European Review for Medical and Pharmacological Sciences 2018; 22: 4075-4079

NF-κB inhibits the differentiation of hysteromyoma cells by reducing myocardin expression

X.-W. XU, L.-J. RUAN, J.-D. YANG, H.-Y. LIN

Department of Gynecology and Obstetrics, The Fifth Affiliated Hospital, Southern Medical University, Guangzhou, P.R. China

Abstract. – OBJECTIVE: To investigate the effect of nuclear factor-kappaB (NF-κB) on the myocardin-mediated differentiation of hysteromyoma cells.

MATERIALS AND METHODS: Expression levels of myocardin in hysteromyoma cells from patients with hysteromyoma were detected. Normal uterine smooth muscle cells were used as control group. Overexpression of myocardin in hysteromyoma cells was achieved through lentivirus infection. Changes in expression levels of uterine smooth muscle cell maker p21, p57, Cyclin D1, PCNA, SM22α, and αSMA were detected. Hysteromyoma cells with lentivirus infection were stimulated by lipopolysaccharide (LPS), and changes in expression levels of myocardin were detected.

RESULTS: Compared with normal uterine smooth muscle cells, the expression level of myocardin in hysteromyoma cells was extremely low, or even undetectable, and expression levels of smooth muscle cell differentiation markers were also minimal, and cells were in the de-differentiated state. Expression of exogenous myocardin can improve the expression of smooth muscle cell differentiation markers to induce cell re-differentiation. LPS stimulation can activate NF-κB to inhibit myocardin expression, thereby inducing cell dedifferentiation.

CONCLUSIONS: NF-κB can inhibit the differentiation of hysteromyoma cells by decreasing the expression level of myocardin.

Key Words:
Hysteromyoma, Smooth muscle, Myocardin, NF-ÐB, Cell differentiation.

Introduction

As a member of SAP transcription factor family, myocardin is one of the activators of serum response factor (SRF) in smooth muscle cells that regulates gene expression in smooth muscle cells[1]. Recent studies have shown that myocardin plays important roles in development, differentiation, and pathological changes of smooth muscle cells[2]. Myocardin can activate the expression of smooth muscle cell differentiation marker genes, including SM22α, calponin, αSMA, etc[3]. In tumor cells, the expression level of myocardin is low or even undetectable, and expression levels of cell differentiation markers are also low, and cells were in the de-differentiated state. The proliferation of those genes will eventually lead to the formation of a tumor, indicating that expression of myocardin is related to the development of some types of tumors[4,5].

NF-κB is a transcription factors family. As an important member of NF-κB, p65 (RelA, NF-κB3) plays an important role in regulating inflammation, immune response, cell proliferation, differentiation, and survival[6]. Infection or inflammation caused by exogenous stimuli can activate p65 signaling pathway to mediate immune response[7].

Myocardin and p65 in cardiomyocytes are both involved in the pathological process of cardiac hypertrophy, but the mechanism of the function of p65 in regulating myocardin-induced pathological changes is still unclear[8]. This research was carried out to detect the expression of myocardin and smooth muscle cell differentiation markers in hysteromyoma cells, and to investigate the changes in cell differentiation caused by overexpression of myocardin in hysteromyoma cells. Moreover, the effect of NF-κB on myocardin-regulated cell differentiation was explored.

Patients and Methods

Cell Isolation and Culture

With patient's agreement, hysteromyoma tumor tissue was collected from patients with hysteromyoma, and uterine smooth muscle tissue was collected from control patients. Tissue was

Corresponding Author: Haiyan Lin, MD; e-mail: 44015582@qq.com

placed in phosphate-buffered saline (PBS) to remove the residual blood. A sterile ophthalmic surgical scissor was used to cut the tissue into small pieces (about 1 mm^3), followed by digestion with Dulbecco's Modified Eagle Medium (DMEM) medium containing 2% collagenase for 1 hour. After that, cells were centrifuged at 1000 rpm for 3 min to remove the supernatant. Digested cells were then used to make single cell suspension and transferred to a T75 tissue culture flask prefilled with DMEM medium containing 10% calf serum. Cells were cultured in an incubator (37°C) containing 5% CO_2. The culture medium was replaced by DMEM medium containing 10% calf serum after cell adhesion.

Lentivirus Infection of Hysteromyoma Cells to Overexpress Myocardin

pLP lentivirus system was constructed to express myocardium. The gene encoding human myocardin was inserted into lentiviral plasmid pLP-VSVG to construct pLP-VSVG-myocardin plasmid. pLP-VSVG-myocardin, pLP1 and pLP2 were co-transfected into 293T cells. After incubation for 48 hours, the lentivirus-containing supernatant was collected. 1×10^6 hysteromyoma cells were inoculated into a six-well plate, and cultured overnight into monolayer cells, followed by incubation with lentivirus-containing supernatant for 72 h to allow lentivirus to infect cells. Cells infected with lentivirus were collected to detect the changes in expression levels of p21, p57, Cyclin D1, PCNA, SM22α, and αSMA by Western blotting. Hysteromyoma cells infected with GFP-expressing pLP-VSVG lentivirus were used as control.

LPS Treatment to Activate NF-κB in Hysteromyoma Cells

LPS (1.0 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) was used to stimulate hysteromyoma cells for 24 hours to activate the transcription factor p65 in the NF-κB signaling pathway. Cells were collected after LPS stimulation to detect the changes in expression level of p65, myocardin, p21, p57, Cyclin D1, PCNA, SM22α, and αSMA by Western blot. Hysteromyoma cells pretreated with NF-κB specific inhibitor (5 μM/ml, Sigma-Aldrich, St. Louis, MO, USA) were treated with LPS for 24h to serve as control.

Western Blot

Cells were collected and lysed by lysate, followed by electrophoresis using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the protein was transferred to polyvinylidene difluoride (PVDF) membrane, followed by blocking with 5% skimmed milk for 2 h. Membranes were then incubated with the corresponding mouse anti-human primary antibodies (1:1000, Sigma-Aldrich, St. Louis, MO, USA) including anti-β-actin, anti-myocardin, anti-p65, anti-p21, anti-p57, anti-Cyclin D1, anti-PCNA, anti-SM22α, and anti-αSMA at room temperature for 1h. After washing, membranes were incubated with the horseradish peroxidase (HRP)-labeled rabbit anti-mouse secondary antibody (1:2500) for 1 hour. HRP enzyme substrate was added and the signals were detected and recorded using Roche Elecsys-2010 chemiluminescence meter (Roche Diagnostics, Indianapolis, IN, USA). Greyscale values were measured, and quantitative analysis was carried out using image J with β-actin as endogenous control to calculate the relative expression level of each protein. All anti-bodies were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical Analysis

Data were expressed as mean ± standard deviation and analyzed by Student's *t*-test. $p<0.05$ was considered to be statistically significant.

Results

Expression of Myocardin and Smooth Muscle Cell Differentiation Markers in Hysteromyoma Cells and Uterine Smooth Muscle Cells

Western blot results showed that expression level of myocardin was very low in hysteromyoma cells but relatively high in uterine smooth muscle cells (Figure 1). A significant difference in expression level of myocardin could be observed between those two types of cells ($p<0.01$). Expression levels of uterine smooth muscle cell differentiation markers p21, p57, PCNA, SM22α, and αSMA in hysteromyoma cells were significantly lower than those in uterine smooth muscle cells ($p<0.05$). The expression level of Cyclin D1 in hysteromyoma cells was also lower than that in uterine smooth muscle cells, but the difference was not significant ($p>0.05$).

Overexpression of Myocardin in Hysteromyoma Cells

As shown in Figure 2, myocardin was highly expressed in hysteromyoma cells after

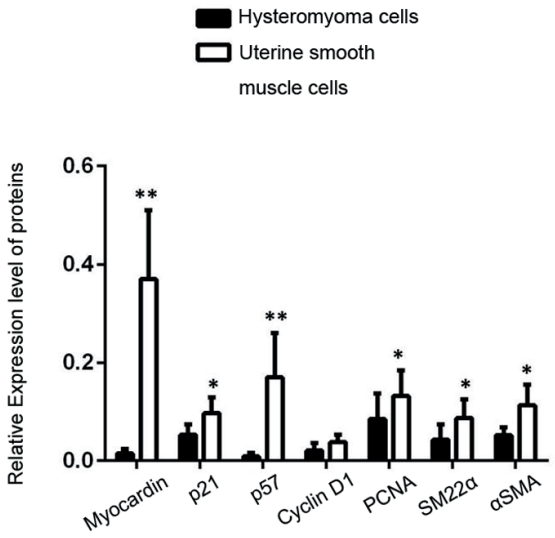

Figure 1. Expression of myocardin and smooth muscle cell differentiation markers in hysteromyoma cells and uterine smooth muscle cells. Expression levels of myocardin and smooth muscle cell differentiation markers in hysteromyoma cells and uterine smooth muscle cells were detected by Western blot. Notes: *compared with hysteromyoma cells, $p<0.05$; **compared with hysteromyoma cells, $p<0.01$

pLP-VSVG-myocardin lentivirus infection. Compared with control hysteromyoma cells, expression levels of smooth muscle cell differentiation markers Cyclin D1, PCNA, SM22α, and αSMA were significantly increased in hysteromyoma cells infected with pLP-VSVG-myocardin lentivirus ($p<0.05$).

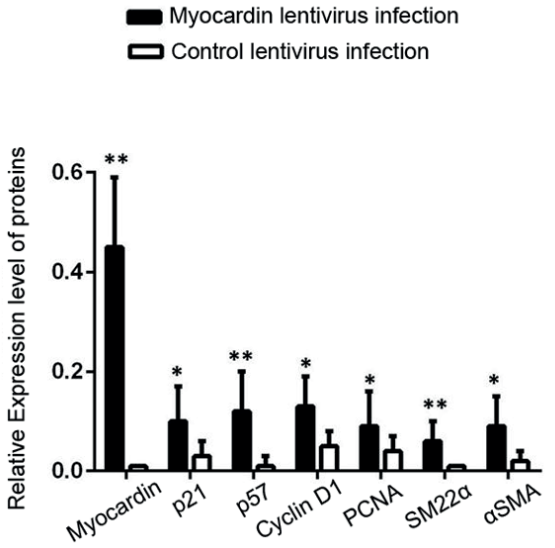

Figure 2. Expression level of smooth muscle cell differentiation markers in hysteromyoma cells after the overexpression of myocardin. Expression levels of smooth muscle cell differentiation markers in hysteromyoma cells detected by Western blot 3 days after infection. Notes: *compared with hysteromyoma cells, $p<0.05$; **compared with hysteromyoma cells, $p<0.01$

p65 Inhibited Myocardin Expression

LPS stimulation can activate NF-κB signaling pathway, so that the expression level of p65 protein was increased after LPS treatment. As shown in Figure 3, the expression level of p65 protein was significantly increased after LPS stimulation in hysteromyoma cells overexpressing myocardin ($p<0.05$).

P65 can inhibit the expression of myocardin in hysteromyoma cells, so as to reduce the expression levels of p21, p57, Cyclin D1, PCNA, SM22α, and αSMA in hysteromyoma cells, leading to the dedifferentiation of hysteromyoma cells. As shown in Figure 4, LPS stimulation can still induce the expression of p65 in hysteromyoma cells treated with NF-κB specific inhibitor Bay11-7082, but Bay11-7082 can inhibit the function of p65. Therefore, the expression of myocardin is still high, which in turn improve the expression of smooth muscle cell differentiation markers, indicating that p65 plays a role in inhibiting myocardin expression after LPS stimulation.

Discussion

Expression of myocardin in the myocardium and smooth muscle cells can activate SRF, so

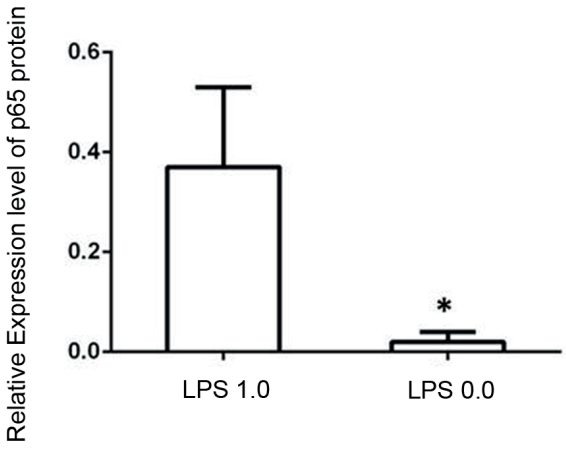

Figure 3. Expression level of p65 in hysteromyoma cells was increased after LPS stimulation. Expression level of p65 in hysteromyoma cells detected by Western blot 24 h after LPS (1.0 μg/ml or 0 μg/ml) stimulation. Notes: *compared with hysteromyoma cells treated with buffer only, $p<0.05$

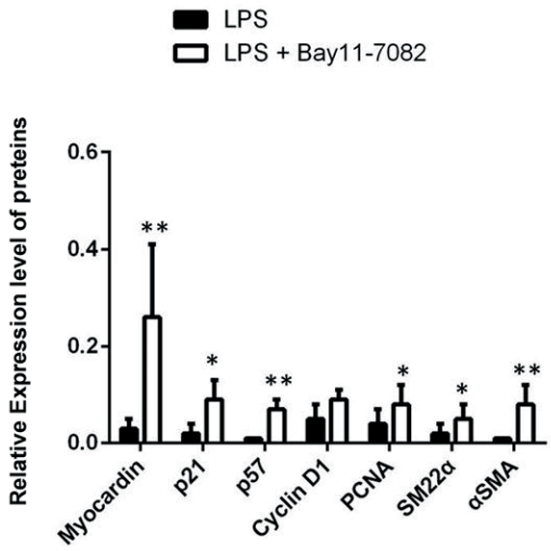

Figure 4. P65 inhibited expression of myocardin and smooth muscle cell differentiation markers in hysteromyoma cells. Expression of myocardin and smooth muscle cell differentiation markers in hysteromyoma cells treated with NF-κB specific inhibitor Bay11-7082 24 h after LPS stimulation. Notes: *compared with hysteromyoma cells treated with LPS only, $p<0.05$; **compared with hysteromyoma cells treated with LPS only, $p<0.01$

myocardin is an important factor in regulating the differentiation of smooth muscle cells. Myocardin regulates smooth muscle cell differentiation by activating the expression of the corresponding differentiation markers[8,9]. This study showed that, compared with smooth muscle cells, expression levels of myocardin and smooth muscle cell differentiation markers were relatively low in hysteromyoma cells, which indicated the low differentiation degree of hysteromyoma cells. This study and previous studies have shown that the expression of myocardin and smooth muscle cell differentiation markers in hysteromyoma cells is low and the degree of cell differentiation is also low[10]. Therefore, the decline in the expression level of myocardin is closely related to the occurrence and development of hysteromyoma. Expression of smooth muscle cell differentiation markers was increased, and cells showed differentiation phenotype after the expression of exogenous myocardin in hysteromyoma cells. These results indicate that the decrease in the expression level of myocardin is related to the dedifferentiation of hysteromyoma cells.

Expression of myocardin and the regulation of myocardin in hysteromyoma cells is still not entirely clear. Researches have shown that SRF can inhibit the activity of myocardin[1]. SRF is highly expressed in smooth muscle cells, leading to low or even undetectable expression levels of myocardin[11,12]. Exogenous factors can inhibit the differentiation of hysteromyoma cells by inhibiting myocardin expression. Studies have shown that NF-κB (p65) can inhibit gene expression in myocardin-activated smooth muscle cells, and p65 can directly interact with myocardin to inhibit the function of myocardin/SRF/CArG complex[13]. But whether p65 affects myocardin-mediated smooth muscle cell differentiation remains unclear. We showed that p65 could down-regulate the expression of myocardin, thereby further reduce the expression of smooth muscle cell differentiation markers, leading to cell dedifferentiation. So, cells showed similar phenotypes to hysteromyoma cells.

Conclusions

This study showed that expression levels of myocardin and smooth muscle cell differentiation markers in uterine leiomyoma cells were low and the degree of cell differentiation was also low, which is the characteristic of the infinite proliferation of tumor cells. After myocardin overexpression, expression levels of differentiation markers were increased and degree of cell differentiation was also increased. However, NF-κB (p65) can inhibit the expression of myocardin in hysteromyoma cells, so as to further inhibit cell differentiation, indicating that the increase in the level of NF-κB (p65) induced by inflammation and other factors may play important roles in the development of hysteromyoma.

Ethical Committee Approval

This study was approved by the Ethical Committee of The Fifth Affiliated Hospital of Southern Medical University (Guangzhou, China).

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, Krieg PA, Olson EN. Activation of cardiac gene expression by myocardin, a tran-

scriptional cofactor for serum response factor. Cell 2001; 105: 851-862.

2) Xing W, Zhang TC, Cao D, Wang Z, Antos CL, Li S, Wang Y, Olson EN, Wang DZ. Myocardin Induces Cardiomyocyte Hypertrophy. Circ Res 2006; 98: 1089-1097.

3) Chen J, Kitchen CM, Streb JW, Miano JM. Myocardin: a component of a molecular switch for smooth muscle differentiation. J Mol Cell Cardiol 2002; 34: 1345-1356.

4) Shats I, Milyavsky M, Cholostoy A, Brosh R, Rotter V. Myocardin in tumor suppression and myofibroblast differentiation. Cell Cycle 2007; 6: 1141-1146.

5) Singh P, Zheng XL. Dual regulation of myocardin expression by tumor necrosis factor-α in vascular smooth muscle cells. PLoS One 2014; 9: e112120.

6) Napetschnig J, Wu H. Molecular Basis of NF-κB Signaling. Annu Rev Biophys 2013; 42: 443-468.

7) Jia WC, Liu G, Zhang CD, Zhang SP. Formononetin attenuates hydrogen peroxide (H2O2)-induced apoptosis and NF-κB activation in RGC-5 cells. Eur Rev Med Pharmacol Sci 2014; 18: 2191-2197.

8) Zhou LF, Zeng W, Sun LC, Wang Y, Jiang F, Li X, Zheng Y, Wu GM. IKKepsilon aggravates inflammatory response via phosphorylation of ERK in rheumatoid arthritis. Eur Rev Med Pharmacol Sci 2018; 22: 2126-2133.

9) Yoshida T, Sinha S, Dandré F, Wamhoff BR, Hoofnagle MH, Kremer BE, Wang DZ, Olson EN, Owens GK. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. Circ Res 2003; 92: 856-864.

10) Milyavsky M, Shats I, Cholostoy A, Brosh R, Buganim Y, Weisz L, Kogan I, Cohen M, Shatz M, Madar S, Kalo E, Goldfinger N, Yuan J, Ron S, MacKenzie K, Eden A, Rotter V. Inactivation of myocardin and p16 during malignant transformation contributes to a differentiation defect. Cancer Cell 2007; 11: 133-146.

11) Tang RH, Zheng XL, Callis TE, Stansfield WE, He J, Baldwin AS, Wang DZ, Selzman CH. Selzman. Myocardin inhibits cellular proliferation by inhibiting NF-κB (p65)-dependent cell cycle progression. Proc Natl Acad Sci U S A 2008; 105: 3362-3367.

12) Wang DZ, Li S, Hockemeyer D, Sutherland L, Wang Z, Schratt G, Richardson J A, Nordheim A, Olson EN. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. Proc Natl Acad Sci U S A 2002; 99: 14855-14860.

13) Yoshida T, Sinha S, Dandre F, Wamhoff BR, Hoofnagle MH, Kremer BE, Wang DZ, Olson EN, Owens GK. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. Circ Res 2003; 92: 856-864.