The Effect of H_2S on PLGF Secretion by Human Vascular Cells

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Abstract

Angiogenesis is the growth of new blood vessels that plays an essential role in development and wound healing as well as during coronary revascularization, which is necessary to repair tissues following a myocardial ischemic event. Arteriogenesis is the increase in diameter and wall thickness of pre-existing blood vessels to provide blood flow to otherwise deprived tissue. This can be beneficial through widening collateral vessels when occlusions and ischemia occur. PLGF is a multifunctional angiogenic and arteriogenic cytokine that is secreted as a compensatory response following vascular occlusion. PLGF is unique in that it drives both physiological (arteriogenesis) and pathological (atherosclerosis) processes. 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. PLGF contributes to early progression of atherosclerosis and serves as a clinical biomarker for cardiovascular disease. Hyperglycemia is a well-established risk factor for cardiovascular disease due to glucose-driven damage to the endothelial cells lining the vessel wall, as well as increased osmolarity in the vessel. H_2S is endogenously produced in the human body by the enzyme cystathionine gamma lyase (CSE) in vascular cells. H_2S has been shown to be pro-angiogenic, anti-inflammatory, and vasodilatory. The effect of H_2S on arteriogenesis, as well as combating atherosclerosis is unknown. Since low levels of hydrogen sulfide (H_2S) have recently been shown to be protective to the cardiovascular system, we hypothesized that treatment of human vascular cells with H_2S would increase secretion of the pro-arteriogenic cytokine, PLGF. Based on conclusions from our first hypothesis, we further hypothesized that hyperglycemia would increase secretion of PLGF and that H_2S treatment would reverse this effect. We found that H_2S decreased PLGF secretion at 6 hours post treatment in human vascular smooth muscle and endothelial cells under basal conditions. In addition, we found that hyperosmolarity-induced PLGF secretion was reversed by H_2S treatment. We conclude that H_2S treatment reduces the secretion of PLGF and therefore protects vascular cells from a PLGF-driven pro-atherogenic environment. Further studies are needed to elucidate the mechanism whereby H_2S affects PLGF secretion under basal and hyperosmolar conditions.

Introduction

About 600,000 people die from heart disease every year, with 385,000 of those deaths resulting from coronary artery disease.[4] Oklahoma has one of the highest rates of cardiovascular disease. There are many different drug therapies available to treat cardiovascular disease through different mechanisms including antiplatelet drugs, anticoagulants, beta blockers, and nitric oxide (NO) donors/vasodilators.[2] Recent studies to better understand cardiovascular disease and potential treatments have focused on important signaling molecules produced by the vessel wall, such as the well-known vasodilator NO, which targets angiogenic and arteriogenic pathways. Hydrogen sulfide (H_2S) has long been recognized as a toxic environmental gas (similar to the initial understanding of NO) that inhibits the final step in the mitochondrial electron transport chain, which blocks ATP production.[5] However, recent evidence shows that H_2S is endogenously

produced at low concentrations by our blood vessels and exerts a number of beneficial properties including anti-inflammatory, anti-oxidative, vaso-relaxant and pro-angiogenic actions.[1]

Angiogenesis is the growth of new blood vessels that plays an essential role in development and wound healing as well as during coronary revascularization, which is necessary to repair tissues following a myocardial ischemic event. Angiogenesis can be pathological in the development of cancer tumors and plaques, but also beneficial in wound healing, embryogenesis, development, and ischemic damage. The angiogenic process promotes important compensatory and repair mechanisms in cardiovascular disease.[7] Arteriogenesis is the increase in diameter and wall thickness of pre-existing blood vessels to provide blood flow to otherwise deprived tissue. This can be beneficial through widening collateral vessels when occlusions and ischemia occur.[9]

Placental Growth Factor (PLGF) is a member of the Vascular Endothelial Growth Factor (VEGF) family that was first discovered in the placenta as a critical growth factor, but was later found to be important in the cardiovascular system. VEGF itself is a cytokine involved in the growth of vascular endothelial cells and is important in both angiogenesis and arteriogenesis. PLGF is a multifunctional angiogenic and arteriogenic cytokine that is secreted as a compensatory response following vascular occlusion. It has been shown in skeletal muscle, post ligation of the femoral artery, to drive the expansion of collateral vessels. While VEGF is also an important cytokine for arteriogenesis, PLGF has been shown to be more robust in its ability to drive arteriogenesis. VEGF has been shown to be essential for life in mice, while PLGF deficiency is not lethal. While this makes PLGF redundant in its ability to drive angiogenesis and arteriogenesis, PLGF is unique in that it drives both physiological (arteriogenesis) and pathological (atherosclerosis) processes. The mechanism whereby this switch occurs from physiological to pathological is an area of active investigation. Therefore, PLGF is a target of interest for cardiovascular disease treatments.[11,7]

Blood flow creates shear stress that acts on the vessel wall, which serves as a signal for arteriogenesis. In previous collaborative studies, our laboratory with P. Lloyd mimicked shear stress on the coronary vessel wall and found that exposure to shear stress drives an arteriogenic response, resulting in an increase in PLGF secretion and an increase in cystathionine gamma lyase (CSE) gene expression in coronary endothelial cells (ECs). This correlation between CSE gene expression in ECs and the increase in PLGF secretion suggested that H_2S plays a role in the process of arteriogenesis.

Despite several benefits of angiogenesis and arteriogenesis, these processes can be pathological, such as in the vascularization of tumors and in atherosclerotic plaque progression, which enhances lesion growth and coronary arterial occlusion.[4] 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.[4] PLGF contributes to early progression of atherosclerosis and serves as a clinical biomarker for cardiovascular disease. Cardiovascular disease is multifaceted with many risk factors that can contribute to progression of the disease. Hyperglycemia is a well-established risk factor for atherosclerosis and cardiovascular disease due to glucose-driven damage to the endothelial cells lining the vessel wall, as well as increased osmolarity in the vessel.[4] Hyperglycemia affects about 10 percent of Americans and more than 13 percent of Oklahomans; thus there is a need for different therapeutics to treat hyperglycemia.[8]

At high concentrations, H_2S is a flammable, toxic, odorous gas that is formed through the breakdown of organic matter and waste materials.[5] This gas can have detrimental effects on human health when exposed to large amounts, possibly resulting in death.[4] However, H_2S is endogenously

produced in the human body by the enzyme cystathionine gamma lyase (CSE) in vascular cells and is the byproduct of cysteine metabolism. H_2S, at low concentrations, has been shown to be pro-angiogenic, anti-inflammatory, and vasodilatory. It is also thought to be anti-atherogenic. Due to these qualities, H_2S donating drugs are in pre-clinical and clinical trials. For example H_2S donating nonsteroidal anti-inflammatory drugs (NSAIDs) are in clinical trials to reduce the detrimental effects NSAIDs have on the gastrointestinal system after long periods of use.[3] However, the effect of H_2S on arteriogenesis, as well as combating atherosclerosis is unknown.

Since low levels of H_2S have recently been shown to be protective to the cardiovascular system, we hypothesized that treatment of human vascular cells with H_2S would increase secretion of the pro-arteriogenic cytokine, PLGF. Based on conclusions from our first hypothesis, we further hypothesized that hyperglycemia would increase secretion of PLGF and that H_2S treatment would reverse this effect. The objectives were to (i) expose vascular cells to H_2S and a H_2S-donating drug (S-diclofenac), (ii) measure PLGF protein secretion and CSE gene expression in H_2S-treated versus untreated cells, (iii) treat human endothelial cells with hyperglycemic conditions in the presence or absence of H_2S and measure PLGF secretion, and (iv) measure PLGF protein secretion in H_2S-treated cells following exposure to hyperglycemic conditions.

Methods

Cell Culture:

Coronary artery smooth muscle cells (CASMC) were obtained from a 51 year old male donor and purchased from Lonza (Wakersville, MD). CASMC were cultured in 6-well plates in a CO_2 incubator (37° C, 5% CO_2) with smooth muscle cell (SmGM) medium until ~85% confluent. CASMC medium was supplemented with 5% fetal bovine serum (FBS) and growth factors to promote healthy, robust growth. Cells were serum starved for 48 hours by reducing the FBS to 1% in order to synchronize cell population growth prior to treatment. This procedure was repeated 3 times in duplicate (n=6). Pooled human umbilical vein endothelial cells (HUVEC) were also purchased from Lonza (Walkersville, MD, USA) and grown in 6-well plates at 37°C in 5% CO_2 until 85% confluent with EBM-2 medium. HUVEC medium was supplemented with growth factors and 2.5% FBS (Lonza). Cells were serum starved for 48 hours by reducing the FBS to 1% in order to synchronize cell population growth prior to treatment. This procedure was performed once in triplicate (n=3).

Drug Treatment:

To treat CASMC with the H_2S-donating drug, S-diclofenac was dissolved in a vehicle containing cell culture medium with 0.5%DMSO, and then degassed with argon to minimize spontaneous oxidation of the S-diclofenac. A final concentration of 50 μM of S-diclofenac was added directly to the medium of each designated well for each time point. To serve as a control for the effect of H_2S alone, cells were treated with 50 μM of sodium sulfide (NaHS), which quickly liberates H_2S. Because DMSO is an anti-oxidant capable of affecting gene and protein expression, a vehicle control (0.5%DMSO in cell culture medium) was added directly to cells. Media were collected on ice into tubes containing proteinase inhibitor at 6h post treatment. Media were also collected from wells containing untreated cells. Media was stored at -80°C until the ELISA. Cell samples were collected on ice at 6 hours post treatment and mixed with Lysis Buffer to lyse the cells, forming CASMC lysates. Cell lysates were stored at -20°C until RNA isolation for downstream qPCR applications. HUVECs were treated with NaHS (degassed in argon) to liberate H_2S. Treatment groups included: 50 μM and 200μM of NaHS, 50 μM Diclofenac which was dissolved in a vehicle containing culture medium with 5%DMSO (degassed with argon), and a

vehicle control (DMSO). 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.

To treat HUVECs in hyperglycemic 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 hyperosmolarity, 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) NaHS for 6 hours. Media were collected in protease inhibitor and stored for ELISA.

Enzyme- Linked Immunosorbent Assay:

To measure the cytokine PLGF secretion by CASMC and HUVEC, sample media harvested from treated cells were added to each well of the Enzyme-Linked Immunosorbent Assay (ELISA) plate, which was coated with the capture antibody of interest (PLGF) and incubated for 2 hours at room temperature. The wells were washed 4X followed by the addition of a detection antibody and incubation at room temperature for 2 hours. The detection antibody is linked to Streptavidin-HRP, which allows for a colorimetric reaction to occur upon the addition of the substrate solution and 20 minute incubation at room temperature. Stop solution was then added to each well and the absorbance was measured at 450 nm using a microplate reader. The absorbance values were used to calculate the pg/mL of protein (VEGF or PLGF) based upon a standard curve generated from known concentrations of the protein of interest that were assayed alongside our samples.

It is important to normalize the secretion (pg/mL) of our protein of interest relative to total protein secretion (mg/mL) thereby controlling for any global changes in translation of total protein following treatment conditions. To normalize the cytokine expression data, sample media were used in the Bicinchoninic Acid Protein Assay (BCA). In short, this assay allows for a colorimetric reaction to occur that measures the total protein present in samples. The BCA assay relies on two reactions. Peptide bonds in protein reduce Cu^{2+} ions from the cupric sulfate to Cu^{+}. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Then, two molecules of bicinchoninic acid react with each Cu^{+} ion, forming a purple-colored product that strongly absorbs light at a specific wavelength. The bicinchoninic acid Cu^{+} complex is influenced in protein samples by the presence of cysteine, tyrosine, and tryptophan side chains. The amount of protein present in a solution can be quantified by measuring the absorption spectra at 540 nm and comparing with protein solutions of known concentration using a microplate reader.

RNA Isolation:

RNA is isolated from the cell lysates through combining guanidine-isothiocyanate lysis with silica-membrane purification. To isolate RNA from coronary artery muscle cells, ethanol was mixed with the lysates to provide ideal binding conditions. Cell lysates were transferred to Qiagen columns onto the RNeasy silica membrane. Lysates were washed with RNA Wash Buffer once and RPE Wash Buffer, twice and then centrifuged after each wash. The fluid in the collection tube was discarded each time. Then, RNase-free water was added to the column and centrifuged. The elution from the collection tube was collected and quantified using a NanoVue reader. These RNA samples were stored at -80°C until cDNA synthesis.

RNA samples were treated with gDNA Wipeout Buffer to remove any genomic DNA contaminating the RNA sample to give pure RNA samples. In order for RNA to be converted to cDNA, the enzyme reverse transcriptase was added to the RNA sample to reverse transcribe the cDNA. This was done by adding a mastermix of Quantiscript RT, Quantiscript RT Buffer, and RT

Primer Mix to the microtubes and by placing the microtubes of mastermix and RNA sample in the G-Storm Cycler for 15 minutes at 42°C then for 3 minutes at 95°C. The cDNA samples were then stored at -20°C until real time qPCR.

Quantitative PCR:

To measure gene expression by coronary artery muscle cells, cDNA samples were added to each well of the qPCR plate, which was followed by the addition of a master mix of SYBR green, dNTPs, Taq DNA polymerase, $MgCl_2$, UNG, ROX Reference Dye and stabilizers, forward primer and reverse primer of the target gene, and molecular grade water. The forward and reverse primers specifically bind to the CSE gene sequence within the sample cDNA, and a DNA polymerase then synthesizes copies of the primer-specific region of the CSE gene. SYBR green is a fluorescent dye which intercalates the DNA as CSE cDNA is amplified. The fluorescence increases as SYBR green intercalates the amplified DNA. This increase in fluorescence reflects an increase in the gene expression of CSE. The fluorescent signal was measured using an Eppendorf RealPlex, and the delta delta Ct method was used to determine the fold change in gene expression relative to the expression of the housekeeping gene, beta actin.

Results

Overall, the levels of PLGF protein secretion were low in CASMCs in this study. Literature indicates that basal PLGF levels are very low in CASMC supporting the low levels of PLGF protein secretion found in this study are comparable to PLGF secretion in HUVECs. However, we found that PLGF protein secretion decreased in CASMCs treated with S-diclofenac, Vehicle, and NaHS compared to the control 6 hours post treatment (Fig. 1). Treatment with the vehicle caused the greatest decrease in PLGF secretion, possibly due to the anti-inflammatory properties of the DMSO-containing vehicle (Fig. 1).

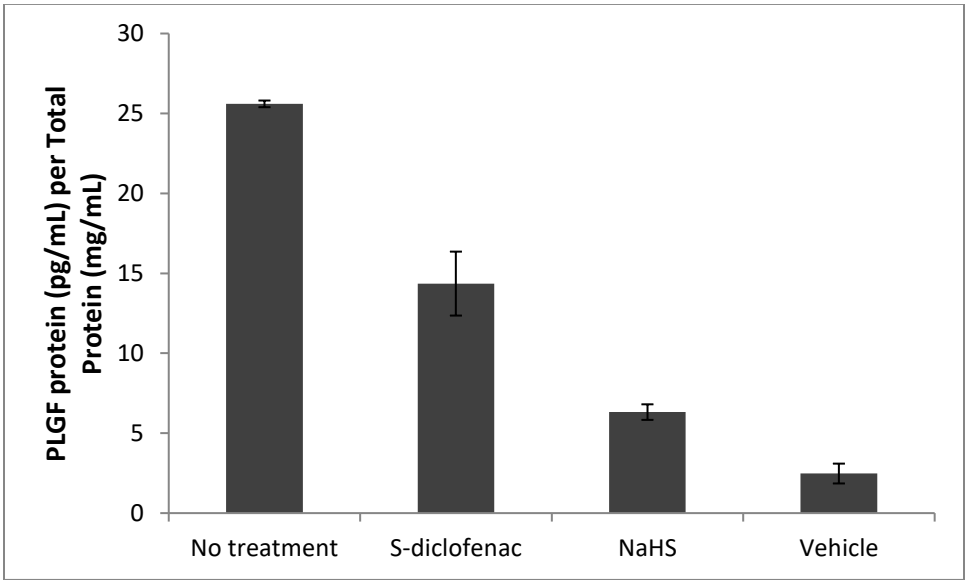

Figure 1. S-Diclofenac and NaHS reduced PLGF secretion by coronary artery *smooth muscle* at 6 hours. S-Diclofenac, NaHS, DMSO, and control treated CASMC media was collected at 6h, and PLGF protein was quantified. There was a decrease in PLGF secretion by S-Diclofenac and NaHS treated cells versus control (n=3).

PLGF protein secretion levels in HUVECs were much higher than CASMCs' PLGF protein secretion levels in this study. We found that PLGF protein secretion decreased in HUVECs treated with S-diclofenac and 200µM NaHS compared to the control at 6 hours post treatment (Fig. 2). There were minor decreases in PLGF secretion in HUVECs treated with the vehicle (DMSO) and 50 µM NaHS compared to the control 6 hours post treatment (Fig. 2).

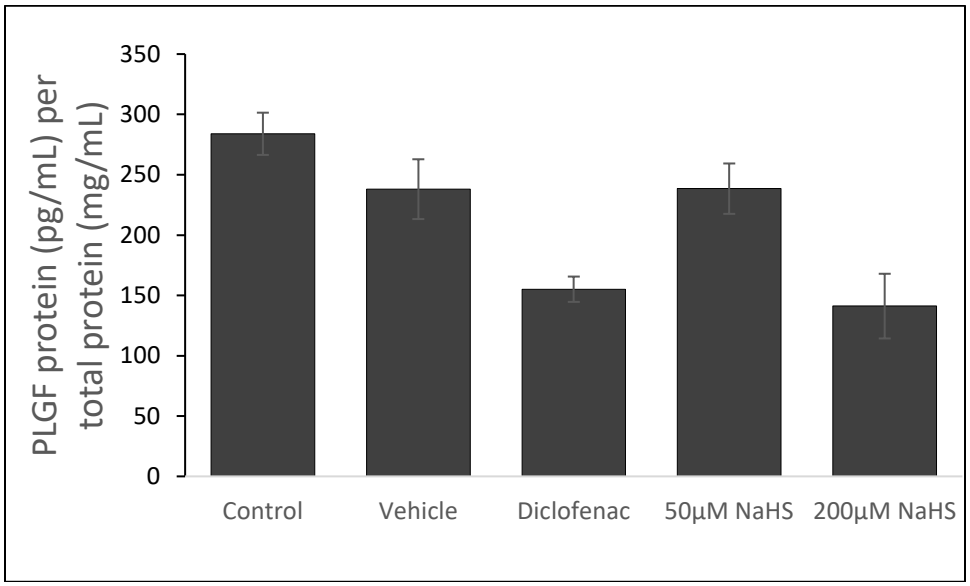

Figure 2. 200µM NaHS and S-diclofenac decreased PLGF secretion by *human primary endothelial cells* at 6 hours. Media were collected from HUVEC treated with S-diclofenac, 50µM NaHS, 200µM NaHS, DMSO, and control for 6h, and PLGF protein was quantified. There was a decrease in PLGF secretion by S-diclofenac and 200µM NaHS treated cells versus control (n=3).

We found that CASMCs treated with S-diclofenac, vehicle, and NaHS 6 hours post treatment showed an increase in CSE gene expression compared to the control (Fig. 3). There was no difference between treatment with S-diclofenac and the vehicle used to make up S-diclofenac (Fig. 3). Therefore, it was unclear if the large increase in CSE gene expression of cells treated with S-diclofenac was due to the anti-inflammatory components of the vehicle or the H_2S donating component of S-Diclofenac itself.

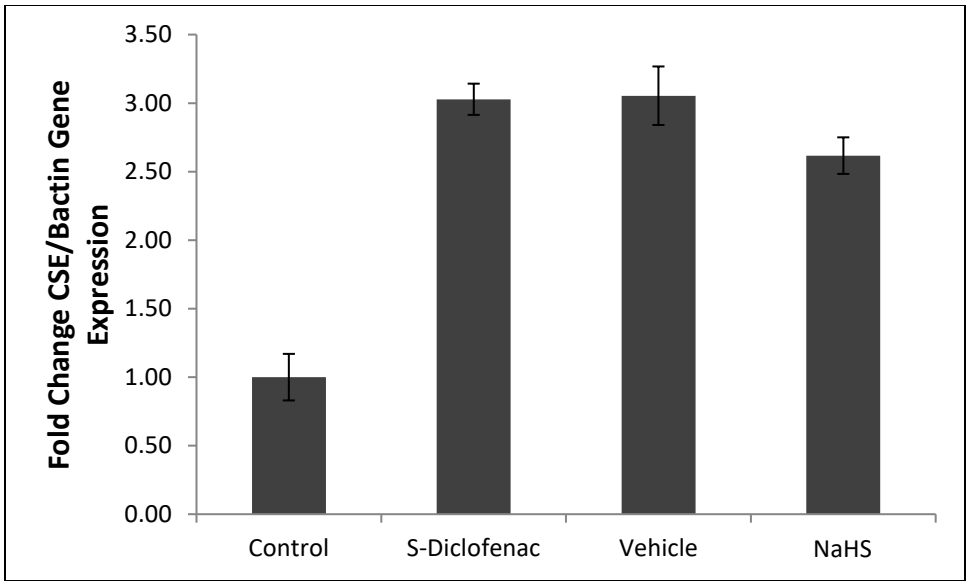

Fig. 3: H_2S increases CSE gene expression by coronary artery *smooth muscle* at 6 hours. Cells treated with s-diclofenac, Vehicle, and NaHS 6 hours post treatment showed an increase in CSE gene expression compared to the control. There was a substantial increase in CSE gene expression for cells treated with S-diclofenac compared to cells treated with NaHS.

Figure 4 is a representation of HUVECs exposed to a diabetic, or hyperosmolar conditions. We found that exposure to mannitol and high glucose without NaHS caused an increase in PLGF secretion compared to the normal glucose (control) after 3 days (Fig. 4). We also found that larger treatments with NaHS caused a reversal of PLGF secretion by HUVECs exposed to high glucose back to PLGF secretion by cells exposed to normal glucose levels (Fig. 4).

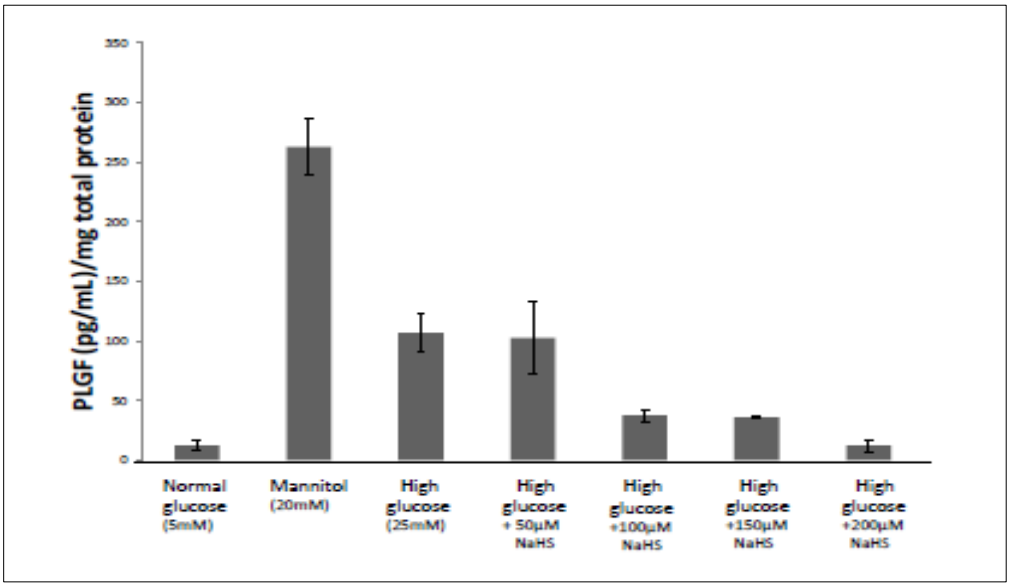

Figure 4. Chronic hyperosmolar conditions increased PLGF secretion by *human endothelial cells* and this effect was reversed by NaHS. HUVEC were treated with either normal glucose (5mM), mannitol (20mM, osmotic control), high glucose (25mM) for 3 days. Varying doses of

NaHS were added to the HUVEC. Media was collected at 6h, and PLGF protein was quantified. There was an unexpected increase in PLGF secretion by mannitol treated cells versus control (n=3).

Discussion

Overall, we determined that exposure to H_2S reduced PLGF secretion in human vascular cells. Based on the literature that demonstrates PLGF is pro-atherogenic the observed decrease in PLGF secretion by vascular cells following exposure to H_2S shows that H_2S potentially exerts an anti-atherogenic effect. Decreased PLGF secretion when exposed to H_2S suggests a reversal of preliminary atherosclerosis, as opposed to having pro-angiogenic properties, as we originally hypothesized. The H_2S donating drug, S-diclofenac, had inconclusive effects on the vascular cells. While a decrease in PLGF secretion was observed in human vascular cells when treated with S-diclofenac, the S-diclofenac vehicle alonealso caused a decrease in PLGF secretion. The vehicle, DMSO, has anti-inflammatory properties, as well. Therefore, it is unclear if the decrease in PLGF secretion observed in human vascular cells when treated with S-diclofenac was due to the S-diclofenac portion or the vehicle portion of the drug.

Exposure to H_2S increased CSE gene expression in CASMCs, suggesting an increase in endogenous H_2S production. Previous data shows that CSE gene expression is increased in coronary ECs following shear stress patterns similar to blood flow in atherosclerotic arteries, suggesting CSE is up-regulated to produce the cardioprotective gas, H_2S. This increase in CSE gene expression in coronary ECs suggests an increase in H_2S gas in the vessel wall. When exposed to H_2S, potentially from coronary ECs, CASMCs increase CSE gene expression to produce H_2S, as well. Together, these findings suggest that H_2S has a potential autocrine effect resulting in a positive feedback loop of H_2S gas in the vessel wall increasing H_2S levels in order to perform its anti-atherogenic role.

In hyperglycemic conditions, mannitol served as the control for hyperosmolarity due to its similar osmolarity to glucose. The increase in PLGF secretion after exposure to mannitol suggests that the hyperosmolarity alone caused the increase in PLGF secretion after exposure to glucose, not the presence of glucose specifically in the media. In hyperglycemic conditions, hyperosmolarity substantially increased the secretion of PLGF by human endothelial cells. This increase in PLGF secretion suggests early progression of atherosclerosis due to hyperosmolar conditions, a risk factor for atherosclerosis. The hyperosmolar-induced increase in PLGF secretion was reversed by H_2S, suggesting that H_2S is a cardioprotective gas against atherosclerosis in both basal and hyperglycemic/hyperosmolar conditions. While treated diabetic patients do not usually have hyperosmolar plasma, this finding could have possible implications for individuals with untreated diabetes who may experience hyperglycemic, hyperosmolar events.

One limitation of this study is HUVECs were used to better understand the effects that H_2S had on the endothelial cells of the vessel wall instead of human coronary artery endothelial cells. HUVECs were used due to cost, but these cells may not best represent the endothelial vessel lining of coronary arteries. To improve this study, human coronary artery endothelial cells may be treated with H_2S in order to measure PLGF secretion and therefore determine whether our results were accurate. Also, there is an insufficient *n* value for statistical analysis to prove the significance of the correlations observed. This experiment needs to be repeated in both basal and hyperglycemic conditions to increase the *n* value for statistical analysis.

Future studies include increasing our *n* value to determine statistical significance of this observed response to H_2S. Also, further studies are also needed to determine the mechanism whereby H_2S affects PLGF secretion under basal and hyperosmolar conditions.

Posters Presented

Homeyer, Sydnee, et al. "The Effect of Hydrogen Sulfide on PLGF Secretion by Human Vascular Cells." 24 Apr. 2015.

Homeyer, Sydnee and Jennifer H. Shaw. "The Effect of a Sulfide Donating Drug on VEGF and PLGF Protein Secretion by Coronary Artery Smooth Muscle." 20 Feb. 2014.

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