Doxycycline Inhibits Matrix Metalloproteinase-2 (MMP-2) Production by Neonatal Pulmonary Artery Endothelial Cells

Student Researchers: E'Lana Shuford-Hopkins; Arlene Bulger BS
Mentors: Namasivayam Ambalavanan, MD;
Joseph B. Philips, III, MD

Persistent pulmonary hypertension of the newborn (PPHN) affects more than 10,000 infants in the United States every year. Abnormal pulmonary vascular remodeling may predispose to PPHN.

Matrix metalloproteinases are a large family of zinc-dependent enzymes that are needed for cell proliferation, cell migration, and changes in the extracellular matrix. MMPs are produced by various cells (vascular smooth muscle, monocytes, endothelial) and are needed for degradation of stiff collagen fibrils; however, overexpression of MMPs can lead to heart failure. There are more than 20 different identified MMPs, which are grouped based on their function or location. MMP-2 is a gelatinase found in both vascular smooth muscle and pulmonary artery endothelial cells. MMP-2 production is required for tissue remodeling and is suspected to play a role in neonatal pulmonary vascular remodeling. Doxycycline, a member of the tetracycline family, is a commonly used antimicrobial agent. It has been shown to inhibit the activity of mammalian collagenases and gelatinases. This inhibition is unrelated to antimicrobial properties. It has been speculated that doxycycline inhibitory effects are due to reduced MMP-2 mRNA stability. We hypothesized that doxycycline will decrease MMP-2 production by neonatal porcine pulmonary artery endothelial cells.

From the Department of Pediatrics, University of Alabama at Birmingham; Birmingham, Alabama.

Methods

The Institutional Animal Care and Use Committee at University of Alabama at Birmingham approved all protocols. Three-day-old piglets were obtained from Snyder Farms in Gadsden, Alabama.

Cell Isolation

Resistance pulmonary artery endothelial cells (RPAE) were cultured from a three-day-old piglet by lavage of the pulmonary arteries with a dilute trypsin solution. Cells were characterized as RPAE by (+) Von Willebrand Factor Staining (VWF), and cobblestone morphology on light microscopy.

Drug Preparation

Doxycycline (Sigma Chemical Company) was dissolved at a concentration of 100mg/mL in phosphate buffered saline (PBS). Stock doxycycline was made sterile using a .2μm 50mL filtration unit.

Cell Culture

Cells were cultured in Eagle's Minimum Essential (MEM, Mediatech, Washington, DC) with 10% fetal bovine serum (hyclone), penicillin-streptomycin (5000 units-5 mg/L), and thymidine (2.4mg/L) in 25cm^2 flask until fully confluent. Cells were plated at 2×10^4 cells per well in three 24-well plates containing Eagle's (MEM). After reaching 75% confluency, MEM media were replaced with Serum Free Media (SFM) and cells were incubated at 37° C, 5% CO_2 for 30 minutes. MEM was replaced again and doxycycline was added to different wells at 5, 10, 20, and 40 micrograms/milliliter. Conditioned media were collected at 24 hours.

Estimation of Matrix Metalloprotinase-2 (MMP-2): Zymography Electrophoresis

Conditioned media were diluted 1:1 in zymogram sample buffer. The wells of a 10% zymogram ready gel were loaded with 27 μl of sample. A recombinant human MMP-2 standard and a protein ladder were also run on the same gel. The gel was run at constant voltage (100V) for approximately 1.5 hours, then placed in a pyrex dish containing renaturation buffer, and incubated using orbital shaking for 30 minutes. After discarding the renaturation buffer the gel was placed in development buffer and incubated overnight at 37° C. The gel was stained in 0.5% Coomassie Blue R-250 (BIO-RAD) for 1 hour, and destained in 40% methanol, 10% acetic acid until the clear bands appeared (30 minutes). Gel was viewed and photographed on a light table.

Matrix Metalloproteinase-2 (MMP-2) Biotrak Elisa

Reagents and standards (0.2, 0.4, 0.8, 1.5, 3, 6, and 12ng/mL) were prepared as described in Elisa reagent preparation section. RPAE doxycycline exposed conditioned media samples were diluted 1:1 in zymogram sample buffer. A microtitre plate coated with MMP-2 antibody was set up with sufficient wells for running of all zero (blanks), standards and samples. After adding 100 μl of assay buffer to all wells, 100 μl of standards and samples were added to their appropriate wells. The microtitre plate was covered with a lid and incubated at 2–8° Celsius overnight. The

wells were then washed 4 times and the remaining ELISA MMP-2 detection reagents were added. After shaking the plate for 20 seconds, the plate was read at 405nm to obtain a time zero value, and then placed at 37°C. A final reading was taken at 5 hours.

Data Analysis

Data was calculated as mean ± SEM. Statistical analysis was done by ANOVA followed by Tukey's multiple comparison test. A P value $<.05$ was considered statistically significant.

RESULTS

There was no change in the morphology of the control pulmonary artery endothelial cells. At 20 micrograms/milliliter of doxcycline, RPAE cells appeared slightly elongated. A significant elongation of cells was noted at 40 micrograms/milliliter of doxycycline.

Gelatin zymography was used to access MMP-2 activity in porcine pulmonary artery endothelial cells. There was no significant difference between control 0, 5, and 10 micrograms/milliliters of doxycycline. There was a partial inhibition of total MMP-2 release into conditioned media at 20 micrograms/milliliter of doxycycline, and full inhibition at 40 micrograms/milliliters of doxycycline. Resistance porcine pulmonary artery endothelial cells (RPAE cells) exposed to 20 and 40 micrograms/milliliters of doxycycline showed a significant decrease in MMP-2 release as compared to control cells and cells exposed to lower concentrations of doxycycline. (Figure 1)

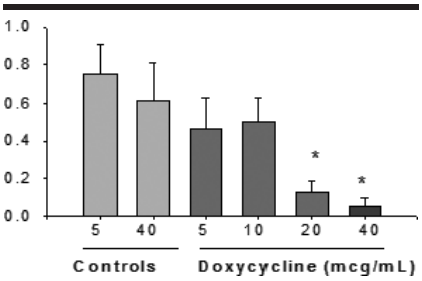

Fig 1. Total MMP-2 by ELISA in conditioned media of resistance porcine pulmonary artery endothelial cells exposed to doxycycline for 24 hours in SFM (mean+SEM; $n = 4$ at each condition) ($P=.01$ by ANOVA; $*P<.05$ vs Control 5)

DISCUSSION

Studies of MMP-2 inhibition have been limited to cancer, connective tissue, inflammatory diseases, and heart failure. In this study, we evaluated if doxycycline would decrease MMP-2 production by pulmonary endothelial cells, in order to determine the feasibility of doxycycline as a therapeutic agent for neonatal pulmonary vascular remodeling, in which gelatinases are known to play a role. Based on gelatin zymography and ELISA, we conclude that inhibition of MMP-2 production by doxycycline is dose dependent. MMP-2 production by porcine pulmonary artery endothelial cells was inhibited at doxycycline concentrations of 20 micrograms/milliliters (partial inhibition), and 40 micrograms/milliliters (full inhibition). Doxycycline is an ideal drug for use because it is relatively safe. Drugs that can block MMP-2 overexpression may offer therapeutic potential in treating persistent pulmonary hypertension of the newborn.