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ISSN: 0015-5659

e-ISSN: 1644-3284

Integrin subunit alpha 5 maintains mitochondrial function in ox-LDL-induced cardiac microvascular endothelial cells via activating the PI3K/AKT signaling pathway

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DOI: 10.5603/fm.95500

Article type: Original article

Submitted: 2023-05-10

Accepted: 2023-09-21

Published online: 2024-05-13

ORIGINAL ARTICLE

Integrin subunit alpha 5 maintains mitochondrial function in ox-LDL-induced cardiac microvascular endothelial cells *via* activating the PI3K/AKT signaling pathway

Xianfeng Wang et al., ITGA5 maintains mitochondrial function in CMECs

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ABSTRACT

Background: Cardiac microvascular endothelial cells (CMECs) assume a pivotal role in the regulation of blood flow, and their impairment precipitates a spectrum of pathological transformations. Our previous study unveiled a notable mitigation of CMECs dysfunction through the intervention of integrin subunit alpha 5 (ITGA5), a member of the integrin protein family. This study delves into the effect of ITGA5 on the mitochondrial function in CMECs and reveals the regulation pathway.

Materials and methods: CMECs were stimulated with oxidized low-density lipoprotein (ox-LDL) to mimic coronary artery disease (CAD). The effects of ITGA5 on diverse facets of CMEC behavior, encompassing viability, apoptosis, angiogenesis, oxidative stress, and

mitochondrial function, was systematically ascertained. Employing the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway as a focal point of investigation, the mediation of this pathway was substantiated utilizing the PI3K inhibitor LY294002.

Results: ITGA5 overexpression exerted a mitigating influence upon the ox-LDL-induced detriment to CMECs, manifested as increased viability, angiogenesis, mitochondrial function, and diminished apoptosis and oxidative stress. The counteraction of these salubrious effects by the administration of the PI3K inhibitor attests to the engagement of the PI3K/AKT signaling pathway.

Conclusions: This study has discerned that ITGA5 activates the PI3k/Akt signaling pathway to orchestrate mitochondrial function and diminish ox-LDL-induced CMEC dysfunction. Thus, the targeted amelioration of this cellular injury emerges as a strategically pivotal endeavor for the prevention and amelioration of this ailment.

Keywords: CAD, AS, integrin, CMEC, mitochondrial function

INTRODUCTION

Coronary artery disease (CAD) stands as a prevalent ailment characterized by the accumulation of lipids and immune cells within the endothelial space of coronary or atherosclerotic arteries [6]. Despite notable strides in pharmaceuticals and technology, CAD persists as the foremost global cause of mortality. It constitutes a multifaceted pathological process influenced by an amalgamation of factors, encompassing genetics, environment, and lifestyle [12]. Elevated levels of circulating low-density lipoprotein (LDL) stand out as a prominent risk factor for CAD, intricately linked to the progression of atherosclerosis [20]. The emergence of oxidized low-density lipoprotein (ox-LDL) stems from through the post-translational oxidative modification of LDL. As LDL traverses the intimal arterial layer, it becomes ensnared beneath the subintimal space. This accrual of LDL beneath the arterial endothelium signifies the primary step in the pathogenesis of atherosclerosis (AS) [15]. Autopsy studies have underscored that lesions bearing heightened deposits of ox-LDL correlate with an augmented risk of plaque rupture [9, 22]. In addition, multiple studies have

affirmed an association between elevated serum ox-LDL levels and the eventual development of cardiovascular disease, frequently heralding an unfavorable prognosis, thus meriting consideration as a clinical biomarker [25].

A pivotal determinant in the development of CAD lies in the impairment of cardiac microvascular endothelial cells (CMECs) [2], the lining of the inner surface of cardiac microvessels. Our preceding investigation has unearthed a notable amelioration of CMECs dysfunction through the action of integrin subunit alpha 5 (ITGA5). Specifically, ITGA5 curbs the inflammatory response induced by ox-LDL and augments angiogenic processes. As a member of the integrin protein family, ITGA5 establishes physical connections between cells and the extracellular matrix, serving as a cell adhesion receptor [18]. It has been reported that integrins are intricately linked to the genesis and progression of AS [3]. Within the context of CAD, the role of integrins in modulating vascular smooth muscle cell (VSMC) migration [4], endothelial cell activation, and leukocyte adhesion [5] has been meticulously explored. However, the realm of integrins within CAD remains an evolving domain, with much remaining to be gleaned regarding the specific functions of distinct integrins in the evolution and advancement of the disease.

Given the association between ox-LDL and mitochondrial calcium overload in human umbilical vein endothelial cells (HUVECs) [23], the present study perseveres in its pursuit to decipher the effect of ITGA5 on ox-LDL-induced mitochondrial function in CMECs, elucidating the intricate regulatory pathway underlying this phenomenon.

MATERIALS AND METHODS

Cell culture and treatment

CMECs (Procell, Wuhan, China) were digested with 0.25% trypsin, inoculated into T25 culture flasks at a ratio of 1:2 for passage, and then maintained in Dulbecco's modified eagle's medium (DMEM, Gibco) at 37 °C/5% CO_2. Medium was supplemented with 10% fetal bovine serum (Gibco), 30 μg/ml endothelial cell growth supplement (ScienCell), 1 U/mL heparin, and penicillin-streptomycin mixture (Gibco) as previously described [1]. To mimic CAD, CMECs were treated with ox-LDL (100 μg/mL, Yeasen, Shanghai, China) for

24 h. Phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (1 mM, CST) treated CMECs for 48 h.

Cell transfection

To achieve ITGA5 overexpression, by means of X-tremeGENE transfection reagent (Roche, Shanghai, China), pcDNA 3.1 plasmids carrying ITGA5 with CMV promoter (HanBio, Shanghai, China) were transfected to the CMECs. Empty plasmids without overexpression-promoting functions served as negative controls (oe-NC). Briefly, CMECs were plated one day before transfection, and when the confluence reached 70%, the mixture of plasmids and transfection reagent was supplemented to the wells. After 48 h at 37°C, transfection efficacy was assessed.

RT-qPCR

RT-qPCR was conducted as described elsewhere [1]. RNA from CMECs was reverse transcribed using Evo M-MLV RT Kit (Accurate, Changsha, China). QuantiTect SYBR Green PCR Kit (Qiagen, Shanghai, China) was used for qPCR. Relative mRNA levels were measured using the ΔΔCt method after normalization to actin. Primer sequences (5'-3') are as follows: for ITGA5, forward, GGCTTCAACTTAGACGCGGA, reverse, GGCCGGTAAAACTCCACTGA; for actin, forward, CTTCGCGGGCGACGAT, reverse, CCACATAGGAATCCTTCTGACC.

Western blotting

Western blotting was conducted as described elsewhere [19]. Proteins from CMECs were quantified using a Nano 3000 protein detector (YPH-Bio, Beijing, China). Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Roche). Membranes were incubated sequentially with skimmed milk, primary antibodies, and HRP-conjugated secondary antibody. Primary antibodies against ITGA5 (A6209), Bcl-2 (A0208), Bax (A12009), and GAPDH (A19056) were obtained from ABclonal. Primary antibodies against p-PI3K (AF3241), PI3K (AF6241), p-Akt (AF0016), Akt (AF6261) and secondary antibody (S0001) were obtained from Affinity Biosciences. Blots were semi-

quantified using ImageJ software after ECL reagent (Millipore) treatment.

Cell Counting Kit-8 (CCK8)

CMECs were plated in a 96-well plate and treated with ox-LDL for 24 h, CCK8 solution (Beyotime, Shanghai, China) was supplemented into each well. After 2 h, absorbance at 450 nm (A_{450}) was recorded using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometry

CMECs were digested and transferred to a tube, collected by centrifugation at 1000 g for 5 min, gently resuspended in PBS. The cell suspension was centrifuged at 1000 g for 5 min again, the supernatant was discarded, and CMECs were suspended in Annexin V FITC binding buffer. Annexin V FITC and propidium iodide (Elabscience, Wuhan, China) were supplemented and incubated with CMECs for 15 min in the dark. Apoptosis was analyzed using flow cytometry (BD FACSCanto, Franklin Lakes, NJ, USA) and FlowJo software.

Angiogenesis assay

Matrigel basement membrane matrix (BD Biosciences) was thawed in a refrigerator at 4°C one day in advance and then diluted 1:1 with cold medium. CMECs were starved in the medium containing 0.2% FBS. Matrigel was added to the pre-cooled cell plate, starved CMECs were seeded on solidified Matrigel and incubated at 37°C for 6 h. The structure of the capillary was observed using a microscope (Olympus).

Caspase 3 activity

Caspase 3 activity assay kit (Beyotime, Shanghai, China) was used for detection. CMECs were digested and collected by centrifugation at 600 g for 5 min, and then washed once with PBS. CMECs were lysed on ice for 15 min and the lysate was centrifuged at 10,000 × g for 15 min at 4°C to collect the supernatant. The sample was added to the buffer, followed by the supplement of Ac-DEVD-pNA (2 mM), and incubated at 37°C for 1 h. A_{405} was determined using a microplate reader.

Oxidative stress

Malondialdehyde (MDA), ROS, SOD, and catalase (CAT) were regarded as indices. The detection was conducted with specific assay kits (Nanjing JianCheng Bioengineering Institute). Briefly, CMECs were lysed on ice for 15 min and the lysate was centrifuged at 10,000 × g for 15 min at 4°C to collect the supernatant. The working solution was configured according to the instructions and added to the samples. Standards were serially diluted with distilled water to create a standard curve. The absorbance was measured by a microplate reader and the content or activity value was calculated according to the standard curve.

Mitochondrial membrane potential

Mitochondrial membrane potential was analyzed using a JC-1 detection kit (Yeasen, Shanghai, China). JC-1 (200×) was diluted with ultrapure water, fully dissolved and supplemented with staining buffer (5×), mixed well and used as JC-1 staining working solution. Cell suspension were incubated with JC-1 staining working solution at 37°C in the dark for 20 min. Upon centrifugation at 600 × g for 3 min, the CMECs were washed twice with staining buffer (1×) and photographed under a fluorescent microscope (Leica).

ATP content

CMEC lysate was centrifuged at 10,000 x g for 15 min at 4°C to collect the supernatant. ATP level was measured with the ATP detection kit (mlbio, Shanghai, China). The ATP working solution was configured according to the instructions and added to the wells and left at room temperature for 5 min, after which the samples or standard was added. A_{636} was recorded using a microplate reader.

Mitochondrial DNA (mtDNA) level

The relative copy number of mtDNA was quantified using nuclear DNA content as a standard. Human mtDNA Copy Number Assay Kit (Takara) was used for RT-PCR. DNA was separated from CMECs using a DNA isolation kit (Vazyme, Nanjing, China) and 20 μL of reaction mixture per PCR tube containing 1 μL forward primer, 1 μL reverse primer, 8 μL

sample, and 10 μL reaction mix was prepared on ice. Following the reaction was completed, the mtDNA copy number was calculated according to the $2^{\Delta Ct}$ method.

Statistical analysis

Statistical differences (mean ± SD, n ≥ 3) between groups were analyzed using unpaired Student's t-test (two groups), and one-way ANOVA followed by Tukey's *post hoc* test (multiple groups) with SPSS 19.0. $P < 0.05$ indicates significant difference.

RESULTS

Impacts of ITGA5 on CMEC viability and apoptosis

ITGA5 mRNA and protein in CMECs decreased significantly under ox-LDL stimulation (Fig. 1A, B). To study its role in pathological conditions, cells were transfected to promote high expression of ITGA5. The efficiency of transfection was verified by RT-qPCR and western blotting experiments (Fig. 1C, D). CMECs highly expressing ITGA5 showed a significant increase in intracellular ITGA5 after ox-LDL treatment compared with the oe-NC group (Fig. 1E, F). The effect of ITGA5 overexpression on cell survival was achieved by assessing its viability and apoptosis. CCK8 experiments showed that ox-LDL stimulation significantly reduced cell viability, while ITGA5 overexpressed cells were less affected (Fig. 1G). The results of flow cytometry showed that the apoptosis rate was significantly increased after ox-LDL treatment, and this increase was not so prominent in the group of ITGA5 overexpressed CMECs (Figure 1H). Caspase3 activity (Fig. 1I) and Bax protein content displayed the same trend, specifically the increase induced by ox-LDL and the restraint upon ITGA5 overexpression, whereas the trend of anti-apoptotic protein Bcl-2 was opposite (Fig. 1J).

Impacts of ITGA5 on CMEC dysfunction and oxidative stress

Ox-LDL also inhibited the angiogenesis ability of CMECs, which was relieved in ITGA5 overexpressing cells (Fig. 2A). Intracellular MDA and ROS significantly increased, SOD and CAT decreased significantly under ox-LDL stimulation, and ITGA5 overexpression could alleviate their surge or decrease (Fig. 2B). JC-1 fluorescent probe was used to indicate the

mitochondrial membrane potential. Compared with the ox-LDL + oe-NC group, Overexpression of ITGA5 reduced JC-1 monomer aggregation and promoted the increase of aggregates, reversing the effect of ox-LDL on membrane potential reduction to a certain extent (Fig. 2C). ATP content (Fig. 2D) and mitochondrial DNA (Fig. 2E) decreased after ox-LDL treatment and was alleviated under the influence of ITGA5 overexpression.

Mediation of PI3K/Akt signaling in regulating CMEC viability and apoptosis

Western blot results showed that ox-LDL inhibited the phosphorylation of PI3K and Akt, while ITGA5 could promote their activation (Fig. 3A). To explore the potential regulatory mechanism of this signaling in ITGA5, the PI3K inhibitor LY294002 was used to treat the CMECs. Compared with the ox-LDL + oe-ITGA5 group, PI3K inhibitor decreased cell viability (Fig. 3B). In addition, PI3K inhibitors also increased the rate of apoptosis (Fig. 3C), increased caspase3 activity (Fig. 3D) and Bax content, and decreased Bcl-2 content (Fig. 3E).

Mediation of PI3K/Akt signaling in regulating CMEC dysfunction and oxidative stress

The effect of the PI3K inhibitor LY294002 on CMEC dysfunction and oxidative stress was next evaluated. Compared with ox-LDL + oe-ITGA5, LY294002 reduced cellular angiogenesis ability (Fig. 4A). In addition, LY294002 also increased the content of MDA and ROS, and decreased the content of SOD and CAT (Fig. 4B). JC-1 aggregates were reduced by LY294002, accompanied by an increase in the monomeric form, indicating that inhibition of the PI3K pathway reduces mitochondrial membrane potential (Fig. 4C). Cellular ATP content (Fig. 4D) and mitochondrial DNA (Fig. 4E) were significantly reduced under LY294002 treatment, reversing the protection of mitochondrial function by ITGA5 overexpression.

DISCUSSION

CAD stands as a substantial contributor to global morbidity and mortality [10, 17]. Central to this cardiovascular landscape, CMECs play a pivotal role in the orchestration of myocardial blood flow regulation. Yet, when these cells are compromised, a cascade of pathological alterations unfolds, encompassing diminished nitric oxide production, heightened oxidative stress, and compromised inflammatory and angiogenic responses [24]. These intricate shifts

lay the foundation for AS, the primary etiology underpinning CAD. Herein, ITGA5 overexpression equipped CMECs with the capacity to fend off ox-LDL-induced apoptosis and concomitantly ameliorated angiogenesis reduction. Impaired angiogenesis is a salient mechanism underpinning CAD progression [14].

Integrins, comprising α/β heterodimeric cell surface receptors, serve as critical conduits in cellular dialogue with the extracellular matrix. Their role transcends signal transduction from the extracellular milieu to the cell interior; they also act as discerning interpreters of intracellular cues [8]. Consequently, integrin signaling is implicated across the spectrum of atherosclerotic evolution, ranging from the inflammatory initiation to the culmination in fibrotic plaque formation. Balance between integrins with different functions governs the entrapment or movement of lipoproteins and growth factors within the arterial wall matrix. If a buildup forms, it culminates in arterial wall thickening—a hallmark of atherosclerosis [16]. ITGA5 mediates such dynamic regulation, which is critical for maintaining CMEC function.

Further, ITGA5 overexpression was ascertained to exert a modulating effect on ox-LDL-induced oxidative stress within CMECs. The acknowledged contribution of oxidative stress to CAD is underscored by the propensity of ROS, notably originating from the mitochondrial respiratory chain [13], to exact damage upon cellular components, including DNA, proteins, and lipids. Moreover, these ROS serve as instigators of signaling cascades that converge on inflammatory processes and cellular dysfunction.

The follow-up investigations corroborated the centrality of the PI3K/AKT signaling axis in the ITGA5-mediated regulation of CMECs. We consider that ITGA5 influences intracellular signaling and alters cell behaviors through the process of “outside-in” signal transmission. Noteworthy instances within diverse systems fortify the PI3K/AKT signaling pathway. Inhibition of p-Akt promotes apoptosis in arterial endothelial cells in a high-fat diet-induced mouse model [7]. PI3K/Akt/mTOR pathway activation safeguards mitochondrial function and inhibits apoptosis in SH-SY5Y cells upon rotenone exposure [11]. The augmentation of ITGA5 expression through kaempferol confers mitigation of oxidative stress and enhancement of mitochondrial function [21]. Collectively, these studies all illustrate the pervasive participation of the PI3K/AKT signaling pathway in mitigating oxidative stress

across various systems, accentuating its salience.

CONCLUSIONS

Overall, this study delineates the capacity of ITGA5 to galvanize the PI3k/Akt signaling pathway, thereby orchestrating the preservation of mitochondrial function and ameliorating the ox-LDL-induced dysfunction of CMECs. In light of the pivotal role played by CMEC injury in CAD development, the strategic targeting of this perturbation emerges as a linchpin strategy in the realm of CAD prevention and treatment. Moreover, interventions aimed at abating oxidative stress, quelling inflammation, and fostering angiogenesis stand poised to fortify the defense of CMECs, ultimately curtailing the risk of CAD.

Article information and declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Authors' contributions

X.W., W.M., and X.M. contributed to the study concept, experiments and analysis. X.W. drafted the manuscript. All authors approve the final version of the manuscript.

Funding

No funding was received.

Conflict of interest: The authors declare that they have no competing interests.

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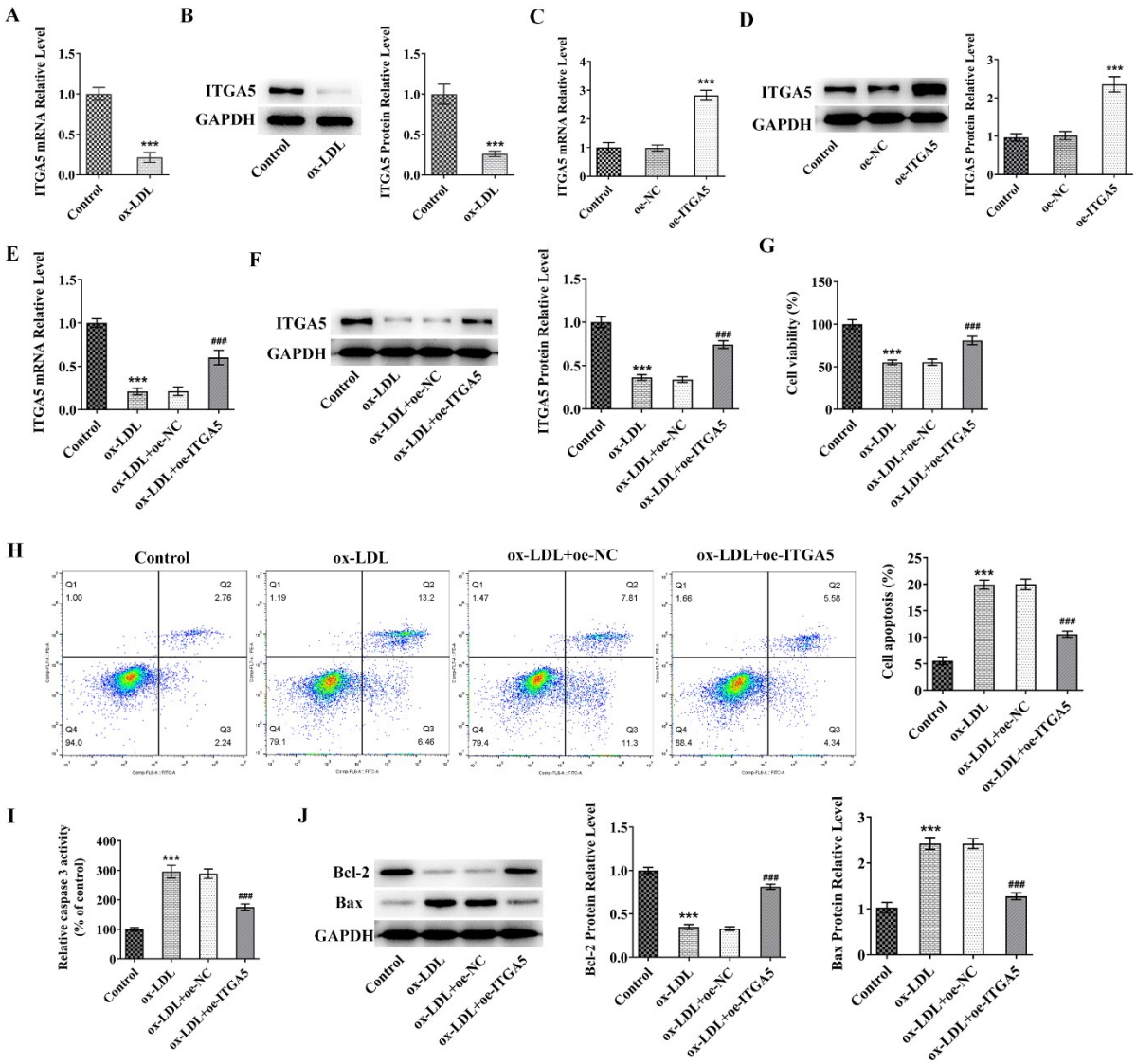

Figure 1. Impacts of ITGA5 on CMEC viability and apoptosis. **A, B.** ITGA5 mRNA and

protein in CMECs upon ox-LDL stimulation. **C, D.** CMECs were transfected to promote high expression of ITGA5. The efficiency of transfection was verified by RT-qPCR and western blotting experiments. **E, F.** ITGA5 mRNA and protein in transfected CMECs with ox-LDL treatment. **G.** CCK8 assay indicated the effect of ox-LDL stimulation and ITGA5 overexpression on cell viability. **H.** Flow cytometry, **I.** Caspase3 activity and **J.** Bax and Bcl-2 protein content indicated the effect of ox-LDL stimulation and ITGA5 overexpression on the apoptosis. $^{***}p < 0.001$ vs control or oe-NC; $^{\#\#\#}p < 0.001$ vs ox-LDL + oe-NC.

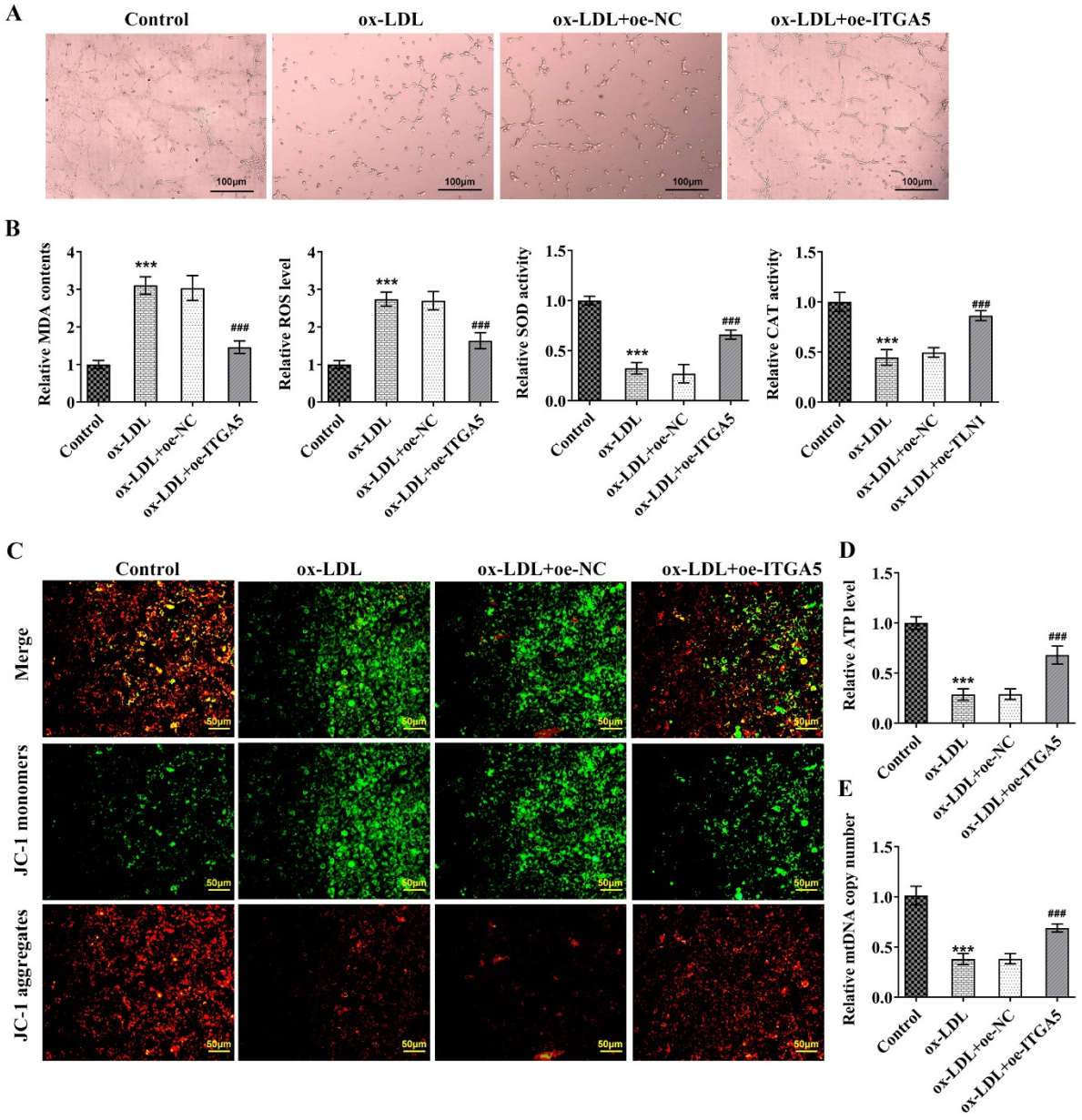

Figure 2. Impacts of ITGA5 on CMEC dysfunction and oxidative stress. **A.** the angiogenesis

ability of CMECs upon ox-LDL stimulation and ITGA5 overexpression. **B.** Intracellular MDA, ROS, SOD and CAT upon ox-LDL stimulation and ITGA5 overexpression. **C.** Mitochondrial membrane potential upon ox-LDL stimulation and ITGA5 overexpression using JC-1 fluorescent probe. **D.** ATP content upon ox-LDL stimulation and ITGA5 overexpression. **E.** mitochondrial DNA upon ox-LDL stimulation and ITGA5 overexpression. $^{***}p < 0.001$ vs control; $^{\#\#\#}p < 0.001$ vs ox-LDL + oe-NC.

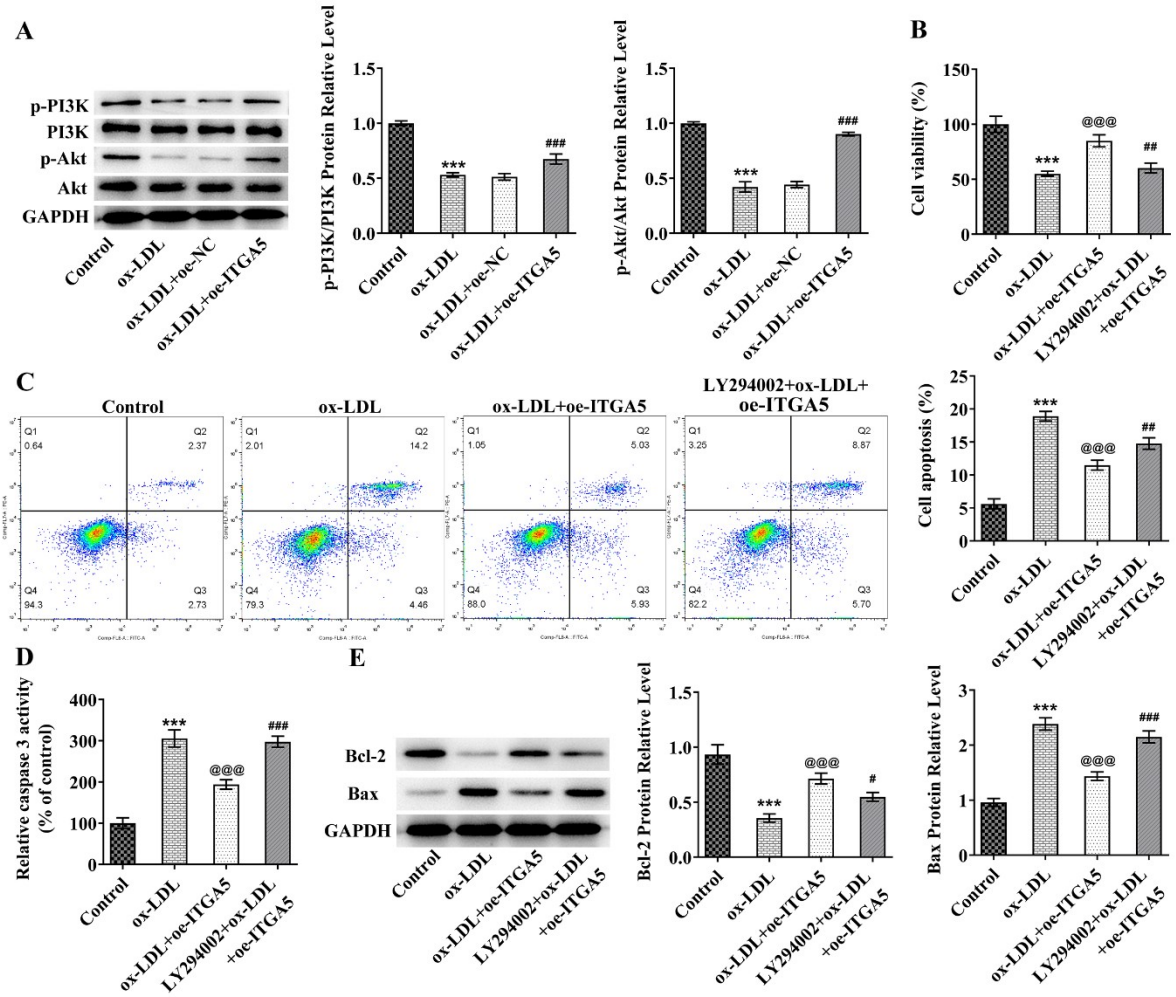

Figure 3. Mediation of PI3K/Akt signaling in regulating CMEC viability and apoptosis. **A.** Western blot results showed the effect of ox-LDL and ITGA5 overexpression on the phosphorylation of PI3K and Akt. **B.** the PI3K inhibitor LY294002 was used to treat the CMECs. Its influences on (**B**) cell viability, (**C**) the rate of apoptosis, (**D**) caspase3 activity and (**E**) Bax and Bcl-2 content were determined. $^{***}p < 0.001$ vs control; $^{@@@}p < 0.001$ vs ox-

LDL + oe-NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs ox-LDL + oe-ITGA5.

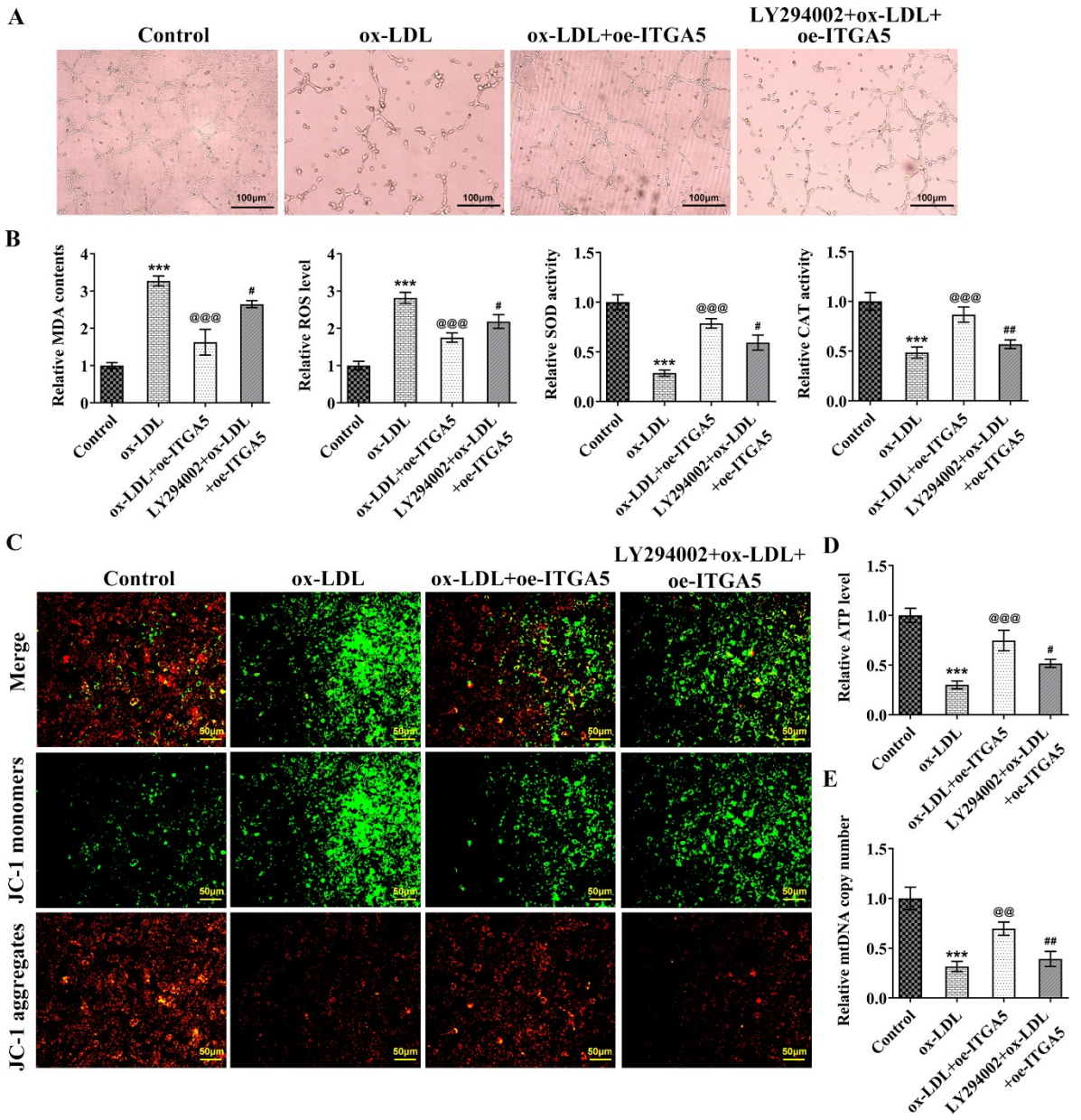

Figure 4. Mediation of PI3K/Akt signaling in regulating CMEC dysfunction and oxidative stress. **A.** The effect of the PI3K inhibitor LY294002 on CMEC angiogenesis ability **B.** The content of MDA, ROS, SOD and CAT. **C.** Mitochondrial membrane potential with JC-1 probe, (**D**) ATP content and (**E**) mitochondrial DNA were determined. $^{***}p < 0.001$ vs control; $^{@@}p < 0.01$, $^{@@@}p < 0.001$ vs ox-LDL + oe-NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs ox-LDL + oe-ITGA5.