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RESEARCH ARTICLE

High Glucose Transglutaminase 2 Promotes YAP/TAZ and Fibroblast Proliferation in Pulmonary Hypertension

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ABSTRACT: Pulmonary hypertension (PH) is a cardiopulmonary disease characterized by uncontrollable cell proliferation of adventitial fibroblasts in the pulmonary artery, leading to increased pulmonary arterial stiffness and eventual right ventricular dysfunction and heart failure. Previous studies have reported a significant role for glycolysis-induced transglutaminase 2 (TG2) in cell proliferation, fibrogenesis and PH. Furthermore, a direct relationship was established between TG2 and YAP/TAZ, molecules that activate extracellular matrix remodeling when triggered by vascular stiffness in cancer cells. Thus, this study sought to determine whether the same relationship between TG2 and YAP/TAZ existed in cell culture models of PH. Utilizing human pulmonary artery adventitial fibroblasts, we conducted a series of three experiments 1) analyzing the effect of high glucose concentrations on YAP and TAZ levels, 2) the effect of TG2 inhibition (ERW1041E) on high-glucose induced YAP and TAZ levels, and 3) the effect of YAP/TAZ inhibition (YAP/TAZ siRNA) on fibroblast proliferation. We found that high glucose significantly induced TG2-mediated YAP and TAZ mRNA expression. Furthermore, YAP and TAZ mediate high-glucose-induced fibroblast proliferation. This confirmed our hypothesis that glycolysis- mediated TG2 activation plays a significant role in YAP/ TAZ upregulation, promoting pulmonary arterial adventitial fibroblast proliferation in response to high glucose. These findings support a novel role for glycolysis-induced TG2 and YAP/TAZ in pulmonary arterial remodeling associated with experimental PH.

KEYWORDS: Biomedical and Health Sciences; Cell, Organ, and Systems Physiology; Cardiovascular; Pulmonary Hypertension; Transglutaminase 2; YAP/TAZ.

Introduction

Pulmonary hypertension (PH) is a proliferative cardiac disease affecting between 50 to 70 million individuals worldwide. The condition may be caused by uncontrollable cell proliferation of smooth muscle cells (SMCs) and fibroblasts in the pulmonary artery, composed of an inner layer of endothelial cells, a middle layer of SMCs, and an outer layer of adventitial fibroblasts. In PH, aberrant pulmonary arterial (PA) remodeling resulted in a rapid buildup of muscle in the arterial walls and increased pressure in the right ventricle of the heart. This adverse remodeling of the PA further causes remodeling of the right ventricles, eventually culminating in heart failure. Currently, there is no effective treatment for PH.

In previous years, a notable role for transglutaminase 2 (TG2) has been identified in PH.[1,2] As a cross-linking enzyme that alters protein function, TG2's post-translationally modified extracellular matrix (ECM) proteins were shown to promote cell growth and migration.[1] This may result in the increased ECM's stability, fibrogenesis, and tissue stiffness. TG2 has also been shown to cross-link between protein glutamine and lysine residues, enabling its contribution to the serotonylation of vascular proteins such as fibronectin.[2] In PH specifically, TG2 was shown to mediate heightened collagen deposition and tissue fibrosis in the PA, which may promote increased vascular resistance and, eventually, right ventricle (RV) pressure. Recently, experiments showed TG2 levels were stimulated by glycolysis, which could be induced through high-glucose concentrations.[1]

More and more researchers acknowledge vascular ECM modifications as an important molecular contributor to PH.[5] During both the early and late stages of PH, uncontrolled collagen and elastin assembly have been detected.[5] However, activities connecting PH vasculature to ECM mechanotransduction, a series of activities allowing cells to perceive and adjust to external stimulants, have only started to be identified.[4,5] In particular, Yes-associated protein 1 (YAP) and TAZ, two regulators of the Hippo signaling pathway, are triggered by ECM rigidity; its activity is directly controlled by cell shape and polarity, which is governed by the cell's cytoskeleton. They can pick up on how cells perceive both themselves and their environment within tissues, communicating with others.[4,5] Its ability to modify cells' actions to an organ's specific needs, combined with its regulation of cell division, allows it to aid tissue growth and repair. In early PH *in vivo* models, it was found that pulmonary vascular stiffness triggers YAP/TAZ, inducing further ECM remodeling and cell proliferation. Thus, when regular pathways fail, abnormal YAP/TAZ induction results in diseases such as fibrosis, cancer, and atherosclerosis.

TG2 is thought to serve as a direct target gene of YAP/TAZ in cancer cells.[6] In response to YAP/TAZ overexpression, TG2 levels significantly increased, and YAP/TAZ knockdown decreased TG2 expression in multiple cell lines. Data analysis showed that mRNA levels of TG2 positively correlated with numerous downstream target genes of YAP/TAZ, including the expression levels of YAP/TAZ. This suggests that TG2 is a direct target of YAP/TAZ, significantly contributing to the hostile transformation in cancer cells activated by YAP/TAZ.[6]

Analyzing these points, we connected TG2's role in PA tissue fibrosis to YAP/TAZ's role in ECM stiffness and the correla-

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tion between TG2 and YAP/TAZ in cancer cells. Therefore, in the present study, we chose to investigate if a similar relationship between YAP/TAZ and TG2 expression is implicated in PH pathogenesis and if these pathways could be explored as potential targets for novel therapies for PH. We hypothesized that high glucose induces glycolysis-mediated TG2 activation and YAP/TAZ upregulation, promoting pulmonary arterial adventitial fibroblast proliferation.

Methods

Materials:

This study utilized human pulmonary artery adventitial fibroblasts (HPAAFs) grown in 6-well cell culture plates. Cells were grown in a fibroblast medium supplemented with fetal bovine serum (FBS) and varying high glucose concentrations. Glucose-free media was used to starve fibroblasts overnight. To test TG2 effects, fibroblasts were pre-treated with varying concentrations of ERW1041E, a known inhibitor of TG2 activity, or vehicle control (dimethyl sulfoxide; DMSO). To test the effects of YAP/TAZ, fibroblasts were transfected with a 20nM concentration of YAP and TAZ siRNA or control scrambled siRNA. The quantitative PCR method was used at the experimental endpoints to determine the mRNA expression of TG2, YAP, and TAZ genes. For PCR analysis, Trizol, nuclease-free centrifuge tubes, DNase I, RNA primers (TG2, YAP, TAZ, and beta-actin), reverse transcriptase enzyme, DNA Polymerase, dNTPs, SYBR Green I dye, and a qPCR machine were used according to previously published methods (1). Cell counting was performed to quantify cell proliferation. This analysis required a hemocytometer chamber with a coverslip, trypan blue, tally counter, pasteur pipettes, and a microscope.

High Glucose Cell Culture:

Seeking to investigate the impact of TG2 activity on YAP/TAZ expression and fibroblast proliferation, this study used high glucose concentrations to stimulate TG2 activity. Three sets of experiments were conducted: one to determine if high glucose concentrations induced TG2 and YAP/TAZ mRNA expression, another to determine the effect of TG2 inhibition on high glucose-induced YAP/TAZ mRNA expression, and lastly to determine the relationship between YAP/TAZ inhibition and high glucose-induced fibroblast proliferation.

The first experimental set mentioned above attempted to discover the effect of high glucose concentrations on TG2 and YAP/TAZ expression. Thus, the study used glucose concentrations of 0, 5.5, 10, 25, and 50 mM as the independent variable and YAP/TAZ expression levels as the dependent variables. The experiment was conducted with HPAAFs, placed in 6-well plates with approximately 300,000 cells per well. After reaching about 70% confluency, fibroblasts were starved overnight with glucose-free media without FBS to ensure all cells began the experiment at the same conditions before glucose introduction. They were then exposed to varying glucose concentrations of 5.5, 10, 25, and 50 mM for 72 hours. Cells receiving no glucose were used as controls. Each of these concentrations was replicated in at least three cell culture wells. PCR analysis was then carried out at the experimental end points to determine TG2 and YAP/TAZ mRNA expression levels.

TG2 Inhibitor:

The second experimental set, as mentioned above, hoped to identify the effect of TG2 inhibition on high glucose-induced YAP/TAZ mRNA expression. Thus, the independent variables were the varying ERW1041E concentrations and high glucose, while the dependent variable was YAP/TAZ expression. The experiment was conducted with HPAAFs, placed in 6-well plates with approximately 300,000 cells per well. After reaching about 70% confluency, fibroblasts were starved overnight with glucose-free media without FBS to ensure all cells began the experiment at the same conditions before glucose introduction. Following pretreatment with vehicle-control (DMSO) or 50uM concentration of known TG2 inhibitor ERW1041E for 1 hour, fibroblasts were cultured with high glucose (25mM) for 72 hours. Each experimental condition was replicated in at least three cell culture wells. PCR analysis was then carried out at the experimental endpoints to determine YAP/TAZ mRNA expression levels.

siRNA Transfection:

The final experimental set mentioned above sought to determine the relationship between the YAP/TAZ pathway and high glucose-induced fibroblast cell proliferation. The present study used YAP/TAZ siRNA inhibition and high glucose as the independent variables and fibroblast proliferation as the dependent variable. It was conducted with HPAAFs, which were cultured in a fibroblast medium. The cell cultures were done in 6 well plates, with approximately 300,000 cells per well. After reaching about 70% confluency, fibroblasts were transfected with a 20nM concentration of control siRNA, YAP, and TAZ siRNA for 6 hours in growth medium without antibiotics. Fibroblasts were cultured in normal (5.5 mM) glucose medium overnight. Cells were then subjected to varying glucose concentrations for 72 hours. Each transfection was replicated in at least three cell culture wells. After 72 hours, cell counting was then carried out at the experimental endpoint to quantify fibroblast growth.

Polymerase Chain React (PCR) Analysis:

At the experimental endpoints of the first and second experiments, cell lysis and total RNA extraction was performed on each individual cell culture well using Trizol. This lysis process was conducted in stabilizing conditions required to maintain its contents. Specifically, it was done with nuclease-free plasticware (tubes, pipettes, etc.) in a neutral pH environment and stored at -20 degrees Celsius to avoid nucleic acid degradation.

With the cell lysate recovered, RNA estimation was first carried out through spectroscopic methods to normalize RNA amounts, taking measurements of RNA concentration and purity. Real-time PCR was then conducted. While the polymerase chain reaction (PCR) is a technique that amplifies DNA fragments after the reaction completes, real-time PCR allows the PCR reaction to be visualized as the reaction progresses. By adding Deoxyribonuclease I, or DNase I, the RNA solution underwent DNase treatment to cleave any genomic DNA strands, leaving RNA with a ribosom-

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ribosomal majority. With DNA removed, reverse transcription was conducted with the addition of targeted RNA primers, the reverse transcriptase enzyme, DNA polymerase to extend the DNA strands, and dNTPs to allow the reaction to occur. SYBR Green I dye was added to the solution to detect the final DNA product.

The qPCR machine was used to conduct real-time PCR, allowing the number of cycles and temperatures per cycle to be constantly set. To denature proteins, the qPCR machine began at 95 degrees Celsius, allowing the primer to begin binding to the DNA strands and beginning DNA replication. At this point, the temperature was lowered to around 72 degrees Celsius to optimize replication. This heating and cooling cycle was replicated in about 45 cycles. Cycle threshold (Ct) values were then used for qPCR analysis.

Cell Counting:

At the experimental endpoint of the third experiment, cell counting was conducted on each individual cell culture well. The cell suspensions were dropped into a hemocytometer chamber using a Pasteur pipette, and capillary action drew the suspension into the chamber. The opposite chamber was similarly filled. Next, the chamber was placed on a microscope, where the 10x objective was used to count the number of cells in a 1mm square area. Cells on the other side of the hemocytometer were similarly counted. To calculate the cells' concentration, we counted the average of all 1mm 2 areas and applied the formula c=n/v, where c was the cell concentration in cells per mL, n was the average number of cells per mm square area, and v was the volume counted.

Results and Discussion

Role of High Glucose in YAP and TAZ mRNA Expression:

When stimulated with higher glucose concentration levels, YAP mRNA expression increased in a glucose-concentration-dependent manner. A significant increase in expression was seen in glucose levels of 10 mM, 25 mM, and 50mM, indicating that high glucose levels serve as a stimulant for YAP expression (Figure 1). TAZ expression showed similar results when met with high-glucose levels, except for a significant change in the presence of 10 mM glucose levels (Figure 1). Nevertheless, high glucose concentrations also stimulate TAZ mRNA expression. However, in the normal glucose concentration level of 5.5mM, YAP and TAZ expression did not significantly differ compared to no glucose (Figure 1). With the confirmation that high glucose concentrations stimulate both YAP and TAZ expression, 25 mM glucose concentrations were used to induce YAP and TAZ expression in each subsequent experiment.

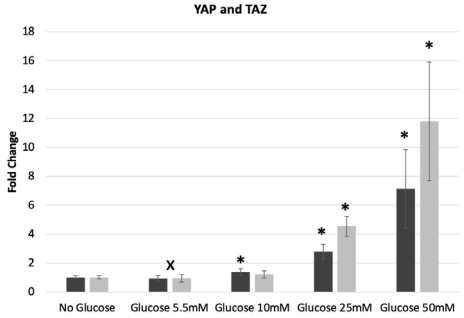

Figure 1: High glucose significantly induced YAP and TAZ mRNA expression in HPAAFs. Dark grey bars denote YAP expression, while light grey bars denote TAZ expression. The reference groups, normal glucose (5.5 mM), are devoted with (x). Significant differences compared to normal glucose (5.5mM) are denoted with (*), utilizing p-values. Error bars depicting standard deviation are represented throughout.

Role of TG2 Inhibition in High-Glucose Induced YAP and TAZ mRNA Expression:

YAP expression significantly increased in the presence of high glucose at a concentration of 25 mM (Figure 2) compared with normal glucose, in line with findings from (Figure 1). More importantly, high glucose-induced YAP expression significantly decreased when treated with a 50 uM concentration of ERW1041E, a known inhibitor of TG2 activity, compared to vehicle control (Figure 2). TAZ expression was affected similarly, with a significant increase in the presence of high glucose at a concentration of 25 mM compared to no glucose (Figure 2). High glucose-induced YAP expression also significantly decreased when treated with ERW1041E at a concentration of 50 uM compared to vehicle control (Figure 2). These results indicate that TG2 activity directly affects the glucose-induced expression of both YAP and TAZ mRNA.

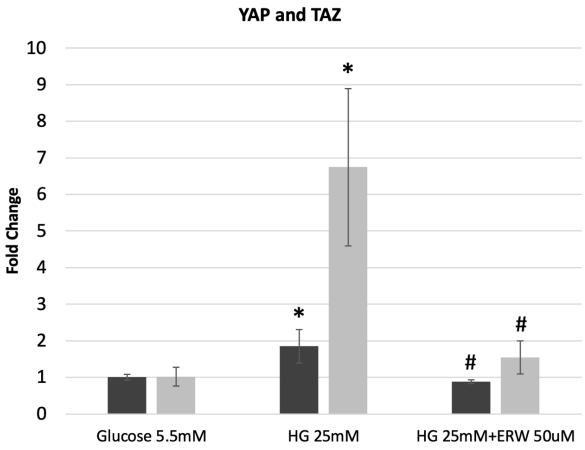

Figure 2: TG2 mediates high glucose-induced YAP and TAZ mRNA expression in HPAAFs. Dark grey denotes YAP expression, while light grey denotes TAZ expression. Significant differences compared to normal glucose (5.5mM) are marked with (*), and significant differences compared to high glucose (25 mM) are denoted with (#), utilizing p-values. Error bars depicting standard deviation are represented throughout.

Role of YAP and TAZ siRNA in High-Glucose Induced Cell Proliferation:

HPAAF Cell number significantly increased in the presence of high glucose compared to normal glucose (Figure 3), suggesting high glucose induces cell proliferation. As there was no significant change when high glucose-induced fibroblasts were transfected with control siRNA (Figure 3), it was further indicated that siRNA did not adversely impact the fibroblast's growth. Fibroblasts transfected with YAP siRNA and TAZ siRNA, which largely inhibited YAP and TAZ translation, showed a significant decrease in fibroblast cell numbers compared to high glucose control siRNA (Figure 3). Thus, this indicates that YAP and TAZ both play a substantial role in high-glucose-induced fibroblast proliferation.

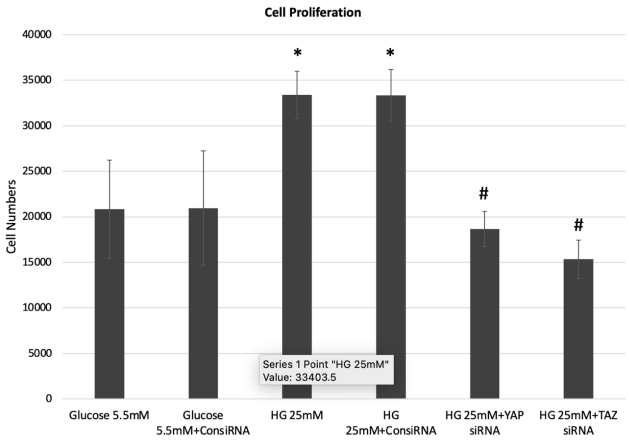

Figure 3: YAP and TAZ mediate high glucose-induced HPAAF cell proliferation. Significant differences compared to normal glucose (5.5 mM) with control siRNA are denoted with (*), and significant differences compared to high glucose (25 mM) with control siRNA are marked with (#), utilizing p-values. Error bars depicting standard deviation are represented throughout.

Discussion

We found that in HPAAFs, high glucose induces YAP and TAZ mRNA expression levels, TG2 activity significantly blocks glucose-induced YAP/ TAZ expression, and YAP/TAZ expression plays a significant role in fibroblast cell proliferation (Figure 4). Figure 4 shows the pathways our study verified, or the pathways between high glucose and YAP/TAZ, TG2 and YAP/TAZ, and YAP/TAZ and fibroblast proliferation. This proves our hypothesis that TG2 and YAP/TAZ are essential in promoting PA adventitial fibroblast growth, suggesting these molecular pathways may contribute to the adverse PA remodeling seen in PH patients.

In future studies, we hope to confirm real-time PCR mRNA analysis of the three experiments through Western Blot protein analysis. As for potential next steps, we suggest looking at YAP/TAZ inhibition's effect on collagen deposition through real-time PCR and Western Blot analysis. Finally, we recommend investigating the impact of TG2 inhibition on YAP/TAZ nuclear translocation through both real-time PCR and Western Blot analysis.

Potential limitations of this study include not conducting a controlled trial with 5.5 mM glucose utilizing YAP/TAZ siRNA. This could have checked if YAP/TAZ siRNA knocked down proliferation in normal glucose conditions. In this case, YAP/TAZ siRNA would have knocked down proliferation in general and not only in response to high glucose concentrations.

Given the role of TG2in collagen deposition and fibrogenesis,[1,2] the impact of YAP/TAZ on ECM,[4,5] and the relationship between TG2 and YAP/TAZ in cancer cells[6]; our current study's findings explore the relationship between TG2 activity, YAP/TAZ expression and fibroblast proliferation. Thus, these studies further advance our understanding of PH pathogenic mechanisms. Furthermore, these studies suggest that TG2 and YAP/TAZ may serve as potential targets for novel therapies for patients with PH.

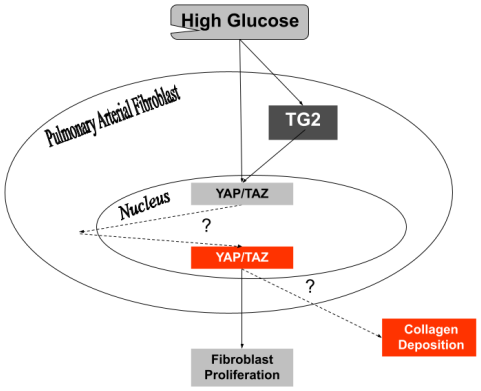

Figure 4: Schematic pathway for high glucose-mediated induction of TG2, YAP/TAZ, fibroblast proliferation, and fibrogenesis in PH. Verified pathways are denoted with filled arrows, while unverified pathways are marked with dotted arrows and (?).

Acknowledgements

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