Int J Clin Exp Med 2017;10(4):6756-6762
www.ijcem.com /ISSN:1940-5901/IJCEM0045500

Original Article

Aldosterone upregulates chemerin via chemokinelike receptor 1-Rho-ROCK-JNK signaling in cardiac fibroblasts

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Received November 30, 2016; Accepted February 6, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: Chemerin is a novel adipokine that affects inflammation, insulin resistance, metabolic syndrome, and cardiovascular diseases. Nonetheless, it is unclear whether chemerin is expressed in cardiac fibroblasts or cardiac fibrosis, as are the effects of chemerin in this process. This study was aimed at investigating the regulation of the chemerin release and its effects on cardiac fibroblasts. Rat cardiac fibroblasts were cultured and exposed to increasing concentrations of aldosterone for 4-48 h. Y27632 was used to block ROCK signaling. PD98059, SB203580, and SP600125 were used to inhibit ERK1/2, p38 MAPK, and JNK signaling pathways, respectively. RT-PCR and western blotting were carried out to determine the expression levels of chemerin. CMKLR1, TGF-β, ROCK1, ROCK2, MYPT1, and JNK were assayed by western blot analysis. Chemerin and CMKLR1 were expressed in cardiac fibroblasts. Aldosterone increased chemerin mRNA and protein levels in a time- and dose-dependent manner in cardiac fibrosis. Spironolactone, Y27632, and SP600125 effectively suppressed the expression of chemerin. Our study revealed that chemerin and CMKLR1 are expressed in cardiac fibroblasts and the expression levels of chemerin are increased in cardiac fibrosis. Thus, chemerin may play an important role in cardiac fibrosis. Our study showed that chemerin may play this role via molecular mechanisms mediated by the CMKLR1-Rho-ROCK1-JNK signaling pathway.

Keywords: Chemerin, CMKLR1 cardiac fibrosis, aldosterone, spironolactone

Introduction

Aldosterone is a steroid hormone that is secreted by the zona glomerulosa of the adrenal cortex. Recent data indicate that aldosterone acts on a variety of cell types affecting cellular mechanisms that mediate important tissue responses, including hypertrophy and fibrosis. Landmark studies have detected the expression of receptors with high affinity for aldosterone in cardiac fibroblasts obtained from human hearts [1]. Several studies have indicated a clear relation between aldosterone and adipokines. A study on Sprague-Dawley rats that were fed high-salt diets revealed an increase in blood pressure, downregulation of aldosterone, and an increase in plasma adiponectin concentrations [2].

Chemerin is a novel adipokine secreted by the liver and white fat tissue. Chemerin is associated with inflammation, insulin resistance, metabolic syndrome, and cardiovascular diseases. To date, all known chemerin functions have been attributed to activation of the G protein-coupled receptor chemokinelike receptor 1 (CMKLR1). Our previous study indicates that chemerin and CMKLR1 are expressed in rat cardiomyocytes and induce insulin resistance [3]. Nonetheless, whether chemerin is expressed in cardiac fibroblasts or contributes to cardiac fibrosis is uncertain. We found that chemerin was expressed more strongly in patients with chronic heart failure and correlated with cardiac function class. Thus, we wanted to exclude feedback interference, and our in vitro experiment confirmed chemerin's relations with myocardial fibrosis. There is a novel finding that chemerin signals through RhoA and Rho-associated protein kinase (ROCK)-dependent pathway for activation of the transcriptional regulator serum-response factor [4].

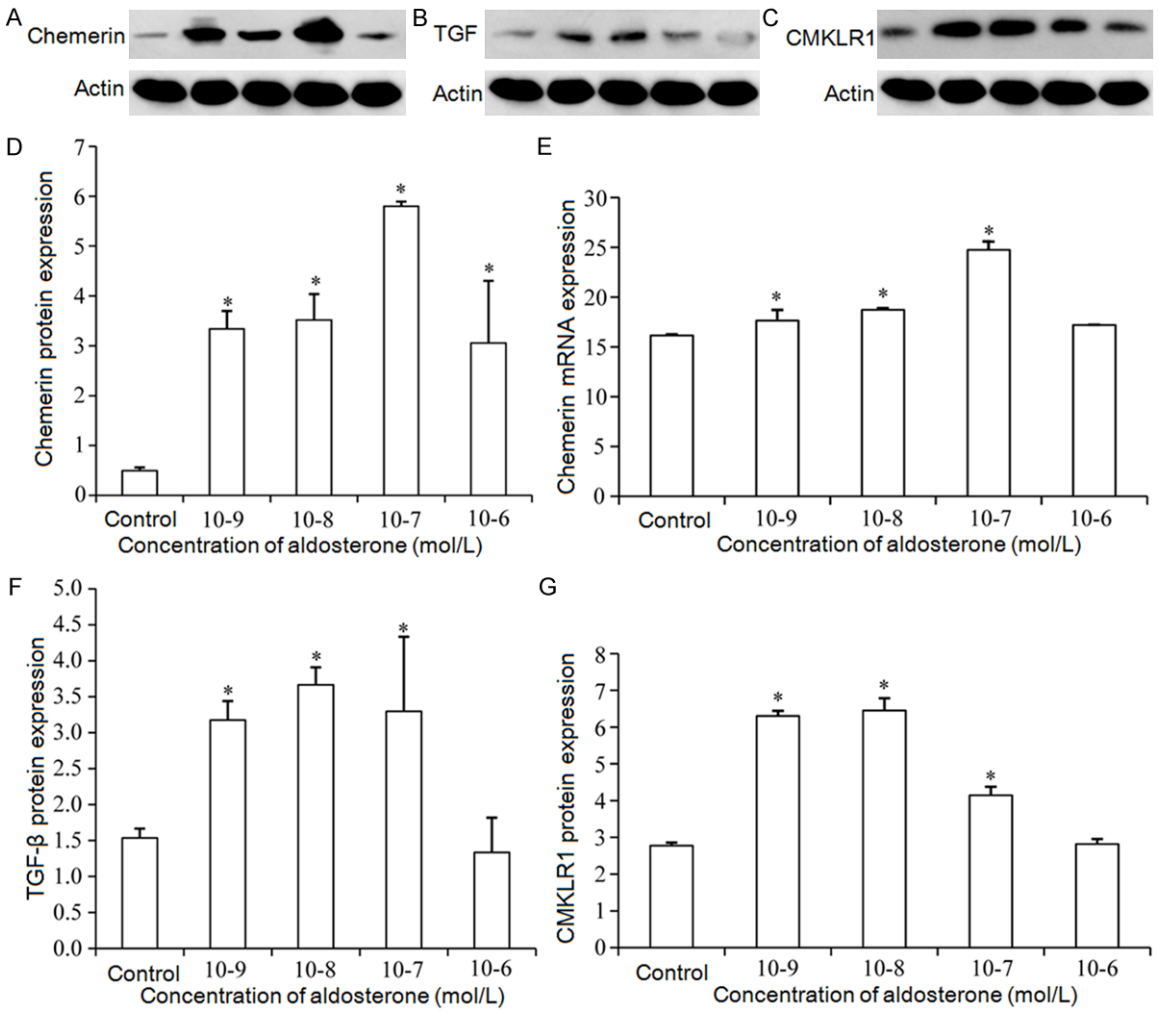

Figure 1. Aldosterone increases chemerin and TGF-β in a dose-dependent manner. Cardiac fibroblasts were treated with aldosterone (10^{-6}-10^{-9} mol/L) for 24 h. Chemerin protein (A, D) and mRNA (E) were evaluated by western blot, and RT-PCR. TGF-β protein levels (B, F) were evaluated by western blot. CMKLR1 protein levels (C, G) were evaluated by western blot. Data are represented as mean ± SD. *$P < 0.05$ compared with control group.

On the basis of the above information, we hypothesized that chemerin is expressed during aldosterone-induced cardiac fibrosis through Rho/ROCK and MAPK signaling pathways and may participate in cardiac fibrosis. These findings will provide novel data regarding the molecular mechanisms leading to the expression of chemerin in cardiac fibrosis and during cardiac remodeling.

Materials and methods

Animals

Two-hundred Sprague-Dawley rats (1- to 3-day-old) were obtained from the Laboratory Animal Centre of Hebei Medical University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Hebei Medical University.

Cell culture

Cardiac fibroblasts were isolated and purified from Sprague-Dawley rats (1- to 3-day old). First, the hearts of Sprague-Dawley rats were isolated and digested in 10 mL of phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO) containing 0.08% trypsin (Sigma Aldrich, St. Louis, MO) for 10 min at 37°C. After each

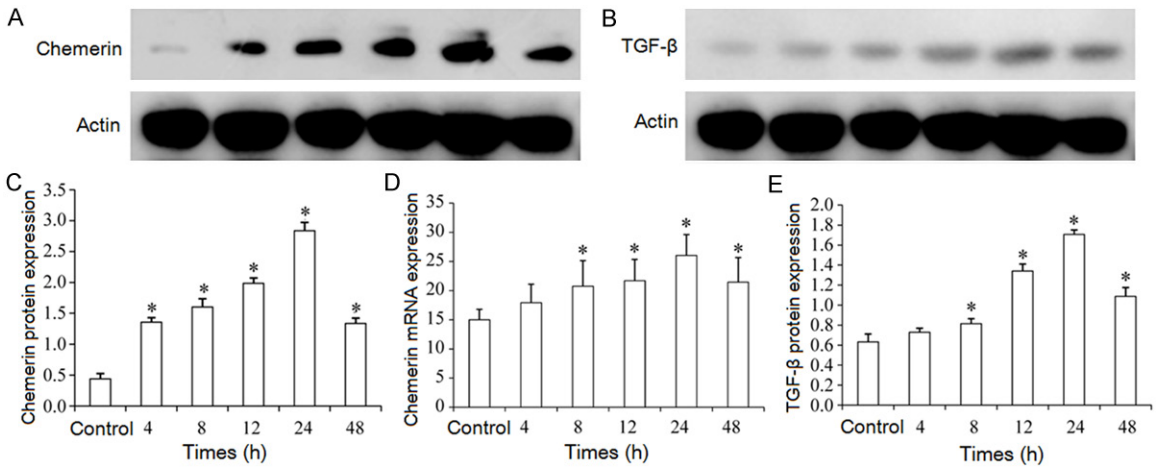

Figure 2. Aldosterone increases chemerin and TGF-β in a time-dependent manner. Cardiac fibroblasts were treated with aldosterone (10^{-7} mol/L) for 4, 8, 12, 24 and 48 h. Chemerin protein (A, C) and mRNA (D) were evaluated by western blot, and RT-PCR. TGF-β protein levels (B, E) were evaluated by western blot. Data are represented as mean ± SD. *$P < 0.05$ compared with control group.

digestion step, the medium containing suspended cells was removed and an equal volume of the Spinner/collagenase (Sigma Aldrich, St. Louis, MO) solution was added. Primary cultures of rat cardiac stromal cells were grown in DMEM (Sigma Aldrich, St. Louis, MO) supplemented with 20% of fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL; Sigma Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere containing 5% of CO_2. Cardiac fibroblasts at the third or fourth passage were used for experiments.

RT-PCR

This analysis of chemerin was performed on RNA extracted with the TaqMan reverse transcription kit (Applied Biosystems). Rat Rarres 2, 144 bp, Cat. No. RQP046621, reference position 102, GenBank NM_001013427.1.

Western blot

Protein samples (30 μg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked. Antibodies against chemerin (1:1000), CMKLR1 (1:500), col3A1 (1:500), col3A2 (1:500), TGF-β (1:1000), ROCK1 (1:1000), ROCK2 (1:1000), MYPT1 (1:1000), p-MYPT1 (1:1000), Jnk (1:1000) and p-Jnk (1:1000), ERK1/2 (1:1000), phospho (p)-38MAPK (1:1000), and p-ERK1/2 (1:1000) (Santa Cruz Biotechnology, America) were used. Blots were incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000), and protein phosphorylation was normalized to the total protein band by densitometry.

Statistical analysis

The SPSS 13.0 software package was used for all statistical analyses. Multiple-group comparison was performed by analysis of variance (ANOVA). The significance level was defined as 0.05.

Results

Effects of aldosterone on chemerin and TGF-β expression levels

To determine whether chemerin and TGF-β are expressed in cardiac fibrosis, cardiac fibroblasts were treated with increasing concentrations of aldosterone (10^{-6} to 10^{-9} mol/L) for 24 h (**Figure 1**). The optimal concentration to increase chemerin and TGF-β expression levels was determined by treating cardiac fibroblasts with aldosterone for varying periods (4, 8, 12, 24, or 48 h; **Figure 2**). Exposure to aldosterone significantly increased chemerin protein (**Figure 1A**, **1D**) and mRNA levels (**Figure 1E**). As we know, TGF-β, contributing to cardiac fibrosis, is believed to stimulate cell growth, apoptosis and differentiation, increase collagen and matrix

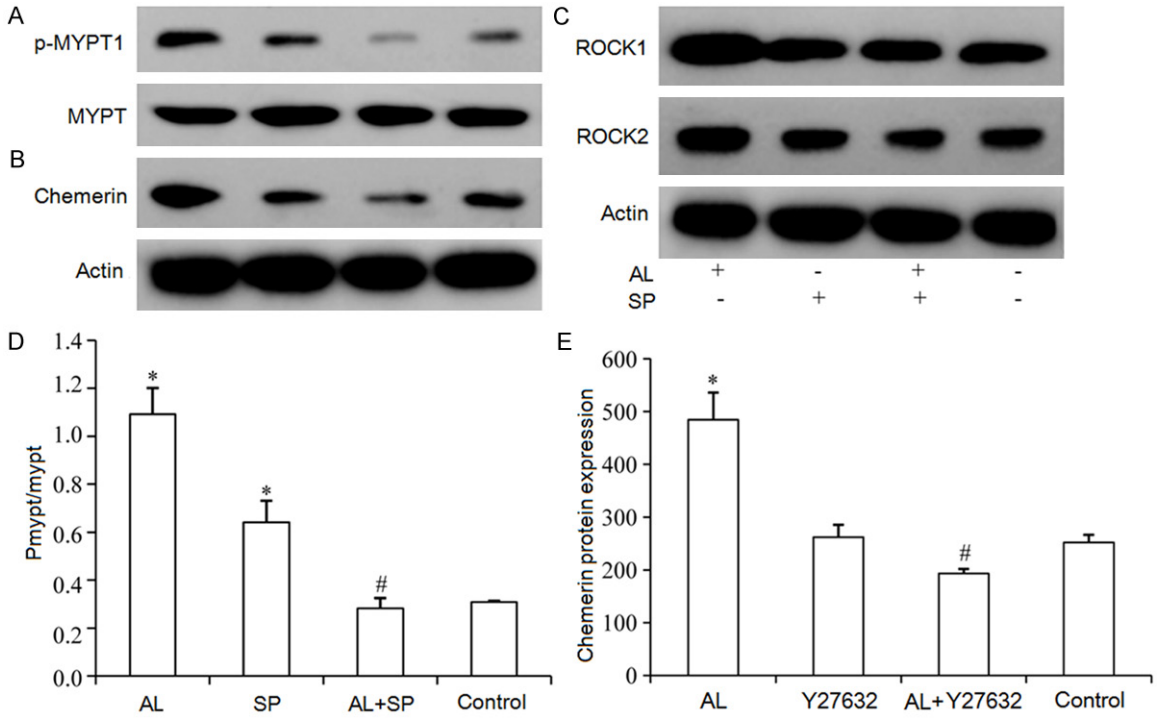

Figure 3. Rho/ROCK signaling is required for aldosterone-induced chemerin expression. Cardiac fibroblasts were stimulated with the aldosterone receptor inhibitor, spirolactone (SP) (10^{-6} mol/L) for 30 min and exposed to aldosterone (10^{-7} mol/L) for 24 h. The level of aldosterone-induced MYPT1 phosphorylation was quantified as p-MYPT1/total MYPT1. Inhibition of ROCK activity by spirolactone (A, D). Chemerin protein expressions in response to Y27632 (B, E). ROCK1 and ROCK2 protein expressions in response to spirolactone (C). Datas are represented as mean ± SD. **P* < 0.05 compared with control group, #*P* < 0.05 compared with aldosterone (AL) group.

protein production, maintain fibroblast viability, and inhibit production of a metalloproteinase that facilitates collagen degradation [5, 6]. Protein levels of TGF-β increased in a dose- and time-dependent manner (**Figure 1B**, **1F**). Thus, chemerin and TGF-β expression levels increased at an optimal concentration of aldosterone (10^{-7} mol/L) after 24 h in aldosterone-induced cardiac fibrosis.

Chemerin functions have been attributed to activation of the CMKLR1. Thus, we wanted to test whether CMKLR1 is expressed in cardiac fibroblasts. Eventually, we found that CMKLR1 protein levels are increased after exposure to aldosterone (**Figure 1C**, **1G**).

Effects of Rho-ROCK signaling on chemerin expression

Phosphorylated myosin phosphatase target subunit 1 (p-MYPT1) levels, which represent activated Rho-ROCK signaling, decreased after Y27632 treatment [7, 8]. Treatment of cardiac fibroblasts with an aldosterone inhibitor, spirolactone, significantly reduced the p-MYPT1/MYPT1 ratio after stimulation with aldosterone (**Figure 3A**, **3D**).

Furthermore, cardiac fibroblasts were pretreated with the ROCK inhibitor, Y27632 (10 μmol/L), for 30 min and then exposed to aldosterone (10^{-7} mol/L) for 24 h. Chemerin protein levels were significantly inhibited during treatment with Y27632 (**Figure 3B**, **3E**). ROCK1 was significantly downregulated by spirolactone, but there was no significant change in ROCK2 (**Figure 3C**).

Effects of the JNK pathway on chemerin expression

Inhibitors of ERK1/2 (PD98059), JNK (SP600125), and p38 MAPK (SB203580) were used to block the MAPK signaling cascade to determine whether there is signaling cross-talk between Rho/ROCK and MAPK. Aldosterone-induced p-JNK was completely inhibited by

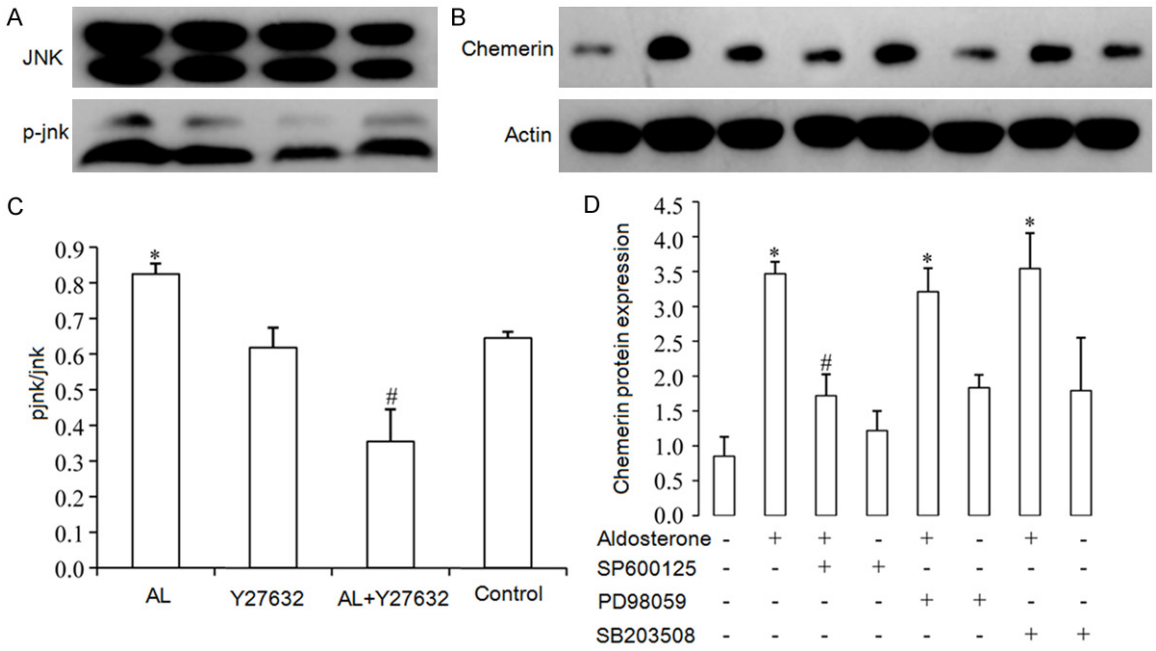

Figure 4. JNK is the downstream mediator of Rho/ROCK activity in chemerin expression. Cardiac fibroblasts were treated with the ROCK inhibitor, Y27632 (10 umol/L) for 30 min and exposed to aldosterone (10^{-7} mol/L) for 24 h. The extent of JNK phosphorylation was quantified by p-JNK/total JNK (A, C). Cardiac fibroblasts were pretreated with PD98059 (10 umol/L), SP600125 (10 umol/L), and SB203580 (10 umol/L) for 30 min and exposed to aldosterone (10-7 mol/L) for 24 h (B, D). Datas are represented as mean ± SD. *P < 0.05 compared with untreated group, #P < 0.05 compared with aldosterone (AL) group.

Y27632 (10 μmol/L; **Figure 4A**, **4C**). Chemerin protein levels were significantly suppressed in the aldosterone+SP600125 group compared with to that in groups aldosterone+PD98059 and aldosterone+SB203580. There were no differences between the PD98059 group and SB203580 group (**Figure 4B**, **4D**).

Discussion

The adipokine chemerin, also known as TIG2 (tazarotene-induced gene 2) or RARRES2 (retinoic acid receptor responder protein 2), is an 18-kDa protein produced mainly in the liver and in adipose tissue but also in many other tissues, including those of the cardiovascular system [9-11]. Studies on human subjects have revealed that chemerin is directly linked to inflammation, obesity, insulin resistance, and cardiovascular diseases. As we know, chemerin has been proposed as a predictive marker of cardiovascular risk [12, 13]. We found that chemerin and hs-CRP are overexpressed in patients with chronic heart failure and correlate with a cardiac function class. Accordingly, we hypothesized that chemerin is associated with heart failure. More studies have shown recently that chemerin and *CMKLR1* genes are both expressed by cells of the cardiovascular system, where their levels have been shown to positively correlate with the severity of cardiac pathologies such as atherosclerosis [14]. Our previous study revealed that chemerin and CMKLR1 are expressed in rat cardiomyocytes and induce insulin resistance [3]. Therefore, we hypothesized that chemerin is expressed in cardiac fibroblasts or contributes to cardiac fibrosis.

Aldosterone plays an important role in the pathogenesis of fibrosis. It can contribute to the accumulation of the extracellular matrix (ECM) by stimulating expression of collagen types I and IV and transforming growth factor [15]. Spironolactone is the most commonly used aldosterone receptor antagonist. Large-scale clinical studies showed beneficial effects of mineralocorticoid receptor blockade on cardiovascular morbidity and mortality in patients with heart failure [16]. Recent clinical studies also showed that spironolactone reduces the combined end point of death or hospitalization

for heart failure in non-African-American patients [17].

In our study, we found that chemerin and CMKLR1 are both expressed in cardiac fibroblasts and are upregulated in a dose- and time-dependent manner in aldosterone-induced cardiac fibrosis. TGF-β protein expression was also upregulated, and we found that chemerin overexpression is blocked by the aldosterone inhibitor spironolactone. Accordingly, we assumed that chemerin may play a critical role in aldosterone-induced cardiac fibrosis. This effect can be attenuated by spironolactone. These findings are consistent with our study on patients with heart failure.

There is a recent report that chemerin signals through a RhoA and Rho-associated kinase (ROCK)-dependent pathway for activation of the transcriptional regulator serum-response factor [4]. It is known that Rho GTPase and its downstream target, ROCK, are implicated in cardiac fibrosis. Inhibition of ROCK attenuates cardiac fibrosis in angiotensin II-induced cardiac hypertrophy in vivo [18]. In human endothelial cells, chemerin induces activation of p38 MAPK, extracellular signal-regulated kinases (ERKs), and phosphoinositide-3-kinase/protein kinase B pathways in a dose-dependent manner. Mitogen-activated protein kinases (MAPKs) are key mediators of signal transduction from the cell surface to the nucleus. c-Jun NH2-terminal kinase (JNK) is one of the major members of MAPKs, and JNK activation is also implicated in cardiac fibrosis. Thus, we tested whether chemerin signals through the Rho-ROCK or MAPKs pathway in cardiac fibrosis.

In summary, using specific inhibitors of JNK and Rho/ROCK, we found that the Rho/ROCK signaling cascade is the molecular mechanism that leads to the aldosterone-induced chemerin expression in cardiac fibrosis. ROCK1 plays an essential role in the process. In addition, aldosterone-induced chemerin expression is downregulated by SP600125.

In conclusion, our study shows that chemerin is expressed in cardiac fibroblasts and is upregulated in cardiac fibrosis. Targeting of chemerin may help to reverse the progression of cardiac fibrosis. Chemerin expression is mediated by the CMKLR1-Rho-ROCK1-JNK signaling pathway in cardiac fibroblasts. Further research is needed to clarify the clinical significance of chemerin in cardiac fibrosis.

Disclosure of conflict of interest

None.

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