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PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330

Int J Hum Genet, 22(4): 242-249 (2022)
DOI: 10.31901/24566330.2022/22.04.841

Study on Thyroxine Preventing Coronary Atherosclerosis through TGFBR1 Signaling Pathway

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KEYWORDS Migration. Proliferation. Thyroxine. Transforming Growth Factor b Receptor 1(TGF-b). Vascular Intimal Hyperplasia

ABSTRACT To explore the mechanism of Thyroxine inhibiting Human Aortic Vascular Smooth Muscle Cell (HASMC) proliferation, migration and vascular intimal hyperplasia through the transforming growth factor β receptor 1(TGFBR1). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium (MTS), 5-ethynyl-2' -deoxyuridine (EdU) staining, Transwell, cell scratch test and Western bloting were performed sequentially to detect the effects of Thyroxine on HASMC and the TGFBR1 signaling pathway. A cell model of TGFBR1 overexpression was constructed to further explore the mechanism of TGFBR1 in Thyroxine inhibiting the activation of HASMC. Thyroxine can inhibit HASMC proliferation and migration, and the activation of TGFBR1 signaling pathway, both in a concentration-dependent manner. Following the overexpression of TGFBR1, the effect of Thyroxine on inhibiting HASMC proliferation, migration and activation of TGFBR1 signaling pathway was partially inhibited. Thyroxine can inhibit the proliferation, migration and vascular intimal hyperplasia of HASMC by inhibiting the activation of TGFBR1 signaling pathway, thereby playing a role in preventing and treating vascular restenosis and related diseases.

INTRODUCTION

Coronary atherosclerotic heart disease is the most usual type of Cardiovascular Disease (CVD) that can seriously endanger human health and life. With the continuous development and progress of treatment technology, treatment methods and related equipment, percutaneous coronary intervention has become an important treatment method for Coronary Atherosclerotic Heart Disease (CHD). However, clinical observations have found that, although the therapy can significantly improve the symptoms of myocardial ischemia, cardiac function, and improve the quality of life and long-term prognosis, in-stent restenosis after PCI greatly limits the benefits of these interventions. In recent years, with the popularization and application of drug-eluting stents in CHD patients, the incidence of intrastent restenosis after PCI has been improved to a certain extent, but patients receiving drug-eluting stents have re-stents after surgery. The incidence of stenosis is still as high as 10 percent. Therefore, how to effectively prevent restenosis after Percutaneous Coronary Intervention (PCI) is still an urgent problem to be solved in the treatment and prognosis improvement of the patients with coronary heart disease (Benjamin et al. 2019; Aringazina et al. 2018; Shlofmitz et al. 2018; Zhang et al. 2018). Normal vascular smooth muscle cells are located in the vascular mesomembrane, and their proliferation ability is very low, and the proliferation index can hardly be detect-

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ed. However, after vascular injury, VSMC can be observed to migrate from the mesomembrane to the intima and proliferate abnormally. For the rat model of acute vascular injury caused by balloon angioplasty, after acute vascular injury, vascular smooth muscle cells can be observed to pass through the inner elastic membrane and enter the intima. Some studies have shown that after the injury of vascular smooth muscle cells, the medial smooth muscle cells begin to proliferate, and the maximum proliferation rate can reach 46 percent within 48 hours after the injury. The proliferation of vascular smooth muscle cells can be observed in the intima on the 4th day after the injury, the maximum proliferation rate can reach 73 percent on the 7th day after the injury, and the intima thickening is the most serious 3-4 weeks after the injury. The results showed that the migration of vascular smooth muscle from the media to the intima and abnormal proliferation were the main causes of intimal hyperplasia. Intimal hyperplasia is an important pathological basis leading to vascular restenosis and other related diseases. It can be seen that it can effectively inhibit the abnormal proliferation and migration of VSMC and prevent vascular restenosis and related diseases (Lu et al. 2020).

Thyroxine can promote metabolism, maintain normal growth and development, and improve body sensitivity. Clinically, it is mainly used to treat cretinism, myxedema and other hypothyroidism. Our research is to explore whether Thyroxine may inhibit Vascular Smooth Muscle Cell (VSMC) proliferation, migration and vascular intimal hyperplasia through inhibiting TGFBR1, thus playing a role in preventing and treating restenosis after PCI.

METHODOLOGY

Cell Grouping and Detection of HASMC Cell Proliferation by MTS Method and EdU

MTS Method

HASMC cells were purchased from the cell bank of the Chinese Academy of Sciences, which was then put into an incubator at 37 ^{o}C with 5% CO_2.The cell viability was tested according to the MTS method, cells in the logarithmic growth phase were taken out, and centrifuged at 800 rpm, Following this,the supernatant was abandoned. Following the counting, the cell was adjusted to 5×10^4/ml and put into a 96-well culture plate with 200 µl per well,and the groupings were as follows: A: Control; B: 5 µM Thyroxine; C: 10 µM Thyroxine; and D: 20 µM Thyroxine. The microplate reader was applied to measure the absorbance (OD value) at a wavelength of 490 nm. Cell viability = [OD-OD (blank)] / [OD (control)-OD (blank)] ×100 percent.

Edu Staining Method

The grouping is the same as that in the MTS method. The staining was operated according to the instructions. The cells (blue) and the EdU-positive cells (red) in 3 fields of view were randomly counted under a fluorescence microscope. EdU-positive percentage = [EdU-positive cells / total number of cells] ×100 percent.

Transwell Cell Migration Test to Detect the Migration Ability of Vascular Smooth Muscle Cells

The grouping is the same as the above. Transwell without Matrigel gel was used for the experiment. The cells used in the routine experiment were adjusted to a concentration of 1×10^5/ml. The cells were grouped as above. Follow the instructions, the number of cells penetrating the chamber was counted under an inverted microscope. The number of migrated cells in each group were counted with ImageJ software.

Western Blot to Detect the Expression of Proliferation, Migration and Apoptosis-related Proteins in Vascular Smooth Muscle Cells

The grouping is the same as above. The operation was conducted according to the instructions. The statistical results were analyzed by ImageJ software.

Model Establishment of Overexpressed TGFBR1 Adenovirus Vector, Grouping and Related Experiments

The cell density is fused to about 60 percent, the number of cells is estimated, and the

amount of Ad-TGFBR1 and ad NC virus required for transfection is calculated. Then, the required Ad-TGFBR1and ad NC virus are added to the prepared cell culture system and cultured in serum-free medium. After transfection for 4h, the culture is continued in normal full medium for 48h.The cells were divided into A: Ad-NC virus group, B: Ad-NC + 20 μM Thyroxine group, C: Ad-TGFBR1 virus group, and D: Ad-TGFBR1 + 20 μM Thyroxine group. The effect of Thyroxine on the activation of HASMC TGFBR1 signaling pathway was detected according to the above method.

Statistical Analysis

The data (mean ± standard deviation) were analyzed using SPSS 20.0 software. One-way ANOVA was used when the data passed the test of homogeneity of variance, and the Bonferroni method was used for pairwise comparison between groups. The Welch robust estimation was employed for uneven variance, and the Dunnett's T3 method was applied for pairwise comparison. $P<0.05$ was considered statistical significance, and each experiment was repeated for 3 times at least.

RESULTS

Thyroxine can Inhibit Cell Proliferation

After 48 hours of treatment with different concentrations of Thyroxine, the proliferation of HASMC was detected by the MTS method. As the concentration increased, the proliferation of HASMC showed a decreasing trend, and in a concentration-dependent manner. As the concentration of Thyroxine increased, the number of red cells decreased gradually. It can be found that the results of EdU staining were consistent with the results of MTS. As the concentration of Thyroxine increased, the proliferation of HASMC decreased gradually, and in a concentration-dependent manner, as shown in Figures 1 and 2.

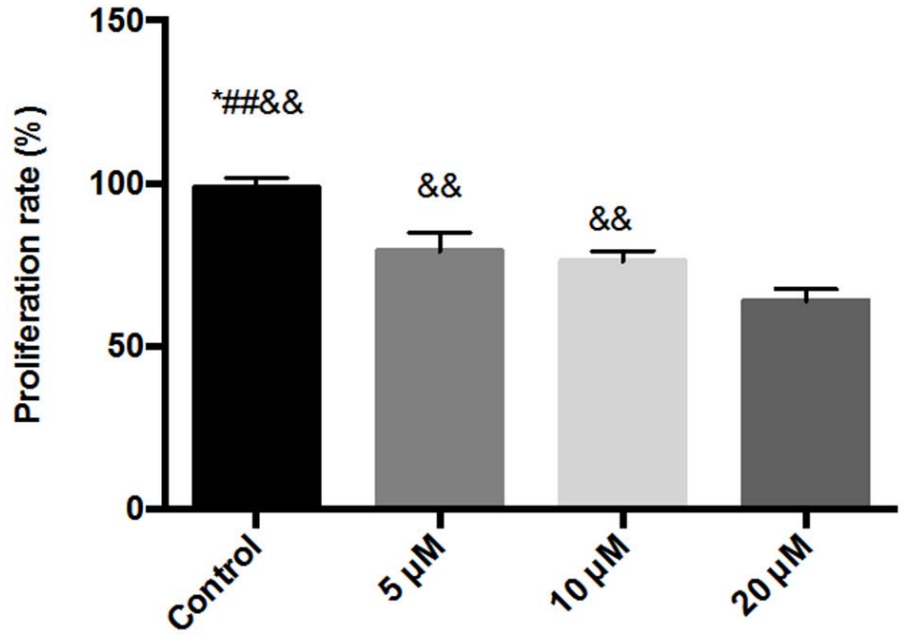

Fig. 1. MTS method to detect HASMC proliferation
**** p<0.01 vs 5 mM * p<0.05 vs 5 mM ## p<0.01 vs 10 mM**
 # p<0.01 vs 10 mM && p<0.01 vs 20 mM

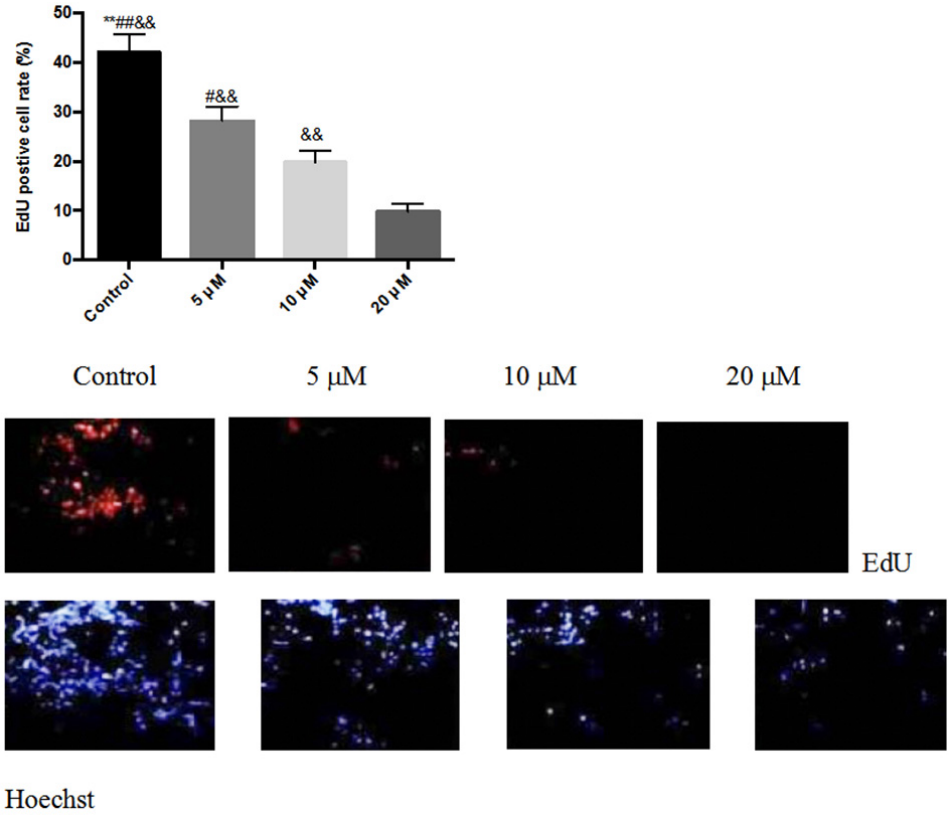

Fig. 2. EdU staining method to detect the proliferation of HASMC
**** p<0.01 vs 5 mM ## p<0.01 vs 10 mM # p<0.01 vs 10 mM**
&& p<0.01 vs 20 mM

Thyroxine can Inhibit HASMC Cell Migration

The Transwell cell migration test was applied to detect the migration ability of HASMC. With the increase of drug concentration, the migration ability of HASMC decreased. There were statistical differences in all groups, and all concentration-dependent, as shown in Figure 3.

Thyroxine Inhibits the Expressions of Proliferation-related Proteins in a Concentration-dependent Manner

It was found that, compared with the Control group, with the increase of drug concentration, the expression of CydinDl and PCNA decreased to varying degrees. The drug inhibited the expression of PCNA and Cyclin D1 in a concentration-dependent manner, as shown in Figure 4.

Thyroxine Inhibited the Expressions of HASMC Cell Migration-related Proteins

The expressions of MMP-2 and MMP-9 proteins were measured. As the drug concentration increased, the expressions of MMP2 and MMP9 proteins were reduced to varying degrees, in a concentration-dependent manner (Fig. 5).

Thyroxine Inhibited TGFBR1 Activation in HASMC Cells

Based on relevant literature research and analysis, in order to further explore whether the

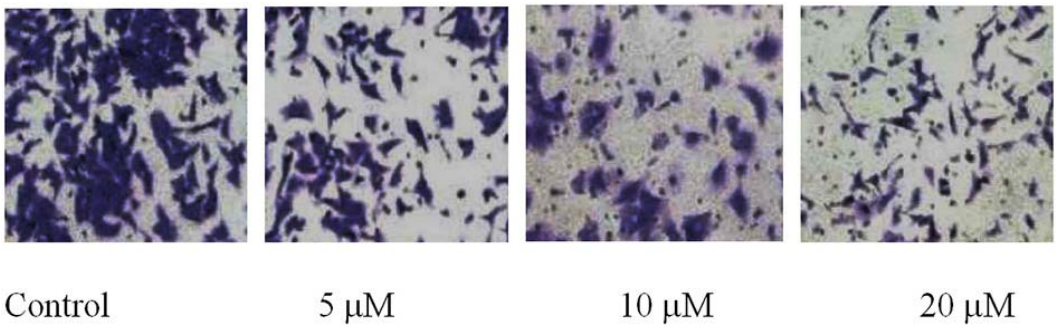

Fig. 3. Thyroxine inhibited the migration of HASMC cells

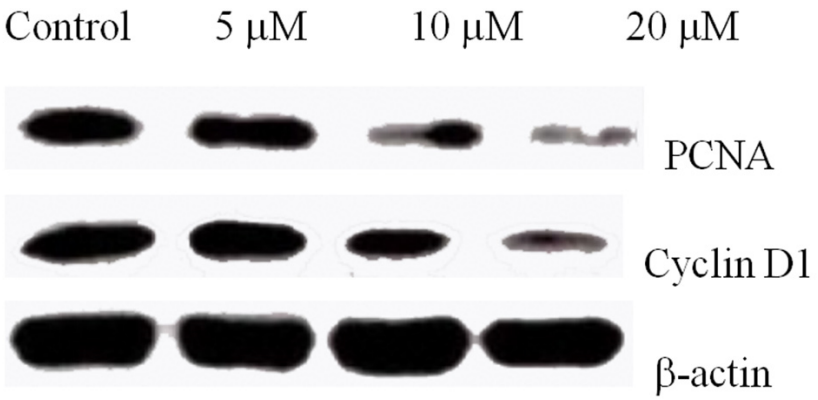

Fig. 4. Thyroxine inhibited the expressions of proliferation-related proteins

mechanism of drugs inhibiting HASMC is related to the TGFBR1 pathway, the researchers detected the expressions of TGFBR1 pathway-related proteins. Compared with the Control group, Thyroxine inhibited TGFBR1 in a concentration-dependent way. Smad-2 and Smad-3 are the downstream signal molecules of TGFBR1. The drug can inhibit the phosphorylation level of Smad-2 and Smad-3 in a concentration-dependent manner, as shown in Figure 6.

Following the Overexpression of TGFBR1, the Inhibitory Effect of Thyroxine on the Activation of TGFBR1 Signaling Pathway is Partially Inhibited

Following the overexpression of TGFBR1 in HASMC, compared with the Ad-NC virus group, the phosphorylation level of TGFBR1 in the TGFBR1 overexpression group was significantly increased. After drug treatment, compared with the Ad-NC + 20 mM Thyroxine group, with TGFBR1 overexpression, the inhibitory effect of the drug on the phosphorylation level of TGFBR1 was partially weakened. Which are the downstream molecules of TGFBRI, it was found that after overexpression of TGFBR1, After drug treatment, compared with the Ad-NC+20 mM Thyroxine group, the inhibitory effect of the drug on the phosphorylation was also partially weakened after the overexpression of TGFBR1. The results further proved that TGFBR1 is the target of Thyroxine, which can inhibit the proliferation and migration of VSMCs by inhibiting the activation of the TGFBRl/Smads, as shown in Figure 7.

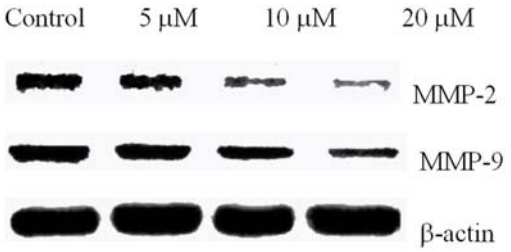

Fig. 5. The effects of Thyroxine on the expressions of cell migration-related proteins

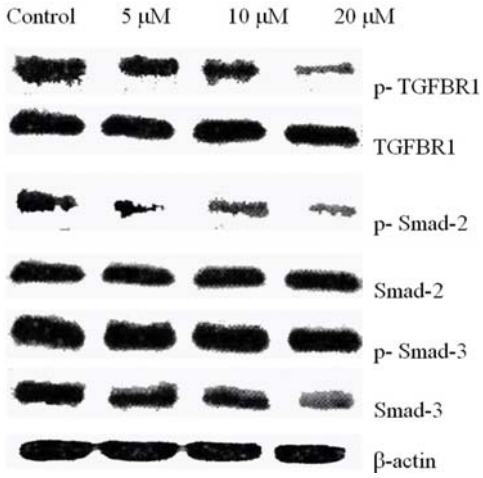

Fig. 6. The effect of Thyroxine on the activation of TGFBR1 signaling pathway in HASMC cells

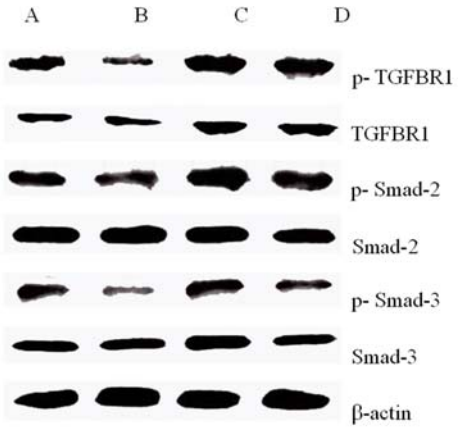

Fig. 7. The effect of Thyroxine on the overexpression of TGFBRI signaling pathway
A: Ad-NC B: Ad-NC+20mM C: Ad-TGFBR1 D: Ad-TGFBR1+20mM

DISCUSSION

HASMC cells are located in the vascular media, with relatively low proliferation ability. The proliferation index is almost undetectable. However, after vascular injury, HASMC can be observed to migrate from the media to the intima and to proliferate abnormally. In classic ultrastructural studies on the rat model of acute vascular injury caused by balloon angioplasty, it is revealed that after acute vascular injury, vascular smooth muscle cells can be observed to penetrate the inner elastic membrane and enter the intima (Sanchez et al. 2019). In addition, the use of 3H thymidine labeling also showed that media smooth muscle cells began to proliferate after vascular smooth muscle cell injury, and within 48 hours after injury, the maximum proliferation rate could reach 46 percent. The proliferation of can be observed in the intima, and the maximum proliferation rate could reach 73 percent on the 7th day after injury, and the thickest vascular intima is at 3-4 weeks after injury. The results showed that vascular smooth muscle migrates from the media to the intima and proliferates abnormally, which is the main reason for the proliferation of the damaged vascular intima. Previous studies have shown that, intimal hyperplasia is an important pathological basis for related diseases such as vascular restenosis. It can be seen that the drug can effectively inhibit the abnormal proliferation and migration of HASMC, thus preventing and treating vascular restenosis and related diseases (Vazhappilly et al. 2019; Mahmoud et al. 2019; Wang et al. 2019).

TGFb is a cytokine that can regulate various functions of cells, including the proliferation and migration of smooth muscle cells (Zou et al. 2018; Han et al. 2018). Previous studies have shown that TGF-b can induce phosphorylation of TGFBR1 at Serl65 after binding to TGFBR2, meaning that the specificity of TGF- receptor signal transduction is regulated by the phosphorylation of TGFBR1 at Serl65 (Torrado et al. 2018; Hong et al. 2018; Li et al. 2020). In this study, the researchers found that Thyroxine can significantly inhibit the phosphorylation level of TGFBR1 (Sed65). Activated TGFBR1 specifically can recognize and phosphorylate this, and then the phosphorylated this are transferred to the nu-

cleus together with Smad4, which activates various transcription programs by binding to Smad elements and regulates a series of biological functions (Zhang et al. 2021; Parichatikanond et al. 2020). Based on this, the researchers further studied the effect of Thyroxine on the phosphorylation of Smad3 and Smad2, and it is found that Thyroxine can significantly reduce the phosphorylation level of this protein. To further determine whether Thyroxine can inhibit the proliferation and migration of VSMCs by inhibiting the Smads signaling pathway through inhibiting TGFBR1, the researchers further measured the expressions of this protein, the downstream signals of TGFBR1. The results suggest that, after the overexpression of TGFBR1, the phosphorylation levels of this protein are also significantly increased, and the inhibitory effect of Thyroxine on the phosphorylation level of this protein is partially eliminated, suggesting that Thyroxine can further inhibit the activation of TGFBR1, thus inhibiting the activation of this protein. The TGFBRl/Smads signal transduction pathway is also the mechanism by which Thyroxine inhibits the proliferation and migration of VSMCs.

CONCLUSION

In conclusion, Thyroxine can inhibit the proliferation, migration and vascular intimal hyperplasia of HASMC by inhibiting the activation of TGFBR1 signaling pathway, thereby playing the role of preventing and treating vascular restenosis and related diseases.

RECOMMENDATIONS

Thyroxine can inhibit HASMC proliferation and migration, as well as the activation of TGFBR1 signaling pathway (PU5) in a concentration-dependent manner. After the overexpression of TGFBR1, the effect of Thyroxine on inhibiting HASMC proliferation, migration and activation of TGFBR1 signaling pathway was partially inhibited.

ABBREVIATIONS

ANOVA: analysis of variance.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This paper does not contain any studies with human participants or animals performed by any of the authors.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analysed during this study are included in this. Further enquiries can be directed to the corresponding author.

FUNDING

The authors have not received any funding support.

COMPETING INTERESTS

There are no potential conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

QL is responsible for the study concepts & design, experimental studies, statistical analysis, manuscript editing; HBY is responsible for the manuscript preparation; XHZ and QYX are responsible for the literature research, data acquisition & analysis; XZ is responsible for the guarantor of integrity of the entire study, definition of intellectual content, clinical studies, manuscript review. All authors read and approved the final manuscript.

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Paper received for publication in March, 2022
Paper accepted for publication in May, 2022