Pakistan J. Zool., vol. 56(1), pp 59-67, 2024 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20220905090943

The Expression and Distribution of MIF, ERK, Akt and Rb/E2F in Pulmonary Hypertension in Broilers

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Article Information
Received 31 March 2021
Revised 12 November 2022
Accepted 09 December 2022
Available online 23 Decembesr 2022 (early access)
Published 04 December 2023

Authors' Contribution
YW designed this research, carried out experimental work and data processing. HL participated in the first draft of the paper. LH helped analyze the data. WW managed and guided the project. All authors have approved this manuscript for publication.

Key words
Broilers, Pulmonary hypertension (PH), Macrophage migration inhibition factor (MIF), Cyclin D1, ERK, Akt

ABSTRACT

Pulmonary hypertension (PH) is a metabolic disorder that occurs in broilers. It is characterized by pulmonary hypertension, right heart failure and a large amount of yellowish liquid accumulated in the body cavity, and has become one of the most causes of non-infectious death in broiler industry worldwide. The macrophage migration inhibition factor (MIF) and related protein and gene expressions in its downstream signal pathways need to be studied to explore the mechanism of MIF participating in PH. In this study, 4-5 weeks broilers with clinical PH were collected as experimental (PH) group, while the healthy broilers were taken as control group. The related mRNA expression levels were determined by qRT-PCR. The protein expressions and distributions were detected by Western blotting and immunohistochemistry. The results showed that, the mRNA expression of *MIF, Raf, ERK, Akt, GSK3β, cyclin D1, Rb, E2F* in PH group were significant higher than that of control. Moreover, the expression levels of MIF, p-Raf, p-ERK, p-Akt, p-GSK3β, cyclin D1, p-Rb, E2F proteins in PH group were significantly higher than that of the control. It can be concluded that, MIF participated in the occurrence of PH, possibly through Raf/ERK and Akt/GSK3β pathways by increasing the expression of cyclin D1, phosphorylation of Rb, and transcription function of E2F, thereby induced cell proliferation, caused pulmonary artery vascular remodeling, and lead to PH. This study aims to provide theoretical basis for finding new therapeutic targets for broiler PH.

INTRODUCTION

Pulmonary hypertension (PH), also known as pulmonary hypertension syndrome (PHS) or ascites syndrome (AS), is a metabolic disorder of broilers characterized by pulmonary hypertension, right heart failure, and accumulation of a large amounts of yellowish liquid in the body cavity. PH is one of the main causes of broiler death, which occurs in broiler flocks worldwide, and has become one of the main causing losses to broiler production (Hassanzadeh *et al*., 2014; Hernandez, 1987; Wideman *et al*., 2007). The formation of PH in broilers is affected by factors, such as management, environment, nutrition and heredity. Some factors include the rapid growth of broilers, relatively weak cardiopulmonary function, and congenital factors related to heredity are easy to cause relative or absolute hypoxia of the body. In addition, improper feeding and management such as poor ventilation, environmental and climatic changes such as winter cold stimulation, nutrient imbalance such as vitamin C deficiency, and other factors diseases cause the body to be in a state of stress and immune dysfunction. These factors will aggravate the degree of hypoxia, lead to changes in the structure and function of the pulmonary artery and the formation of PH (Al-Masri and Hassanzadeh, 2010; Daneshyar *et al*. 2012; Hu *et al*., 2017).

In the development of PH in broilers, the increase of pulmonary artery pressure is the core index, and vascular remodeling is a very critical step. The structural changes of vascular remodeling are mainly reflected in the following aspects. The proliferation, migration and transformation of vascular endothelial cells (VECs) that constitute the intima. The proliferation of pulmonary artery smooth muscle cells (PASMCs) and the thicker of tunica media, and the middle membrane becomes thicker. Fibroblasts also proliferate and secrete extracellular matrix, which gathers around the vascular wall. And various immune cells outside the pulmonary artery secrete cytokines, growth

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0030-9923/2024/0001-0059 $ 9.00/0

factors, chemokines, etc. These changes eventually lead to smaller diameter of pulmonary artery, lower blood flow velocity, and lower elasticity, leading to the occurrence of PH. Therefore, the proliferation of cells constituting the pulmonary artery is the main reason for pulmonary vascular remodeling (Wideman *et al*., 2013; Mohammad *et al*., 2014).

Macrophage migration inhibitory factor (MIF) is a pleiotropic proinflammatory cytokine involved in the regulation of inflammation and cell growth. MIF plays a key role in the upstream of signal cascade and can significantly reduce inflammatory cell infiltration and other inflammatory cytokines, including interleukin-1 (IL-1), IL-6, IL-8, IL-12, interferons (IFNs), reactive oxygen species (ROS), nitric oxide (NO) and tumor necrosis factor-α (TNF-α) (Morrison and Kleemann, 2015; Calander and Roger, 2003). It was found that MIF could cause the activation of many signal cascades and play a key role in cell proliferation through the extraceller signal regulated kinase (ERK) and protein kinase B (Akt) signal pathways (Zhang *et al*., 2017). MIF could also effectively regulate Rb (retinoblastoma protein)/E2F (E2F transcription factor) pathway, and MIF deficient cells showed E2F dependent growth abnormalities (Petrenko and Moll, 2005). Overall, MIF may lead to two main characteristics of pulmonary hypertension, the pulmonary artery remodeling caused by perivascular inflammation and cell proliferation (Jalce and Guignabert, 2020).

Our previous study (Li *et al*., 2017) found that the thickness of pulmonary artery intima and media were increased in PH broilers; moreover, MIF was involved in the pathogenesis and its mechanism may be that it upregulates the expression of cyclin D1 by activating ERK pathway. However, whether there are other proteins and genes involved in ERK pathway, and whether Akt and Rb/E2F participate in the process of broiler PH, need to be further investigated.

Therefore, in this study, the ERK, Akt and Rb/E2F pathways were analyzed, to explore the genes and proteins related to pulmonary artery remodeling in PH broilers, and tried to further explain the mechanism of MIF participating in PH and analyzed the role of MIF. This study aims to find a new breakthrough point for broiler PH treatment.

MATERIALS AND METHODS

Main reagents

RNAiso Plus reagent, Prime Script™ RT Master Mix and SYBR Green RT-PCR Kit were purchased from Takara Bio (Dalian, China). Anti-β-actin, ECL Western blotting substrate and goat anti-mouse IgG-HRP were purchased from Solarbio Life Sciences (Beijing, China). Anti-phospho-Raf1 (Ser338), Anti-phospho-MAPK1/MAPK3 (Tyr204), Anti-Rb, Anti-phospho-Rb1 (Ser807), Anti-E2F antibodies were purchased from Sango Biotech Co., Ltd. (Shanghai, China). Polyclonal Antibodies (GSK-3β, Raf, and cyclin D1), and Lamin B monoclonal antibody were purchased from Proteintech Group (Wuhan, China). Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb, Phospho-GSK-3β (Ser9) (5B3) Rabbit mAb, and p44/42 MAP Kinase (L34F12) Mouse mAb were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-Akt1/2/3 antibody was from Abcam (Cambridge, UK). SABC-POD (Goat IgG) Kit, DAB chromogenic kit, and goat anti-rabbit IgG/HRP were purchased from Boster Biological Technology (Wuhan, China).

Animal materials and samples collection

A sufficient number of broilers at 4–5 weeks old of the same batch were purchased from Shanxi Elephant Agriculture and Animal Husbandry Group (Lyuliang, China). Two groups were set: in the pulmonary hypertension (PH) group, 20 broilers with PH were selected according to the naturally occurring population. The criteria for autopsy were that the abdominal cavity contained more than 50 mL of clear and transparent light yellow or brown liquid, and the ascites heart index (AHI) of ascites was >0.25. In the control group (N group), 20 healthy broilers from the same source were used.

During sampling, the upper part of the right lung was cut and put into a cryotube for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis. The remaining parts of the right lung were washed with cooled normal saline, dried with sterilized filter paper, then separately packed into cryotubes and stored in liquid nitrogen for immunoblot analysis. The middle part of the left lung were taken and fixed with Bouin's solution. After dehydration and transparency, they were embedded in paraffin wax and preserved for immunohistochemistry (IHC) analysis.

qRT-PCR analysis

The lung tissue was ground with liquid nitrogen, and the total RNA was extracted from each sample using Trizol reagent and its concentration was determined. The cDNA was synthesized as follows: at 37°C for 15 min from 0.8 μg total RNA with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 85 °C for 5 s, under detailed system 5× Primescript RT Mix 2.0 μL, total RNA 1.0 μL, RNase Free dH_2O 7.0 μL according to the manufacturer's instructions. According to the sequence of *Gallus gallus* genome from NCBI, the primers of the target genes were designed using Primer 5.0, and synthesized by BGI TECH (Shenzhen, China). Their details were shown in Table I.

Table I. Premiers used for qRT-PCR.

Gene	Accession No.	Sequence 5`→3`
MIF	NM_001305091.1	F: GCAGCCTCTACAGCATTGG R: TCTAACGGGCAGCACGAG
Raf	NM_205307.2	F: GAAAATAGGAGACTTTGGTCTAGC R: ATCTGACTGAAAACTGAACGGA
ERK1/2	NM_204150.1	F: AGCAAGCTTTAGCCCATCCA R: CCTTCGGCAAGTCATCCAAT
Akt	NM_205055.1	F: CTGATGATGCCAAGGAGATT R: TGGTCAGGAGGAGTGATTGT
GSK3β	XM_015294935.1	F: CCATGTGCAGAGGATGTGGC R: GCAGGGCTGAGTGTGTCCAA
Cyclin D1	NM_205381.1	F: CAACAGCGAGAGCCACGTAA R: TTCCATCGTTCAGTTTCTGCTT
Rb	NM_204419.1	F: GGACAGGGATGTGCTGAGATTG R: TGCCATAGGTAGCCATGACAAT
E2F	NM_205219.1	F: CTTCCTGGCGGATGAGTTCA R: ACGTCCCCTTGGCAGTGA
β-actin	L08165.1	F: GATGGACTCTGGTGATGGTGTTAC R: TTGATGTCACGCACAATTTCTCTC

qRT-PCR reactions were performed using the Applied Biosystems 7500 fast Real-time PCR system (Bio-Rad). Each 10 μL reaction contained: 3.2 μL ddH_2O, 0.4 μL forward primer, 0.4 μL reverse primer, 1.0 μL cDNA template, 5.0 μL SYBR Premix Ex Taq TM II (2×). The PCR program was as follows: 95°C for 5 min, 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. β-actin was used to normalize the relative expression levels of the target genes via the $2^{-\Delta\Delta CT}$ method. Three technical replicates were analyzed for each biological replicate.

Immunoblot

About 100 mg lung tissue were grinded in liquid nitrogen, lysed with RIPA. After ice bath was performed for 30 min, centrifuged at 12000 rpm under 4°C for 15 min. The supernatant was used to determine the protein concentration, added loading buffer, and denatured in a boiling water bath for 8 min. Polyacrylamide gel electrophoresis was performed, then electrically transferred to nitrocellulose (NC) membrane, sealed with 5% skimmed milk powder for 2 h (Fetterer and Barfield, 2003). The primary antibody (anti-MIF 1:500; anti-β-actin 1:4000; Anti-p-Raf, Anti-Raf, Anti-p-ERK, Anti-ERK, Anti-p-Akt, Anti-Akt, Anti-p-GSK-3β, Anti-GSK-3β, Anti-cyclin D1, Anti-p-Rb, Anti-Rb, Anti-E2F, Anti-Lamin B diluted according to the manufacturer's instructions) was added, incubated in a shaker at 4°C overnight. Then the membrane was washed with TBS-T for 10 min, repeated for 3 times. After that, secondary antibody (TBS-T dilution ratio 1:3000) was added, incubated in shaking table at room temperature for 1 h, then the membrane was washed with TBS-T for 10 min, repeated for 3 times. ECL substrate for western blotting was applied and images were taken with gel imaging system. And densitometric analysis of immunoblot was performed by ImageJ software (NIH, Bethesda, MD, USA).

Immunohistochemistry (IHC) staining

The sample of lung tissue was analyzed followed by the instruction of the SABC-POD (Rabbit IgG) kit to investigate the expression and distribution of MIF, p-Raf, p-ERK, p-Akt, p-GSK3β and cyclin D1 proteins in lungs of the PH groups. The diluent PBS was used as the negative control, and 9% H_2O_2 was used to block the endogenous peroxidase. Sodium citrate solution was used for heat repair, then sealed with 5% BSA (bovine serum albumin), added with primary antibody, incubated overnight in a wet box at 4°C, and stained with DAB staining. The paraffin block containing lung tissue was sliced and dewaxed; the paraffin sections were dropped with 9% H_2O_2 to block endogenous peroxidase, placed in a wet box at room temperature for 15 min, and rinsed with PBS. Then they were placed in sodium citrate repair solution in a 95°C water bath for 15 min, cooled at room temperature, and rinsed with PBS (Rowson *et al*., 2003). Paraffin lung sections were blocked with 5% BSA in a wet

box at 37°C for 45 min. Then, 5% BSA was erased, and primary antibody dilution (MIF = 1:150), antibody diluent PBS was used as a negative control, placed in a wet box at 4°C overnight, rewarmed at room temperature for 30 min, and washed with PBS. The secondary antibody and SABC solution in the kit were added to the sections successively, and then the sections were washed with PBS, and then the sections were colored with DAB (yellow).

Data analysis

Data are presented as mean ± standard deviation (SD). The differences between PH group and control group were tested by Student's t-test using SPSS 23.0 (IBM SPSS Inc., Armonk, NY, USA). All figures were constructed using R software v3.4.4 (The R Foundation, Vienna, Austria).

RESULTS

Expression of MIF and related genes in downstream pathway in lungs of PH broilers

Figure 1 shows the expression of *MIF*, and *Raf, ERK, Akt, GSK-3β, cyclin D1, Rb* and *E2F* genes in downstream signal pathways in the lungs of PH broilers. Compared with normal groups, *MIF,* which functioned as a class of pleiotropic immune regulatory cytokines with unique structure, was significantly increased in the PH groups (P <0.01). For the ERK pathway, the *Raf* and *ERK* mRNA was significantly increased (P <0.01) than the normal groups, which indicated that the ERK pathway was highly activated. For the Akt pathway, the mRNA expression of *Akt* and *GSK3β* were significantly increased in the PH groups (P <0.01) than the normal groups, which suggested that Akt/GSK3β pathway played a role in the process of PH boilers. For the cell cycle related mRNA, *cyclin D1*, *Rb* and *E2F,* were significantly increased in the PH groups (P <0.01) than the normal groups, which showed that cell cycle was controlled in PH broilers.

Expression and distribution of MIF and related proteins in downstream pathway in lungs of PH broilers

According to western blot and immunohistochemical analysis, the MIF, p-Raf, p-ERK, p-Akt, p-GSK3β, cyclin D1, Rb and E2F proteins showed a basic expression and distribution in the lungs of broilers (Figs. 2 and 3). Western blotting showed that in the lungs of PH broilers, the expression of p-Raf and p-ERK in MIF and ERK pathways, p-Akt and p-GSK3β in AKT pathway, cyclin D1, Rb, E2F proteins in Rb/E2F pathway were significantly higher than that in healthy broilers (P <0.01) (Fig. 2). This indicated that the Raf/ERK, Akt/GSK3β and Rb/E2F pathway were highly activated.

IHC result showed that, in PH broilers, MIF protein was mainly expressed in the tunica media and intima of pulmonary artery (Fig. 3 a3). P-Raf and p-ERK were mainly colored in the intima and media of blood vessels (Fig. 3 b3, c3). P-Akt was mainly colored on the outer and outer sides of the tunica media (Fig. 3 d3). P-GSK3β and cyclin D1 were colored in different degrees in the inner, middle and outer membranes of the vascular wall (Fig. 3 e3, f3). Compared with healthy broilers (Figs. 2 and 3), the expression level of all proteins increased, with obvious visual differences.

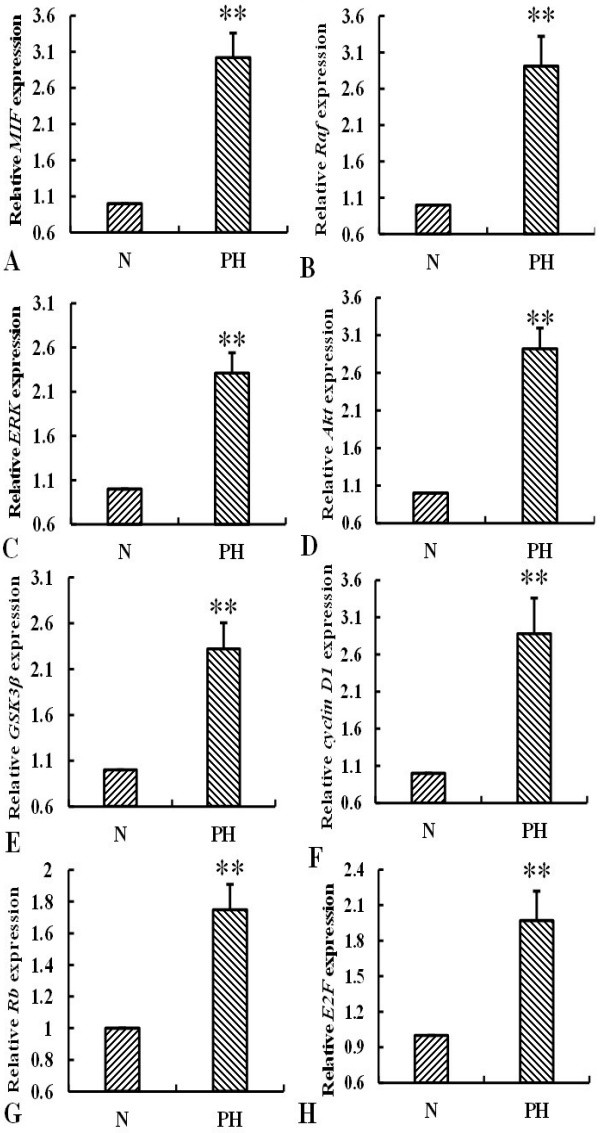

Fig. 1. mRNA expression of *MIF* (A), *Raf* (B), *ERK* (C), *Akt* (D), *GSK3β* (E), *cyclin D1* (F), *Rb* (G), *E2F* (H) in the lungs of PH broilers (n = 3, compared with healthy lungs, ** P <0.01). *MIF*: migration inhibition factor.

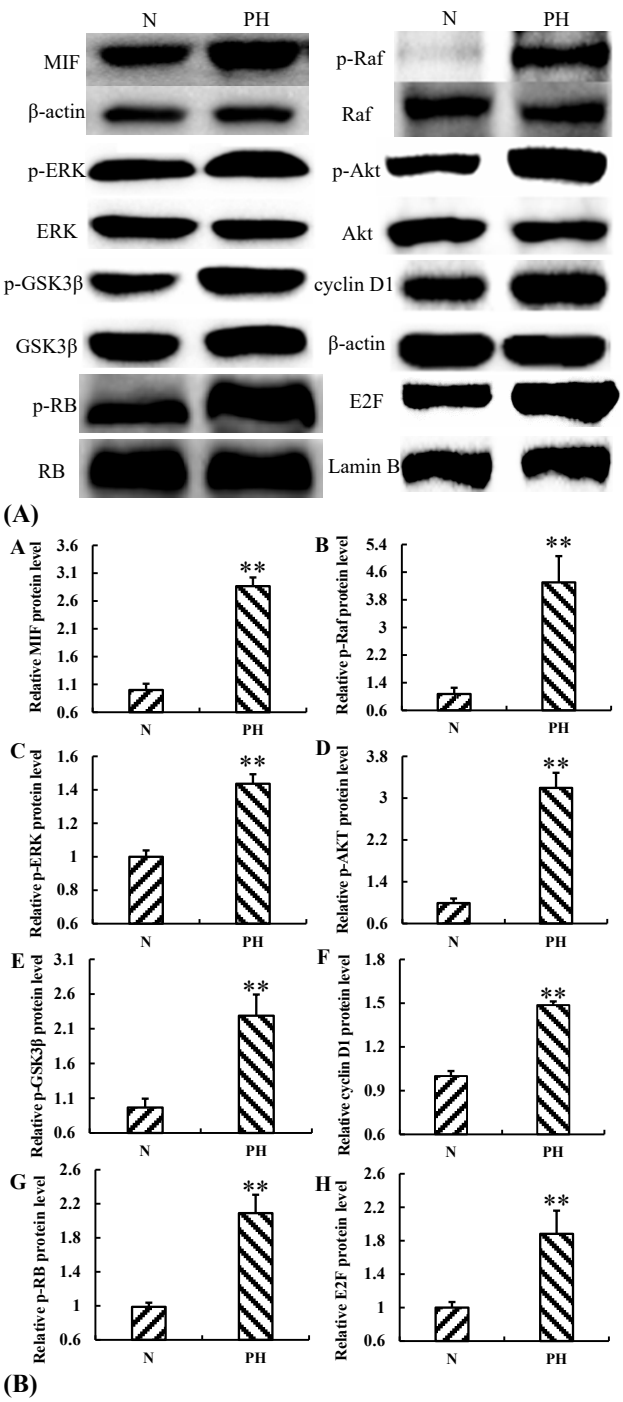

Fig. 2. Western blotting analysis of protein expression in PH broilers lung. (a) The effect of PH broilers lung on MIF, Raf, ERK, Akt, GSK3β, cyclin D1, Rb, E2F protein expression. (b) Western blotting analysis of the test results: (n = 3, compared with healthy broilers lung, ** P <0.01).

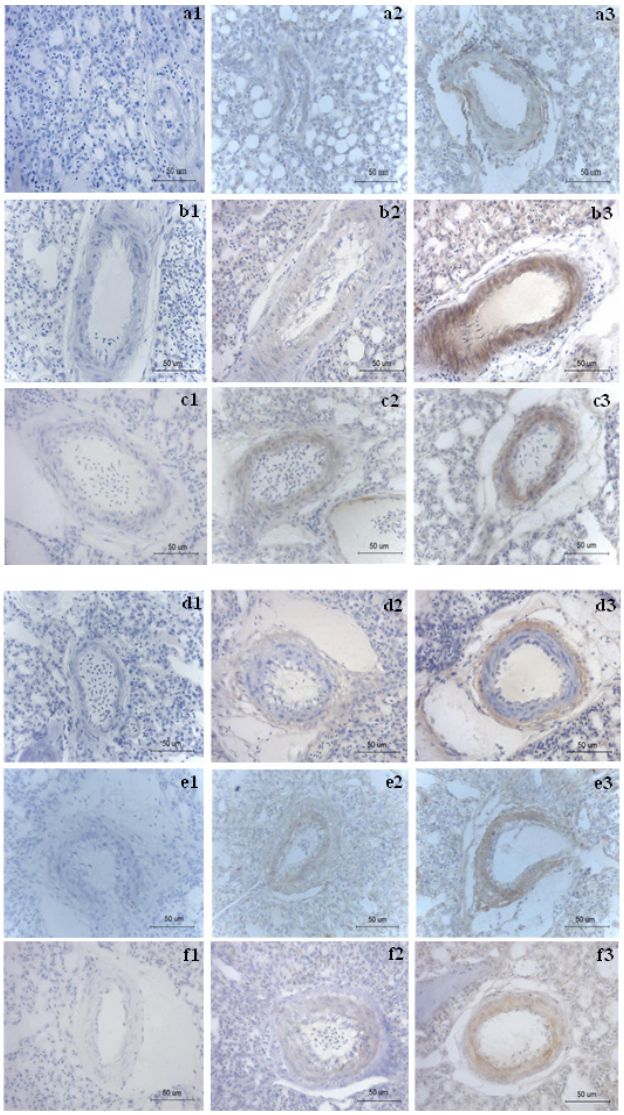

Fig. 3. Immunohistochemical analysis of protein in broiler lungs (magnification: 400×). a, MIF; b, p-Raf; c, p-ERK; d, p-Akt; e, p-GSK3β; f, cyclin D1; 1, negative control with PBS; 2, the lungs of healthy broilers; 3, the lungs of PH broilers.

DISCUSSION

For cell proliferation, the first step is to implement the synthesis of relevant DNA, which transforms the cell cycle from G1 phase to S phase. E2F transcription genes include cell cycle related genes, such as cyclin A, D, E, and so on, thus it is a key factor in cell cycle regulation. The transcription function of E2F is regulated by retinoblastoma protein (Rb). Rb plays a controlling role in the regulation of G1/S transformation. Dephosphorylated

Rb binds E2F to control its transcription function, and the cell cycle stops in G1 phase. The hyperphosphorylated Rb (p-Rb) is separated from E2F to enable it to play its transcription function, regulate the cycle process into S phase, and cell proliferation occurs (Singh *et al*., 2010; Kwon *et al*., 2017). In this experiment, the mRNA levels of *Rb* and *E2F* in the lungs of PH broilers were significantly increased, and the protein expressions of p-Rb and E2F were significantly increased compared with those of healthy broilers, indicating that the Rb protein content was increased. Moreover, Rb undergoes hyperphosphorylation, which breaks the binding with E2F. Free E2F starts the transcription of cell cycle genes, further expresses related proteins, and realizes the transformation from G1 phase to S phase.

The hyperphosphorylation and dephosphorylation of Rb are regulated by cyclins and cyclin dependent kinases (CDKs). The combination of cyclin D1 and CDK4/6 is an important step in inducing the hyperphosphorylation of Rb. After hyperphosphorylation of Rb, the inhibition of G1 phase is relieved, and the function of E2F as a transcription factor is activated, entering the division phase (Coqueret, 2002; Roskoski, 2019). At present, the mechanism of cyclin D1 in PASMCs proliferation has been studied. After silencing cyclin D1, pulmonary vascular remodeling was significantly reduced (Zeng *et al*., 2013). Research on rat vascular smooth muscle cells using gene chip technology showed that, miR-146a may promote the proliferation of rat vascular smooth muscle cell (VSMC) by up regulating the level of cyclin D1 (Wei *et al*., 2021). In this study, IHC and Western blotting were used to detect the expression of cyclin D1 in the lung of broilers. The expression of cyclin D1 protein in the lung of PH broilers was significantly higher than that of healthy broilers. qRT-PCR showed that *cyclin D1* mRNA was significantly increased. The experimental results are similar to those above. Therefore, in broiler PH, cyclin D1 acts as the high phosphorylation factor of Rb, resulting in the separation of Rb and E2F. Free E2F starts the expression of related genes and participates in cell proliferation.

The expression level of cyclin D1 is regulated by many factors, among which Akt and ERK pathways are the main pathways regulating cell proliferation and participate in the upstream signal pathway of cyclin D1 expression regulation. Previous studies showed that activation of Akt and ERK signaling pathways could achieve the proliferation of PASMCs (Xiong *et al*., 2016; Li *et al*., 2020). Interference of Akt and ERK signaling pathways can inhibit the expression of cyclinD1 and prevent the proliferation of primary rat VSMC (Chen *et al*., 2011). Rats with pulmonary hypertension also regulated the expression of cyclin D1 through the induction of Akt and ERK signaling pathways and finally regulated the apoptosis of endothelial cells of small vessels (Suzuki *et al*., 2013).

In this study, from the IHC results, the ERK pathway related proteins p-Raf and p-ERK are mainly expressed in the pulmonary vascular middle membrane, while the Akt pathway related proteins, p-Akt, are mainly expressed in the pulmonary vascular outer membrane, p-GSK3β is mainly expressed in the outer and middle membranes. The results of IHC and Western blotting showed that, the protein expression levels of p-Raf and p-ERK in the ERK pathway of PH broilers were significantly higher than those of healthy broilers (P <0.01), and the mRNA expressions of Raf and ERK were significantly higher. While the protein expression level p-Akt, p-GSK3β in Akt pathway increased significantly (P <0.01). The mRNA expressions of *Akt* and *GSK3β* were increased. The results are basically consistent with those of human and mammalian studies. This indicated that, Raf/ERK participated in the pulmonary vascular remodeling of broiler PH by inducing the level of cyclin D1; Akt/GSK3β by inducing the level of cyclin D1, it participates in the pulmonary vascular remodeling of broiler PH, p-Akt is expressed in the adventitia fibroblasts of the vascular wall, and its induced active substance acts on PAMSCs after release, causing this change.

ERK and Akt pathways are regulated by many inflammatory factors, including MIF. Studies have shown that, the knockout of MIF could significantly reduce the proliferation of melanoma cells, increase apoptosis, affect and reduce the S phase of cell cycle, reduce the expression of cyclin D1 and the reduction of Akt phosphorylation (Oliveira *et al*., 2014). *In vitro*, MIF enhanced the resistance of temozolomide (TMZ) to sensitive cells by enhancing cell proliferation and inhibiting apoptosis, through PI3K/Akt signaling pathway (Wei *et al*., 2021). MIF also stimulates pathophysiological processes through ERK pathway, such as cell proliferation that constitutes the intima, media and adventitia of blood vessels (Zhang *et al*., 2012a). Moreover, the recombinant MIF induced rat corneal angiogenesis *in vivo* and changed endothelial cell morphology, also showed ERK and Akt dependent (Amin *et al*., 2003). In hepatocellular carcinoma (HCC) cells, the expression of MIF was positively correlated with cyclin D1 and tumor severity. Knockdown of MIF reduced tumor growth rate, and the expression levels of cyclin D1, p-Akt and p-ERK were also down-regulated (Huang *et al*., 2014). MIF can also induce the activity of ERK pathway, the subsequent cyclin D1 level and the progression from G1 to S phase (Swant *et al*., 2005). MIF098, an antagonist of MIF, inhibited the proliferation and migration of PASMC by regulating MAPK/ERK1/2 signals and cell cycle related proteins (Huang *et al*., 2019).

In this study, it was found that the expression of MIF in lung of PH broilers was significantly higher than that of healthy broilers by IHC and western blotting, and MIF was mainly expressed in intima and media. qRT-PCR showed that *MIF* mRNA level was increased. This shows that during the formation of PH, the endothelial cells of the intima can be stimulated to release MIF, which together with the MIF released by the smooth muscle cells of the middle membrane can stimulate the middle membrane, leading to the activation of ERK and PI3K/Akt pathways. Previous studies have confirmed the role of MIF in pulmonary hypertension. MIF and its signal transduction may contribute to the progress of pulmonary vascular remodeling associated with pulmonary hypertension (Jalce and Guignabert, 2020; Zhang *et al*., 2012b). Therefore, the MIF secreted by the intima and media of pulmonary vascular wall in PH broilers acts on PASMCs, and increases the expression of cyclin D1 through ERK and Akt pathways. After phosphorylation of Rb, E2F plays a transcriptional function, thereby inducing the proliferation of pulmonary artery wall cells, causing pulmonary vascular remodeling, and leading to pulmonary hypertension in broilers.

CONCLUSION

In conclusion, compared with that of healthy broilers, the protein expression levels of MIF, p-Raf, p-ERK, p-Akt, p-GSK3β, cyclin D1, p-Rb, and E2F in lungs of PH broilers were increased significantly. Moreover, qRT-PCR showed that the mRNA expression of *MIF, Raf, ERK, Akt, GSK3β, cyclin D1, Rb, E2F* increased significantly, suggesting that MIF acted on pulmonary vascular cells, and increased the expression of cyclin D1 through ERK and Akt pathways, and Rb phosphorylation, E2F transcription. Thus induced cell proliferation, caused pulmonary artery remodeling, and lead to PH. This study is expected to provide a basic reference for the treatment of PH.

Funding

This research was supported by the Natural Science Foundation of Shanxi Province (No. 2014011028-2).

Ethical compliance (IRB approval)

Research experiments conducted in this article with animals or humans were approved by the Institutional Animal Care and Use Committee of Shanxi Agricultural University (Approval No. 002006) following all guidelines, regulations, legal, and ethical standards as required for humans or animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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