Angiotensin II induces the exocytosis of galectin-3 via integrin αv/AKT/NF-κB signaling pathway

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Abstract. – OBJECTIVE: To explore the role of integrin αv in Angiotensin II (Ang II)-induced exocytosis and endocytosis of galectin-3 (gal-3) in vascular smooth muscle cells (VSMCs).

MATERIALS AND METHODS: A primary culture of mouse VSMCs was established by the enzymatic digestion of aorta. Adeno-Cre was used to specifically knockdown integrin αv. VSMCs were treated with Ang II, LY294002 (inhibitor of AKT signaling pathway), and Bay11-7082 (inhibitor of nuclear factor-kappa B, NF-κB), respectively. Endocytosis of His-tagged gal-3 was analyzed by immunofluorescence. The Western blot was performed to detect the protein level in cell supernatant and lysate.

RESULTS: Ang II increased the exocytosis of gal-3 and activated AKT and NF-κB signaling pathways. The knockdown integrin αv effectively decreased the activation of AKT and NF-κB signals and the exocytosis of gal-3 induced by Ang II, but it had a little effect on the endocytosis of gal-3. Ang II increased the phosphorylation of AKT and NF-κB through integrin αv. AKT is the upstream signal of the NF-κB signaling pathway. LY294002 and Bay11-7082 could decrease Ang II-induced exocytosis of gal-3 in VSMCs.

CONCLUSIONS: Ang II, depending on integrin αv/AKT/NF-κB signaling pathway, induced the exocytosis of gal-3.

Key Words:
Angiotensin II, Galectin-3, Integrin αv.

Introduction

The renin-angiotensin system (RAS) plays a vital role in the cardiovascular system. It is not only an endocrine system, but also serves local paracrine and autocrine functions in tissues and organs. Angiotensin II (Ang II), the primary effector molecule of this system, virtually regulates the function of all organs, including heart, kidney, and vasculature. Ang II could regulate blood pressure by adjusting salt/water homeostasis and vasoconstriction. In heart failure patients, Ang II also plays a key role in cardiac hypertrophy and remodeling[1].

Galectin-3 (gal-3) was found in the cytoplasm and extracellular matrix. It has two kinds of forms, an extra- and intra-cellular lectin, and the extracellular gal-3 could be endocytosed into the cytoplasm and become the intracellular gal-3[2]. In the [illegible] cells, extracellular gal-3 binding to integrin β1 resulted in activation of apoptosis processes including cytochrome release and caspase-3 activation[3]. In the breast carcinoma cells, gal-3 was also found to be internalized via a caveolae-like pathway[4].

Gal-3 has also been found in the plasma sample of the human beings[5] and is regarded as an important fibrosis related protein. Gal-3 was upregulated in some kinds of cancers[6], mediated the interaction between the tumor cell and extracellular matrix, promoted cancer progression and tumor metastasis[7-9]. Gal-3 is a strong independent predictor of cardiovascular death in heart failure (HF) patients[10]. In an HF mice model, gal-3 induced cardiac fibroblast proliferation and collagen deposition[11]. Gal-3 was upregulated in the acute period of myocardial infarction (MI), and suggested, as a novel informative biomarker, to predict the adverse outcomes in MI patients[12-14]. The vascular smooth muscle cells (VSMCs) are one of the main important cells in the artery wall; the activation of VSMCs could enter into the *intima*, transform to be foam cells, and finally deteriorate the pathological process of atherosclerosis.

Integrin, as a cell surface receptor, could mediate the process of endocytosis and exocytosis, however, the effects of different subtypes of integrin in affecting endocytosis and exocytosis are various. Endocytosis of integrin β1 depend-

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ed on gal-3 which triggered the glycosphingolipid (GSL)-dependent biogenesis of a morphologically distinct class of endocytic structures, termed clathrin-independent carriers (CLICs)[15]. In the immature rat the Sertoli cells, calcium uptake, and exocytosis depended from the integrin αvβ3[16]. Integrin αvβ3 mediated exocytosis of mucin which was induced by the *Entamoeba histolytica* cysteine proteinase 5 in colonic goblet cells[17]. The active Matrix metalloproteinase-2 (MMP-2) induced the secretion of VEGF-A via integrin αvβ5 instead of integrin β1 in the vascular endothelium[18]. Integrin αvβ3 could also mediate the internalization of cRGDfK modified gold nanoparticles (cRGDfK-PEG-AuNPs), but it has a little effect on the endocytosis of PEG-conjugated gold nanoparticles (PEG-AuNPs)[19].

Integrins have been found to mediate the functions of gal-3 in many different cells. Gal-3 could increase lateral mobility of integrin receptors, the cluster size of integrin, and cell migration in HeLa cells[20]. Integrin β1 also mediated gal-3 and induced the production of the inflammatory cytokines in pancreatic stellate cells (PSCs)[21]. Gal-3 activated the outside-in integrin signaling by promoting cell migration and matrix remodeling in metastatic cancer cells[22]. Integrin αvβ3 was the major galectin-3-binding protein in the endothelial cells, and anti-αv, anti-β3 and anti-αvβ3 integrin antibodies could significantly inhibit gal3-induced cell migration and capillary tubule formation[23].

Ang II plays an important role in the process of vascular fibrosis, and gal-3 has also been regarded as a fibrosis-related protein[24]. Integrins are the cell surface receptors which are related to the cardiac fibrosis[25]. However, our knowledge about the endocytosis and exocytosis of gal-3 was still limited, and the role of integrin αv in this process should also be further determined.

Materials and Methods

Reagents

The Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Lonza (Basel, Switzerland), fetal bovine serum (FBS) and penicillin/streptomycin (penicillin=100 U/ml and streptomycin=100 microg/mL) were purchased from Gibco (Rockville, MD, USA). His-tagged gal-3 was purchased from ATGen (Los Angeles, CA, USA). The primary antibody against phospho-AKT (No. CST-4060), AKT (No. CST-9272), phospho-NF-κB (No. CST-3033), NF-κB (No. CST-3034), and GAPDH (No. CST-2118) were acquired from the Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-gal-3 (No. ab76245) and anti-integrin αv (No. ab179475) were obtained from Abcam (Cambridge, MA, USA). Bay11-7082 (NF-κB inhibitor) was from Calbiochem (Billerica, MA, USA). The His-tagged antibody was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals came from commercial sources.

Cell Culture

We generated the loxP-floxed integrin αv mice in which the exon 4 was flanked by two loxP sequences. The primary culture of mouse VSMCs was established by the enzymatic digestion of the aorta[26]. The primary cultures were maintained in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin, Gibco, Carlsbad, CA, USA). The cells between the 5th and 15th passages were used in these experiments. The animal use was in compliance with the principles of the declaration of Helsinki and was authorized by the local Ethical Committee Prevention and Wellbeing of Animals of the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Comité d'Ethique Lorrain en Matière d'Experimentation Animale (CELMEA).

Adeno-Cre Interference

The integrin αv expression was inhibited by the transfection with Adeno-Cre. Briefly, $5x10^5$ of VSMCs per well were cultured in 6-well plates up to 75% confluence. The cells were then transfected with 0.5 µl GFP or adeno-cre/hole using 2 ml DMEM. The process of transfection was performed in the absence of antibiotics. After 48 h incubation at 37°C, the cells were used for other experiments.

Immunofluorescence

The immunofluorescence staining and confocal laser microscopy. The cells ($5x10^5$) were seeded on the flame-sterilized coverslips and placed into 24-well tissue culture plates. The integrin αv were knocked down by Adeno-Cre for 48 h. Then, the cells were incubated with His-tagged gal-3 for different times (30 min, 1h, 2h). The cells were subsequently fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 20 min, blocked with 1% bovine serum albumin for 1 h, and incubated with specific primary antibody overnight at 4°C. The cells

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were incubated with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (Invitrogen Life Technologies) for 1 h at room temperature. The nuclei were stained with DAPI (1:1,000) for 5 min at room temperature. The protein expression levels of His-tagged gal-3 were then quantified using the confocal laser scanning microscope.

Western Blot

The secreted proteins in the supernatants of the culture were precipitated by trichloroacetic acid-sodium deoxycholate/acetone (TCA-DOC/ acetone). The cells were lysed in a lysis buffer containing 50 mM Tris (pH 7.6), 250 mMNaCl, 3 mM EDTA, 3 mM EGTA, 0.5% NP40, 2 mM dithiothreitol, 10 mM sodium orthovanadate, 10 mM NaF, 10 mM glycerophosphate, and 2% of protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 30 min on ice. The protein concentrations were measured with the BCA Protein Assay (Pierce, Rockford, IL, USA). The lysates (20 μg) were electrophoresed on 4-15% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Hybond-ECL, GE Healthcare, Velizy-Villacoublay, France). The membrane was blocked with 5% nonfat dry [illegible] in TBST (Tris-Buffered Saline+0.1% Twee[illegible]0) buffer (100 mM NaCl, 10 mM Tris-HCl, pH [illegible] and 0.1% Tween-20) for 1 h at room temperatu[illegible] The blots were then incubated [illegible] dilute[illegible] primary antibodies in TBST [illegible] 4°C [illegible]rnight, and then washed twice with T[illegible] buf[illegible] room temperature and incubat[illegible] for 1 [illegible] the appro-priate peroxidase-conjugated secondary antibody (1:3000 dilution). To quantify the protein, the band intensity was assessed by Image J.

Statistical Analysis

All data are expressed as the mean ± SEM. The statistics were performed using the Statistical Product Service Solution (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA). Th[illegible] One-way ANOVA followed by the Student[illegible]ewm[illegible]euls post-hoc analyses were also u[illegible] when app[illegible]ri-ate. For the data that did no[illegible] the norm[illegible]ty test; the non-parametric [illegible]NOV[illegible]Kruskal[illegible]al-lis test) was used. A [illegible]lue of p<[illegible] w[illegible] con-sidered statistically [illegible]gnifica[illegible]t. All [illegible]riments were performed at l[illegible] th[illegible] times in a triplicate assay.

[illegible]esults

[illegible]g II Increased the Exocytosis of Gal-3

[illegible]o investig[illegible]e whether Ang II induces the se-c[illegible]on of gal-[illegible] we used a different concentration of A[illegible]I (0, [illegible]0, 200, 500 nM) to deal with cells for 6h. [illegible] analyzed the immunoblots of the su-[illegible]atant cultures of VSMCs. We found that Ang [illegible]d increase the secretion of gal-3 into the cellular medium after the treatment for 6h. The expression of gal-3 in VSMCs was also detected by Western blot, but we did not observe any significant changes after the treatment of Ang II for 6h (Figure 1).

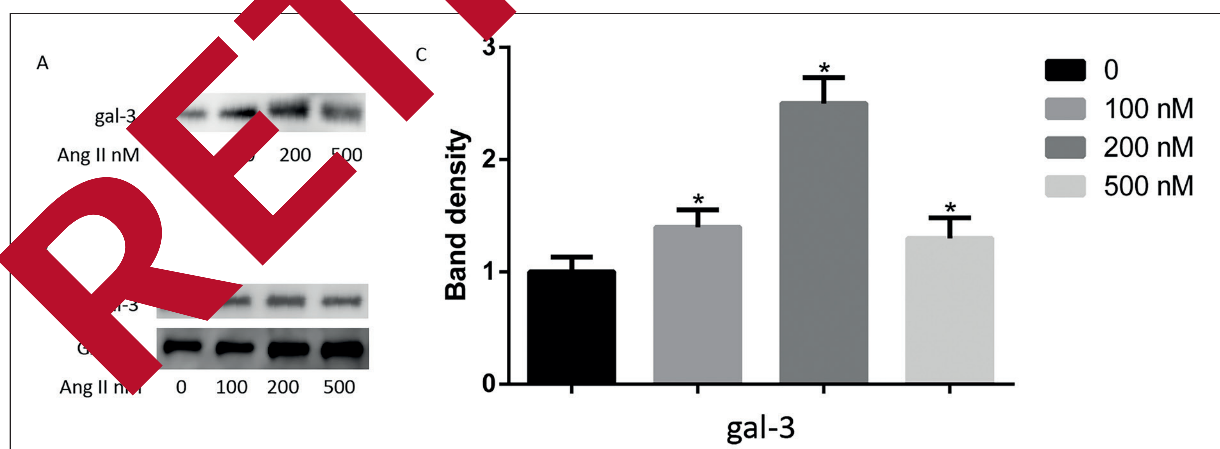

Figure 1. Ang II increased the exocytosis of gal-3 in VSMC. ***A,*** The different concentrations of Ang II (0-500 nM) were used to deal with the cells for 6 h, the culture supernatants of VSMC were analyzed by the Western blot. Densitometric measurements of replicate results are given in the right panel ***(C)***. The band density of the native VSMCs was chosen as a reference for relative expression and set to 1. *p<0.05 compared with any other concentrations. The data (mean±SEM) were obtained from three independent experiments. ***B,*** VSMCs were treated with Ang II over a range of concentrations (0-500 nM) for 6 h, and the expression of gal-3 in VSMCs was measured by the Western blot.

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Integrin αv Mediated the Exocytosis of Gal-3, but it Has Little Effect on the Endocytosis of Gal-3

Integrin αv is a cell surface receptor, and has also been proved to mediate the process of endocytosis[15], It was our interests to observe the effects of integrin αv in regulating the exocytosis and endocytosis of gal-3. We specifically knocked down the integrin αv by using Adeno-Cre, which reduced the expression of integrin αv protein of 85%. The knockdown integrin αv also significantly inhibited the exocytosis of gal-3 induced by Ang II. By using the method of immunofluorescence, we observed the endocytosis of His-tagged gal-3 for up to 2 h, however, the knockdown integrin αv has a little effect on the endocytosis of gal-3 (Figure 2).

Ang II Could Activate the NF-κB Signaling Pathway Through Integrin αv

Integrin αv, as the cell surface receptor, has a variety of downstream signaling pathways[25]. We analyzed the activation of AKT and NF-κB signaling pathways. We found that Ang II could increase the phosphorylation of AKT and NF-κB signal. To demonstrate whether integrin αv is necessary to mediate the activation of AKT and NF-κB signal in the presence of Ang II, we infected the cells with GFP or Adeno-Cre vectors to knockdown the integrin αv expression in VSMCs before Ang II treatment. In integrin αv knockdown cells, Ang II could not induce the activation of NF-κB and AKT at all. To confirm whether phosphorylation of AKT could result in Ang II-induced activation of NF-κB signaling pathway, we also used LY294002 to specifically block AKT signal. We found that NF-κB could not be phosphorylated by Ang II after blocking AKT signaling pathway (Figure 3).

Ang II Could Induce the Exocytosis of Gal-3 Through AKT/NF-κB Signaling Pathway

AKT and NF-κB signals have been proved to relate the process of exocytosis[27,28]. To explore the role of NF-κB and AKT in Ang II-induced exocytosis of gal-3, we specifically blocked NF-κB or AKT signal by using Bay11-7082 or LY294002, and then we used Ang II to deal with the cells again. We found that Ang II could not increase the exocytosis of gal-3 again after blocking AKT or NF-κB signaling pathway (Figure 4).

Discussion

Ang II is a pivotal protein in RAS which could adjust the functions of vessels. Gal-3 could be secreted by some kinds of cancer cells into the serum and extracellular matrix and has been regarded as a fibrosis related protein[24,29]. The level of gal-3 in the serum was upregulated in the coronary heart disease patients and has been suggested as a diagnostic and prognostic biomarker in MI patients[30-32]. In this article, we found that Ang II increased the exocytosis of gal-3 depending on integrin αv/AKT/NF-κB axis.

AKT and NF-κB signal are the downstream signaling pathways of integrin αv. The serine/threonine kinase AKT affected the integrin-induced mechanotransduction, and mediated the shear stress-induced translocation of transient receptor potential vanilloid 4 (TRPV4)[33]. In the B cells, the knockdown integrin αv could affect the duration time and nuclear localization of NF-κB[34]. In our research, we also found that Ang II could increase the phosphorylation of AKT and NF-κB through integrin αv, and AKT is the upstream signaling pathway of NF-κB in VSMCs.

Integrin αv mediated the exocytosis of gal-3 induced by Ang II. AKT and NF-κB signaling pathways are related to the exocytosis. In the human umbilical vein endothelial cells, the hypoxia-induced vesicular release of ATP was abolished by LY294002[35]. AKT could mediate insulin-dependent glucose transporter 4 (GLUT4) exocytosis via tomosyn[28]. The NF-κB signaling pathway is related to the production of various cytokines and chemokines[36,37]. The NF-κB signal pathway mediated the endocytosis of epirubicin (EPI)-loaded folic acid-conjugated pullulan acetate (FPA/EPI) nanoparticles in the Kupffer cells (KCs)[38]. The functional NF-κB directly bound to the promoter of small GTPases Rab27A which regulated the autocrine and paracrine cytokines by monitoring the exocytosis of the extracellular vesicles[27]. In accordance with these researches, we also found that after specifically blocking AKT or NF-κB signal, Ang II could not effectively increase the secretion of gal-3 in VSMCs at all.

Conclusions

We showed that the integrin αv/AKT/NF-κB axis mediates Ang II-induced gal-3 exocytosis

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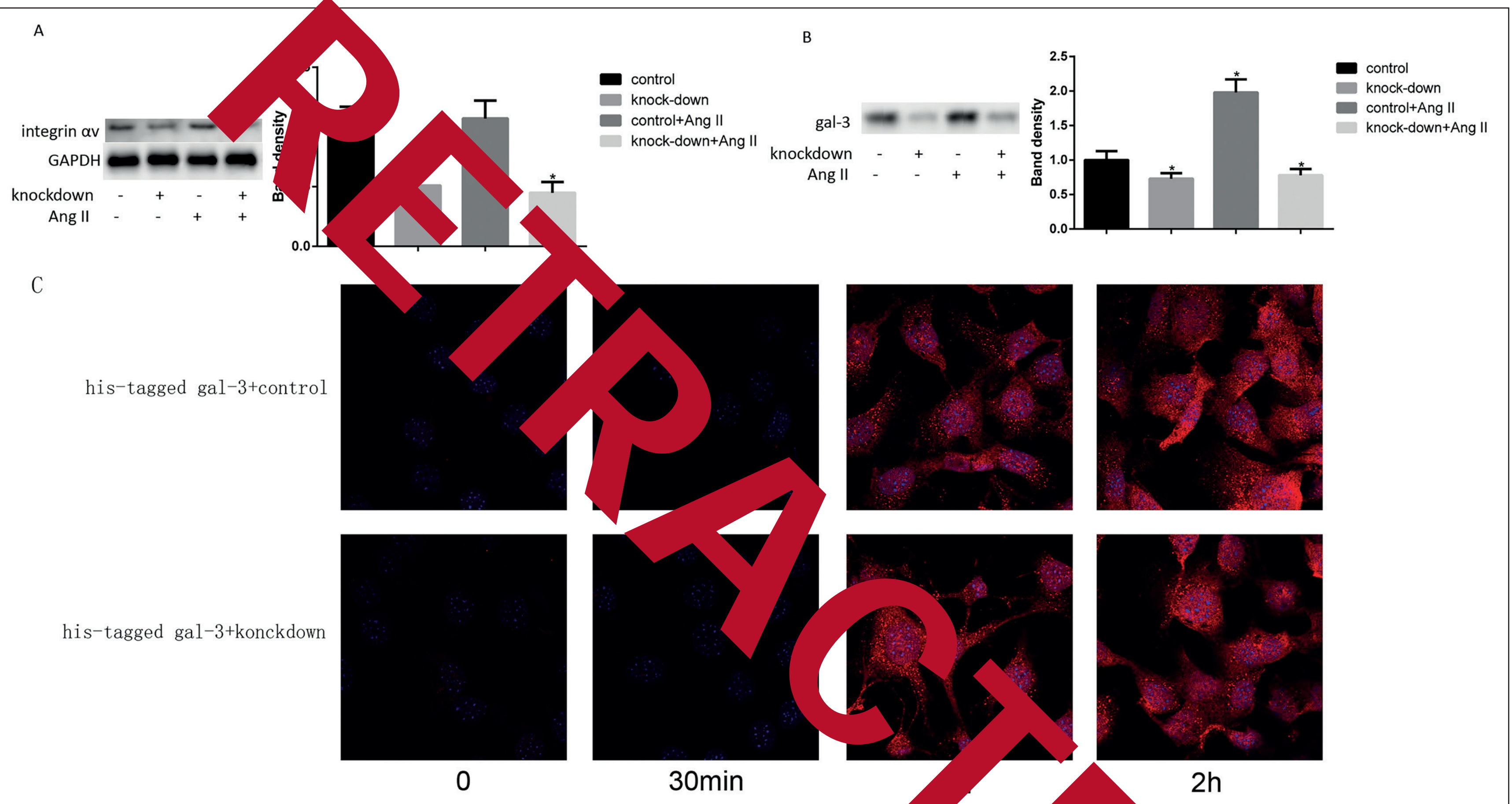

Figure 2. Integrin αv mediated the exocytosis of gal-3, but it has little effect on the endocytosis of gal-3. ***A,*** After tra[illegible] with ei[illegible] GFP (control) or cre (knock-down) for 48 h, VSMCs were incubated for 6 h in the absence or presence of Ang II (200 nM). The expression of integrin αv [illegible]MC[illegible]s analyzed by Western blot. The densitometric measurements of the replicate results are given in the right panel. The band density of the native VSMCs was c[illegible]n as a reference [illegible] relative expression and set to 1. *p<0.05 compared with any other concentrations. The data (mean±SEM) were obtained from three independent expe[illegible]nts. ***B,*** Afte[illegible] with either GFP (control) or cre (knock-down) for 48 h, the VSMCs were incubated for 6 h in the absence or presence of Ang II (200 nM), the culture s[illegible]rnata[illegible]VSMCs [illegible]e analyzed by Western blot. The densitometric measurements of the replicate results are given in the right panel. The band density of the native VSMCs were [illegible]n as a refere[illegible] for the relative expression and set to 1. *p<0.05 compared with any other concentrations. The data (mean±SEM) were obtained from three independent e[illegible]ments. ***C,*** Af[illegible]ansfection with either GFP (control) or cre (knock-down) for 48 h, the VSMCs were incubated with His-tagged gal-3 (10 µg/ml) for up to 2 h, the confocal [illegible]ges of VS[illegible] were used anti his-tag (red) antibodies, followed by the DAPI nuclear counter staining (blue) which are shown. The merged images containing all markers are a[illegible]gnification, × 630).

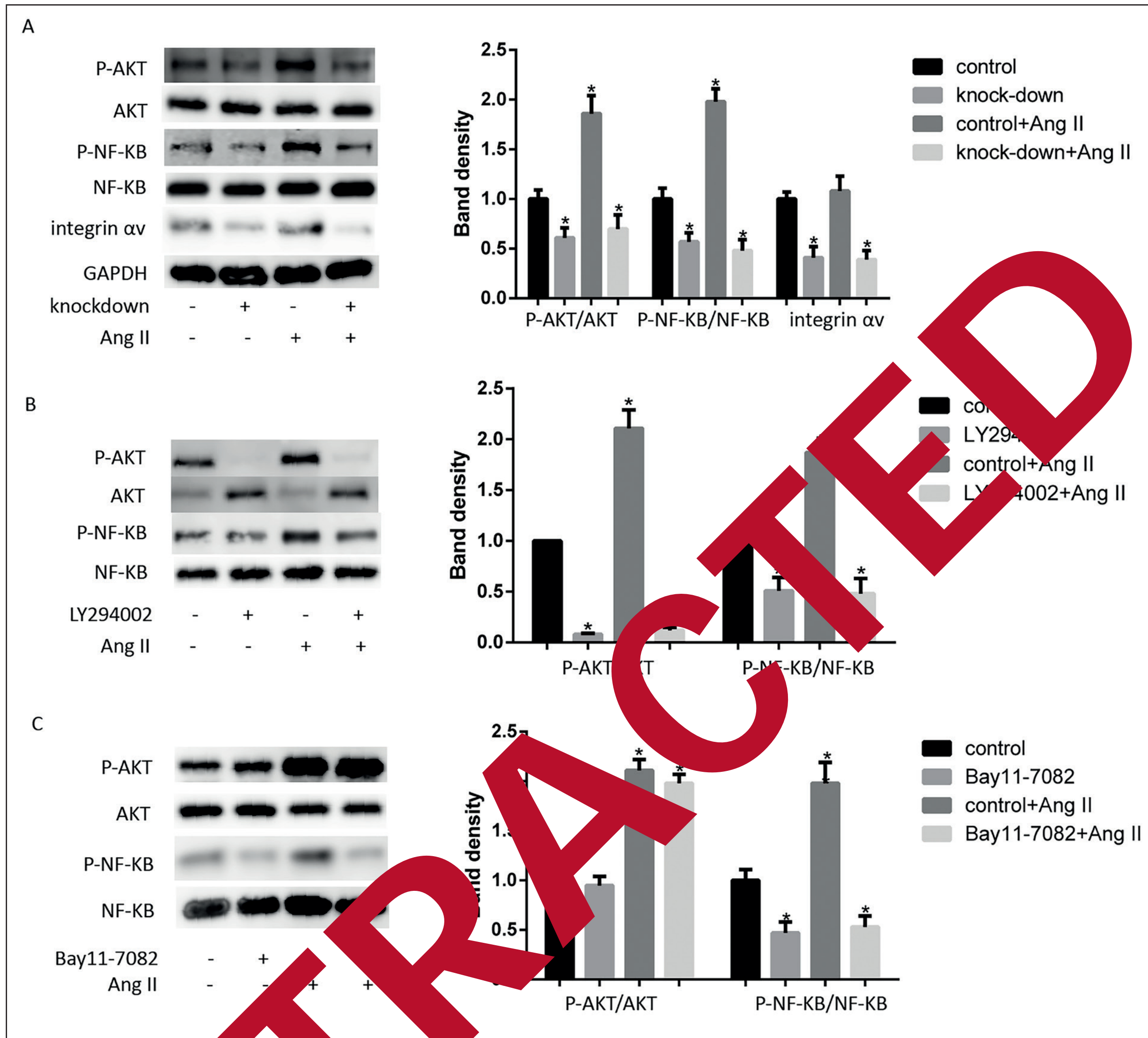

Figure 3. Integri[illegible]-mediated A[illegible]-induced the activation of AKT and NF-κB signaling pathways. ***A,*** After transfection with either GF[illegible] cre for 48 h, VS[illegible] were incubated in the absence or presence of Ang II (200 nM), the expression of p-AKT, AK[illegible] NF-κB, NF-KB and in[illegible]egrin αv were measured by Western blot. The GAPDH expression was used for the protein level [illegible]ali[illegible]on. ***B,*** After the addition of a specific AKT signaling pathway inhibitor (LY294002), the VSMCs were incubated [illegible] absence [illegible] presence of Ang II (200 nM), the expression of p-AKT, AKT, p-NF-κB and NF-κB were meas[illegible] Weste[illegible]t. T[illegible]ensitometric measurements of the replicate results are given in the right panel. The cells were tr[illegible]ecte[illegible]h GF[illegible] as a reference for relative expression and set to 1. *p<0.05 *vs.* any other treatments. The data [illegible]an±SE[illegible] were obta[illegible]d from three independent experiments. ***C,*** After the addition of a specific NF-κB inhibitor (Bay11-[illegible] VS[illegible] incubated in the absence or presence of Ang II (200 nM). The expression of p-AKT, AKT, p-NF-κB and NF[illegible]ed by the Western blot. The densitometric measurements of the replicate results are given in the right panel. [illegible] cells were transfected with GFP chosen as a reference for relative expression and set to 1. *p<0.05 *vs.* any other treatme[illegible]e data (mean±SEM) were obtained from three independent experiments.

in VSMCs. Ang II could induce the phosphorylation of AKT by integrin αv and then, the activation of the NF-κB signal and the activation of AKT and NF-κB signaling pathway could further affect the exocytosis of gal-3. As smooth muscle cells play an important role in a variety of plausible mechanisms of atherosclerosis, integrin αv/AKT/NF-κB axis may be used as a valuable target for future therapeutic strategies designed to inhibit the VSMCs activation.

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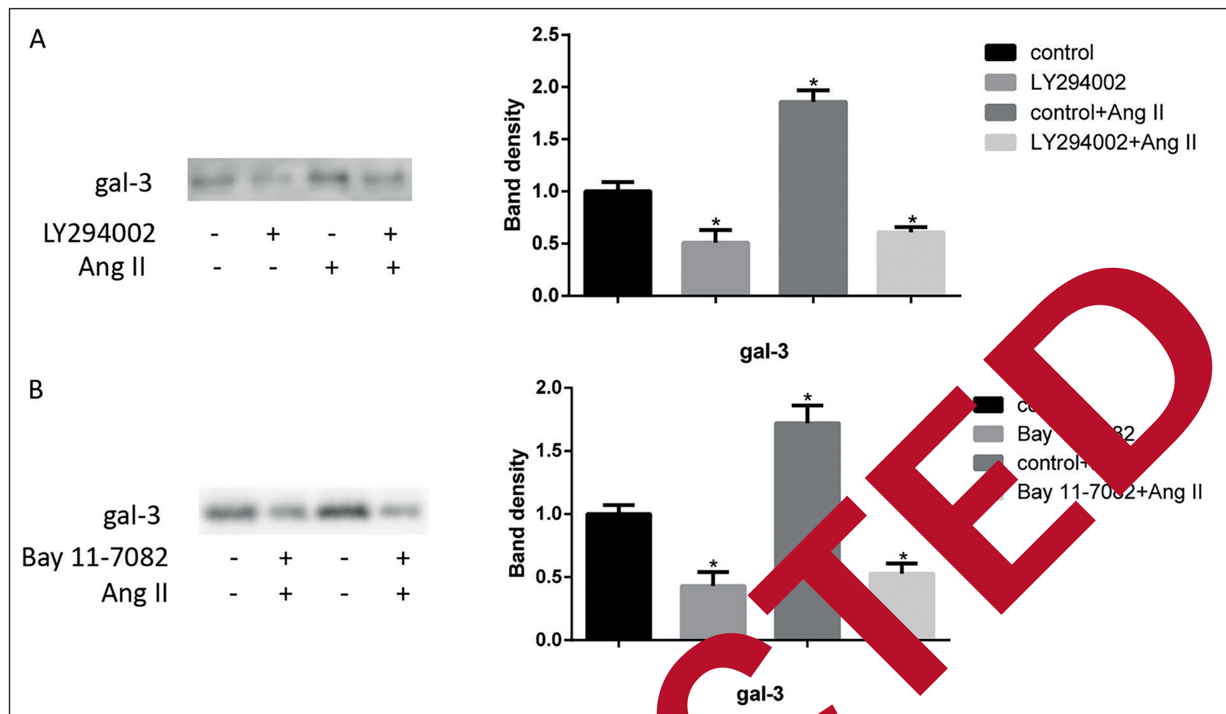

Figure 4. Ang II could induce the exocytosis of gal-3 through AKT/[illegible]κB signal[illegible] pathway. ***A,*** The cells were treated with LY294002 for 1 h; then, VSMCs were incubated in the [illegible]ence or pre[illegible] of [illegible] II (200 nM). The culture supernatants of VSMC were analyzed by Western blot. ***B,*** The cells w[illegible] with Bay[illegible]7082 for 1 h; then, VSMCs were incubated in the absence or presence of Ang II (200 nM). The culture s[illegible]nata[illegible]VSMC were analyzed by Western blot. The densitometric measurements of the replicate results are given in the [illegible]t pan[illegible]nd density of the native VSMCs were chosen as a reference for relative expression and set to 1. *p<0.05 [illegible]her treatments. The data (mean±SEM) were obtained from three independent experiments.

Conflict of Interest

The Authors declare that t[illegible]ave no con[illegible]f interests.

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