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Title: Hyperosmolar state results in increase in PLGF secretion

Abstract:

Diabetes mellitus and heart disease are prevalent in the United States and around the world. Especially with the aging population, it is vital to understand the potential risks associated with diabetes, specifically type 2 diabetes mellitus. Hyperglycemia is an established risk factor for heart disease. Our study aims to understand the effects of hyperglycemia on secretion of PLGF, a biomarker for heart disease. In our study, we tested cells in both a hyperglycemic and healthy environment, and used mannitol as a control for hyperosmolarity. We hypothesized that treatment of human vascular cells with H_2S would decrease secretion of the pro-atherogenic cytokine, PLGF. We further hypothesized that hyperglycemia (a risk factor for cardiovascular disease) would increase secretion of PLGF by vascular cells and that H2S treatment would reverse this effect.

We found that in both mannitol and hyperglycemic environments, PLGF secretion increased significantly. This implies that hyperosmolarity itself rather than specifically hyperglycemic conditions increase PLGF secretion. Additionally, we found that hydrogen sulfide treatment in increasing doses reversed the increase in PLGF secretion upon hyperglycemic conditions. This supports our hypothesis that treatment of human vascular cells with hydrogen sulfide would decrease secretion of PLGF, and that hyperglycemia would increase secretion of PLGF. These findings are potentially applicable to the hyperosmolar hyperglycemic state sometimes associated with uncontrolled diabetes mellitus. Further understanding of this mechanism could lead to potential clinical treatment of this condition using H_2S donating drugs.

I. Beginning goals and objectives:

Rationale for our study

Heart disease is the number one killer in the US, with coronary heart disease being the most common type. Coronary heart disease kills over 370,000 people every year in the US[1]. Although prevalent, the distribution of deaths annually varies regionally. Oklahoma has one of the highest rates of death from coronary heart disease[1]. Hyperglycemia also has a high incidence rate in Oklahoma and is an established risk factor for heart disease, as it is can contribute to the formation of atherosclerotic plaques in coronary arteries[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[1]. This is a result of irritants such as hyperlipidemia, high blood pressure, smoking, and hyperglycemia. The development of atherosclerotic plaques over time is supported by the formation of microvessels within the plaques. This formation of new blood vessels is known as “angiogenesis”.

PLGF is a multi-functional, angiogenic cytokine that contributes to the early progression of atherosclerosis ("atherogenic") although the mechanism is not fully understood. Likewise, PLGF is now an established clinical biomarker for cardiovascular

disease[4]. We are interested in ways to potentially minimize its effects on the progression of atherosclerosis.

Potential for cardio-protective therapeutic drugs has increased with recent discovery of the importance of gaseous mediators in the body. Examples of these include nitric oxide, carbon monoxide, and hydrogen sulfide (H_2S). These gaseous mediators are endogenously produced at low concentrations, and each participates in vasodilation and cardioprotection. Hydrogen sulfide, although commonly known as a respiratory poison, has actually been recently discovered to act (at physiological concentrations) as a cardioprotectant in the body. H_2S is endogenously produced at low concentrations by the enzyme cysthathionineγlyase (CSE) in our blood vessels and exerts a number of properties including anti-atherogenic,anti-oxidative , and vasodilatory action[2]. The effect of hydrogen sulfide on PLGF is unknown, however the protective properties of this gas make it a strong candidate to potentially alleviate the atherogenic effect of PLGF. Since hyperglycemia is a common risk factor for atherosclerosis, we choose to mimic diabetic conditions in vitro to test the effect on PLGF secretion and the impact of treatment with hydrogen sulfide. We also tested vascular cells from healthy versus diabetic donors to assess CSE gene expression, which would suggest changes in endogenous production of hydrogen sulfide.

Background on hyperosmolar hyperglycemic state

Hyperosmolar hyperglycemic state (HHS) is a serious acute complication associated with diabetes mellitus type 2[5]. This state is often associated with patients with concomitant illness that reduces fluid intake[5]. This could include infection, but could also include dehydration, altered mental state, or most commonly cardiovascular disease[3,5]. HHS is usually an issue in older patients, and exerts a higher mortality rate relative to diabetic ketoacidosis (DKA). HHS often can be difficult to differentiate between the original illnesses, rendering it almost impossible to diagnose[3].

HHS is characterized by hyperglycemia, plasma hyperosmolarity, and dehydration in patients[5]. Additionally, it can cause disorientation in mental state[5]. Clinically, one-third of DKA and HHS cases overlap, which implies that both states of diabetes mellitus differ only in amount of dehydration and presence of acidosis[3].

Hyperosmolar hyperglycemic state occurs in the absence of ketoacidosis, however it follows the same pathway as DKA[5]. The difference is the cause of HSS is hyperglycemia rather than ketoacidosis[5]. DKA takes hours to develop, but HHS takes days, as it is more extreme[6]. Hyperglycemia in diabetes mellitus combined with a rise in plasma proteins cause the hyperosmolar state[3]. Due to this state, antidiuretic hormone is released, relieving renal water loss[3]. This also causes stimulation in thirst[3]. In HHS, drinking water cannot compensate for renal water loss[3]. This severe dehydration causes hypovolemia, leading to hypotension and impaired tissue perfusion[3]. The end stage of HHS is coma due to electrolyte disturbances in result of hypvolemia[3]. Severe hyperosmolarity causes a stimulation of renin-angiotensin-aldosterone system and can lead to a total shutdown of the renal system[3]. In this state, oliguria prevents excretion of glucose, which in turn exacerbates the hyperglycemic state of the patient[3].

Our predictions

We hypothesized that treatment of human vascular cells with H_2S would decrease secretion of the pro-atherogenic cytokine, PLGF. We further hypothesized that hyperglycemia (a risk factor for cardiovascular disease) would increase secretion of PLGF by vascular cells and that H2S treatment would reverse this effect.

II. Experiments performed:

Culturing cells

Primary pooled 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% CO_2 until 85% confluent then sub-cultured into 6 well plates for experimental procedures. HUVEC were cultured in 6 well plates for three 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. Following the three day period, all cells were treated with varying doses (0, 50, 100, 150, 200 uM) of sodium hydrosulfide (NaHS), which liberates hydrogen sulfide gas, for six hours. Culture media was collected on ice in tubes containing protease inhibitor for quantification of secreted PLGF, an atherogenic cytokine. In addition, cells were harvested and lysed to isolate RNA for downstream gene expression studies.

ELISA

Sample medium was 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 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 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. In addition, in order to normalize the secretion (pg/mL) of our target protein in relation to the overall protein (mg/mL) to account for overall changes in protein concentration, we used Bicinchoninic Acid Protein Assay (BCA). This assay induces a colorimetric reaction that measures total protein in the samples. This depends on two reactions. First, the peptide bonds in the protein must reduce the Cu^{2+} ions to Cu^{+} from the cupric sulfate. This amount of reduction is in proportion to the total amount of protein in the solution. Second, two molecules of bicinchoninic acid then react with the Cu^{+} ions. This reaction induces a purple color, which absorbs light at a specific wavelength. This complex is reliant upon presence of side chains of cysteine, tyrosine, and tryptophan. The total protein present was then quanitified. We quantify this by measuring the spectra at 540 nm. We then compared these with the spectra known concentrations of protein solutions using a microplate reader.

RNA isolation & cDNA synthesis

In order to measure the expression of CSE, RNA was isolated from diabetic versus nondiabetic cells by our collaborator (Dr. Nabil Rashdan) using RNeasy columns from Qiagen; then we reverse transcribed the RNA into cDNA. .

In order to synthesize cDNA we first treated the cDNA with gDNA Wipeout Buffer. This removes any genomic DNA that could potentially contaminate the sample of RNA. To convert the RNA sample to cDNA, we added the enzyme reverse transcriptase to reverse transcribe the cDNA. To do this, we added a mastermix of Quantiscrupt RT, Quanitscript RT Buffer, and RT Primer Mix to microvials and added RNA samples. We then places the microtubes into the G-Storm Cycler for 15 minutes at 42°C then for 3 minutes at 95°C. After synthesis of cDNA, we stored these samples at -20°C until real time qPCR.

qPCR

In order to measure gene expression by the donor cells, we used quantitative real time PCR (qPCR). We first made a master mix containing SYBR green (an intercalating dye), forward primer and reverse primer for the target genes (CSE and beta-actin), and molecular grade water. The primers bind to CSE gene in the cDNA. DNA polymerase synthesizes copies of this specific region of the CSE gene. SYBR green dye is fluorescent and intercalates the DNA while the CSE cDNA is amplified using the Eppendorf Mastercyler. This fluorescence increases during this process anddirectly correlates with the increase of CSE gene expression. The signal was analyzed using EppendorfRealPlex software. We used the delta delta Ct method to analyze the data of both the EC and CASMC expression of CSE in relation to the housekeeping gene beta actin.

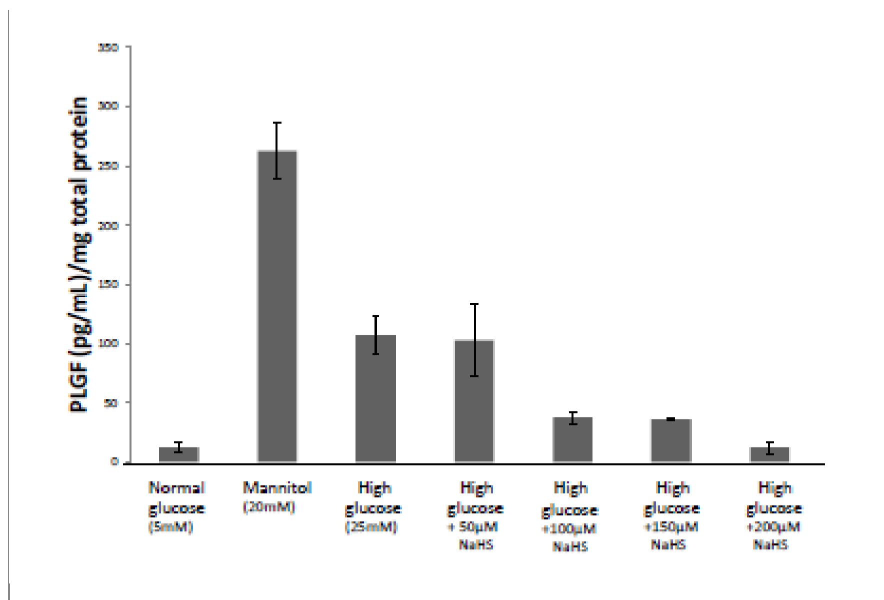

Figure 1. PLGF protein secretion by human endothelial cells (EC) following chronic hyperglycemic/hyperosmolar conditions. EC were treated with either normal glucose

(5mM), mannitol (20mM), high glucose (25mM) for 3 days. Then varying doses of NaHS were added to the EC. Six hours later, medium was collected and secreted PLGF protein was measured by ELISA (n=2).

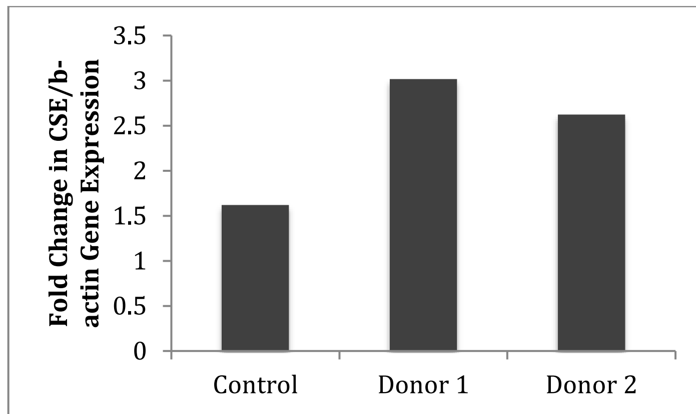

Figure 2. CSE expressed by endothelial cells (EC) in diabetic donors.

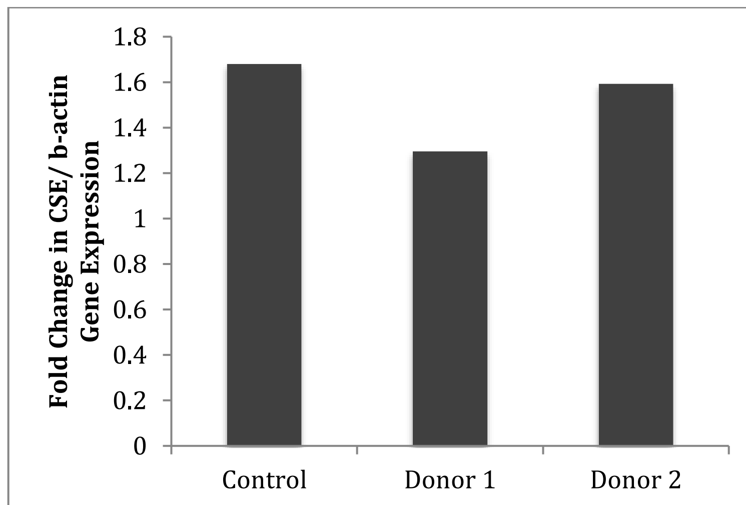

Figure 3. CSE expressed by Coronary Artery Smooth Muscle Cells (CASMC)in diabetic donors.

Interpretation of Data

We previously found 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, H_2S. In this study, hyperosmolarity substantially increased the secretion of PLGF by human endothelial cells. Hyperglycemic conditions produced an increase in PLGF protein expression, but this may be due strictly to changes in osmolarity rather than a glucose-specific response.Induction of PLGF protein secretion was abolished by treatment with 100-200μM NaHS, which liberates H_2S gas. Other studies show that hyperosmolarity drives oxidative pathways, suggesting that the observed induction of PLGF expression by hyperosmolarity in our study may be due to oxidative stress[9]. Published data from our labs[8] show that PLGF expression is regulated by

p38MAPK and JNK, which are key players in oxidative stress pathways and support our findings herein.

In addition, using cells from diabetic donors, we have found an increase in CSE gene expression in endothelial cells of diabetic donors. We suggest that this increase in expression is due to the correlation of hyperglycemia with atherosclerotic conditions, which increases the need to dilate the vessels, a task that could theoretically be accomplished through an increase in endogenously produced hydrogen sulfide. These results could potentially be applied to HHS conditions. To specifically apply our findings to HHS, future studies may include measuring the effect of H_2S treatment onEC exposed to mannitol alone or mannitol plus hyperglycemic conditions. This could potentially help us to understand the pathway of HHS, and potential treatment to decrease severity of or stop HHS in uncontrolled diabetes mellitus.

References:

1. "Heart Disease Facts." *Centers for Disease Control and Prevention*. http://www.cdc.gov/heartdisease/facts.htm
2. Cheung et al. "Anti-atherogenic effect of hydrogen sulfide by overexpression of cystathionineγlyase (CSE) gene." *PLOsOne*, Nov 2014, vol 9: 11 e113038.
3. Hemphill, R. "Hyperosmolar hyperglycemic state." *Medscape,* Aug 03, 2016
4. Mieke Dewerchin and Peter Carmeliet. "PLGF:A multitasking cytokine with disease-restricted activity." *Cold Spring HarbPerspect Med* 2012; 2:a011056.
5. Rybka J and Mistrik J. "Hyperosmolar hyperglycemic state" *Vnitr Lek* 2015 May;61(5):451-7
6. Scott, A.R. "Management of hyperosmolar hyperglycaemic state in adults with diabetes." *Diabet. Med.* 32, 714–724 (2015)
7. Shaw, Jennifer H., Lingjin Xiang, Anu Shah, Wei Yin, and Pamela G. Lloyd. "Placenta Growth Factor Expression Is Regulated by Hydrogen Peroxide in." *American Journal of Physiology*. American Physiological Society, 1 Dec. 2010. Web. 17 Feb. 2014. <http://ajpcell.physiology.org/>.
8. Jennifer H. Shaw and Pamela G. Lloyd. "Post-transcriptional regulation of placenta growth factor mRNA by hydrogen peroxide." *Microvascular Research* 84 (2012), 155-160
9. Suda et al. "Protection of human endothelial cells from oxidative stress." *Circulation* DOI: 10.1161/hc0802.104324