Introduction

About 600,000 people die from heart disease every year, with 385,000 of those deaths resulting from coronary heart disease. Oklahoma has one of the highest rates of cardiovascular disease in the US.1

Atherosclerosis is a gradual process whereby arteries become increasingly narrow due to the presence of fatty plaques in the arterial wall that protrude into the vessel space and compromise blood flow to distal regions.2

Hyperglycemia is an established risk factor for atherosclerosis due to glucose-driven damage to the endothelial cells (EC) lining the vessel wall, as well as increased osmolarity in the vessel.3

PLGF is an angiogenic cytokine that contributes to the early progression of atherosclerosis (“atherogenic”) and is a clinical biomarker for cardiovascular disease.4

Evidence shows that H2S is endogenously produced at low concentrations by our blood vessels and exerts a number of vaso-relaxant actions4. Other studies show CSE overexpression is anti-atherogenic4.

CSE is endogenously produced by cystathionine gamma lyase (CSE) in vascular cells from a PLGF-driven pro-atherogenic environment. Further studies are needed to elucidate the mechanism whereby H2S affects PLGF secretion under basal and hyperosmolar conditions.

Methods

Culturing Primary Human Coronary Artery Smooth Muscle Cells and Primary Human Umbilical Vein Endothelial Cells: Human Coronary Artery Smooth Muscle Cells (CASM) were obtained from a 53 year old male donor and purchased from Lonza (Walkersville, MD). CASM were grown in SmGM medium supplemented with growth factors and 5% FBS in 6-well plates in a CO2 incubator (5% CO2) until ~85% confluent. Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, MD, USA) and grown in EBM-2 medium supplemented with growth factors and 2.5% FBS (Lonza). Cells were cultured at 37°C in 5% CO2 until 85% confluent.

Treatment of Coronary Artery Smooth Muscle Cells: Cells were treated with 50 μM of NaSH (degassed with argon), which liberates H2S gas. S-diclofenac was prepared in a vehicle containing cell culture medium with 5%DMSO, then degassed with argon. 50 μM of S-diclofenac was added directly to the medium of each designated well and 5% DMSO, as a vehicle control. Media was collected into tubes containing protease inhibitor at 6 hours post treatment and stored for ELISA. Media was also collected from wells containing untreated cells. Following media collection, CASMCs were lysed and stored at -80°C for downstream RNA isolation.

Treatment of Human Primary Endothelial Cells with H2S: Cells were treated with NaSH (degassed in argon) to liberate H2S. Treatment groups included: 50 μM and 200μM of NaSH, 50 μM S-diclofenac in a vehicle containing culture medium with 5%DMSO (degassed with argon) and a vehicle control. Treatments were added directly to the medium of each designated well. Media was collected into tubes containing protease inhibitor at 6 hours post treatment and stored for ELISA. Media was also collected from wells containing untreated cells.

Treatment of Human Primary Endothelial Cells with Hyperosmolar Conditions: Cells were cultured in 6 well plates for 3 days. Each day cells were treated with fresh media containing either normal glucose (5mM) as a control, mannitol (20mM) to control for hyperosmolality, or high glucose (25mM) to mimic chronic hyperglycemia. After the 3 day period, all cells were treated with varying doses (0, 50, 100, 150, 200 μM) NaSH for 6 hours. Media were collected in protease inhibitor and stored for ELISA.

Enzyme-Linked Immunosorbent Assay (ELISA): Sample media was added to each well of the Enzyme-Linked Immunosorbent Assay (ELISA) plate, which is coated with the capture antibody of interest (PLGF). Nineteen wells were prepared in duplicate and incubated for 2 hours at room temperature. Samples were washed 4X; detection antibody was added and incubated for 1 hour. The detection antibody is linked to Streptavidin-HRP which allows for a colorimetric reaction to occur upon the addition of substrate solution and 20 minute incubation at room temperature. Stop solution was then added to each well and the absorbance was measured at 450nm.

Real-time quantitative PCR (qPCR): SMCs were harvested for RNA isolation using RNeasy columns (Qiagen) and human CSE gene expression was measured using PerfeCTa SYBR FastMix (Quanta Biotecnologies). Data were normalized to the expression of housekeeping gene, β-actin.

The goals of this study were to:

1) Expose vascular cells to H2S and a H2S-donating drug (S-diclofenac).
2) Measure PLGF protein secretion and CSE gene expression in H2S-treated versus untreated cells.
3) Treat human endothelial cells with hyperglycemic conditions in the presence or absence of H2S and measure PLGF secretion.

Conclusions

• Expose to H2S reduces PLGF secretion in human vascular cells, suggesting H2S has an anti-atherogenic effect.
• Expose to H2S increases CSE gene expression in human vascular cells, suggesting an increase in endogenous H2S production.
• Previous data shows that CSE gene expression is increased following shear stress patterns similar to blood flow in atherosclerotic arteries, suggesting CSE is up-regulated to produce the cardioprotective gas, H2S.
• In this study, hyperosmolarity substantially increased the secretion of PLGF by human endothelial cells.
• The hyperosmolar-induced increase in PLGF secretion was reversed by H2S.

Future Directions

• Future studies include increasing our n value to determine statistical significance of this observed response to H2S.
• We will measure CSE gene expression in HUVEC treated with H2S to assess the effect on endogenous production of H2S.
• Further studies are needed to determine the mechanism whereby H2S affects PLGF secretion under basal and hyperosmolar conditions.

References:

5. “Treatment of Human Primary Endothelial Cells with H2S: Cells were treated with NaSH (degassed in argon) to liberate H2S. Treatment groups included: 50 μM and 200μM of NaSH, 50 μM S-diclofenac in a vehicle containing culture medium with 5%DMSO (degassed with argon) and a vehicle control. Treatments were added directly to the medium of each designated well. Media was collected into tubes containing protease inhibitor at 6 hours post treatment and stored for ELISA. Media was also collected from wells containing untreated cells.”
6. “Treatment of Human Primary Endothelial Cells with Hyperosmolar Conditions: Cells were cultured in 6 well plates for 3 days. Each day cells were treated with fresh media containing either normal glucose (5mM) as a control, mannitol (20mM) to control for hyperosmolality, or high glucose (25mM) to mimic chronic hyperglycemia. After the 3 day period, all cells were treated with varying doses (0, 50, 100, 150, 200 μM) NaSH for 6 hours. Media were collected in protease inhibitor and stored for ELISA.”