Mir-146a Inhibits IFN-γ Production Via Suppressing TLR4/IRAK-1/NF-κb Expression in Pulmonary Arterial Smooth Muscle Cells

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Abstract

Purpose: The microRNA-146a (miR-146a) could regulate proliferation of vascular smooth muscle cell and inhibits inflammation of airway, but its role in inflammation of pulmonary arterial smooth muscle cell (PASMC) hasn’t been reported. We aim to explore the effect of miR-146a on regulating inflammatory signaling in the study.

Methods: Primary PASMCs were separated from rats. Cells were stimulated by lipopolysaccharides (LPS). miR-146a was transfected into cells with plasmid. miR-146a expression in PASMCs was assessed by real-time PCR. The protein expression of TLR4, phosphorylated-IRAK-1, phosphorylated-IKK, phosphorylated-IκB and NF-κB (P65) in PASMCs was analyzed using western blotting. The level of IFN-γ was detected using ELISA.

Results: The protein expression of TLR4, phosphorylated-IRAK-1, phosphorylated-IKK, phosphorylated-IκB and NF-κB (P65) in PASMCs was increased when induced by LPS, which was reversed by miR-146a. The level of IFN-γ in supernatant of PASMCs was higher in LPS-treated group than controls, which was decreased in cells with miR-146a overexpressed.

Conclusion: miR-146a could attenuate LPS-induced IFN-γ production, and activation of TLR4, IRAK-1 and NF-κB in PASMCs, which might provide novel target on the therapy of pulmonary hypertension.

Introduction

Pulmonary hypertension (PH) is a hemodynamic and pathophysiologic syndrome from increased blood pressure within pulmonary arteries, which prevalence is approximately 10 % in general population. Its prognosis is depressed that the one-year mortality is approximately only 15% [1]. Pulmonary arterial smooth muscle cell (PASMC) participates in PH through activating inflammatory signaling, such as NF-κB pathway [2,3]. However, the precise mechanisms of inflammation in PASMC are not very clear.

Recent studies have showed that microRNA-146a (miR-146a) could regulate proliferation of vascular smooth muscle cell from aortic artery [4,5] In addition, miR-146a could reduce inflammation in airway by targeting on IRAK-1 [6]. However, the role of miR-146a in inflammation of PASMC hasn’t been reported. Interestingly, miR-146a was found to contribute to inhibiting lipopolysaccharides (LPS)-induced activation of TLR4/IRAK1/NF-κB signaling in monocytes [7]. Similar finding was showed in intestine epithelial cells [8]. These findings suggested that miR-146a could inhibit the activation of TLR4/IRAK1/NF-κB signaling in inflammation. Moreover, our previous work found that LPS could induce the activation of TLR4/IRAK1/NF-κB signaling, resulting in an increased production of IFN-γ in PASMCs [9]. Thus, in this study, we explore the role of miR-146a in regulating IFN-γ production and TLR4/IRAK1/NF-κB signaling activation in PASMCs.
Methods

Cell Culture and Transfection

Male Wistar rats (8-10 weeks old, weighing 280±20 g) were obtained from experimental animal center of Guilin Medical University. All experimental procedures were approved by the Animal Care and Use Committee of the Affiliated Hospital of Guilin Medical University. Rats were anesthetized with 5% isoflurane by inhalation in oxygen and killed by cervical dislocation. The small vascular was separated from the 3rd level or lower artery branch of pulmonary lobe segments, and then was minced to small pieces and digested by 0.2% type I collagenase for 20 min at 37 °C in water. Digestion was stopped by adding 10% FBS (GIBCO, MA, USA)). The primary PASMCs were cultured in DMEM medium containing 10% FBS at 37°C in 5% CO2. Seven days later, PASMCs at passages 3-6 were used to conduct the experiments. Cells were cultured in serum-free medium 30min prior to transfection.

The primary PASMCs were identified using immunohistochemistry with α-SM-actin staining (Figure 1). The slides of cells were fixed by 4% paraformaldehyde for 20 min and incubated in 0.6% H2O2 for 30 min to quench endogenous peroxidase activity. The slides were incubated with primary mouse anti-rat antibody against α-SM-actin (dilution 1:100, BM0002, BOSTER, Wuhan, China) at 4 °C overnight, and then were incubated with horseradish peroxidase conjugated goat anti-mouse IgG antibody (BA1001, BOSTER, Wuhan, China) at room temperature for 20 min. After washes with PBS for three times, 3’3-diaminobenzidine-tetrahydrochloride was applied on the slides as a chromogen for 1–5 min, and were then by haematoxylin for 5–10 min. The transfection of miR-146a was performed with plasmid (Genechem, Shanghai, China) and lipofectamine2000 (Invitrogen, MA, USA) according to the manufacturer’s instruction. When six hours after transfection, LPS-induced cells were stimulated with LPS(1μg/ml) (Sigma, MO, US) for 20 min. The loaded proteins (15μg) were separated on a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring onto PVDF membranes. After blocking with 5% non-fat dried milk for 2 h at room temperature, the membranes were incubated with mouse anti-rat antibodies against TLR4 (dilution 1:500; Abcam, MA, USA)), IKK (dilution 1:5000; Abcam, MA, USA), β-actin (dilution 1:1000; ZSGB-Bio, Beijing, China), rabbit anti-rat antibodies against IRAK-1 (dilution 1:500; Abcam, MA, USA), I kB (dilution 1:1000; Abcam, MA, USA), and NF-κB (p65) (dilution 1:1000; CST, MA, USA) overnight at 4 °C, and then were incubated with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit(dilution 1:5000) for 1 h. Finally, the blots were developed with the ECL Plus reagents (Bio-Rad, USA).

Quantitative Real-Time PCR

PASMCs (1×106 cells/well) were plated into six-well plates and incubated overnight in a humidified incubator at 37 °C in 5% CO2. Total RNA was extracted using the RNA simple Total RNA Kit according to the manufacturer’s protocol, and RNA was reverse transcribed into cDNA. A quantitative real-time polymerase chain reaction (PCR) was performed using Hairpin-itTM microRNA and U6 snRNA Normalization real time-PCR quantitation kit (GenePharma, Shanghai, China)) with ABI PRISM 7500 Sequence Detection System (Thermo Fisher Scientific, Inc, Carlsbad, California, USA) in accordance with the manufacturer’s protocol. The 20μl PCR reactions (with 10μl Real-time PCR Master Mix, 0.4μl microRNA-146a /U6 snRNA specific Primer set, 0.2μl microRNA-146a /U6 snRNA specific Probe, 0.4μl ROX reference dye, 0.2μl Taq DNA polymerase, 2μl miRNA RT product and 6.8μl PCR H2O) were undergone 3 min at 95 °C, then 40 cycles of 12 s at 95 °C and 40 s at 62 °C. RT and PCR primer sequences are as follows: mir-146a RT:

GTCGTATCCATGGTGCTGAGTGACTGGAT
GGAGAACAACCTTCTCA
miR-146a FP: GGCGTGAGAACTGAATGG
GGAT ACGACAACCCAT
miR-146a RP: TCGTGAGTCGGCAATTG
GAAT T CCA
U6 RT: CGGTTCACGATTTTG CGGTTCGTCA
U6 FP:
CGCTACGACGATTTAATAAAT
U6 RP: CGGTTCGAC GAA TTTGCGTGCAT

The level of mRNA expression was reported as fold change using the 2−△△ CT method. Every sample was triplicated.

Western Blot Analysis

PSMCs were treated with 100μl RIPA and PMSF (100:1) for 30 min on ice, and then centrifuged at 12000 × g (4 °C) for 20 min. The loaded proteins (15μg) were separated on a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring onto PVDF membranes. After blocking with 5% non-fat dried milk for 2 h at room temperature, the membranes were incubated with mouse anti-rat antibodies against TLR4 (dilution 1:500; Abcam, MA, USA)), IKK (dilution 1:5000; Abcam, MA, USA), β-actin (dilution 1:1000; ZSGB-Bio, Beijing, China), rabbit anti-rat antibodies against IRAK-1 (dilution 1:500; Abcam, MA, USA), I kB (dilution 1:1000; Abcam, MA, USA), and NF-κB (p65) (dilution 1:1000; CST, MA, USA) overnight at 4 °C, and then were incubated with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit(dilution 1:5000) for 1 h. Finally, the blots were developed with the ECL Plus reagents (Bio-Rad, USA).

TATCCAGTGCTGCTGAGATCGCGCAATTGGACTGGAT
AGCACAACCCAT, miR-146a FP: GGCGTGAGAACTGAAT
TCCA, miR-146a RP: TCGTGAGTCGCAATTG;
U6 RT: CGGTTCACGATTTTG CGGTTCGTCA;
U6 FP: CGCTACGACGACATAATCAAAAT, U6 RP: CGGTTCGAC TTTG CGGTTCAT

The level of mRNA expression was reported as fold change using the 2−△△ CT method. Every sample was triplicated.

Western Blot Analysis

PSMCs were treated with 100μl RIPA and PMSF (100:1) for 30 min on ice, and then centrifuged at 12000 × g (4 °C) for 20 min. The loaded proteins (15μg) were separated on a 10% SDS-polyacrylamide gel electrophoresis (SDS-
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**Enzyme-Linked Immunosorbent Assay**

Enzyme-linked immunosorbent assay (ELISA) was used to detect the level of IFN-γ in cell culture supernatants according to the protocol of ELISA kit (Elabscience, Wuhan, China). Each sample was repeated in three wells. Briefly, in 96-well plates, 100μl sample and 100μl biotinylated detecting antibody (50μl cells and 50μl Detection reagent A) were incubated for 1 h at 37 °C, followed by incubation with 100μl Horseradish-peroxidase (HRP) conjugated working solution for 30 min at 37 °C. Subsequently, plates were incubated with substrate solution as a chromogen for 15 min without light. The optical density (OD) was measured at 450 nm using a microplate reader (TECAN, Switzerland).

**Statistical Analysis**

All statistical analyses were performed using SPSS 21.0 (IBM SPSS Inc., Chicago, IL, USA). Group data are expressed as mean ± std. deviation (SD). Significant differences were evaluated using an independent-samples t-test, and multiple groups were compared using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test or the Games–Howell test. p-values < 0.05 were considered to be statistically significant.

**Results**

**miR-146a Inhibits TLR4 Expression in PASMCs**

When PASMCs were transfected with miR-146a, the expression of miR-146a was respectively increased about 6-fold at the 24th hour and 18-fold and at the 48th hour (Figure 1A). This demonstrated the successful transfection of miR-146a. Moreover, the expression of miR-146a was significantly induced by LPS after 24-hour administration (Figure 1B). That effect was time-dose dependent. Furthermore, the protein expression of TLR4 in PASMCs was detected after miR-146a transfection. TLR4 expression was increased in LPS group compared with controls, whereas it was reversed when transfected with miR-146a (Figure 2). Thus, miR-146a could inhibit TLR4 expression in PASMCs.

**miR-146a Inhibits IRAK-1 Activation in PASMCs**

The activation of IRAK-1 in PASMCs was detected by Western blotting. The protein expression of phosphorylated-IRAK-1 (Figure 3) was increased when treated with LPS. However, it’s reduced in cells with miR-146a overexpression. These findings suggest that miR-146a could inhibit IRAK-1 activation in PASMCs.

**miR-146a inhibits IKK activation in PASMCs**

The activation of IKK, IκB and NF-xB (P65) in PASMCs was detected by western blotting. The protein expression of phosphorylated-IKK (Figure 4), phosphorylated-IκB (Figure 5) and NF-xB (P65) (Figure 6) was increased when treated with LPS. However, it’s reduced in cells with miR-146a overexpression. These findings suggest that miR-146a could inhibit IKK activation in PASMCs.

**miR-146a inhibits the secretion of IFN-γ in PASMCs**

The level of IFN-γ in the supernatant of PASMCs culture...
Figure 2: miR-146a inhibits TLR4 expression in PASMCs. PASMCs were transfected miR-146a expressing plasmids. TLR4 expression in PASMCs was increased in LPS group, whereas it was reversed when transfected with miR-146a. The representative images are shown in left panel and quantitative analysis results are shown in right panel. **: p<0.01.

Figure 3: miR-146a inhibits IRAK-1 activation in PASMCs. PASMCs were transfected miR-146a expressing plasmids. Phosphorylated-IRAK-1 expression in PASMCs was increased in LPS group, whereas it was reversed when transfected with miR-146a. The representative images are shown in left panel and quantitative analysis results are shown in right panel. * p<0.05, ** p<0.01.

Figure 4: miR-146a inhibits IKK activation in PASMCs. PASMCs were transfected miR-146a expressing plasmids. Phosphorylated-IKK expression in PASMCs was increased in LPS group, whereas it was reversed when transfected with miR-146a. The representative images are shown in left panel and quantitative analysis results are shown in right panel. * p<0.05, ** p<0.01.

Figure 5: miR-146a inhibits IκB phosphorylation in PASMCs. PASMCs were transfected miR-146a expressing plasmids. Phosphorylated-IκB expression in PASMCs was increased in LPS group, whereas it was reversed when transfected with miR-146a. The representative images are shown in left panel and quantitative analysis results are shown in right panel. **: p<0.01.
Figure 6: miR-146a inhibits NF-κB (p65) expression in PASMCs. PASMCs were transfected with miR-146a expressing plasmids. NF-κB (p65) expression in PASMCs was increased in the LPS group, whereas it was reversed when transfected with miR-146a. The representative images are shown in the left panel, and quantitative analysis results are shown in the right panel. **: p<0.01.

Figure 7: miR-146a inhibits the secretion of IFN-γ in PASMCs. PASMCs were transfected with miR-146a expressing plasmids. Six hours after transfection, the cells were treated with LPS (1μg/ml) until 48 hours post-transfection. The cell culture medium was assessed by ELISA. (Figure 7) illustrates that the level of IFN-γ was higher in the LPS group than controls. In contrast, it’s decreased in cells with miR-146a overexpression. These findings indicate that miR-146a could inhibit IFN-γ secretion in PASMCs.

Discussions

Our study shows that miR-146a could attenuate LPS-induced IFN-γ production, TLR4 expression, and activation of IRAK-1 and NF-κB in PASMCs. The present study confirmed our previous finding that LPS could induce IFN-γ production, [9] and further found that miR-146a could significantly inhibit LPS-induced IFN-γ production. In vascular smooth muscle cells, IFN-γ could stimulate NF-κB activation, leading to inflammation [10]. Those findings suggest that in vascular smooth muscle cells, IFN-γ may be not only an effector of LPS stimulation, but also a stimulator in the process of inflammation. It may play a key role in positive feedback of inflammation. Thus, it’s meaningful to disturb that feedback for reducing inflammation in PH treatment. The miR-146a may be a potential target since it could reduce LPS-induced IFN-γ production in PASMCs as it’s found in our study.

TLR4 is a crucial signaling in promoting inflammation of vascular smooth muscle cells [11-14]. Our study found...
that TLR4 expression in PASMCs was increased in LPS group, whereas it was reversed when transfected with miR-146a. The miR-146a could regulate TLRs and downstream signaling through TNF receptor-associated factor 6 and IL-1 receptor-associated kinase [15]. Thus, our findings suggest that miR-146a could suppress LPS-induced TLR4 expression in PASMCs.

Furthermore, TLR4 could activate NF-κb singling in LPS-induced inflammation of vascular smooth muscle cells from thoracic aortas [16,17]. Similarly, IRAK-1 is also the downstream of TLR4 in vascular smooth muscle cells from thoracic aortas [18]. In pulmonary vascular smooth muscle cells, TLR4 could activate IRAK-1/NF-κB singling in the present study. This study showed that when miR-146a was overexpressed, the LPS-induced activation of IRAK-1 and NF-κB singling in PASMCs was inhibited. Since TLR4/IRAK-1/NF-κB singling in PASMCs could be activated by LPS and then lead to IFN-γ production, [9] we supposed that miR-146a could attenuate the activation of TLR4/IRAK-1/NF-κB singling, resulting in the decreased production of IFN-γ. Therefore, miR-146a may be a potential therapeutic target on inflammation of pulmonary artery, which may provide novel avenues in the therapy of PH.

Conclusion

In conclusion, miR-146a could attenuate LPS-induced IFN-γ production via inhibiting TLR4/IRAK-1/NF-κB pathway in pulmonary arterial smooth muscle cells, which might provide novel target on the therapy of PH.

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Disclosure of Interest

The authors report no conflict of interest.

References

1. McLaughlin VV, Archer SL, Badesch DB, Barst RJ, Farber HW, et al. (2009) ACCF/AHA clinical expert consensus document on pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents. J Am Coll Cardiol 53: 1573-1619.
2. Jin H, Wang Y, Zhou L, Liu L, Zhang P, Deng W, et al. (2014) Melatonin attenuates hypoxic pulmonary hypertension by inhibiting the inflammation and the proliferation of pulmonary arterial smooth muscle cells. J Pineal Res 57(4): 442-450.
3. Price LC, Shao D, Meng C, Perros F, Garfield BE, et al. (2015) Dexamethasone induces apoptosis in pulmonary arterial smooth muscle cells. Respir Res 16: 114.
4. Sun SG, Zheng B, Han M, Fang XM, Li HX, et al. (2011) miR-146a and Krüppel-like factor 4 form a feedback loop to participate in vascular smooth muscle cell proliferation. EMBO Rep 12(1): 56-62.
5. Cao J, Zhang K, Zheng J, Dong R (2015) MicroRNA-146a and -21 cooperate to regulate vascular smooth muscle cell proliferation via modulation of the Notch signaling pathway. Mol Med Rep 11(4): 2889-2895.
6. Huang Y, Crawford M, Higuera-Castro N, Nana-Sinkam P, Ghandi SN (2012) miR-146a regulates macrophage transendothelial migration and pressure-induced inflammation in small airway epithelium. PASEB J 26(9): 3351-3364.
7. Loubaki L, Chabot D, Paré I, Drouin M, Babin R (2017) MiR-146a potentially promotes IV Ig-mediated inhibition of TLR4 signaling in LPS-activated human monocytes. Immunol Lett 185: 64-73.
8. He X, Zheng Y, Liu S, Shi S, Liu Y, et al. (2018) MiR-146A protects small intestine against ischemia/reperfusion injury by down-regulating TLR4/TRAF6/NF-κB pathway. Cell Physiol 233(3): 2476-2488.
9. Wang P, Han X, Mo B, Huang G, Wang C (2017) LPS enhances TLR4 expression and IFN-γ production via the TLR4/IRAK-1/NF-κB signaling pathway in rat pulmonary arterial smooth muscle cells. Mol Med Rep 16(3): 3111-3116.
10. Hsieh CY, Hsu MJ, Hsiao G, Wang YH, Huang CW, Chen SW, et al. (2011) Andrographolide enhances nuclear factor-κappa B subunit p65 Ser536 dephosphorylation through activation of protein phosphatase 2A in vascular smooth muscle cells. J Biol Chem 286(8): 5942-55.
11. Cao X, Zhang L, Chen C, Wang Q, Guo L, et al. (2017) The critical role of ABCG1 and PPARγ/LXRα signaling in TLR4 mediates inflammatory responses and lipid accumulation in vascular smooth muscle cells. Cell Tissue Res 368(1): 145-157.
12. Liu N, Liu JT, Ji YY, Lu PP (2010) C-reactive protein triggers inflammatory responses partly via TLR4/IRF3/NF-κB singling pathway in rat vascular smooth muscle cells. Life Sci 87(11-12): 367-374.
13. Ji Y, Wang Z, Li Z, Liu J (2010) Modulation of LPS-mediated inflammation by feno-fibrate via the TRIF-dependent TLR4 signaling pathway in vascular smooth muscle cells. Cell Physiol Biochem 25(6): 631-640.
14. Ji YY, Liu JT, Liu N, Wang ZD, Liu CH (2009) PPARα activator feno-fibrate modulates angiogenesis in LPS-induced inflammatory responses in vascular smooth muscle cells via the TLR4-dependent signaling pathway. Biochem Pharmacol 78(9): 1186-1197.
15. Staniszewski J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, et al. (2008) Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. Arthritis Rheum 58(4): 1001-1009.
16. Meng Z, Yan C, Deng Q, Gao DF, Niu XL (2013) Curcumin inhibits LPS-induced inflammation in rat vascular smooth muscle cells in vitro via ROS relative TLR4-MAPK/NF-κB pathways. Acta Pharmacol Sin 34(7): 901-911.
17. Yuan J, Liu J, Gao X, Liu J, Yang H, et al. (2014) Palmitate induces interleukin-8 expression in human aortic vascular smooth muscle cells via Toll-like receptor 4/nuclear factor-κB pathway (TLR4/NF-κB). J Diabetes 6(1): 33-41.
18. Guo L, Chen CH, Zhang L, Cao XJ, Ma QL, et al. (2015) IRAK1 mediates TLR4-induced ABCA1 downregulation and lipid accumulation in VSMCs. Cell Death Dis 206: e1949.
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