Int J Clin Exp Pathol 2017;10(4):4191-4197
www.ijcep.com /ISSN:1936-2625/IJCEP0046643

Original Article

CXCR4 was involved in andrographolide inhibits atherosclerosis by inactivating the PI3K/AKT/NF-κB signaling pathway

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Received December 14, 2016; Accepted January 20, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Atherosclerosis is one of the major diseases that seriously impacts human health, which has been conceived as a complex process. In this study, we sought to explore the role and the underlying mechanism of Andrographolide (Andro) in Atherosclerosis progress. The CCK8 and flow cytometry were respectively used to explore the effect of Andro on cell proliferation and apoptosis. Then, we detected the effect of Andro on inflammatory factors. Next, the connection of Andro and CXCR4 and the underlying mechanism was researched. Our study proved that Andro could promotes cell proliferation and inhibits cell apoptosis. And, Andro significantly inhibits the expression of TNF-α, IL-6, endothelian 1 (ET-1), MMP-9 and malondialdehyde (MDA). Further, we have also confirmed that CXCR4 was involved in the Andro regulation progress in atherosclerosis inhibition by inactivating the PI3K/AKT/NF-κB signaling pathway. All these findings suggest that Andro inhibits atherosclerosis and may be involved in progression of atherosclerosis and could be a new therapeutic target for this disease.

Keywords: Andro, CXCR4, atherosclerosis, PI3K/AKT/NF-κB, inflammation

Introduction

Atherosclerosis, a leading cause of morbidity and mortality, results from chronic vascular inflammation which is a complex process driven by many cell types such as vessel cells, immune cells and endothelial progenitor cells [1-3]. The feature of atherosclerotic lesion is intimal fibrofatty plaques, which involves in a complex integration of cholesterol penetration, inflammatory cell infiltration, vascular smooth muscle cell (VSMC) migration, and neovascular invasion [4-6].

Inflammation is an important process in atherosclerosis, leading to plaque rupture and acute coronary syndrome [7, 8]. Previous study demonstrated that IL-35 improved immune suppression in atherosclerotic mice, thus suggesting IL-35 a novel therapeutic target for atherosclerosis [8].

Moreover, previous study showed that the atherosclerosis causes progressive deterioration of affected vessels and remains the leading cause of heart disease worldwide, despite the advances in contemporary therapies, the molecular mechanisms underlying the pathophysiology and effective therapeutic strategies of atherogenesis are not sufficient [3, 9].

Andro, a natural bicyclic diterpenoid lactone of andrographis paniculata, has been found to exert anti-inflammatory properties by regulated the expression of inflammatory factors such as TNF-α, IL-6 [10, 11]. Moreover, it has been reported to exhibit potent anti-inflammatory properties through inhibition of inflammatory cell infiltration via supression of NF-κB in several diseases [12, 13].

Oxidized low density lipoprotein (oxLDL) induced the atherosclerosis by triggering the endothelial cell damage and accelerate the lipid accumulation and proinflammatory response [14, 15]. Meantime, Atorvastatin has been demonstrated to delay the procession of atherosclerosis

and improve the stability of atherosclerotic plaques [16]. In the present study, we adopted different ways to contrast how Andro control the atherosclerosis process by inhibiting the inflammatory response. Atherosclerosis coculture of endothelial cells (EC) and smooth muscle of culture cells (SMC) is a frequently used atherosclerosis model, so we choose this cocultivation method to explore the role of CXCR4 in the regulation of PI3K/AKT/NF-κB of atherosclerosis. In detail, CCK8 and flow cytometry assay were applied to test the role of Andro on cell viability and apoptosis. Then, we further researched the effect of Andro on inflammatory factors and the connection of Andro and CXCR4. We found Andro promotes cell proliferation and inhibits cell apoptosis, additionally, it regulates CXCR4 and PI3K/AKT/NF-κB signaling pathway in anti-inflammatory progress.

All of our efforts will provide theoretical basis and new insights into the treatment of atherosclerosis.

Materials and methods

EC monocultures and preparation of EC-SMC-MC co-cultures

Human umbilical vein smooth muscle cells and human umbilical artery endothelial cells were incubated separately with smooth muscle conditioned medium and enriched culture medium, containing 5 (v/v) fetal calf serum at 37°C in a 5% CO_2 and 95% air-humidified atmosphere. THP-1 cells were cultured in 90% RPMI-1640, at 37°C and 5% CO_2. 1×10^5 THP-1 cells/well were added to the upper chamber of the insert and incubated for 30 min at 37°C and 5% CO_2. To prepare an EC-SMC co-culture for use as a model of the arterial wall, Millicell insert units were placed in sterile tissue culture dishes and inverted, so that the outside of the membrane faced upward. The SMCs were seeded onto this outer membrane surface at a density of 1×10^5 cells/cm^2. The SMCs became adherent following 6 h of culture, and the insert units were placed in a 24-well culture plate in an upright position at 37°C and 5% CO_2. The ECs were plated onto the inner surface of the membrane at a density of 1×10^5 cells/well. These dishes were co-incubated for different durations at 37°C and 5% CO_2 to form an EC-SMC co-culture system, the EC-SMCs were co-cultured for 3-6 days. In addition, monocultures of ECs or SMCs were also prepared using the same method as the control. The supernatants from the upper and lower chambers of the insert were obtained, and the phosphatidylethanolamines (PE) membrane was cut to detect the markers and determine the EC-SMC co-culture condition. The EC-SMC were co-cultured for 6 days and incubated with 100 μg/ml oxLDL for 4 h at 37°C and 5% CO_2. The MCs were subsequently added into the upper chamber of the insert at a density of 1×10^5 cells/cm^2, and the mixture was co-cultured for another 20 h. The EC-SMCs were co-cultured for 6 days and the MCs were directly added to continue the co-culture for 20 h at 37°C and 5% CO_2, which was used as a control to determine the inducer and sensitive marker of the inflammatory reaction in this model of atherosclerosis.

CCK8 cell assay

The EC-SMCs were co-cultured for 6 days and incubated with 100 μg/ml oxLDL, 10 μmol/L atorvastatin and 10 μmol/L andrographolide, for 4 h at 37°C and 5% CO_2. To determine the effect of the drug on the inflammatory reaction in atherosclerosis, the MCs were subsequently added and co-cultured for another 20 h at 37°C and 5% CO_2. CCK8 reagent (0.5 mg/ml) was then added to the cell culture medium and incubated at 37°C for 1 h. The absorbance was evaluated at 450 nm using a microplate reader.

Apoptosis assay

Cell apoptosis were examined by flow cytometry. For the cell cycle assay, the transfected cells were collected and fixed in 70% ethanol at 4°C for 16 h and then stained with propidium iodide (PI, sigma, usA) at 4°C for 30 min in the dark. Cell apoptosis assay was performed by using phycoerythrin (Pe)-Annexin V apoptosis detection kit. The apoptotic rate and cycle distribution were measured by using a FACsCalibur flow cytometer.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-6, endothelian 1 (ET-1), MMP-2, and malondialdehyde (MDA) were determined using a commercially available ELISA kit. The co-culture incubation media were collected following the removal of floating cells through centrifugation for 5 min at 4°C, using

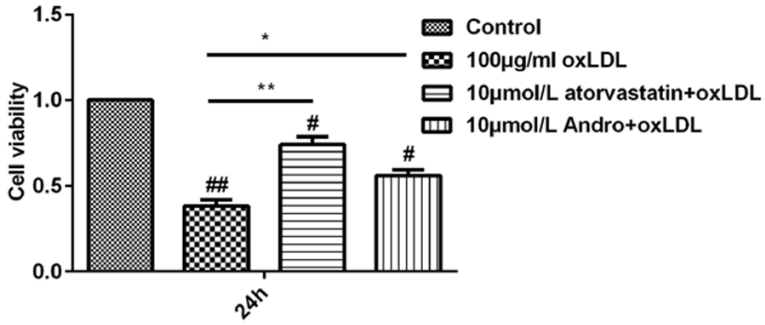

Figure 1. Effects of oxLDL, atorvastatin and Andro on cell viability of EC-SMC-MC. #Means significance compared with the control group, different drugs joining can make significant changes in the cell survival rate. *Means obvious difference compared with oxLDL group.

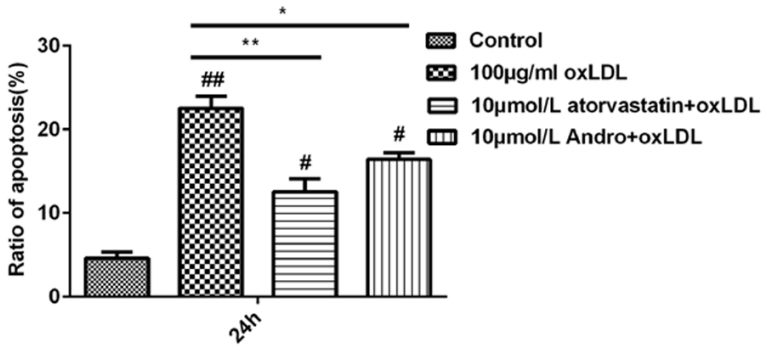

Figure 2. Effects of oxLDL, atorvastatin and Andro on cell apoptosis of EC-SMC-MC. #Means significance compared with the control group, different drugs joining can make significant changes in the cell apoptosis rate. *Means obvious difference compared with oxLDL group.

an Eppendorf 5424R centrifuge (Eppendorf, Hamburg, Gemany). The absorbance was measured at 450 nm using a multiplate spectrophotometer.

CXCR4 siRNA

Specific siRNA was used for CXCR4 knockdown in cells, and stable transfectants at the gene and protein levels selected using Lipofectamine 2000. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of total RNA was performed using a SYBR Premix Ex Taq II Reagent Kit and gDNA Eraser reverse transcriptase. Cells treated with non-silencing scrambled siRNA and transfection reagent were used as controls.

Realtime-PCR

Total RNA was extracted from cultured cells and used to synthesize cDNA using a SYBR Premix Ex Taq II Reagent Kit and gDNA Eraser reverse transcriptase (Takara, Japan). For polymerase chain reaction (PCR) analysis, samples were normalized to β-actin expression by calculating ΔCT (CT target gene-CT actin).

Western blot

Hippocampal neuron cells were collected, and lysed for 30 min in lysis buffer containing 1 mM phenylmethanesulfonyl fluoride. The cells were then centrifuged at 12000 rpm for 10 min at 4°C. Protein concentrations in the cell lysates were determined by the BCA protein assay. Equal amounts of cell lysates were separated by 10% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes and blocked with 1% bovine serum albumin with 0.05% Tween 20 in PBS for 30 min, followed by incubation with Santibodies, and with β-actin as a control, at 4°C overnight. After three washes with TBST, the membranes were incubated with alkaline phosphatase-goat anti-rabbit IgG or alkaline phosphatase-rabbit anti-goat IgG for 1 h. Then, we detected the immunoreactive signals by using Western Blue stabilized substrate alkaline phosphatase. The air-dried membranes were imaged using an image analyzer. Band intensities were measured using the Image J v1.50 software.

Statistic analysis

Results are presented as mean ± SEM. Differences among groups were analyzed using repeated measures one-way ANOVA comparison tests. A value of $P < 0.05$ was considered to be significant.

Results

Andro promotes cell proliferation

We tested the cell viability of EC-SMC-MC under different conditions, with oxLDL treatment as

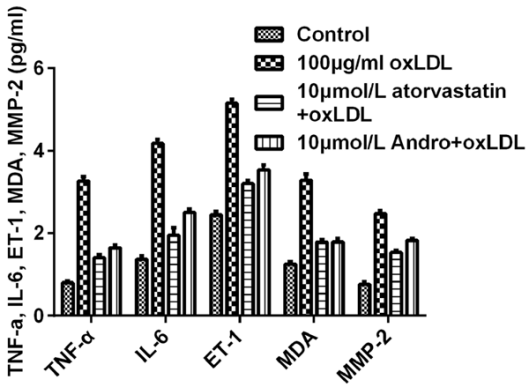

Figure 3. Effects of oxLDL, atorvastatin and Andro on inflammatory factors of EC-SMC-MC. #Means significance compared with the control group, different drugs joining can make significant changes in the cell inflammatory factors expression. *Means obvious difference compared with oxLDL group.

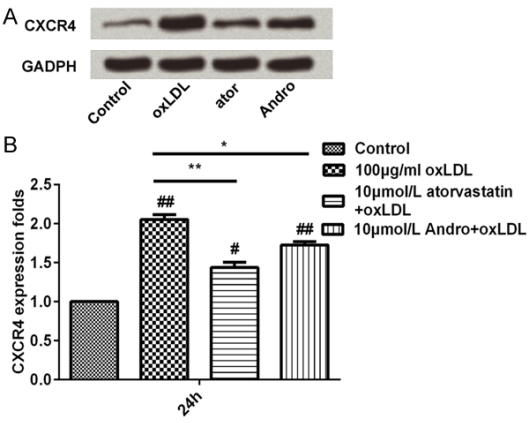

Figure 4. Expression level of CXCR4 after drugs interfere. #Means significance compared with the control group, different drugs joining can make significant changes in the CXCR4 expression. *Means obvious difference compared with oxLDL group.

the model group, atorvastatin and Andro as the treated groups. The results were shown in **Figure 1**. We can read from the cell viability results that oxLDL reduced the cell survival rate obviously, while atorvastatin and Andro can obviously promote the cell survival rate.

Andro inhibits cell apoptosis

Next, we tested the cell apoptosis of EC-SMC-MC under different conditions, with oxLDL treatment as the model group, atorvastatin and Andro as the treated groups. The results were shown in **Figure 2**. The results demonstrated that oxLDL induced the cell apoptosis obviously, while atorvastatin and Andro can obviously inhibit the cell apoptosis rate.

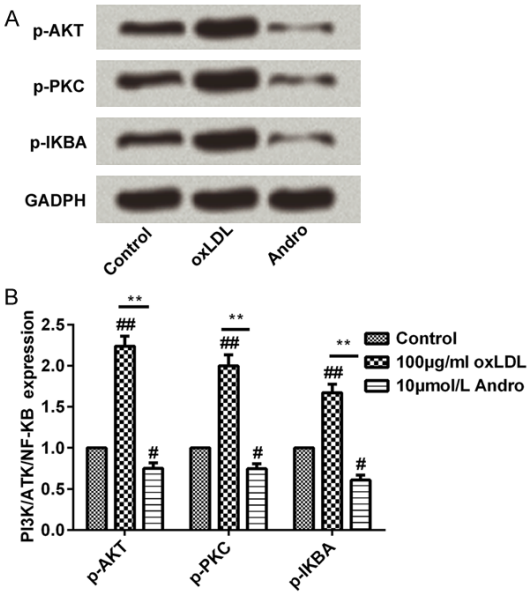

Figure 5. Effects of Andro on phosphorylation protein expression of PI3K/AKT/NF-κB singaling pathway. #Means significance compared with the control group. *Means obvious difference compared with oxLDL group.

Andro downregulates inflammatory factors expression by ELISA assay

Then, in order to detect the expression of inflammatory factors under different conditions, we used ELISA to detect the expression of TNF-α, IL-6, endothelian 1 (ET-1), MMP-9 and malondialdehyde (MDA). As described before, oxLDL served as the model group, atorvastatin and Andro as the treated groups. The results were shown in **Figure 3**. The results demonstrated that oxLDL promotes the expression of inflammatory factors and induced inflammatory, while atorvastatin and Andro can obviously inhibit the inflammatory factors expression. So, we can confirm that Andro can protect cell from inflammatory response.

Andro downregulates CXCR4 expression

Real time-PCR and western blot assays were used to detect the expression of CXCR4 in different conditions. oxLDL served as the model group, atorvastatin and Andro as the treated group as described above. The results were shown in **Figure 4**. From this part of results, we

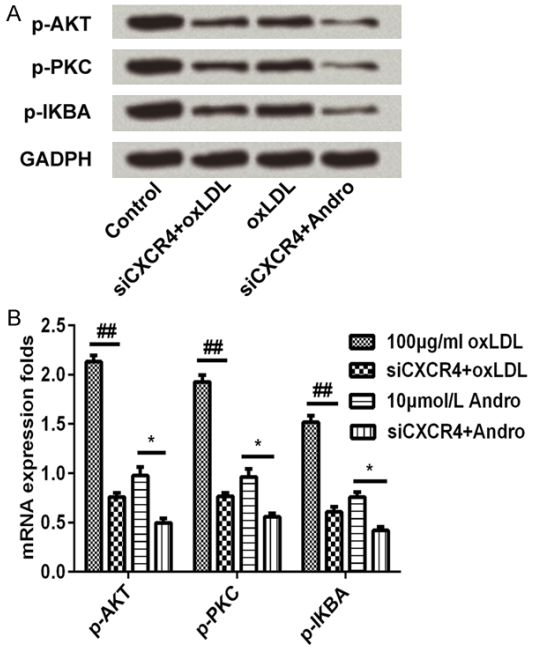

Figure 6. Effects of CXCR4 on PI3K/AKT/NF-κB signaling pathway. #Means significance compared with the oxLDL group. *Means obvious difference compared with Andro group.

can get the conclusion that oxLDL promotes the expression of CXCR4, while atorvastatin and Andro can obviously inhibit the CXCR4 expression.

Andro inhibits PI3K/AKT/NF-κB signaling pathway

The results above verified andro plays a protective role, the following test will investigate the related pathways of CXCR4 regulation. In this part of research, realtime-PCR and western blot were applied to detect the related protein expression in PI3K/AKT/NF-κB singaling pathway, with the oxLDL as positive control, Andro as treatment group. The results shown in **Figure 5** tell that Andro can inhibit the PI3K/AKT/NF-κB singaling pathway.

Silence of CXCR4 inhibits PI3K/AKT/NF-κB signaling pathway

In order to detect the role of CXCR4 in regulating PI3K/AKT/NF-κB pathways, the PI3K/AKT/NF-κB related genes expression was investigated after the CXCR4 expression was inhibited, with the oxLDL as positive control, Andro as treatment group. The results shown in **Figure 6** tell that CXCR4 can regulate the pathway proteins expression, reducing the occurrence of apoptosis pathway.

Discussion

Atherosclerosis, a chronic and progressive disease, is a leading cause of heart attacks, strokes, peripheral vascular disease, endothelial dysfunction, diabetes mellitus, hypertension, and hypercholesterolemia, which is predicted to be the primary cause of death in the world by 2020 [17, 18]. As a chronic inflammatory disease of the arterial wall, the mechanism and clinical study of atherosclerosis research before has got some achievement and make great progress, but for the cure and therapy of atherosclerosis, it is far from sufficient [19, 20].

Andro, a kind of diterpenoid isolated from various plants of the genus Andrographis [21]. Andro has been reported many kinds of properties, such as anti-inflammatory, anti-cancer, anti-hyperglycaemic, anti-fertility, antiviral and cardio protective [22-24]. The study of Ji X, et al., proved andro inhibited Akt/NF-κB signaling pathway and demonstrated that andro is protective against the progression of experimental DN by inhibiting inflammation, oxidative stress and fibrosis [25].

CXCR4 is a stromal-derived-factor-1 specific chemokine receptor, participating in cancer progression and neurodegenerative diseases by regulating cell growth, apoptosis and invasion [26, 27]. Moreover, CXCR4 plays a key role in recruitment of inflammatory cells to inflammation sites at the beginning of the disease process, so modulating of CXCR4 was recognised a new strategy for anti-inflammatory [28].

In this study, we aimed to investigate the role of andro and the connection between andro and CXCR4 in the atherosclerosis progress. We mainly confirmed that andro affects EC-SMC-MC proliferation and the expression of inflammatory cytokines by regulating the CXCR4. Moreover, result shows that andro inhibits the occurrence of apoptosis mainly by regulating the PI3K/AKT/NF-κB signaling pathways. Meantime, WB testing found that andro inhibits CXCR4, and by adjusting the PI3K/AKT/NF-κB signaling pathways regulating proliferation and differentiation of EC- SMC- MC.

The inflammatory cytokines influences EC-SMC-MC and promotes atherosclerosis, and andro inhibits the EC-SMC-MC apoptosis by suppressing CXCR4 mediated inflammatory response, playing a cell protection role. All these shown the andro can relieve andrographolide by inhibiting PI3K/AKT/NF-κB signaling pathway during inflammatory. In conclusion, andro can inactivate PI3K/AKT/NF-κB signaling pathways by regulating the CXCR4 and make EC-SMC-MC have anti-inflammatory function.

Taken together, our study make clear of the role of andro in the atherosclerosis, and find it closely connected with CXCR4 expression. All these findings suggest that andro inhibits atcherosderosis by inactivating the PI3K/AKT/NF-κB signaling pathway and could be a new therapeutic tool for this disease.

Disclosure of conflict of interest

None.

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