World Scientific Research Journal Volume 7 Issue 5, 2021
ISSN: 2472-3703 DOI: 10.6911/WSRJ.202105_7(5).0001

Phosphodiesterase 4 Inhibitor Plays An Anti-fibrosis Effect in Ligamentum Flavum Fibroblasts

Likang Wu[1, a] and Guoqiang Song[1, b, *]

[1]School of Pharmacy & School of Medicine, Changzhou University, Changzhou, PR, China

[a]18000061@smail.cczu.edu.cn, [b]sgq@cczu.edu.cn.

Abstract

Objective: Phosphodiesterase 4 inhibitor has anti-inflammatory and anti-fibrosis effects. We applied Rolipram, a phosphodiesterase 4 inhibitor, to ligamentum flavum fibroblasts to investigate its anti-fibrotic effect and involved signaling pathways. Methods: Transforming growth factor-β1 (TGF-β1) was used to stimulate fibroblasts of the ligamentum flavum to make a fibrotic cell model. Fibrotic cells were treated with Rolipram and WAY-262611. Western blotting was used to detect the expression of TGF-β1, collagen I (Col I) and collagen III in the cells and to examine changes in β-catenin signaling pathways. Results: TGF-β1 stimulates fibroblast fibrosis and increase the expression of collagen I, collagen III and endogenous TGF-β1. Rolipram blocks this change, which is related to the suppression of activated p-β-catenin. Conclusion: Rolipram has a good anti-fibrosis effect in vitro, which may be beneficial to the treatment of ligamentum flavum hypertrophy. We propose a new idea here, but a lot of research is needed for its specific application.

Keywords

Phosphodiesterase; Ligamentum flavum; Fibrosis; TGF-β1.

1. INTRODUCTION

Cyclic adenosine 3'5'-monophosphate (cAMP) is one of second messengers involved in multiple cellular processes. The content of cAMP is controlled by the synthesis of adenylate cyclase (ACs) and the hydrolysis of phosphodiesterase (PDE) [1]. The PDE family has 11 isoforms and many subtypes. Their expression and function are different in different tissues and organs [2]. Researchers develop specific PDE inhibitors to restore normal physiological functions by adjusting the content of second messengers. Among them, Among them, PDE4 inhibitors are used for disease treatment due to their effect of inhibiting the release of inflammatory factors [3]. Roflumilast is used to treat COPD and apremilast is used to treat psoriatic arthritis. More small molecule compounds with the activity of inhibiting PDE4 are being developed [4]. At the same time, the indications of PDE4 inhibitors are also being explored.

Ligamentum flavum (LF) hypertrophy is a complex degenerative disease of the spine, which occurs frequently in the elderly [5]. A variety of factors together cause the LF hypertrophy. Transforming growth factor-β1 (TGF-β1) is upregulated in the hypertrophic LF tissue and stimulates fibroblast fibrosis [6], which is a key process of LF hypertrophy. The application of substances with anti-fibrotic activity to LF hypertrophy may become a new treatment method. We applied Rolipram, a PDE4 inhibitor, to LF fibroblasts to investigate the anti-fibrotic effect. This study is an exploration to expand the indications of PDE4 inhibitors and provide new ideas for the treatment of LF hypertrophy.

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202105_7(5).0001

2. MATERIALS AND METHODS

2.1. Compounds and Drugs

Rolipram (CAS: 61413-54-5, CSNpharm, Shanghai, China) was dissolved in a small amount DMSO. It was dilute more than 1000 times with complete medium and the final concentration was 50 nM. WAY-262611 (CAS: 1123231-07-1, CSNpharm, Shanghai, China) is an agonist of the Wnt/β-catenin pathway and the final concentration was 5 μM. Rh-TGF-β1 (CST, #8915, USA) was dissolved in 20mM citrate with a pH =3.0. The final concentration was 5 ng/mL.

2.2. LF Fibroblast Culture

LF fibroblast was presented by Professor Xu from Shanghai Changzheng Hospital. The cells were cultured in DMEM medium containing DMEM (HyClone, USA) supplemented with 10% fetal bovine serum (FBS; Transgen, Beijing, China), 100 U/mL penicillin and 100 pg/mL streptomycin (HyClone).

2.3. Western Blot Analysis

Cell samples were lysed using RIPA buffer containing a protease inhibitors cocktail (Epizyme, Shanghai, China). Lysates were heated in 95 °C for 10 min in protein sample loading buffer (Epizyme). Total cell lysates were separated using 10% SDS-PAGE (Epizyme) and transferred to an Immobilon-P Transfer Membrane (Millipore, USA). Membranes were blocked using 5% nonfat milk dissolved in Tris-buffered saline and then incubated with TGF-β (CST, #3711, USA), Col3A1 (CST, #30565), phospho-β-Catenin (CST, #4176) or collagen Ⅰ (Abcam, ab34170, USA) antibodies. The following day, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Transgen). GAPDH (Transgen) and β-tubulin (Transgen) were used as loading controls. Finally, membranes were visualized using an ECL Western Blotting Detection Reagent (Epizyme).

2.4. Sirius Red Stain and Photo

The drug-treated LF cells were grown in 6-well plates. After fixing by 4% paraformaldehyde solution for 15 min at RT, cells were stained with Sirius red stain kit (X-Y Biotechnology, China) according to the instructions. The results were visualized using a microscope (OLYMPUS) equipped with a digital camera.

3. RESULTS

3.1. TGF-β1 Stimulates LF Fibroblast Fibrosis

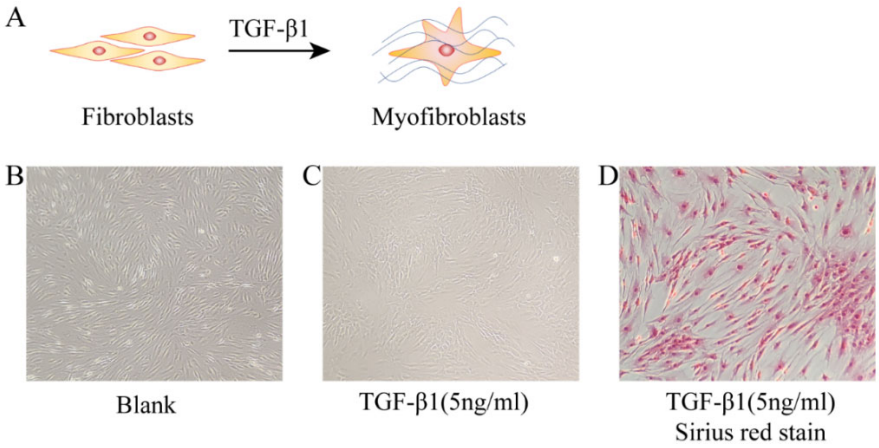

Figure 1. The state of fibroblasts after TGF-β1 stimulation. (A) Fibroblasts differentiate into myofibroblasts. (B) Normal LF fibroblasts. (C) Add recombinant protein TGF-β1 (5 ng/mL, 24h) to the culture medium, the state of fibroblasts. (D) The C was stained with Sirius red.

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202105_7(5).0001

Fibroblasts differentiate into myofibroblasts under the stimulation of TGF-β1 (5 ng/mL) (Figure 1A). The normal ligamentum flavum cells are spindle-shaped, well-arranged and the cell boundaries are clear (Figure 1B). Activated fibroblasts change their spindle-shaped morphology, increase collagen secretion and blur the cell boundaries. The cell proliferation capacity is strengthened and grows in an aggregated state (Figure 1 C-D).

3.2. Rolipram Blocked the Expression of Several Fibrosis Markers Stimulated by TGF-β1

TGF-β1 stimulates fibroblast fibrosis and increases the expression of collagen fibers. Compared with the control group, the expression of Col I and III of fibroblasts increased after TGF-β1 stimulation (Figure 2B-C). Fibroblasts also express TGF-β1. After exogenous TGF stimulation, the expression of endogenous TGF-β1 increases (Figure 2D). After Rolipram treatment, fibrosis was improved and the cells no longer showed a state of aggregate growth (Figure 2A). But there are still differentiated myofibroblasts. Rolipram limits the proliferation capacity of activated fibroblasts. In addition, Rolipram blocked the up-regulation of Col I, III and endogenous TGF-β1 expression stimulated by TGF-β1.

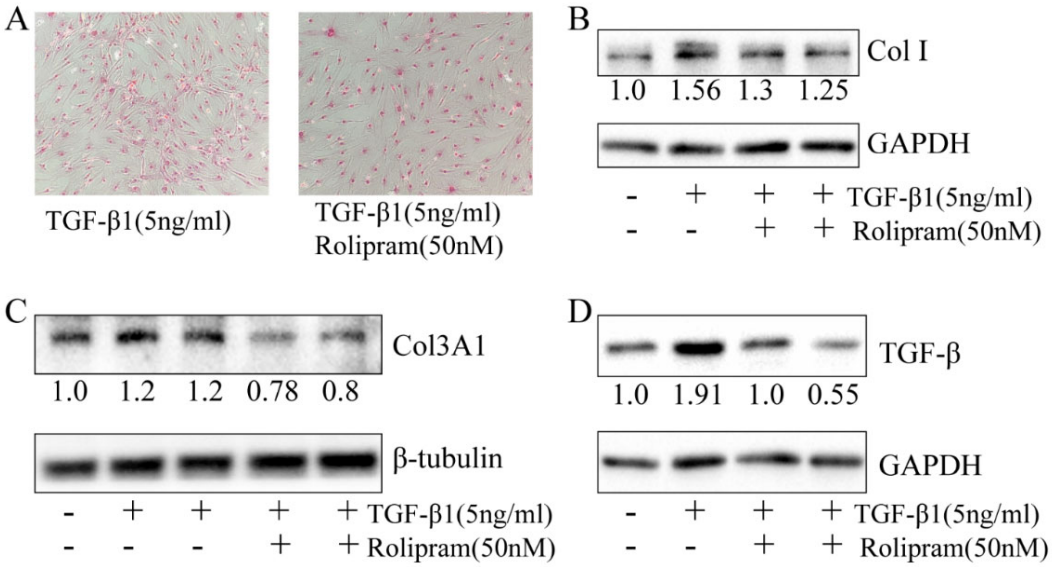

Figure 2. Rolipram treatment improves fibrosis. (A) TGF stimulates fibroblast fibrosis and was blocked by Rolipram. (B) The expression of Col I after cell intervention. Take GAPDH as the internal reference. (C) The expression of Col III after cell intervention. Take β-tubulin as the internal reference. (D) The expression of TGF-β1 after cell intervention. Take GAPDH as the internal reference.

3.3. Rolipram Plays An Anti-Fibrotic Effect by Restoring P-B-Catenin Signal

After fibroblasts are stimulated by exogenous TGF-β1, the expression of endogenous TGF-β1 increases. Rolipram blocked this change (Figure 3A-b). In order to explore the pathway that Rolipram exerts its anti-fibrosis effect. Activation of β-catenin signal with WAY-262611 increased the expression of endogenous TGF-β1 (Figure 3A-c). We tested the phosphorylation of β-catenin. The expression of p-β-catenin increased after TGF-β1 stimulation and was restored by Rolipram (Figure 3B).

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202105_7(5).0001

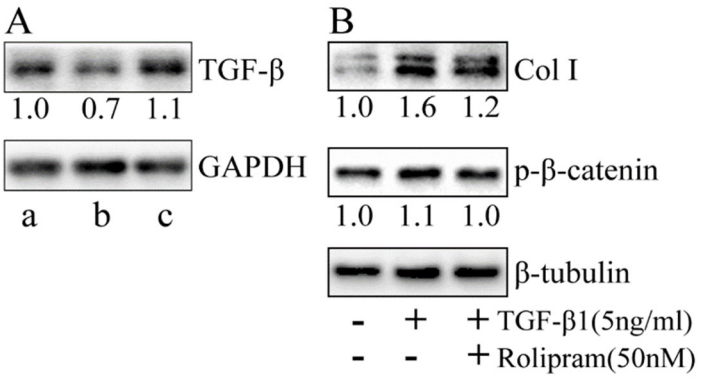

Figure 3. The pathway through which Rolipram exerts its anti-fibrotic effect. (A) The expression of C in fibroblasts after different treatments. a). TGF-β1 (5ng/ml). b). TGF-β1 (5ng/ml)+Rolipram (50nM). c). TGF-β1 (5ng/ml)+WAY-262611 (5μM). Take GAPDH as the internal reference. (B) After the treatment of TGF-β1 (5ng/ml) and Rolipram (50nM), the expression of Col Ⅰ and p-β-catenin. Take β-tubulin as the internal reference

4. DISCUSSION

Fibrosis is an important step in wound healing. After epidermal trauma, a large amount of extracellular matrix forms scars to prevent bacterial invasion [7]. If this extracellular matrix deposition cannot be controlled, it can cause fibrotic diseases. In the hypertrophic LF tissue, macrophage infiltration and new blood vessels increased [8]. Macrophages and vascular endothelial cells release large amounts of TGF-β1 [9]. TGF-β1 is an important fibrosis-promoting factor, which promotes cell proliferation, migration and secretion of a large amount of extracellular matrix [10]. In our study, recombinant TGF-β1 was used to stimulate fibrosis of LF fibroblasts, and a fibrotic cell model was made. The expression of Col I and III increased under the stimulation of TGF-β1 (figure 2B-C). The normal LF is composed of most elastic fibers and a small amount of collagen fibers [11]. In the process of LF hypertrophy, elastic fibers are lost and collagen fibers accumulate. The increased collagen fibers are mainly Col I and III [12]. TGF-β expression is low in normal fibroblasts. After being stimulated by exogenous TGF-β1, the ability of fibroblasts to express endogenous TGF-β1 is increased [13]. This is also one of the reasons for the increased expression of TGF-β1 in the hypertrophic LF.

We apply Rolipram to the cell model of fibrosis. Rolipram improves the fibrotic changes stimulated by TGF-β1. It suppresses the increase of Col I and III and also suppresses endogenous TGF-β1 (figure 2). But for the differentiated myofibroblasts, Rolipram only limited the proliferation function and did not reverse the myofibroblasts to become fibroblasts. Rolipram inhibits PDE4 activity and mediates downstream effector molecules by increasing the intracellular cAMP content [14]. In Togo's research, PDE4 inhibitors inhibited TGF-β1 stimulated lung fibroblasts fibrosis by promoting prostaglandin-E2 (PGE2) [15]. In Ding's study, restoring cAMP by Rolipram may ameliorate renal fibrosis by blocking the TGF-β pathway [16]. Katarzyna develops and synthesizes a series of compounds that inhibit Pan-PDE activity. The compound has an inhibitory effect on the extracellular matrix deposition stimulated by TGF-β1. cAMP-response element binding protein (CREB) activated by cAMP regulates gene transcription and reduces the transcription of fibrotic genes mediated by TGF-β1/Smad2/3 [17].

In the liver fibrosis model stimulated by lipopolysaccharide, Rolipram inhibited the activation of Smad3 of TGF-β signal to exert anti-fibrosis effect [18]. In addition to the classic TGF-β/Smad pathway, TGF-β1 may also function through other pathways [19]. Compared with simple TGF-β1 stimulation, the co-stimulation of TGF-β1 and WAY-262611 increased the endogenous TGF-

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202105_7(5).0001

β1 expression of fibroblasts more. The activation of Wnt/β-catenin signal is conducive to TGF-β. Rolipram restored the elevated p-β-catenin. This shows that Rolipram blocks the fibrosis of TGF-β1 by inhibiting the phosphorylation of β-catenin.

5. CONCLUSION

PDE4 inhibitors can inhibit fibrosis stimulated by TGF-β1. Rolipram inhibits the expression of Col I and III and inhibits the proliferation of activated fibroblasts. At the same time, it also inhibits the expression of endogenous TGF-β1. This effect is related to blocking the activation of β-catenin pathway.

ACKNOWLEDGMENTS

Thanks to Professor Xu from Shanghai Changzheng Hospital for his help on this study.

REFERENCES

[1] A.T. Bender, J.A. Beavo. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev, Vol. 58(2006) No. 3, p.488-520.

[2] N. Musheshe, M. Schmidt, M. Zaccolo. cAMP: From Long-Range Second Messenger to Nanodomain Signalling. Trends Pharmacol Sci, Vol. 39(2018) No. 2, p.209-222.

[3] X. Yang, N. Kambe, R. Takimoto-Ito, et al. Advances in the pathophysiology of atopic dermatitis revealed by novel therapeutics and clinical trials. Pharmacol Ther, (2021), p.107830. (epub online)

[4] Z. Chu, Q. Xu, Q. Zhu, et al. Design, synthesis and biological evaluation of novel benzoxaborole derivatives as potent PDE4 inhibitors for topical treatment of atopic dermatitis. Eur J Med Chem, (2021), p.113171. (epub online)

[5] Y. Hori, A. Suzuki, K. Hayashi, et al. Long-term, Time-course Evaluation of Ligamentum Flavum Hypertrophy Induced by Mechanical Stress: An Experimental Animal Study. Spine (Phila Pa 1976), (2020). (epub online)

[6] J.B Park., H. Chang, J.K. Lee. Quantitative analysis of transforming growth factor-beta 1 in ligamentum flavum of lumbar spinal stenosis and disc herniation. Spine (Phila Pa 1976), Vol. 26(2001) No.21, p.492-495.

[7] P. Pakshir, B. Hinz. The big five in fibrosis: Macrophages, myofibroblasts, matrix, mechanics, and miscommunication. Matrix Biol, Vol. (2018) p.68-69:81-93.

[8] T. Saito, M. Hara, H. Kumamaru, et al. Macrophage Infiltration Is a Causative Factor for Ligamentum Flavum Hypertrophy through the Activation of Collagen Production in Fibroblasts. Am J Pathol, Vol. 187(2017) No. 12, p.2831-2840.

[9] M. Löhr, J.A. Hampl, J.Y. Lee, et al. Hypertrophy of the lumbar ligamentum flavum is associated with inflammation-related TGF-β expression. Acta Neurochir (Wien), Vol. 153(2011) No. 1, p.134-41.

[10] M. Lodyga, B. Hinz. TGF-β1 - A truly transforming growth factor in fibrosis and immunity. Semin Cell Dev Biol, Vol. 101(2020), p.123-139.

[11] K. Sairyo, A. Biyani, V.K. Goel, et al. Lumbar ligamentum flavum hypertrophy is due to accumulation of inflammation-related scar tissue. Spine (Phila Pa 1976), Vol. 32(2007) No. 11, p.340-347.

[12] Y.L. Cao, Y. Duan, L.X. Zhu, et al. TGF-β1, in association with the increased expression of connective tissue growth factor, induce the hypertrophy of the ligamentum flavum through the p38 MAPK pathway. Int J Mol Med, Vol. 38(2016) No. 2, p.391-398.

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202105_7(5).0001

[13] J.W. Hur, T. Bae, S. Ye, et al. Myofibroblast in the ligamentum flavum hypertrophic activity. Eur Spine J, Vol. 26(2017) No. 8, p.2021-2030.

[14] G.S. Baillie, G.S. Tejeda, M.P. Kelly. Therapeutic targeting of 3',5'-cyclic nucleotide phosphodiesterases: inhibition and beyond. Nat Rev Drug Discov, Vol. 18(2019) No. 10, p.770-796.

[15] S. Togo, X. Liu, X. Wang, et al. PDE4 inhibitors roflumilast and rolipram augment PGE2 inhibition of TGF-{beta}1-stimulated fibroblasts. Am J Physiol Lung Cell Mol Physiol, Vol. 296(2009) No. 6, p.959-969.

[16] H. Ding, F. Bai, H. Cao, et al. PDE/cAMP/Epac/C/EBP-β Signaling Cascade Regulates Mitochondria Biogenesis of Tubular Epithelial Cells in Renal Fibrosis. Antioxid Redox Signal, Vol. 29(2018) No. 7, p.637-652.

[17] K. Wójcik-Pszczoła, G. Chłoń-Rzepa, A. Jankowska, et al. A Novel, Pan-PDE Inhibitor Exerts Anti-Fibrotic Effects in Human Lung Fibroblasts via Inhibition of TGF-β Signaling and Activation of cAMP/PKA Signaling. Int J Mol Sci, Vol. 21(2020) No. 11, p.4008.

[18] L. Gobejishvili, S. Barve, K. Breitkopf-Heinlein, et al. Rolipram attenuates bile duct ligation-induced liver injury in rats: a potential pathogenic role of PDE4. J Pharmacol Exp Ther, Vol. 347(2013) No. 1, p.80-90.

[19] T.M. Holm, J.P. Habashi, J.J. Doyle, et al. Noncanonical TGFβ signaling contributes to aortic aneurysm progression in Marfan syndrome mice. Science, Vol. 332(2011) No. 6027, p.358-361.