Materials

Ox-LDL was purchased from Solarbio (Beijing, China). Gal-3 was purchased from Peprotech (BioGems, Rocky Hill, NJ). The ELISA kits of interleukin (IL)-6, IL-8, IL-1β, CXCL1, and CCL-2 were purchased from MultiSciences (Lianke, Hangzhou, China). GTP-RhoA specific inhibitor Y-27632 and JNK inhibitor SP600125 were purchased from Targetmol (Target Molecule Corp, Shanghai, China).

2.3 | Cell culture

HUVECs were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). They were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

2.4 | Cell groups division

The HUVECs were first divided into four groups: (a) control group; (b) Gal-3 group, cells treated with 250 ng/ml Gal-3 for 48 hr; (c) ox-LDL group, cells treated with 150 μg/ml ox-LDL for 6 hr; (d) Gal-3 + ox-LDL group, cells treated with 250 ng/ml Gal-3 for 48 hr before treatment of ox-LDL for 6 hr. Furthermore, cells were further divided into five groups: (a) ox-LDL group; (b) Gal-3 + ox-LDL group; (c) control-siRNA + Gal-3 (250 ng/ml + ox-LDL (150 μg/ml); (d) integrin β1-siRNA + Gal-3 (250 ng/ml + ox-LDL (150 μg/ml), cells transfected with integrin β1 for 72 hr before treatment of 250 ng/ml Gal-3 for 48 hr, followed by treatment with 150 μg/ml ox-LDL for 6 hr; (e) JNK inhibitor (SP600125) + Gal-3 + ox-LDL, cells treated with 20 μM SP600125 for 1 hr before treatment with 250 ng/ml Gal-3 for 48 hr, followed by treatment with 150 μg/ml ox-LDL for 6 hr.
2.5 | Cell viability analysis

The viability of HUVECs was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays. HUVECs were seeded in a 96-well plate and treated according to the requirements of the different groups. The cells were treated with 100 μg/ml MTT for 4 hr in a 37°C incubator. Subsequently, dimethyl sulfoxide (DMSO, 200 μl/well) was added to dissolve the formazan crystals. The absorbance at 490 nm was detected using a microplate reader (Bio-Rad, Inc, Hercules, CA).

2.6 | Annexin V-FITC/propidium iodide assay

HUVECs were seeded in a 6-well plate and treated according to the requirements of the different groups. Apoptosis was determined by flow cytometry. In summary, the differently treated cells were harvested and washed three times with cold phosphate-buffered saline (PBS). Subsequently, 10^6 cells were resuspended in the binding buffer (500 μl) and incubated with Annexin-V-FITC (10 μl) and propidium iodide (PI) (5 μl) for 15 min at 37°C in the dark. Finally, the fluorescent intensities were detected with a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA, USA) and apoptosis rates were analyzed within 1 hr.

2.7 | WB analysis

HUVECs were lysed in RIPA buffer. Equivalent amounts of total protein (20 mg) were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in TBST containing 5% nonfat milk for 2 hr at room temperature. Subsequently, the membranes were incubated with the following primary antibodies at 4°C overnight: integrin β1 (1:1000, cat.24693, abcam), RhoA (1:1000, cat.6352, Affinity), phosphorylation of RhoA (GTP-RhoA) (1:500, cat.211164, abcam), JNK (1:1000, cat. AF6319, Affinity), p-JNK (1:1000, cat. AF3320, Affinity), ICAM-1 (1:1000, cat. DF7413, Affinity) and VCAM-1 (1:1000, cat.DF6082, Affinity), NF-κB P65(1:1000, AF5006, Affinity), PhosphoNFκB P65(1:1000, AF2006, Affinity), IKKα (1:1000, AF6012, Affinity), IκBβ (1:1000, bs-4880R, Bioss, China), Phospho-IκBβ(1:1000, bs-3232R, Bioss, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. After incubation with horseradish peroxidase-conjugated secondary antibodies, the blots were visualized using enhanced chemiluminescence kit (ECL, Perkin Elmer Life Sciences, Boston, MA). The bands were quantified and analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD) and were normalized to GAPDH.

2.8 | Cell staining for immunofluorescence microscopy

The treated cells were grown on coverslips, fixed with 3% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature and blocked with 3% goat serum for 20 min at room temperature. Then cells were incubated with integrin β1 antibody (1:500, abcam) overnight at 4°C. The next day, cells were washed in 0.1 mol/L PBS for 3 times and then incubated with fluorescein-conjugated affinity goat antirabbit IgG (1:500) for 3 hr at 37°C and subsequently stained the nuclei with DAPI. Finally, cells were observed using an Olympus BX51 fluorescence microscope (Tokyo, Japan).

2.9 | Small interfering RNA assay

5 × 10^5 cells/ml were seeded in a 6-well plate and incubated for 24 hr. Plasmid and small interfering RNA (siRNA) transfection were carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions, when the cell confluence reached approximately 70%. Integrin-β1-siRNA (target sequence 5′-CCACA-GACAUUACAUAAUU- 3′) and negative control (NC) siRNA (HP GenomeWide siRNA; Qiagen, Hilden, Germany) were prepared and transfected at 10 nM for 48 hr. Subsequently, the transfected cells were analyzed by WB.

2.10 | Enzyme-linked immunosorbent assay

Supernatant of HUVECs of different groups were detected using corresponding enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The expression of Gal-3, using ELISA, was analyzed in eight blood specimens from patients with AS and eight blood specimens from the control group without AS. Furthermore, the expression of pro-inflammatory cytokines (IL-6, IL-8 and IL-1β) and chemokines (CXCL-1 and CCL-2) was assessed as well.

2.11 | Statistical analysis

Data were presented as the mean ± SD. Differences between groups were analyzed by one-way analysis of variance using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). P < 0.05 was considered to indicate statistical significance.

3 | RESULTS

3.1 | Protein expression of gal-3, integrin β1, RhoA, and GTP-RhoA in AS

We assessed the protein level of Gal-3, using ELISA, and found Gal-3 expression to be significantly higher in blood specimens from AS patients. (Figure 1a) Furthermore, the protein expression of Gal-3, integrin β1, RhoA and GTP-RhoA in the tissue of 4 AS plagues and four normal splenic artery was determined by WB. Gal-3, integrin β1, and GTP-RhoA levels were markedly elevated in AS tissue compared with the control group, (Figure 1b) while the RhoA level underwent almost no change. The results showed that although Gal-3, integrin β1 and GTP-RhoA were expressed in both normal and abnormal conditions, they were prominent in AS vessels. Considering this, we further investigated the role of Gal-3 in the AS and the underlying molecular mechanisms among the three factors.
3.2 | Galectin-3 decreases viability and promotes cell apoptosis of ox-LDL-treated HUVECs

To investigate the effects of Gal-3 on the cell viability in ox-LDL-induced HUVECs, the cells were exposed to 250 ng/ml Gal-3, 150 μg/ml ox-LDL or their combination and the MTT assay was performed. We further measured apoptosis by flow cytometry. As shown in Figure 2a, ox-LDL treatment significantly reduced cell viability as compared with the control group. The ox-LDL-induced reduction in cell viability was strengthened by Gal-3. As shown in Figure 2b,c proportion of apoptotic cells was markedly increased in the ox-LDL group, which was further significantly improved by additional Gal-3 treatment.

3.3 | Gal-3 promotes ox-LDL-induced inflammatory responses via NF-κB activation and enhances the expression of related inflammatory factors, chemokines, and adhesion molecules

Activation of the NF-κB pathway is widely recognized as characteristic of inflammation. To explore whether Gal-3 aggravate ox-LDL-induced inflammatory responses in HUVECs, we first detected the expression of p65 NF-κB after ox-LDL or Gal-3 alone or in combination, the result revealed that the single treatment obviously increased its expression and the effect of the combination was greater (Figure 3a,b). However, there was no change in the total
protein of p65 NF-κB. We further assessed whether NF-κB activation induced by Gal-3 was associated with the regulation effect of IKKα and IKKβ, two highly related IκB kinases. Similar to the above results, the phosphorylation levels of IKKα and IKKβ (p-IKKα and p-IKKβ) were significantly increased, and the combined effect was stronger (Figure 3a,b). Additionally, we also evaluated the effect of Gal-3 on inflammatory factors. As shown in Figure 3c,d compared to the control group, the protein levels of pro-inflammatory factors (IL-6, IL-8, and IL-1β) and chemokines (CXCL-1 and CCL-2), using ELISA, were markedly increased following ox-LDL treatment. Moreover, the expression of these cytokines was significantly increased in the combination of Gal-3 and ox-LDL group. As shown in Figure 3e, the adhesion molecules VCAM-1 and ICAM-1 were notably upregulated in the ox-LDL group compared to the control group, and their expression was significantly elevated by the addition of Gal-3 treatment. These results suggest that Gal-3 promotes HUVECs injury via ox-LDL-induced inflammation.

3.4 | Galectin-3 promotes the expression of integrin β1, GTP-RhoA and p-JNK in ox-LDL induced HUVECs

To determine whether integrin β1 was involved in the effect of Gal-3, integrin β1 protein was measured by IF. Integrin β1 was moderately increased following exposure to Gal-3 or ox-LDL alone. However, a combination of them resulted in significantly higher expression as compared to single treatment. (Figure 4a) We also detected the change of integrin β1 protein expression using WB and the result was consistent with IF. (Figure 4b) In addition, to test whether RhoA or JNK molecule was involved in Gal-3 effect, the levels of RhoA, JNK, GTP-RhoA and p-JNK were further assessed after Gal-3 and ox-LDL treatment. The result indicated that single treatment obviously enhanced the levels of GTP-RhoA and p-JNK in the HUVECs cells, whereas their total levels remained unchanged. Moreover, treatment of Gal-3 combined with ox-LDL induced higher GTP-RhoA and p-JNK expression as compared to single drug treatment. (Figure 4c)
Galectin-3 aggravates ox-LDL induced HUVECs injury by activating integrin β1-RhoA-JNK pathway

To investigate the role of integrin β1-RhoA-JNK pathway in the ox-LDL induced HUVECs cell injury, we knocked down integrin β1 using siRNA in ox-LDL-treated HUVECs and the decreased integrin β1 expression was confirmed by WB and IF (Figure 5a, b and c). Next, the MTT assay and flow cytometry were performed to evaluate cell viability and apoptosis (Figure 5d, e and f). Integrin β1 knockdown and the JNK inhibitor, SP600125, significantly increased cell viability and decreased the rate of cell apoptosis, suggesting reducing Gal-3 induced HUVECs cell injury. Moreover, downregulation of integrin β1 markedly reduced GTP-RhoA and p-JNK. To explore whether RhoA was involved in the regulation of integrin β1 on JNK, we chose RhoA specific inhibitor, Y-27632 to suppress its expression. We found that Y-27632 markedly retarded JNK activation, while Gal-3 promoted JNK expression. Meanwhile, Y-27632 partially inhibited the upregulated expression of p-JNK induced by Gal-3. (Figure 5g,h) These results suggest that Gal-3 promotes ox-LDL induced HUVECs cell injury by activating integrin β1-RhoA-JNK pathway.
3.6 Gal-3-induced inflammatory pathway NF-κB activation and expression of related cytokines, chemokines, and adhesion molecules play a promotional role in ox-LDL-induced HUVECs inflammatory injury by activating integrin β1-RhoA-JNK pathway

To explore whether Gal-3 induced HUVECs injury via integrin β1-RhoA-JNK pathway is associated with inflammation, we first assessed the inhibition of integrin β1 and JNK on NF-κB activation, the results indicated that siRNA integrin β1 or SP600125 significantly inhibited the phosphorylation expression of p65, IKKα and IKKβ. (Figure 6a,b). Furthermore, the levels of pro-inflammatory cytokines (IL-6, IL-8, and IL-1β) and chemokines (CXCL-1 and CCL-2) were then assessed by ELISA. Data in Figure 6c,d demonstrated that integrin β1 knockdown or JNK inhibition significantly decreased the level of IL-6, IL-8, IL-1β, CXCL-1, and CCL-2. Moreover, either integrin β1 knockdown or JNK inhibition significantly downregulated the expression of the adhesion molecules VCAM-1 and ICAM-1. (Figure 6e)

4 DISCUSSION

Endothelial cells play a key role in maintaining vascular homeostasis in response to various stimuli. However, vascular injuries, such as angioplasty, diabetes, hypertension, and immune-mediated damages, can lead to endothelial cell dysfunction, which has been considered to be the first cellular event in the pathogenesis of AS. Many studies have revealed an association between endothelial dysfunction and chronic inflammation (Cho et al., 2018; Chrysohoou et al., 2018; Stolberg et al., 2018). Endothelial dysfunction, because of inflammation, contributes to the pathogenesis of AS. Recent evidence suggests that Gal-3 involves in the pathogenesis of inflammatory disorders (Knights et al., 2016; Lu et al., 2017; Oever et al., 2013) and the inactivation of Gal-3 or therapeutic modulation of the protein levels has been shown to halt the progression of AS (Dumic, Dabic, & Flögel, 2016; MacKinnon et al., 2013). In this study, we investigated the effects of Gal-3 on endothelial injury. Our study is the first to show that Gal-3 promotes ox-LDL induced inflammation-associated HUVECs injury by activating integrin β1-RhoA-JNK signaling pathway.
Gal-3 has been shown to be involved in cell growth, differentiation, and apoptosis (Tian et al., 2015). Studies have also shown that the expression of Gal-3 is implicated in a variety of processes associated with heart failure (Cho et al., 2018; Chrysohoou et al., 2018; J. B. Li et al., 2017; Stolberg et al., 2018; Wang et al., 2011; Zhang et al., 2013, 2017). Activation of the NF-κB pathway is widely recognized as characteristic of inflammation. It is thought that NF-κB activation is controlled by IKK complex, which can regulate its nuclear translocation and transactivation. IKKα and IKKβ mediate Ser536 phosphorylation in the p65 transactivation domain. Several studies have demonstrated that NF-κB is involved in the development of various inflammatory cardiac pathologies, namely ischemic heart disease (Huang,
endothelial injury. The effects of integrin β1 and JNK obviously reduced cell injury, led to inhibition of NFkb inflammatory pathway and the decrease of related inflammatory factors in HUVECs. Together, our research suggests that Gal-3 promotes ox-LDL-induced HUVECs injury by integrin β1/RhoA-JNK pathway.

In conclusion, this study is the first to demonstrate that Gal-3 treatment significantly exacerbates ox-LDL-induced HUVECs injury. We also found that Gal-3 promoted ox-LDL-induced inflammatory injury through activation of the integrin β1-RhoA-JNK pathway. However, this study has shortcomings. Our blood samples and CEA specimens were not abundant enough, and further in vivo experiments and more detection of human specimens may be better to explain this. Moreover, our future research is needed to determine the role of other factors besides inflammation in the pathogenic role of Gal-3 in AS.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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