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Acute hyperglycemia increases sepsis related glycocalyx degradation and endothelial cellular injury: A microfluidic study

Lawrence N. Diebel*, David M. Liberati, Jonathan V. Martin

Michael and Marian Ilitch Department of Surgery, Wayne State University, Detroit, MI, USA

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ABSTRACT

Background: Hyperglycemia promotes vascular inflammation; however its effect on endothelial dysfunction in sepsis is unknown. Microfluidic devices (MFD) may closely mimic the *in vivo* endothelial cell microenvironment. We hypothesized that stress glucose concentrations would increase sepsis related endothelial injury/activation.

Methods: Human umbilical vein endothelial cell (HUVEC) monolayers were established in microfluidic channels. TNF was added followed by glucose. Endothelial glycocalyx (EG) integrity was indexed by shedding of the EG components as well as thickness. Endothelial cell (EC) injury/activation was indexed by soluble biomarkers. Intracellular reactive oxygen species (ROS) was by fluorescence.

Results: TNF increased glycocalyx degradation and was associated with biomarkers of EC injury. These vascular barrier derangements were further increased by hyperglycemia. This may be related to increase ROS species generated followed by the combined insults.

Conclusion: MFD technology may be a useful platform to study endothelial barrier function and stress conditions and allow preclinical assessment of potential therapies.

Background

The Third International Consensus for Sepsis (Sepsis-3) defined sepsis as life threatening organ dysfunction caused by a dysregulated host response to infection.[1] The critical role of the microcirculation in tissue oxygenation is known and is a major determinant of organ dysfunction in sepsis.[2–4] In this regard there is increasing evidence that adverse effects of sepsis on endothelial and glycocalyx integrity impair the microcirculation and lead to organ dysfunction.[5,6] Stress hyperglycemia is common in critically ill and septic patients and has led to targeted strategies of glycemic control. The association between hyperglycemia and poor outcomes is most evident in patients without a prior history of diabetes.[7–9]

A causal relationship between hyperglycemia and outcomes in the critically ill remains speculative; however existing studies have shown that diabetes associated hyperglycemia causes endothelial functional and structural changes. *In vitro* cell culture models and *in vivo* animal studies have also demonstrated that acute hyperglycemia also may result in endothelial dysfunction and glycocalyx degradation resulting in activation of the coagulation system.[10–15] The role of stress hyperglycemia on sepsis induced endothelial cellular injury and glycocalyx degradation has not been previously studied. Cell culture models using standard laboratory conditions are inadequate to address this question. In vivo animal studies of the microcirculation are exceedingly complex and difficult to control.

Microfluidic technology has emerged as a promising model to characterize the endothelial response to sepsis and other proinflammatory conditions including hyperglycemia.[16] We hypothesized that stress glucose concentrations would increase sepsis related endothelial injury/activation and endothelial glycocalyx degradation. This was studied using a microfluidic device (MFD) *in vitro*.

Methods

HUVEC culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza Walkersville, Inc. (Walkersville, MD). Cells were grown in a 75 cm^2 flask using complete media (EGM-2 BulletKit,

* Corresponding author. Michael and Marian Ilitch Department of Surgery, 6C University Health Center, 4201 Saint Antoine Street, Detroit, MI, 48201, USA.
E-mail addresses: ldiebel@med.wayne.edu (L.N. Diebel), dliberat@med.wayne.edu (D.M. Liberati), jvmartin@med.wayne.edu (J.V. Martin).

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Lonza). Time to subculture is 5–9 days with media changes every 2 days. Cells are subcultured at 85% confluence using 2 ml of 0.5% trypsin-EDTA (Life Technologies, Carlsbad, CA). A new culture flask is prepared and the remaining cells are used to seed the microfluidic channels of a BioFlux 48 well plate (Fluxion Bio) that has been primed and coated with 100 μg/ml fibronectin (Fisher Scientific) for 1 h at room temperature. Monolayers were formed within the microfluidic channels after overnight perfusion of the cells with complete media at a shear force of 1 dyne/cm^2.

Well plate Microfluidic Device System (MDS)

The main components of the MDS include BioFlux plates, a pressure interface device, a controller instrument and software for the instrument control and image analysis (Fig. 1). The BioFlux plates contain an array of microfluidic flow channels on a well plate format which are connected to inlet and outlet wells. The pressure interface device covers the top of the well plate and applies a controlled pneumatic pressure from the control instrument. This serves to drive the fluid or perfusate through the microfluidic channels at a user-defined flow rate. A glass coverslip in the bottom of the microfluidic channels allows imaging by microscopy. The MDS software allows control of the flow settings as well as other parameters including image analysis. Thus the microfluidic device allows study of the glycocalyx barrier function under flow induced shear stress under controlled experimental conditions.

Experimental design

Once confluent HUVEC monolayers are formed, cells are exposed to TNF at either 25 or 50 ng/ml for 60 min at 37 °C. Subsets of cells that have exposure to TNF are perfused with either control media or media with glucose concentrations of 80 and 200 mg/dl. The perfusate was collected 60 and 180 min after exposure to the varying glucose concentrations (N = 5 for each group). In separate experiments 10 ng/ml lipopolysaccharide (LPS) was substituted for TNF and experiments carried out as outlined above. Glycocalyx shedding was assessed by measuring both syndecan-1 release and hyaluronic acid (HLA) present in cell culture supernatants. Endothelial cell activation/injury was determined by soluble thrombomodulin (sTM) and angiopoietin-1 and angiopoietin-2 (Ang-1/Ang-2). Glycocalyx injury was also assessed by staining cells with Fluorescein isothiocyante conjugated wheat germ agglutinin (FITC-WGA, Sigma Aldrich, St. Louis, MO) antibody and visualizing the glycocalyx using a fluorescent microscope and performing image analysis using Volocity software to quantitate glycocalyx thickness by measuring fluorescent intensity. Reactive oxygen species (ROS) produced by the endothelial cell monolayer were measured using a cell permeant dye and fluorescent microscopy.

Determination of soluble thrombomodulin (sTM) levels in HUVEC supernatants

Quantikine Human Thrombomodulin Immunoassay was used for the determination of sTM in the perfusate of HUVEC cultures exposed to varying glucose concentrations as outlined in experimental design above. The ELISA was performed according to the manufacturer's instructions (R & D Systems, Inc., Minneapolis MN). Briefly, the precoated plate containing a monoclonal antibody specific for human TM is incubated with fifty microliters of culture supernatants or the provided standards. After washing, the plate was further incubated with the TM conjugate and finally the substrate. Finally, the color reaction was stopped using 2 N H_2SO_4 and the optical density measured at 450 nm using a spectrophotometer.

Quantification of human angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2)

Quantikine Human Angiopoietin −1 Immunoassay kit and Human Angiopoietin-2 Quantikine ELISA kits from R & D Systems, Inc. Minneapolis, MN were used to measure ANG-1 and ANG-2 in HUVEC culture supernatants. Fifty microliters of HUVEC culture supernatants were transferred directly to microplate test strips of the ELISA plate. Further assay procedures were followed and

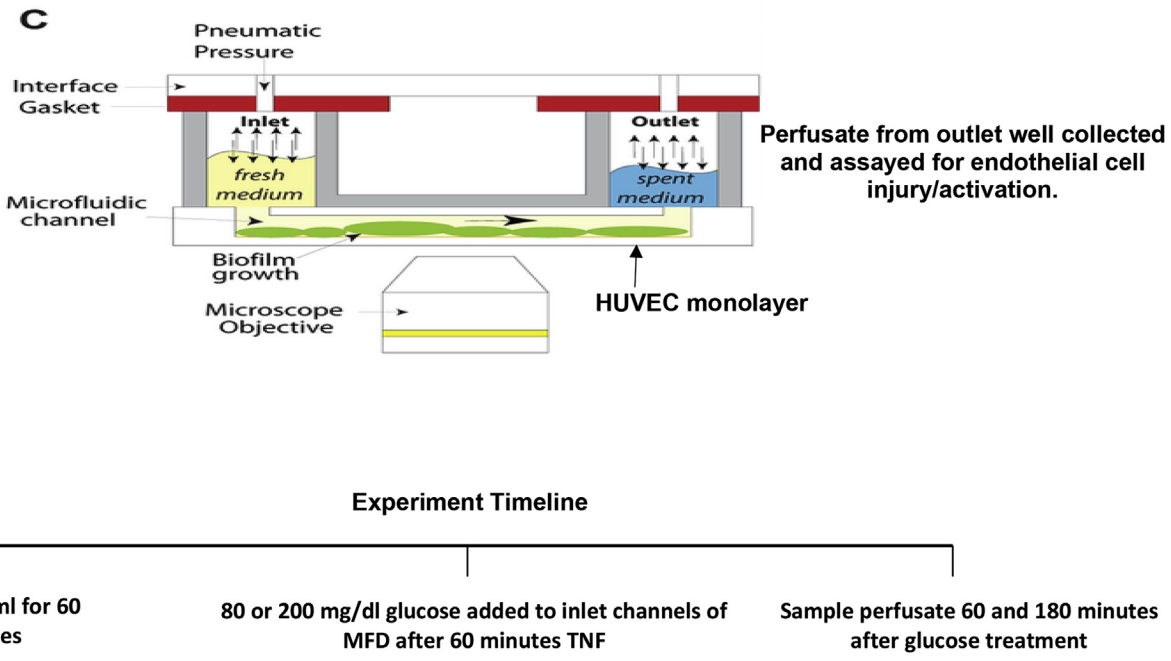

Fig. 1. Microfluidic Device System. Schematic representation of the microfluidic channel and device and description of the experimental timeline.

completed. The absorbance at 450 nm was measured using a spectrophotometer and ANG-1 and ANG-2 levels were calculated using a standard curve of recombinant human ANG-1 and ANG-2 provided.

Syndecan-1 and hyaluronic acid analysis

Quantitative measurement of Syndecan protein and hyaluronic acid in HUVEC was accomplished using the Syndecan-1 Human ELISA kit (Abcam, Cambridge, MA) and the Hyaluronic Immunoassay kit from R & D Systems, Inc., Minneapolis, MN respectively. Standards and unknown samples are added to the microplate wells and assay procedures followed. The optical density is determined using a microplate reader set to 450 nm and the concentration of syndecan-1 and HLA in the supernatants is calculated using a standard curve.

Reactive oxygen species measurement

The cell permeable fluorescent dye 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, ThermoFisher) was used to measure the intracellular ROS level. Briefly, prior to carrying out the experimental conditions outlined above the cultured endothelial cells in the microfluidic channels were incubated in culture medium with 10 μM H_2DCFDA for 45 min to establish a stable intracellular level of the dye. Subsets of cells were then exposed to TNF and varying glucose concentrations. As the presence of ROS builds within the cells, the H_2DCFDA contained within the cytoplasm is oxidized and becomes fluorescent. Therefore, the fluorescent intensity of the cell is a measure of the intracellular ROS level. Immediately before measuring the fluorescent intensity, the cells are washed with phosphate buffered saline (PBS) to remove any extracellular H_2DCFDA dye. The fluorescent images were captured using a Leica TCS SP5 inverted fluorescent microscope and a 20× objective.

Fluorescent imaging and thickness of glycocalyx

HUVEC were cultured in endothelial cell growth medium supplemented by growth factors (EGM-2 BulletKit) Lonza. Before cell seeding, microchannels were coated with human fibronectin (100μg/ml, Fisher Scientific) for 1 h at room temperature. HUVEC suspensions were seeded into the outlet wells of a 48 well BioFlux plate and infused into the microchannel network using the BioFlux 200 system (Fluxion Biosciences, Inc.). Cells were cultured overnight with complete media under flow conditions (shear force of 1 dyne/cm^2) or static conditions. Live cell staining was performed inside the microfluidic channels using wheat germ agglutinin (WGA) conjugated with fluorescein isothiocyanate (FITC, Sigma). Briefly, endothelial cell cultures treated with TNF and media containing varying glucose concentrations had FITC-WGA infused into the microchannel network using the BioFlux 200 system and cells were allowed to incubate for 30 min. The cells were washed 2× and fresh culture medium was added and cells were examined under a fluorescent microscope. Image analysis was performed using Volocity software at the Microscopy, Imaging and Cytometry core facility at Wayne State University (Detroit, MI). Measurement of the glycocalyx thickness was achieved by XYZ image stacks of the endothelial cell layer using a Leica TCS SP5 microscope and a 20× objective.

Statistical analysis

An analysis of variance with a post hoc Tukey test was used to analyze the data. Statistical significance was inferred at P values of less than 0.05. All data are expressed as mean ± SD.

Results

The effects of glucose concentration and/or treatment with TNF on endothelial cell activation/injury are shown in Table 1. Stress concentrations of glucose or TNF alone had similar effects on increasing sTM, endothelial cell ROS generation and increasing Ang-2 with concomitant decrease in Ang-1 levels. IL-1 concentrations were increased 1.2–2 fold. A synergistic effect of glucose with TNF on these endothelial biomarkers was noted, particularly with the 200 mg/dL "stress" glucose concentration. The effects of TNF and acute hyperglycemia on endothelial cell ROS production are shown in Fig. 2. The most intense staining, indication of ROS production was noted in the TNF +200 mg/dL glucose group.

The effects of increasing glucose concentration and/or TNF exposure on the endothelial glycocalyx layer are shown in Fig. 3. Glycocalyx degradation was indexed by shedding of its syndecan-1 (syn-1) and hyaluronic acid (HLA) components. TNF (50 ng/ml) and glucose (200 mg/dL) treatment led to a similar amount of shedding of the syn-1 and HLA glycocalyx components. This was associated with a 22–27% reduction in the thickness of the glycocalyx layer. The most profound effect on the glycocalyx layer was noted in the 200 mg/dL glucose + TNF group; there was approximate 4-fold increase in syn-1 shedding and 10-fold increase in HLA shedding. This was accompanied by a 42% reduction in the thickness of the glycocalyx layer.These findings are corroborated by fluorescent staining of the glycocalyx layer in the different treatment groups (Fig. 4).

The effects of increasing TNF concentration combined with the stress glucose concentration are demonstrated in Table 2. Endothelial biomarker concentrations responded in a similar fashion as TNF was increased from 25 to 50 ng/ml combined with stress levels of glucose (200 mg/dL). The findings noted with lipopolysaccharide (LPS) were similar to the results with TNF. Similar findings were found with the glycocalyx layer (Fig. 5). A synergistic effect between high glucose concentration and increasing TNF

Table 1
The effect of glucose concentration on endothelial cell activation and injury. Perfusate was collected at 60 and 180 min after exposure to varying glucose concentrations ± TNF and used to assess HUVEC injury. Soluble thrombomodulin (sTM), reactive oxygen species (ROS) produced by the HUVEC monolayer, angiopoietin-2 (Ang-2) and the cytokine IL-1 show significant increases in their levels after exposure to stress concentrations of glucose and/or TNF. A concomitant decrease is seen in Ang-1 levels as Ang-2 levels increase.

	TM (pg/ml)	ROS (intensity)	Ang-1 (pg/ml)	Ang-2 (pg/ml)	IL-1 (ng/ml)
HUVEC control (ON flow)	23.9 ± 1.9	24.5 ± 0.08	492 ± 8.7	147 ± 2.3	27.1 ± 1.6
80 mg/dL glucose	26.2 ± 2.5	27.8 ± 1.3	485 ± 6.7	151 ± 1.9	30.3 ± 1.1
200 mg/dL glucose	47.4 ± 2.8*	68.3 ± 1.5*#	277 ± 3.1*	210 ± 3.6*#	36.8 ± 2.3*
TNF 50 ng/ml	51.8 ± 3.1*	53.7 ± 1.6*	262 ± 1.9*	180 ± 4.1*	58.3 ± 5.1*
TNF 50 ng/ml + 80 mg/dL glucose	71.6 ± 5.1*#	68.3 ± 1.4*#	210 ± 1.9*#	261 ± 4.4*#	62.7 ± 5.4*
TNF 50 ng/ml + 200 mg/dL glucose	89.9 ± 6.3*#	80.8 ± 2.4*#	175 ± 4.8*#	335 ± 5.9*#	82.9 ± 6.6*#

*$p < 0.05$ vs. HUVEC control, #$p < 0.05$ vs. TNF 50 ng/ml.

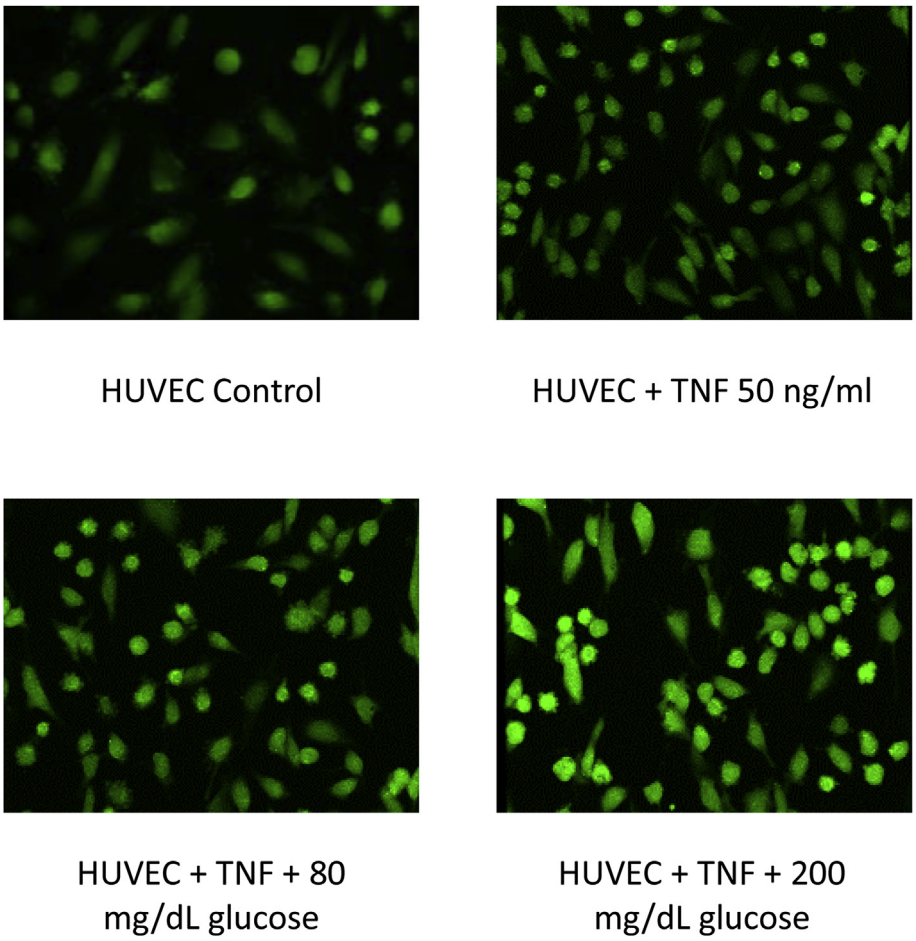

Fig. 2. Effect of TNF and acute hyperglycemia on ROS production. The fluorescent images were captured using a cell permeable fluorescent dye (H2DCFDA) and the Leica TCS SP5 inverted fluorescent microscope and a 20× objective.

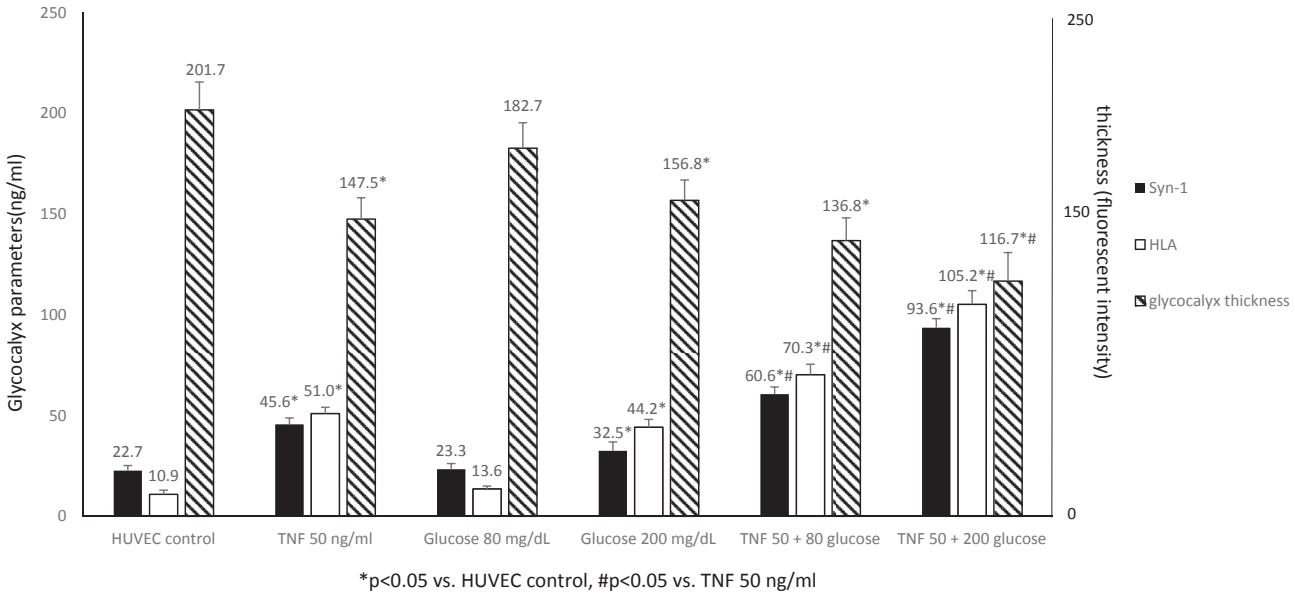

Fig. 3. Effect of TNF and hyperglycemia on glycocalyx shedding and thickness. ELISA kits were used to quantitate syndecan-1 and hyaluronic acid levels present in the perfusate collected from HUVEC treated with TNF 50 ng/ml followed by the addition of varying concentrations of glucose. Three-dimensional XYZ image stacks were acquired and processed and analyzed using Volocity cellular imaging and analysis software to assess glycocalyx thickness by measuring fluorescent intensity.

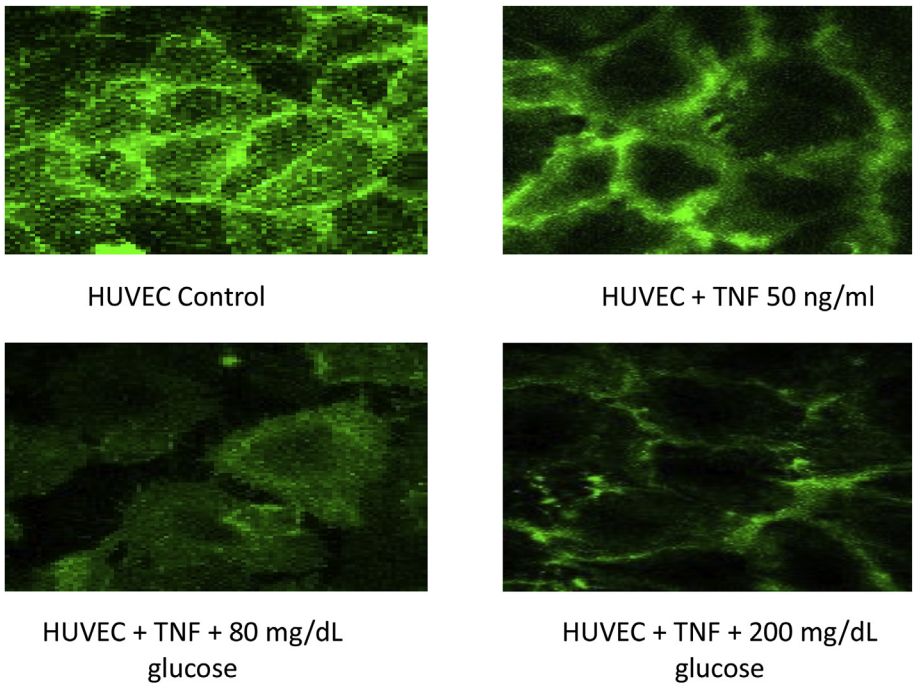

Fig. 4. Glycocalyx staining with FITC-WGA antibody. Images of the glycocalyx on the endothelial surface layer stained with FITC-conjugated wheat germ agglutinin. Acquired XYZ image stacks were processed using the Leica TCS SP5 microscope and a 20× objective.

concentration was noted with glycocalyx degradation as indexed by shedding and resultant thickness of the glycocalyx layer. The glycocalyx degradation noted following high glucose + LPS were similar to findings with the TNF (50 ng/ml) group.

Discussion

Hyperglycemia is common in critically ill patients with and without known diabetes.[17] The long term consequences of hyperglycemia are well known. This led to the interventional trials of glycemic control in the critically ill.[18] The results of these studies suggested that a "one size fits all" approach in regard to glycemic control in the critically ill was inappropriate.[9] Results of tight glycemic control included hypoglycemia (which was independently associated with increased risk in mortality) and ultimately, that there was no consistent evidence that this therapy improved outcomes. This led to modifications of glycemic control guidelines in the critically ill. The 2016 International Guidelines for the Management of Sepsis now recommends a protocolized approach to blood glucose management in septic patients with an upper blood glucose level of >180 mg/dL rather than an upper limit of <110 mg/dL.[19]

Glycemic control in septic patients was initiated in part to improve the deleterious effects of hyperglycemia on certain aspects of host defense. These include impaired polymorphonuclear cell function, increased proinflammatory cytokine production and endothelial cellular activation/injury. More recently the endothelial glycocalyx has been recognized as a critical compound of the microvascular barrier as it regulates vascular integrity and functions in the pathophysiology of sepsis.[5,20] The most convincing evidence of the apparent harm of hyperglycemia in sepsis is in patients with no known history of diabetes.[21] Why hyperglycemia may lead to poorer outcomes in the non-diabetic patient with "stress related" hyperglycemia is uncertain. This may be related to variables other than hyperglycemia itself and include glycemic variability, hypoglycemic episodes, and any treatment effects due to insulin or oral hypoglycemic agents.

It has been demonstrated that acute hyperglycemia leads to endothelial dysfunction and degradation of the glycocalyx layer in cell culture models and in animal studies.[22–24] Furthermore Azcutia and colleagues[10] demonstrated that a proinflammatory environment was necessary for elevated glucose levels to promote vascular inflammation in a study using HUVEC *in vitro* and rat microvessels *in vivo*. This suggested to us that high glucose concentrations may exacerbate sepsis induced vascular endothelial activation/injury and glycocalyx degradation. We used a microfluidic model of the microcirculation to study this *in vitro*.

Microfluidic devices (MDS) better mimic the complex environment of the microcirculation as opposed to standard endothelial cell

Table 2
Effect of TNF concentration or LPS and hyperglycemia on endothelial cell injury. Increased TNF levels and LPS lead to higher sTM, ROS, IL-1 and Ang-2 levels which increase glycocalyx degradation.

	TM (pg/ml)	ROS (intensity)	Ang-1 (pg/ml)	Ang-2 (pg/ml)	IL-1 (ng/ml)
TNF 25 ng/ml	26.7 ± 2.2	41.4 ± 1.0	490 ± 5.8	155 ± 1.6	40.2 ± 3.5
TNF 25 ng/ml + 200 mg/dL glucose	56.2 ± 3.4*	73.0 ± 2.8*	265 ± 2.8*	215 ± 3.8*	43.2 ± 4.2
TNF 50 ng/ml	51.8 ± 3.1*	53.7 ± 1.6*	262 ± 1.9*	180 ± 4.1*	58.3 ± 5.1*
TNF 50 ng/ml + 200 mg/dL glucose	89.9 ± 6.3*#	80.8 ± 2.4*#	175 ± 4.8*#	335 ± 5.9*#	82.9 ± 6.6*#
LPS 10 ng/ml	55.7 ± 4.1*	55.9 ± 2.9*	255 ± 3.2*	185 ± 3.9*	63.8 ± 5.8*#
LPS 10 ng/ml + 200 mg/dL glucose	96.7 ± 6.1*#	86.4 ± 3.1*#	164.6 ± 4.4*#	351.5 ± 6.0*#	89.8 ± 7.6*#

*$p < 0.05$ vs. TNF 25 ng/ml, #$p < 0.05$ vs. TNF 25 ng/ml + 200 mg/dL glucose.

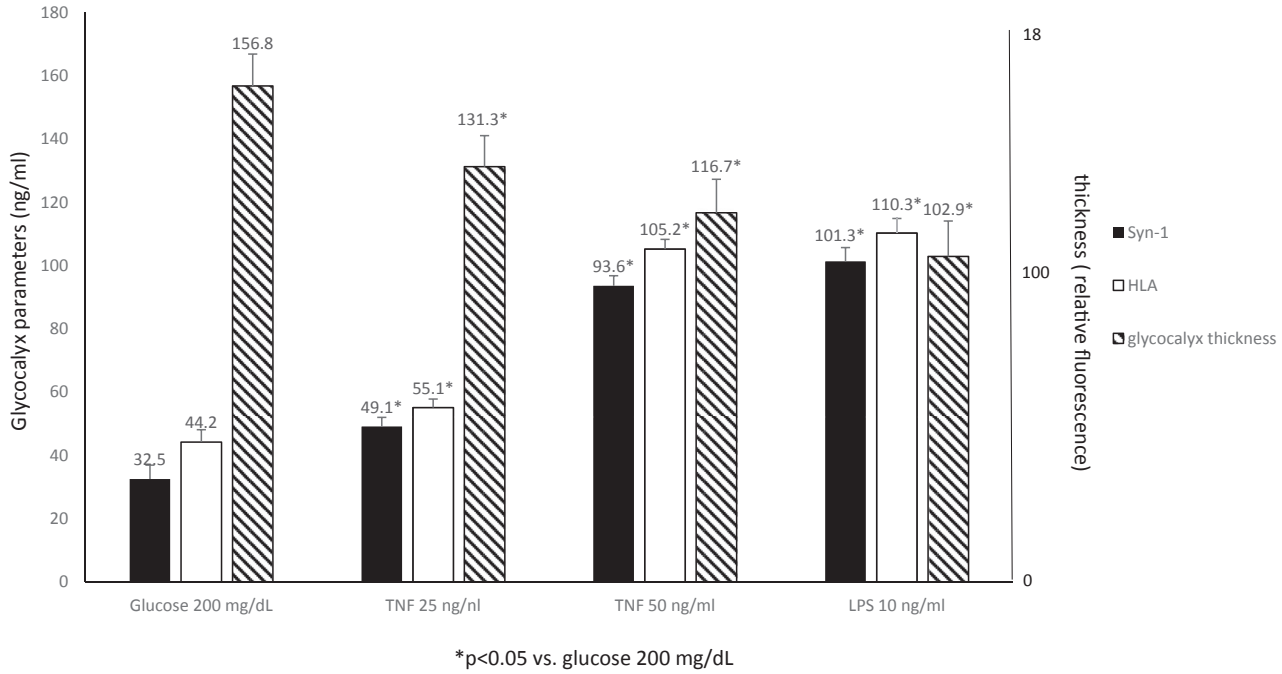

Fig. 5. Effect of TNF concentration or LPS and hyperglycemia on glycocalyx shedding and thickness. ELISA kits were used to quantitate syndecan-1 and hyaluronic acid levels present in the perfusate collected from HUVEC treated with TNF and LPS followed by the addition of 200 mg/dL glucose. Fluorescent intensity was measured using Volocity cellular imaging and analysis software to quantitate glycocalyx thickness.

cultures. Further, the microenvironment in microfluidic channels may be more tightly controlled than in animal models, offering a high throughput model of the microcirculation in sepsis. In our study, several biomarkers of endothelial activation/injury demonstrated that high glucose and septic challenges (TNF or LPS) had a synergistic effect with increasing sTM and Ang-2 (and a relative increase in Ang-2/Ang-1 ratio). Of note changes in the Angiopoietin tie 2 pathway lead to increased vascular permeability and a breakdown of the glycocalyx and as such may be a biomarker of sepsis severity.[25,26]

There are a number of biomarkers of endothelial activation/dysfunction that have been used to index decrease severity or prognosis in sepsis. These include mediators of endothelial function (Ang-2 and 1), components of the coagulation pathway (thrombomodulin) and soluble cell surface adhesion molecules. There is no ideal biomarker. However, we chose Ang-2/1 as these best reflect endothelial function and thrombomodulin as it interacts with the coagulation system. Glycocalyx degradation was indexed by "shedding" of its syn-1 and HLA, as well as thickness measurements. A synergistic effect of high glucose and septic challenge on the glycocalyx parameters was also noted. Additionally the endothelial and glycocalyx derangements were similar between TNF and LPS suggesting that a variety of inflammatory insults may impact these barriers. These barrier derangements also varied with the severity of the inflammatory insult (TNF concentration). Finally endothelial cell ROS generation was enhanced when stress concentrations of glucose were combined with the septic challenges. ROS production may be a therapeutic target, as it is associated with microvascular dysfunction in both sepsis and diabetes.[27–29]

There are several limitations to our study. First hyperglycemia was defined by a single concentration, 200 mg/dL. However this concentration was chosen based on the current glucose treatment threshold of the International Guidelines for Management of Sepsis (2016). Second the stress response to sepsis includes significant increases in plasma concentrations of epinephrine and norepinephrine. Johannson et al.[30] have identified an association between sympathoadrenal activation and endothelial damage in septic patients and thus the addition of catecholamines may have impacted the results of our study.

Finally blood components were not included in our study; sepsis and/or hyperglycemic damage the glycocalyx can lead to exposure of ICAM and other leukocyte receptors on the endothelial cell surface which allow WBC adhesion and further barrier injury.[31] In addition plasma resuscitation has been found to be protective against sepsis induced endothelial injury and glycocalyx degradation.[32] The microfluidic platform is a useful model for the preclinical evaluation of the endothelial vascular barrier in sepsis with or without hyperglycemia. It may allow the evaluation of therapeutic interventions to protect the barrier against these insults.

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