ASSESSMENT OF IN VITRO ISCHEMIC STROKE-LIKE INJURY AND MATRIX METALLOPROTEINASE 9 RESPONSES IN HUMAN BRAIN VASCULAR CELL MODELS

BY

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Abstract

Loss of vascular integrity and successive opening of the blood-brain barrier (BBB) are key compromising events that lead to a vicious cascade of secondary injuries following acute ischemic stroke (AIS). Matrix metalloproteinase 9 (MMP-9) has been strongly implicated as a potential culprit in mediating the proteolytic breakdown of components integral such as tight junctional proteins to the BBB and inflammatory response with adhesion molecules post-stroke; however, studies have mainly focused on neurons as a therapeutic target, rather than the structural and biochemical barrier of the cerebrovasculature. Therefore, the focus of this thesis was then to investigate the expression of adherence and junctional proteins, as well as levels and activity of MMP-9 in male human brain microvascular endothelial cells (HBMECs) following normoxia (21% O_2) or hypoxia plus glucose deprivation (HGD, 1% O_2) exposure at P7 for 3 h, timepoint determined from our previous TEER studies revealing deterioration of endothelial barrier as early as 3 h. Concomitantly, MMP-9 levels and activity were assessed in human brain vascular smooth muscle cells (HBVSMCs) following the same exposure conditions as well as treatment to MMP-9 inhibitor JNJ0966 (0.5 μM), to determine if HGD induced MMP-9 activity could be altered using this novel compound following in vitro ischemic-like injury. Following HGD exposure, cell density and morphology were altered. In addition, a simultaneous decrease in tight junction proteins claudin-5 and zonula occludens 1 (ZO-1) was observed, while HGD exposure resulted in an increase in vascular cell adhesion molecule 1 (VCAM-1) levels in HBMECs, as observed via standard immunoblotting. In both HBMECs and HBVSMCs, HGD also increased MMP-9 protein levels and increased MMP-9 activity assessed via western blot and zymography respectively. However, although there was a trend, the MMP-9 inhibitor, JNJ0966, did not alter HGD-induced MMP-9 activity in HBVSMCs. In conclusion, HGD induced barrier integrity loss occurred by

mediating decreased tight junction expression and concomitant MMP-9 activation in human cerebrovasculature. Mechanism(s) by which HGD regulates enzymatic activity of MMP-9 following ischemic-like injury warrants further investigation.

Introduction

Vascular dysfunction and the opening of the blood-brain barrier (BBB) are key compromising events that lead to a vicious cascade of secondary injuries following acute ischemic stroke (AIS). Existing treatments for AIS, though effective, are limited by strict patient inclusion criteria (Sussman & Connolly, 2013). Endovascular clot retrieval is available primarily at large stroke centers that, however, carry high risk of morbidity, while the only pharmacological FDA approved therapeutic, tissue plasminogen activator (tPA), elicits increased hemorrhagic risk, BBB disruption, and matrix metalloproteinase (MMP-9) activation (Alberts, 1998; Derex & Nighoghossian, 2008). Understanding the molecular mechanisms driving the pathophysiology at the level of the cerebrovasculature following AIS remains of importance for the development of novel therapeutics that may prevent against irreversible neuronal damage and worsened stroke patient outcomes. MMP-9 has been strongly implicated as a potential culprit in mediating this BBB breakdown, particularly because of its ability to degrade proteins and particularly tight junctions stabilizing the BBB and its increased presence during stages with pronounced BBB integrity loss (Hallenbeck & Dutka, 1990; Jung et al., 2010; Latour et al., 2004; Rosenberg et al., 1998; Wang & Lo, 2003). Vascular injury then initiates endothelial activation, which is characterized by cell adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 at the endothelium surface to enhance inflammation (Stanimirovic et al., 1997). Non-selective inhibition of MMP-9 by broad-spectrum MMP inhibitors has demonstrated therapeutic potential by reducing infarct volume and BBB permeability loss within ischemic stroke models that are, however, therapeutically unviable due to dose-limiting toxicities and musculoskeletal side effects (Vandenbroucke & Libert, 2014). This may be attributed to the highly conserved sequence and structural similarities across the various MMPs. Thus, synthesis of selective MMP inhibitors has been technically challenging. However, with the recent discovery of the small selective MMP-9 inhibitor JNJ0966, this novel inhibitor provides an unprecedented ability to evaluate highly selective MMP-9 inhibition in attenuating BBB integrity loss following ischemic stroke.

JNJ0966 is a highly selective MMP-9 inhibitor that prevents activation of the MMP-9 zymogen (inactive form) by preferential binding to the intermediate, inactivated MMP-9 form and blocks subsequent generation of catalytically active MMP-9 (Liu et al., 2019; Scannevin et al., 2017). JNJ0966 demonstrates no effect over the catalytic activity of other MMP forms and has likewise been shown to be able to penetrate the BBB, decrease clinical disease scores observed in mouse experimental autoimmune encephalomyelitis models (*in vivo* model for human neuroinflammatory disorders, i.e., multiple sclerosis), and suppress metastasis in human gastric cancer cells (Liu et al., 2019; Scannevin et al., 2017). To my knowledge, JNJ0966 has not yet been evaluated in AIS models nor in cerebrovascular cells. Therefore, one of the aims of this thesis was to address whether the novel MMP-9 inhibitor, JNJ0966, will alter ischemic like induced MMP-9 activation.

The evaluation of the role of MMP-9 and its potential as a therapeutic target is of prime interest for treating AIS and for suppressing the destruction of the BBB. Focus on the protective cerebrovascular layer mediating the decisive BBB breakdown is of importance, as a therapeutic target. As vascular cells are among the first cells impacted by ischemic stroke, they are important target for treating AIS. Studying the impact of ischemic-like injury on cerebrovascular integrity and activation as well as address the therapeutic potential of MMP-9 inhibition for BBB preservation at the cerebrovascular level is significant for improving therapeutic options and patient outcomes during stroke.

In this study I investigated whether HGD alters MMP-9 activity and levels in a primary human brain microvascular endothelial and vascular smooth muscle cell model and determined whether treatment by MMP-9 inhibitor JNJ0966 alters MMP-9 activation and activity induced by AIS-like injury using an *in vitro* AIS cell model. Data generated from this study provides clinically relevant information, as MMP-9 may be a promising target in the therapeutic treatment of AIS at the vascular level.

Literature Review

MMP-9 Biology and Signaling Pathways

MMPs consist of a family of extracellular zinc-dependent endopeptidases that in concert regulate ECM degradation and remodeling and are essential for normal human development and the progression of various pathological process. Structure amongst the MMPs is highly conserved, consisting of domains for signal peptide, latency, catalysis, and substrate recognition. Within the catalytic domain of MMP-9, a Zn^{2+}-containing active site in general coordinates proteolytic activity. Like most MMPs, MMP-9 is synthesized as a zymogen that require proteolytic removal of its pro-peptide regions that confers latency by blocking zinc ion binding in the active site via a cysteine residue within a sulfhydryl group (Birkedal-Hansen et al., 1993; Chaturvedi & Kaczmarek, 2014). Cleavage of the pro-peptide results in a water molecule to take the place of cysteine, freeing the zinc co-factor for hydration and successive catalytic MMP-9 activity (reviewed in (Birkedal-Hansen et al., 1993; Murphy & Nagase, 2008; Sternlicht & Werb, 2001). Activation by pro-peptide cleavage is then executed by proteases, such as tPA, plasmin, trypsin, as well as other MMPs, e.g. MMP -1, -2, -3, and -14 (Fridman et al., 1995; Knäuper et al., 1997; Nagase, 1997; Ogata et al., 1992; Pepper, 2001).

Active MMP-9 inhibition and bioavailability downregulation occurs at its C-terminal hemopexin domain, serving as a binding site for endogenous, broad spectrum MMP inhibitors tissue inhibitor of metalloproteinases (TIMPs), with preferential non-covalent linkage to TIMP-1 (Van den Steen et al., 2006). TIMP-1 uniquely forms a stable heterodimer complex with MMP-9 proenzyme at its hemopexin domain that interferes with MMP-9 activation and found to be basally expressed in the BBB microenvironment, allowing for MMP-9 to be predominantly found in its inactivated form (Van den Steen et al., 2006). Notably exceptions that are the TIMP-1-free MMP-9 multimers secreted by neutrophils that are more stable than its monomer form (Ardi et al., 2007). MMP-9 is positively regulated at the transcriptional level by multiple factors at its promoters, including activator protein-1 (AP-1), nuclear factor κB (NF-κB), and specificity protein 1 (Sp-1) (Crawford & Matrisian, 1996; Murthy et al., 2012; Wasylyk et al., 1991; Wu et al., 2009). MMP-9 can be found in multiple cell types within the neurovascular unit, such as endothelial, smooth muscle, and macrophagic cells. Thus, complexity of the MMP-9 enzyme and its coordination within the BBB is accentuated by its transcriptional regulators and multiple representations in which the enzyme undertakes, including its latent and active enzyme forms as well as those from dimerization.

Overview of MMP-9 in Stroke Pathophysiology

AIS is a cerebrovascular disease that results in devastating and debilitating neurological injury that remains a leading cause of death and of long-term disability in the United States and worldwide. Stroke onset occurs by a cerebral artery occlusion that leads to cerebral infarction and the cessation of blood flow. Ischemic and hypoxic conditions then result in the early loss of neuronal function and place the ischemic area vulnerable to permanent neuronal cell death. A cascade of secondary injuries then follow by the disruption of the BBB, further aggravating injury and contributing to increased edema development, excitotoxity, vascular dysfunction, neuroinflammatory response, hemorrhagic risk, or even death (del Zoppo et al., 1998; Romanic et al., 1998; Rosenberg et al., 1996; Simard et al., 2007). The development of novel therapeutics for AIS remains urgent for the immediate prevention and treatment of ensuing secondary injury responses, such as hemorrhagic transformations and cerebral edema that ultimately lead to irreversible neuronal damage/death and worsened patient outcomes (Barr et al., 2010; Birkedal-Hansen et al., 1993; Latour et al., 2004; Mun-Bryce & Rosenberg, 1998; Pfefferkorn & Rosenberg, 2003; Sternlicht & Werb, 2001).

Loss in BBB integrity is a key to the vicious cycle of secondary injuries that follow the initial ischemic attack by weakening the cerebrovascular structural integrity and permitting entry of peripheral immune cells into the brain that contribute to hemorrhagic risk, cerebral edema development, and neuroinflammatory responses, as illustrated in Figure 1 (del Zoppo et al., 1998; Romanic et al., 1998; Rosenberg et al., 1996; Simard et al., 2007). Given their abilities to degrade the extracellular matrix and tight junction components stabilizing the BBB, matrix metalloproteinases (MMPs) have been implicated in BBB permeability alternations post-stroke (Birkedal-Hansen et al., 1993; Mun-Bryce & Rosenberg, 1998; Sternlicht & Werb, 2001). In particular, MMP-9 has been strongly linked to the biphasic opening of the BBB following stroke (Chaturvedi & Kaczmarek, 2014; Khokha et al., 2013; Turner & Sharp, 2016). The first, reversible BBB opening occurs within hours of onset, attributed to increased MMP-2 and -9 levels, while late, irreversible disruption occurs 24-48 h following stroke in conjunction to increased MMP-9 levels that persists for days and results in the complete degradation of the basal lamina and tight junction components of the BBB. Evaluation of the role of MMP-9 and its potential as a therapeutic target is of prime interest for treating AIS for suppressing the destruction of the BBB (Rosenberg et al., 1998).

An early and significant event in AIS on the inflammatory response includes peripheral immune response activation and recruitment, eliciting an influx of lymphocytes and neutrophils, endothelial activation, and reactive oxygen species (ROS) production (Romanic et al., 1998; Rosenberg et al., 1996). Neutrophil-mediated inflammation prompts elevated MMP-9 to the injury by increased deposits of inactive pro-MMP-9 granules, absent of TIMP-1 expression (Masure et al., 1991; Opdenakker et al., 1991). MMP-9 is able to enhance inflammation by truncation of major human neutrophil chemoattractant interleukin-8 (IL-8) to a tenfold more potent form, establishing a positive feedback loop for neutrophil recruitment and proMMP-9 secretion during immune response following stroke (Masure et al., 1991). Such poststroke inflammatory responses further involve the release of damage-associated molecular patterns (DAMPs) by ischemic brain cells that activate local microglia and recruited macrophage immune cells and stimulate the release proinflammatory cytokines that work on upregulating MMP-9, ROS, inducible nitric oxide synthase (iNOS), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Kim et al., 2014; Martynov & Gusev, 2015; Turner & Sharp, 2016). Consequently, endothelial activation initiated by DAMPs exposure is concerted at the vascular injury site, resulting to upregulation in cell adhesion molecule expression, i.e. intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, and integrins rapidly at the endothelium surface (Emsley & Tyrrell, 2002; Zhang et al., 1998). Thus, neutrophils with complementary receptors in the acute phase are increasingly recruited and adhere to the endothelium that may cause microvascular obstruction and extravasation of the immune cells through the BBB and into the brain parenchyma (del Zoppo et al., 1991; Eldahshan et al., 2019; Hallenbeck, 1996; Kim et al., 2014). In summary, MMP-9 plays a critical role in the immune response by enhancing peripheral immune cell recruitment, ROS generation, pro-inflammatory cytokine synthesis, and adhesion molecule expression within the cerebral vasculature.

MMP-9 in the Cerebrovasculature

The cerebrovasculature is a crucial homeostatic organ of the BBB, in playing a pivotal role in maintaining selective permeability and separation between the circulatory system and CNS. Upon its breakdown during AIS, the BBB is no longer able to uphold its role as a front-line organ and protectant against injury, allowing for the ensuing prolonged and robust inflammatory response (Dirnagl et al., 1999). Protection of vascular cells is critical for the regulation of nutrients and neuroprotectant supply to the jeopardized tissue, which can be detrimental for recovery and repair following stroke. Damage caused by ischemic stimuli elicits endothelial cell dysfunction to occur, causing these cells to swell or detach from the underlying basement membrane that connects endothelial cells with astrocytes and pericytes within the neurovascular unit, resulting in increased BBB permeability and loss in intercellular communication and brain homeostasis. Key transmembrane proteins that construct the BBB seal include claudins, occludin, and zonula occludens proteins ZO-1, -2, and -3, which undergo altered distribution or loss in ischemic cerebral microvessels in animal stroke models (Asahi et al., 2001; ElAli et al., 2011; Liu et al., 2009; Yang et al., 2007). Endothelial dysfunction is also defined by diminished capacity to generate nitric oxide (NO), which plays a role in mediating vasodilation and inhibiting platelet aggregation, thrombus formation, expression of adhesion molecules and leukocyte attractant chemokines, and vascular smooth muscle proliferation that when lost are pathophysiological features of stroke (Egashira, 2002). Interactions between the endothelium and vascular smooth muscle are integral to regulating and maintaining cerebral vascular flow and tone (Dirnagl et al., 1999). Thus, endothelial dysfunction may be a major determinant in tissue damage extent and relevant focus for attenuating BBB integrity loss following AIS.

In combination with other subsequent consequences of endothelial dysfunction, such as loss in transport regulation and hyperpermeability, BBB disruption permits the entry of free water and serum into the cerebral space, increasing vasogenic edema formation and hemorrhagic risk (Andjelkovic et al., 2019). Active MMP-9 itself delivered through the immune response is able to degrade components of the cerebrovascular endothelium's surrounding basal lamina, composed of laminin, type IV collagen, fibronectin, heparin sulfate, and other ECM components, as well its tight junction proteins, such as occludin, claudin-1, claudin-5, and zonula occludens-1, that served as clefts interconnecting the endothelium layer. These tight junctions are integral BBB structural components that seal the gaps between adjacent endothelial cells and thus restrict and regulates low-paracellular permeability in and out of the cerebral space (Wolburg & Lippoldt, 2002).

Increased vascular MMP-9 levels are associated with worsened stroke patient outcomes and unsuccessful recanalization, while low levels is associated with stable collateral flow in patients with large vessel occlusion (Heo et al., 2003; Mechtouff, Bochaton, Paccalet, Crola Da Silva, Buisson, Amaz, Bouin, et al., 2020). Clinically in select studies, elevated MMP-9 has been observed following tPA treatment and development of intracerebral hemorrhage following thrombectomy (Mechtouff, Bochaton, Paccalet, Crola Da Silva, Buisson, Amaz, Derex, et al., 2020). In rodent models of focal cerebral ischemia, increased MMP-9 protein levels are likewise observed during the acute phase of stroke (2-24 h), coinciding with the openings of the BBB (Hamann et al., 2002; Kelly et al., 2006; Sumii & Lo, 2002). Degradation of the ECM proteins by MMP-9 can affect cell survival and lead to anoikis and apoptosis, brought on by the loss in integrin signaling and communication of cell-to-cell survival signals with the ECM and between the network of once anchored cells (Gu et al., 2002; Liebner et al., 2011). Particularly at the basal lamina, connection between the astrocyte end feet and endothelial cells can be disrupted by cleavage of transmembrane receptor ß-dystroglycan (Agrawal et al., 2006). As a result of the extravasation of peripheral immune cells and increased MMP-9 activity, cerebral edema formation and increased intracranial pressure compounding to aggravated tissue damage following stroke. Brief periods of treatment with MMP inhibitors could then be sufficient to reduce subsequent loss of neurological functions, on account of reducing neutrophil infiltration, oxidative stress, brain edema, neurotoxicity, and vascular dysfunction.

Pharmacological Inhibition of MMP-9 in Stroke

Despite intensive efforts in synthesizing selective MMP-9 inhibitors for potential clinical use, the structural similarities and sequence conservation across the MMPs and particularly MMP-2 have obstructed the generation of highly specific MMP-9 inhibitors, thus resulting in the poor selectivity and broad-spectrum nature of most MMP-9 inhibitors discovered. Studies have, however, shown broad-spectrum MMP inhibitors have demonstrated to reduce severity during experimental stroke, in the association with reduction of brain hemorrhage volume, reduced neurobehavioral disability, and increased survival (Copin et al., 2011; Liu et al., 2012; Skiles et al., 2004; Sumii & Lo, 2002; Walz & Cayabyab, 2017; Yang et al., 2007; Yang & Rosenberg, 2015). Broad-spectrum MMP gelatinase inhibitor selective to MMP-9 and MMP-2, batimastat (BB-94), has been shown to mitigate neutrophil invasion, microglia activation, and BBB integrity loss, in rat lacunar infarction models (Liu et al., 2009; Walz & Cayabyab, 2017). In addition, potent, non-selective MMP inhibitor, SB-3CT, demonstrated reduced degradation of tight junction components in HGD models (Liu et al., 2012). Although these inhibitory compounds are effective in decreasing MMP-9 expression *in vitro*, it remains to be fully understood how the MMP-9 molecularly facilitates

BBB opening following ischemic stroke. By advancing our understanding in these areas of stroke pathology, the development of novel therapies effectively decreasing the risk and severity of cerebral ischemia may be achieved.

Among the aforementioned clinical studies, these unspecific MMP-9 inhibitors have demonstrated proof of therapeutic effect in AIS models alongside MMP-9 inhibition, in reducing infarct volume, tight junction degradation, neutrophil recruitment, and BBB permeability, thereby reinforcing the need to generate and evaluate highly specific MMP-9 inhibitors. While much effort has gone into the evaluation of MMP-9 inhibitors over the decades, the field is still far away from a therapeutic breakthrough. Thus, more work remains to be done to test if novel MMP inhibitors with greater specificity and minimized toxicity are a viable therapeutic strategy for acute ischemic stroke. Recently discovered, the small molecule JNJ0966 is a highly selective MMP-9 inhibitor that has been shown to be able to penetrate the BBB and decrease clinical disease scores observed in *in vivo* model for human neuroinflammatory disorders (Liu et al., 2019; Scannevin et al., 2017). The mechanism of inhibition by JNJ0966 acts by disorienting residues within the activation loop of proMMP-9 and allosteric perturbation of the second cleavage event that generates catalytically active MMP-9, allowing for an inactive intermediate form by formed (Scannevin et al., 2017). Novel inhibitor JNJ0966 presents an unprecedented ability to pharmacologically evaluate highly selective MMP-9 inhibition in attenuating BBB integrity loss following ischemic stroke.

Overall Hypothesis: Hypoxia plus glucose deprivation (HGD; *in vitro* model of ischemia) will alter vascular cell integrity and concomitantly increase MMP-9 activity in human brain cerebrovascular cells. The novel selective inhibitor, JNJ0966, will attenuate MMP-9 activation.

Aim 1: Investigate human brain microvascular endothelial cell (HBMEC) and vascular smooth muscle cell (HBVSMC) density and morphology following normoxia or HGD exposure.

Aim 2: Assess adhesion molecules and tight junction protein levels, BBB related proteins, in brain cerebrovascular cells following HGD and normoxia exposure.

Aim 3: Determine whether HGD induces MMP-9 levels and or activity alters following HGD in cerebrovascular and whether the selective MMP-9 inhibitor JNJ0966 alters this response.

Materials and Methods

Cerebrovascular Endothelial Cell Culture. Primary male human brain microvascular endothelial cells (HBMECs; ACBR1 376 V (2023370), 376.02.02.01.2F, Cell Systems, Kirkland, WA) are purchased commercially and used in these studies. Subcultures are received cryopreserved at passage 3 and passed to passage 7 for treatment. Sex is verified by demonstrating expression of the SRY gene, a male cell marker. Endothelial cell origin is verified via anti-Von Willebrand Factor. Cells will be grown in a 5% CO_2-95% room air atmosphere at 37°C in phenol red-free basal medium (Complete Classic Medium, 4Z0-500, Cell Systems) activated with CultureBoost^{TM} (Cell Systems) containing 10% FBS. Figure 2 captures representative brightfield image of cultured HBMECs.

Cerebrovascular Smooth Muscle Cell Culture. Primary male human brain vascular smooth muscle cells (HBVSMC; H-6085, F11062014/17, Cell Biologics, Chicago, IL) of adult origins are purchased commercially and received cryopreserved at passage 3. Cells are grown to passages 6 to 7 for cell treatment. Cells are grown in 5% CO_2-95% room air atmosphere at 37°C in phenol red-free basal medium (16-405-CV, Corning) supplemented with smooth muscle grown supplement kit containing insulin, human epidermal growth factor (hEGF), human fibroblastic growth factor (hFGF), gentamycin/amphotericin, and 5% FBF (cc-3182, Lonza). Figure 2 captures representative brightfield image of cultured HBVSMCs.

In vitro Stroke-Like Injury Model. Hypoxia Plus Glucose Deprivation (HGD) exposure is an *in vitro* ischemic-like injury model for stroke. Prior to drug treatment and HGD, glucose containing medium is replaced with modified DMEM (A14430-01, ThermoFisher Scientific) media free of L-glutamine, phenol red, sodium pyruvate, and D-glucose. In select experiments, cells were then pretreated to the JNJ0966 or vehicle and placed in humidified, hypoxic sub chamber (BioSpherix) at 1% O^2 and CO_2 at 37°C for 3 hr. Normoxic plates containing their respective cell culture medium for cell type had their media replaced and were incubated in separate Co2 incubator at 21% O^2 and 5% CO_2 at 37°C for 3 hr. Following compound treatments/ HGD exposure for 3 h, plates of cells were placed on ice and harvested for media and cell lysate with lysis buffer solutions. Protein lysates and media collected underwent experiments described further. Experimental timeline illustrated in Figure 2.

Drug and Treatment Protocol. All solutions were freshly prepared on the day of treatment and applied to cell culture model 15-20 minutes before normoxia or hypoxia plus glucose deprivation (HGD) exposure. MMP-9 inhibitor, JNJ0966 (HY-103482, MCE) was prepared as a stock solution with dimethyl sulfoxide (DMSO; vehicle) and diluted to a working solution in Dulbecco's phosphate buffer solution (DPBS; 21-031-CV, Corning) on day of experiments, then diluted to final working concentration (0.5 µM) in cell culture medium. Control groups were treated to the DMSO/ DPBS solution as the vehicle. During preparation and treatment, solutions were best maintained at 37°C. Cells are treated with JNJ0966 or vehicle at 80% to 95% confluency and not beyond passage 7.

Crystal Violet Staining. HBVSMC structure and morphology were visualized using crystal violet staining. Cells at passage 6 were seeded onto coverslips coated with attachment factor (4Z0-201, Cell Systems) within 6-well plates and grown to 80-90% confluent the next day. Treatment groups included normoxia or HGD exposure for 3 h. After exposure, cells were placed on ice and washed twice with ice-cold DPBS. Cell fixation then occurred with -20°C 100% methanol for 10 min and removed, for room temperature incubation with 0.5% crystal violet solution (C0775) for 10 min. Following incubation, cells were then washed with nano-pure H_2O and left overnight to dry. After removing excess water, coverslips were mounted onto slides and sealed with nail polish. Images were obtained using automated inverted digital microscope (BZ-X800, Keyence).

Immunocytochemistry. HBVSMCs at 80-90% confluency at passage 6 were seeded onto glass coverslips coated with attachment factor (4Z0-201, Cell Systems) within 6-well plates, which were grown to 80-90% confluency. Treatment groups were divided into normoxia or HGD for 3 h. Following treatment, plates were placed on ice, media aspirated, and cells washed once with ice cold DPBS. Cell fixture occurred with 4% formaldehyde (10 min) and subsequently washed twice for 4 min with DPBS at room temperature. Cell permeabilization then occurred with 0.1% TritonX-100 (T9284) for 10 min and blocked in 2% bovine serum albumin (BSA) for 15 min at room temperature. HBVSMCs were then assessed for inflammation using anti- NF-κB p65 (Cell Signaling, 3033S) at concentrations of 1:1600 in 2% BSA, by incubation overnight at 4°C. Following incubation, cells were then washed with 2% BSA for three times at 5 min each, succeeded with incubation with secondary antibody, goat anti-rabbit Alexa Fluor 488 (A11001, ThermoFisher Scientific), at 1:6000 in 2% BSA for 1 h at room temperature shielded from light. Afterwards, wells were quickly washed with 2% BSA then DPBS for 5 min washes three times. Negative control was included by incubating the coverslips with secondary antibody only.

Coverslips were then mounted onto slides, utilizing mounting medium with DAPI (H-1200, Vector Laboratories). Coverslips were then sealed using nail polish. Images were captured using an inverted digital microscope (BZ-X800, Keyence).

Western Blot Protocol and Analysis. Protein levels were assessed by western blotting in both HBMECs and HBVSMCs. Following HGD/ normoxia exposure and, in select cases, JNJ0966 drug treatment, cells were harvested in 0.25% Trypsin (15090046, Gibco) and lysed in ice-cold lysis buffer solution (50 mM Tris·HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40) containing protease inhibitors (20 μM pepstatin, 20 μM leupeptin, 0.1 U/ml aprotinin, 0.1 mM PMSF, 1 mM DTT), phosphatase inhibitors (25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate), 1 mM EDTA, and 1 mM EGTA, then homogenized via small glass homogenizing rods for 30-40 seconds each tube. Each tube was then sonicated via 12 strokes. Samples were then centrifuged (Fisher Accuspin Micro 17R) at 13,300 RPM for 10 minutes at 4°C before collection of the supernatant containing cell lysate. Total protein concentrations of each sample were determined and standardized using the PierceTM bicinchoninic acid (BCA) protein assay kit (23225, Thermo Scientific) according to manufacturer's protocol and measured on a Safire II (Tecan) plate reader. Samples standardized by total protein concentration were then diluted in Tris-glycine SDS sample buffer with reducing agent β-mercaptoethanol (10%) and boiled for 5 min. Diluted, standardized samples and fluorescent standards (LICOR Biosciences) were then loaded onto 8% or 10% polyacrylamide SDS-PAGE gels, depending on the size of the protein of interest. The protein samples underwent SDS-PAGE separation in homemade running buffer (25 mM Trizma base, 190 mM glycine, 0.1% SDS) at 85 V for 20 minutes and successively 125 V for 1 hour through mini PROTEAN Tetra electrophoresis system (Bio-Rad Laboratories). Proteins separated on gel were then transferred to nitrocellulose membrane using the same apparatus at 0.25

Amps for 1 hour. Membranes were blocked with 3% nonfat dry milk in TPBS solution (10 mM Tris, pH 8.0, 150 mM NaCl, 1% Tween 20) for 1 hour at room temperature, to prevent non-specific binding. Membrane was then washed twice with TPBS and incubated with select primary antibodies at 4 °C overnight. Primary antibodies for western blot included VCAM-1 (sc-13160, Santa Cruz Technology; 1:500 in TPBS), Claudin-5 (4C3C2, ThermoFisher; 1:1000 in TPBS), ZO-1 (5406S, Life Technologies; 1:1000 in TPBS), and MMP-9 (3852S, Cell Signaling Technology; 1:500 in TPBS) at their respective dilutions. Membranes were then washed five times with TPBS for 5 min each wash and incubated with secondary antibodies, goat anti-rabbit or anti-mouse IRDye 800CW dye (LI-COR), for 1 h at room temperature under gentle agitation. Blots were then washed with TPBS five times once more and imaged on the Odyssey Classic infrared imager (LI-COR). Following imaging, membranes were exposed to β-actin primary antibody (Sigma-Aldrich, St. Louis, MO) at 1:15000 dilution in TPBS for overnight incubation at 4°C and corresponding goat anti-mouse IRDye 680LT (LI-COR) for visual verification of equal loading of protein and loading control. Data were then analyzed using ImageStudio 3.0 software (LICOR).

Zymography and MMP-9 Activity Analysis. To determine the effect of HGD and/or JNJ0966 treatment on proteolytic MMP-9 activity, primary cells were treated with JNJ0966 (HY-103482, MCE) and/or under normoxia or HGD treatment for 3 hours, with MMP-9 activity evaluated using non-denaturing, PAGE-based zymography. Zymography was performed accordingly to manufacturer's protocols (Life Technologies). Cells were grown and harvested at 80-95% confluence at passage 6 or 7. Cell lysates and culturing media were collected after normoxia or HGD treatment for 3 hours. Proteins in both media and cell lysate were then standardized using steps previously described and then subjected to electrophoresis for 2 h at 4°C through 10% polyacrylamide gels (ZY00100BOX, Thermo Scientific) that are copolymerized with 1 mg/mL of gelatin. After electrophoresis, the gels were incubated in 1X Novex™ Zymogram Renaturing Buffer (LC2670, Thermo Scientific) at room temperature to remove the SDS, then incubated overnight at 37°C in 1X Novex™ Zymogram Developing Buffer (LC2671, Thermo Scientific) for 2-3 days. Gels were then stained with Coomassie blue for 2 hours and destained in solution of acetic acid and methanol. Proteolytic activity was visualized as white bands against dark blue background of intact gelatin and imaged on molecular imager (1708195EDU, BioRad) with white light conversion screen (1708289EDU, BioRad). A representative blot illustrating gelatinase activity from cell media is shown in Figure 3.

Reagents and Antibodies

All reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise noted

Table 1. Primary antibodies used in immunoblotting protocol

Protein	Type	Source	Catalogue #	Host	Concentration
ß-actin	Monoclonal	Sigma Aldrich, Darmstadt, Germany	A5441	Mouse	1:15000 TPBS
Claudin-5	Monoclonal	ThermoFisher, Waltham, MA	4C3C2	Mouse	1:1000 TPBS
MMP-9	Polyclonal	Cell Signaling Technology, Danvers, MA, USA	3852S	Rabbit	1:500 TPBS
VCAM-1	Monoclonal	Santa Cruz, Dallas, TX	sc-13160	Mouse	1:500 TPBS
ZO-1	Polyclonal	Life Technologies	5406S	Rabbit	1:1000 TPBS

Statistical Tests. Samples from each treatment group were run on the same Western blot for direct comparison and treatments were repeated for statistical analysis (n= 6 - 7). Data from Western blots will be expressed as an optical density ratio relative to vehicle. We will conduct two-way

ANOVA analysis of ZO-1, claudin-5, MMP-9, and proMMP-9 band densities compared to a loading control, β-actin. Values are means ± SEM. $P < 0.05$ was considered significant. All statistical computations were run using GraphPad Prism 8 (GraphPad Software).

Results

HGD exposure altered HBVSMC and HBMEC density and morphology

HBVSMC and HBMEC cell density and morphology was visually assessed following normoxic and HGD exposure. Figure 4 illustrates the representative images of the HBVSMC and HBMEC cells stained with crystal violet at 40X magnification. Visual assessment of cells following HGD appeared to decrease in cell density. In addition, an alteration in HBVSMC and HBMEC morphology was also noted.

NF-κB expression levels were increased following HGD

To characterize whether an inflammatory marker was altered during HGD exposure, HBVSMCs lysate was probed for the proinflammatory transcription factor, nuclear factor kappa B (NF-κB). Anti-NF-κBp65 (Ser536) was used to detect NF-κB using immunocytochemistry. NF-κB is a key transcription factor previously characterized in vascular smooth muscle cells that is activated by inflammation and oxidative stress (Bellas et al., 1995). NF-κB is likewise a transcription factor of MMP-9 (Bond et al., 2001). Figure 5 illustrates the representative immunocytochemical images of NF-κB taken at 40X magnification. Levels of NF-κB increased in HBVSMC following HGD, suggesting that HGD induced an inflammatory response.

Adhesion molecule VCAM-1 expression levels increased in HBMEC following HGD

As reactive oxygen species have been reported to increase vascular cell adhesion molecule 1 (VCAM-1) levels which in turn activate MMP-9, we observed that VCAM-1 was expressed under normoxic conditions and expression levels increased following HGD exposure (Stanimirovic et al., 1997). This increase following HGD, suggests indirectly HBMEC activation at the level of the cell *in vitro* (Figure 6).

Tight junction protein levels decreased in HBMEC following HGD exposure

Tight junction proteins are integral components to stabilizing the BBB and maintain vascular integrity (Wolburg & Lippoldt, 2002). Following stroke, these key transmembrane proteins are altered or lost in animal stroke models, contributing to a compromised BBB (Asahi et al., 2001; ElAli et al., 2011; Liu et al., 2009; Yang et al., 2007). In our studies we observed that HBMECs expressed tight junction proteins claudin-5 and zonula occludens 1 (ZO-1) and HGD decreased these protein levels. These data suggest that HGD may alter endothelial integrity by regulating tight junction protein levels (Figure 7).

MMP -9 levels were not altered following ischemic-like injury in HBMEC; however, pro-MMP-9 levels decreased and MMP-9 to proMMP-9 ratio increased

Intracellular MMP-9 protein levels were of interest to evaluate potential transcriptional and translational alterations inflicted by stroke-like injury. Following stroke, increased serum levels of

MMP-9 have demonstrated potential to serve as a stroke diagnostic biomarker and linked to increased infarct size, hemorrhagic risk, and worsened stroke patient outcome (Dagonnier et al., 2021; Zhong et al., 2017). Here we observed a decrease in intracellular pro-MMP-9 (inactive enzymatic form) levels and increased ratio of the MMP-9 to pro-MMP-9 following HGD. MMP-9 levels were not altered following ischemic-like injury in HBMEC cell lysate (Figure 8). These results may be suggestive of increased secretion of pro-MMP-9 and/or MMP-9 activity into the extracellular.

Although there was a trend, MMP-9 protein levels were not different following HGD in HBVSMC cell lysate and the selective MMP-9 inhibitor JNJ0966 had no effect

Cerebrovascular VSM cells involving *in vivo* stroke animal models demonstrate transcriptional upregulation of angiotensin AT_1 receptors following stroke, which markedly increase MMP-9 expression at the ischemic region (Maddahi et al., 2009). Thus, MMP-9 forms were likewise evaluated within the HBVSMC cell lysate following normoxia and HGD conditions. We observed that MMP-9 forms, both active and inactive, were not different following HGD. (Figure 9) This may suggest that MMP-9 expression in HBVSMC may be regulated under a temporal-specific response, distinct from HBMEC under *in vitro* stroke-like injury model. JNJ0966 treatment of HBVSMC under normoxia and/or HGD did not exhibit an effect MMP-9 form levels. Although a trend was noted for decreased MMP-9 and pro-MMP-9 protein levels following HGD and JNJ0966 treatment, a larger sample size of each treatment group is needed to further evaluation of HGD and its impact on MMP9 protein levels and JNJ066 in HBVSMCs.

Extracellular MMP-9 activity in HBMEC media increased following HGD

Zymographic analysis of extracellular media of HBMECs following normoxia or HGD exposure revealed that HGD increased MMP-9 activity (Figure 10). Upregulated MMP-9 activity in media may indicate increased endothelial dysfunction and loss in vascular integrity brought on by the proteolytic enzyme. Likewise, increase activation or cleavage of pro-MMP-9 may have occurred under HGD conditions, as pro-MMP-9 levels in HBMEC cell lysate decreased following HGD (Figure 8). Further evaluations must be made of the HBMEC cell lysate following same conditions to fully characterize MMP-9 activity.

Extracellular HGD-induced MMP-9 activity in HBVSMC cell lysate and there was a trend for JNJ0966 to inhibit this response

To characterize impact of MMP-9 inhibitor JNJ0966 in HBVSMC MMP-9 activity following HGD, zymographic assays of HBVSMC cell lysate from cells treated with and without JNJ0966 following HGD were assessed. By visual observation, HGD induced MMP-9 activity appears to have been attenuated following JNJ0966 treatment (Figure 11). Further samples must be evaluated before statistical tests may be performed. MMP-9 activity of HBVSMC media likewise must be performed, to characterize MMP-9 at HBVSMC level.

Discussion

In this study, we utilized an *in vitro* stroke-like injury model called HGD and assessed the impact of HGD on HBMECs and HBVSMCs cell density, morphology, transcription marker expression and MMP-9 levels and activity. We demonstrated that HGD 1) increased in adhesion molecule VCAM-1 protein levels, 2) markedly decreased tight junctions ZO-1 and claudin-5 protein levels, and 3) decreased pro-MMP-9 cytosolic levels and increased MMP-9 to pro-MMP-9 ratio. The effect of the selective MMP-9 inhibitor, JNJ0966, was then evaluated at the HBVSMC level to assess inhibitory response. JNJ0966 treatment did not attenuate active MMP-9 protein levels.

Vascular damage after acute ischemic stroke injury becomes apparent with increased vascular inflammation and cell death (Fagan et al., 2004; Zuloaga & Gonzales, 2011). Hypoxic and nutrient-deficient pathological conditions have previously been observed to stimulate both the endothelium and vascular smooth muscle toward programmed cell death (Garbuzova-Davis et al., 2014; Lee et al., 2019; Wang et al., 2018; Wendt et al., 2021; Zhao et al., 2020; Zille et al., 2019). Studies have further demonstrated that hypoxic exposure can lead to oxidative stress and particularly the formation of reactive oxygen species that contribute to vascular cell death (Bao et al., 2019; Szocs, 2004; Zhang et al., 2014). Thus, following ischemic-like injury conditions of HGD, I predicted HGD exposure would induce greater cell death and decreased cell count. These pathological consequences were brought about for verification with the staining of HBMECs and HBVSMCs following HGD exposure in which a lower cell count was visually observed (Figure 4). Moreover, HGD exposure in my studies suggest an increased presence of inflammatory mediator NF-κB in HBVSMCs by visual observation (Figure 5). NF-κB is a transcription factor to numerous inflammatory genes, including MMP-9, and a necessary factor for cytokine-mediated upregulation of MMP-9 in vascular smooth muscle cells (Bond et al., 2001; Harari & Liao, 2010; Zhang et al., 2004). Excess MMP-9 has likewise been linked to being consequential to vascular stroke pathology, particularly in extracellular matrix degradations, vascular tone alterations, and overall worsened stroke outcomes (Rosell et al., 2008; Rosell et al., 2006; Turner & Sharp, 2016).

The cerebrovasculature is then a target promptly affected by ischemic injury, and thusly strategies to protecting the cerebrovasculature may be relevant to inhibiting enhanced cell death and exacerbated vascular inflammation following stroke.

Maintenance of the cerebral endothelial is important to cerebral blood flow regulation and BBB integrity during physiological conditions (Sandoo et al., 2010). Following ischemic stroke, the pathological conditions of endothelial activation and BBB integrity loss are observed and primarily mediated by the upregulation of adhesion molecules and loss of tight junction proteins respectively (Chen et al., 2009; Danton & Dietrich, 2003; Stanimirovic et al., 1997; Wolburg & Lippoldt, 2002). Cessation of blood flow following stroke results in immediate cellular damage and initiation of an inflammatory response (Yilmaz & Granger, 2008). Proinflammatory cytokines such as TNF-α and IL-1ß are produced during stroke injury and induce cell surface expression of adhesion molecules in vascular endothelial cells (McHale et al., 1999; O'Carroll et al., 2015). Moreover, studies have previously demonstrated in rodent stroke models that cerebral inflammation is associated with activation of the endothelium and its consequential, upregulation of adherent cell surface adhesion molecules that mediate the recruitment of peripheral inflammatory cells to the ischemic area (Frijns & Kappelle, 2002; Stanimirovic et al., 1997; Wang & Lo, 2003). Another prominent feature of ischemic stroke is the disruption of the BBB that is characterized by decreased expression of tight junctional proteins in microvascular endothelial cells (Ronaldson & Davis, 2015; Yang et al., 2019). Tight junctional proteins are essential to maintaining the seal of the BBB by interconnecting the cerebral endothelium and forming the continuous and impermeable BBB barrier, as such the loss of tight junction protein results in edema formation and unchecked influx of water and solutes to the CNS (Abdullahi et al., 2018). Here is this study we sought to measure protein level alterations to adhesion molecule VCAM-1 and tight junction proteins claudin-5 and

ZO-1 following HGD exposure in HBMECs, predicting increased levels of VCAM-1 in parallel to decreased tight junction proteins (Figure 6 and 7). Such predictions were verified by immunoblotting results, in which HGD induced increased VCAM-1 and decreased tight junction levels in HBMEC cell lysate, suggesting confirmation to endothelial activation and loss in endothelial barrier integrity induced by ischemic-like injury. Therefore, it can be concluded that *in vitro* stroke-like injury of HGD exposure may recapitulate the pathophysiological conditions of vascular inflammation and integrity loss through HGD-induced increase in adhesion molecules VCAM-1 and loss in tight junction proteins claudin-5 and ZO-1.

Given its implications linked to worsened stroke outcome previously discussed, MMP-9 was then evaluated at the extracellular level in the HBMEC media, and HBMEC media was observed to undergo HGD-induced upregulation of MMP-9 activity (Figure 10). In HBVSMC, MMP-9 activity following JNJ0966 treatment visually appeared to have decreased, following HGD exposure (Figure 11). Target inhibition of MMP-9 via inhibitor JNJ0966 in HBVSMC was investigated and observed to exhibit no difference in MMP-9 activity and levels following ischemic-like injury. Evaluation of the novel inhibitor remains to be characterized in both vascular cell types—extracellular and intracellular levels-- and *in vivo* animal models. Thus, much work remains to be addressed in regard to the inhibitory impact of JNJ0966 and its therapeutic potential following stroke-like injury.

It is critical to assess if MMP-9 inhibitors can be attractive therapeutic targets to advance the treatment of AIS, given the destructive role MMP-9 plays early on during stroke. Failure of broad-spectrum MMP inhibitors of the past point to the need to evaluate and assess novel MMP-9 specific inhibitors as beneficial targets in the inflammatory AIS phase that may be more clinically viable. Despite the long history of MMP non-specific inhibitors and failure in phase III clinical trials, knowledge gaps in MMPs remains a crucial issue. The reassessment of the biological functions MMP-9 that followed revealed the duality of the enzyme in the context of stroke, dependent on spaciotemporal context, to contribute to both vascular dysfunction and successive vascular remodeling and repair. With the identification of the selective MMP-9 inhibitor JNJ0966 that can be used with minor adverse musculoskeletal effects, we are only beginning to understand the impact of the novel molecule in the larger context of ischemia-like injury. Future work includes expanding upon the understanding of the underlying molecular mechanisms and functional effects of MMP-9 attenuation by JNJ0966 in additional stroke-injury models *in vivo*.

Figure 1. Pathological timeline of cerebrovascular dysfunction and integrity loss during acute ischemic stroke.

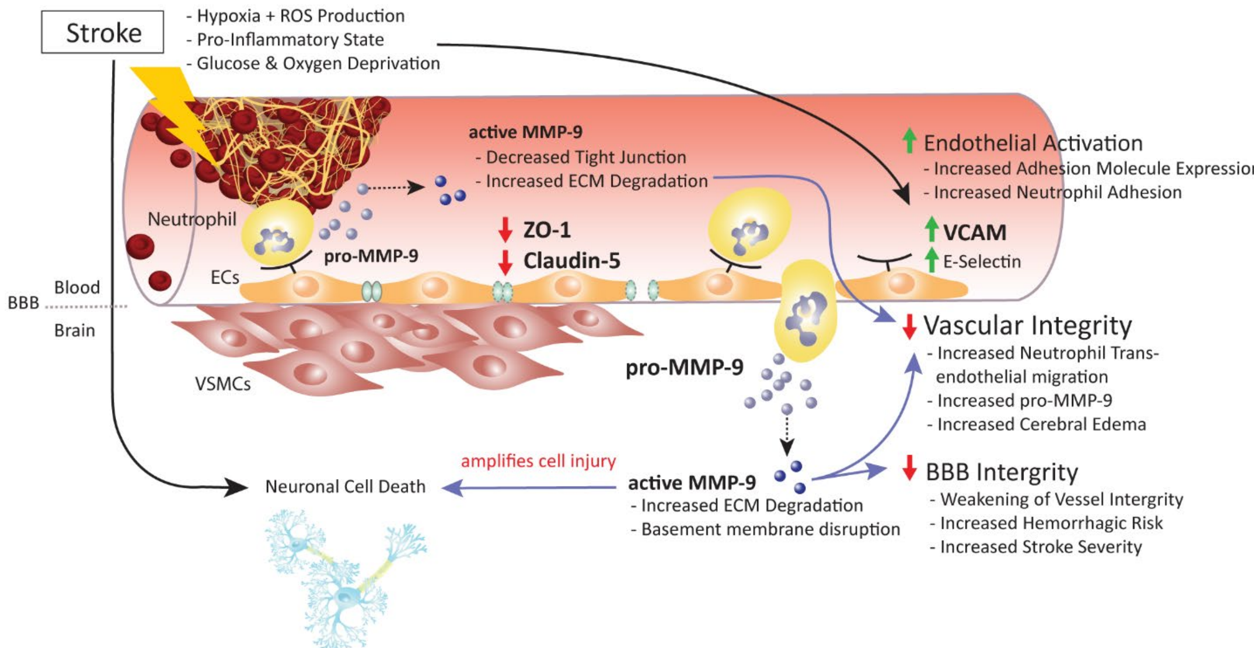

Figure 1. Pathological timeline of cerebrovascular dysfunction and blood brain barrier (BBB) integrity loss during acute ischemic stroke. Initial injury of arterial occlusion induces coagulation, recruitment of immune cells neutrophils, and hypoxic and glucose deprived conditions, leading to oxidative stress, excitotoxicity, and inflammation, causing neuronal cell death and microglial activation that enhances pro-inflammatory cytokine production. Neutrophil migration and adhesion to the cerebral endothelium, allows for release of proMMP-9. Active MMP-9 then can degrade components of the extracellular cell matrix (ECM) and tight junctions stabilizing the BBB, as well as stimulate neutrophil recruitment. Neutrophil infiltration then aggravates BBB breakdown by release of pro-MMP-9 and MMP-9 activity, to enhance risk of cerebral edema formation, weakening of vascular integrity, hemorrhagic risk, and overall stroke severity. Active MMP-9 can degrade ECM components and stimulate MMP-9 release in resident brain cells. (modified from review Li et al., 2020, *Neuroscience*).

Figure 2. Experimental timeline of HBMEC and HBVSMC normoxia or HGD exposure and drug treatment.

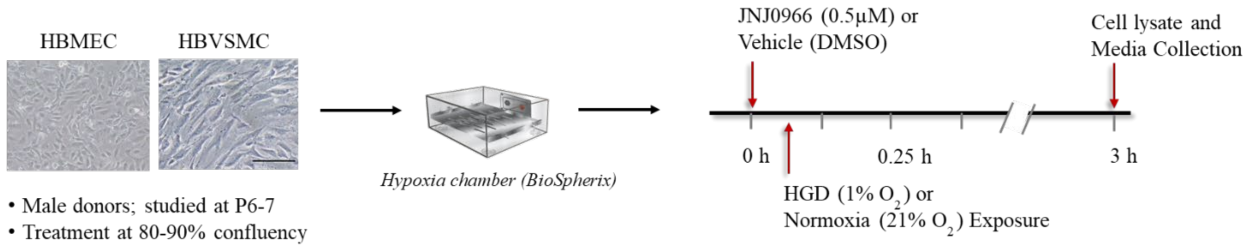

Figure 2. Timeline of cell culture with human brain microvascular endothelial cell (HBMEC) and human brain vascular smooth muscle cell (HBVSMC) and, in some select cases, treatment with JNJ0966 (0.5µM) or vehicle (DMSO), preceding normoxia or HGD exposure for 3 h. Primary cells are cultured and treated at passage number 6 or 7, at 80-90% confluency. Drug treatment of JNJ0966 (0.5µM) or vehicle (DMSO) precedes normoxia exposure at 21% O_2 or HGD occurring in hypoxia chamber at 1% O_2 and 5% CO_2 with N_2 balance at 37°C Representative brightfield captures of HBMEC and HBVSMC depicted (leftmost panel). Bar scale = 50 µm.

Figure 3. Representative zymography of HBVSMC cell lysate

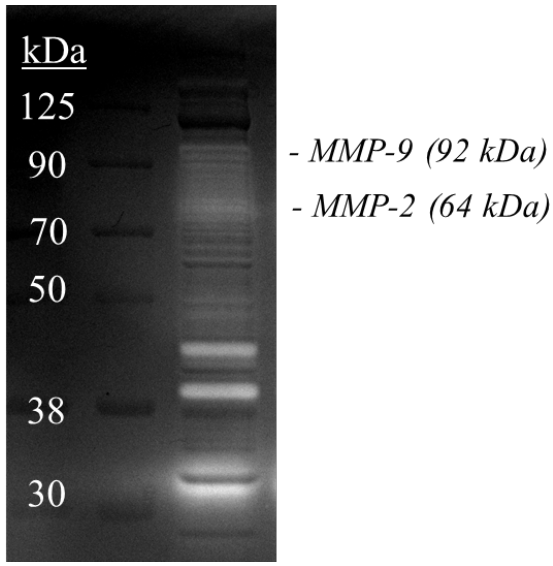

Figure 3. Representative zymography gel of HBVSMC cell lysate depicted here. Proteolytic activity is represented by white bands against dark background with intact gelatin. Expected MMP ranges for gelatinases MMP-9 (92 kDa) and MMP-2 (64 kDa) are delineated.

Figure 4. Crystal violet stained HBVSMC and HBMEC following normoxia and HGD exposure.

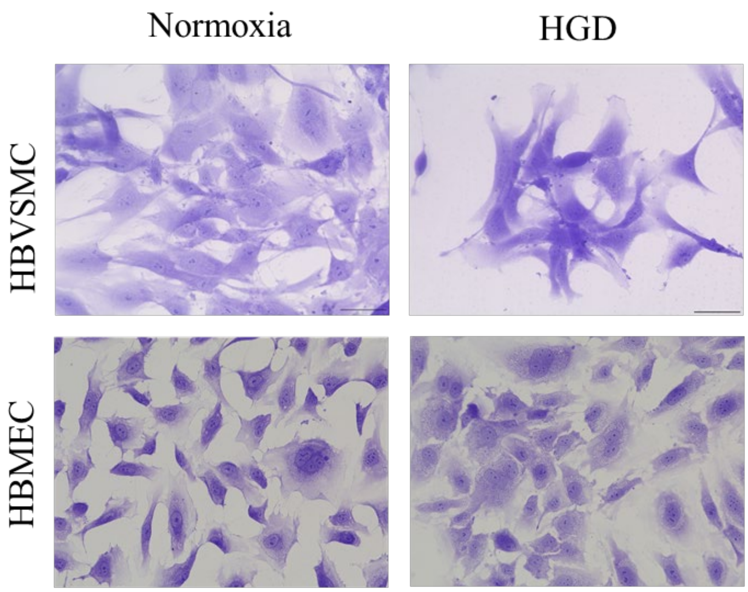

Figure 4. Representative 40X zoomed in images of crystal violet stained human brain vascular smooth muscle cells (HBVSMC) and human brain microvascular endothelial cells (HBMEC) following normoxia and hypoxia plus glucose deprivation (HGD) exposure after 3 h. Following HGD exposure, cells visually appeared to decrease in count and change in morphology. Scale Bar = 50µm.

Figure 5. NF-κB expression in HBVSMC following HGD.

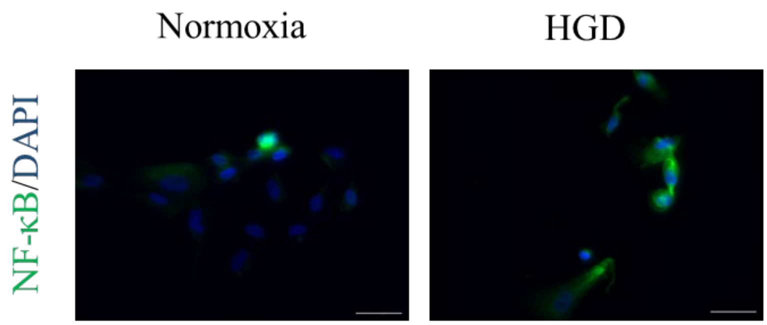

Figure 5. Representative immunocytochemical images demonstrating the expression of phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB, green) p65 in human brain vascular smooth muscle cells (HBVSMC) following exposure to either hypoxia plus glucose deprivation (HGD) or normoxia for 3 h. Nucleus is labeled with 4',6-diamidine-2'-phenylindole 1122 dihydrochloride (DAPI; blue). Imaged at 40X magnification. Scale Bar = 50 μM.

Figure 6. Adhesion molecule VCAM-1 expression increased in HBMEC following HGD.

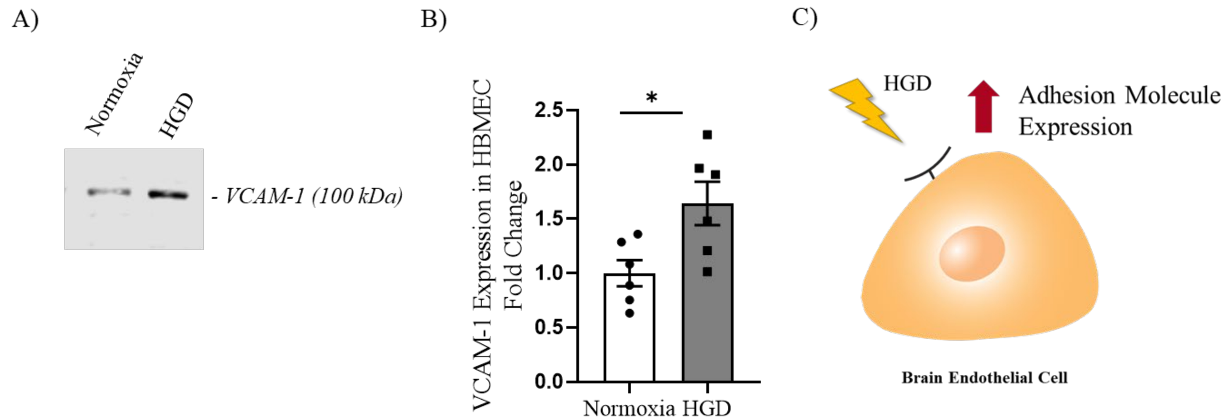

Figure 6. A) Representative western blot of vascular cell adhesion molecule (VCAM-1) levels in primary human brain microvascular endothelial cells (HBMECs) cell lysate treated with either normoxic or hypoxia plus glucose deprivation (HGD) conditions for 3 hours. B) Summary of protein analysis for VCAM-1 band intensity expressed as a fold changed to normoxia. *P=0.0239. Mean ± SEM. n=6/group. C) Figure simplistically summarizes increase in VCAM-1 in endothelial cell following HGD compared to normoxia.

Figure 7. Tight junction protein levels decreased in HBMEC following HGD exposure.

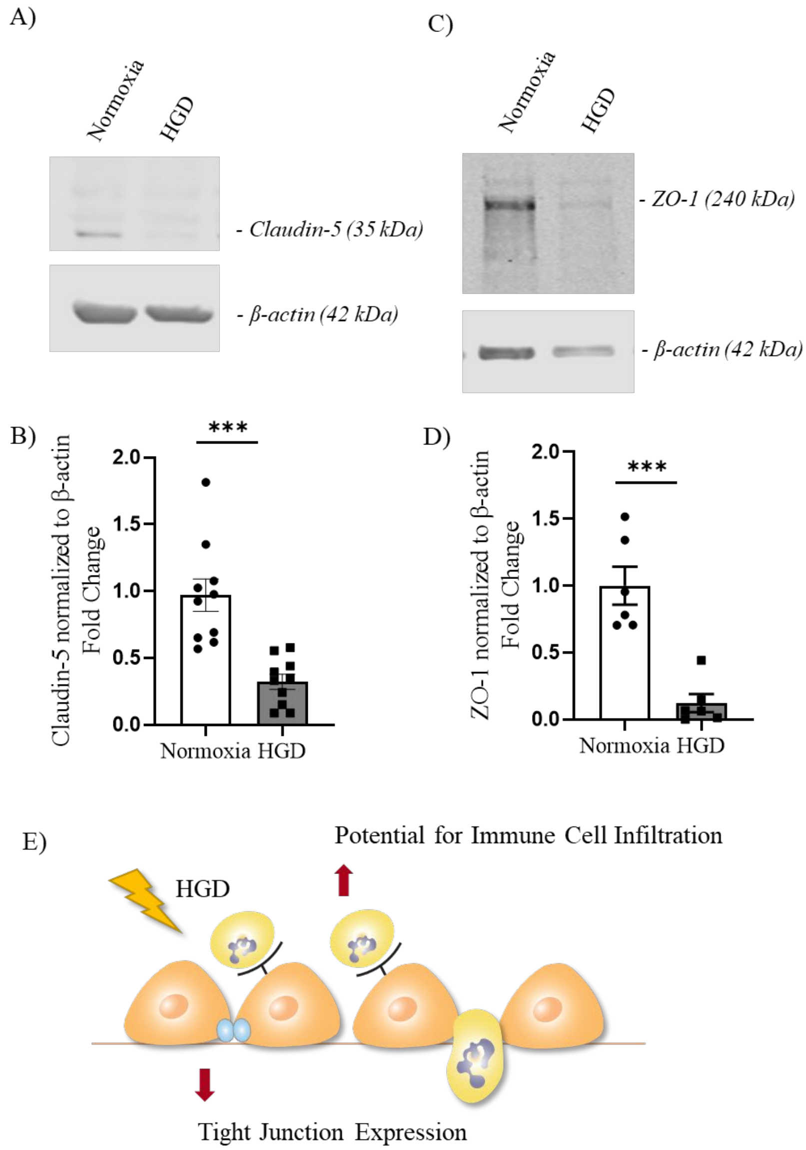

Figure 7. A) Representative western blot of Claudin-5 levels in primary human brain microvascular endothelial cells (HBMECs) cell lysate treated with either normoxic or hypoxia plus glucose deprivation (HGD) conditions for 3 hours. B) Representative western blot of zonula occludens 1 (ZO-1) levels in HBMECs treated with either normoxic or HGD for 3 hours. C.) Summary of the protein analysis for Claudin-5 band intensity normalized to β-Actin (loading control) band intensity and expressed as a fold changed to normoxia (bottom). ***P=0.0004. Mean

± SEM. n=10/group. D) Summary of the protein analysis for ZO-1 band intensity normalized to β-Actin (loading control) band intensity and expressed as a fold changed to normoxia. ***P=0.0008 vs Normoxia. Mean ± SEM. n=6/group. E) Figure simplistically summarizes decrease in Claudin-5 and ZO-1 in endothelial cell following HGD compared to normoxia. Decrease in tight junction protein presence suggests increased potential for immune cell infiltration, relevant to *in vivo* models.

Figure 8. MMP-9 levels were not altered following ischemic-like injury in HBMEC; however, pro-MMP-9 levels decreased and MMP-9 to pro-MMP-9 ratio increased.

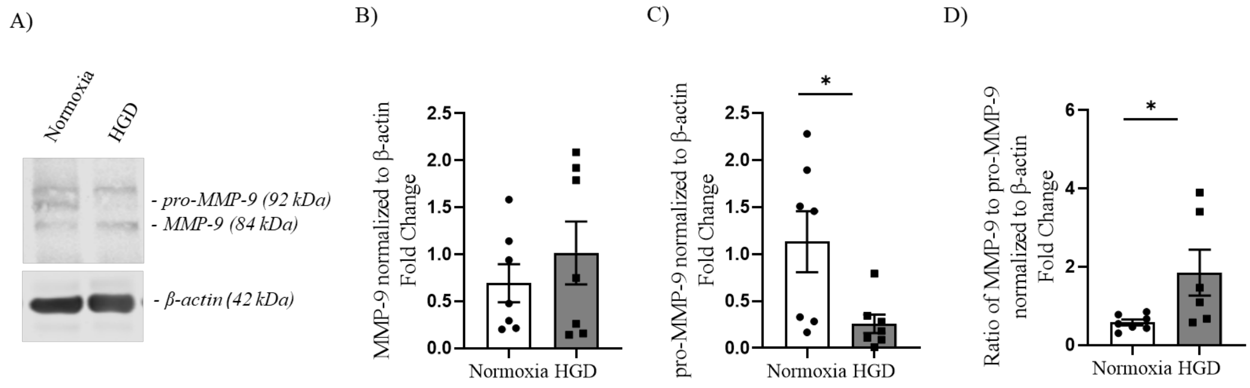

Figure 8. A) Representative western blot of zymogen matrix metalloproteinase 9 (pro-MMP-9) and active MMP-9 in primary human brain microvascular endothelial cells (HBMECs) cell lysate exposed to either normoxic or hypoxia plus glucose deprivation (HGD) conditions for 3 hours B) Summary of protein analysis for MMP-9 band intensity expressed as a fold changed to normoxia. Mean ± SEM. n=7/group. C) Summary of protein analysis for pro-MMP-9 band intensity expressed as a fold changed to normoxia. Normoxia vs HGD *P=0.0364. Mean ± SEM. n=7/group. D) Summary of MMP-9 to pro-MMP-9 protein ratio normalized to ß-actin as a fold changed to normoxia. Normoxia vs HGD *P=0.0221. Mean ± SEM. n=7/group.

Figure 9. MMP-9 protein levels were not different following HGD in HBVSMC cell lysate, and the selective MMP-9 inhibitor JNJ0966 had no effect.

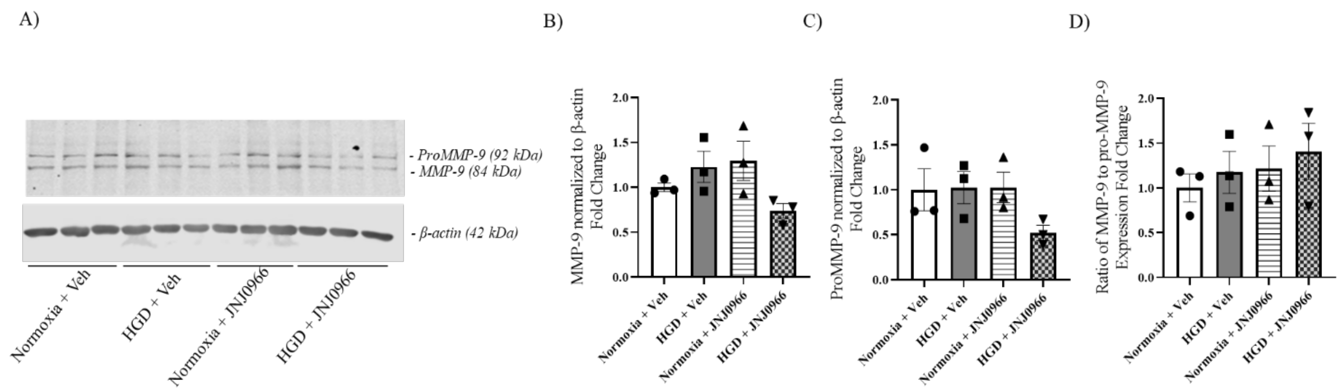

Figure 9. A) Representative western blot of zymogen matrix metalloproteinase 9 (pro-MMP-9) and active MMP-9 in primary human brain vascular smooth muscle cell (HBVSMC) cell lysate exposed to either normoxic or hypoxia plus glucose deprivation (HGD) conditions ± JNJ0966 (0.5 μM) for 3 hours. B) Summary of protein analysis for MMP-9 band intensity expressed as a fold changed to normoxia. Mean ± SEM. n=3/group. C) Summary of protein analysis for pro-MMP-9 band intensity expressed as a fold changed to normoxia. D) Summary of MMP-9 to pro-MMP-9 protein ratio normalized to ß-actin as a fold changed to normoxia.

Figure 10. Extracellular MMP-9 activity in HBMEC media increased following HGD.

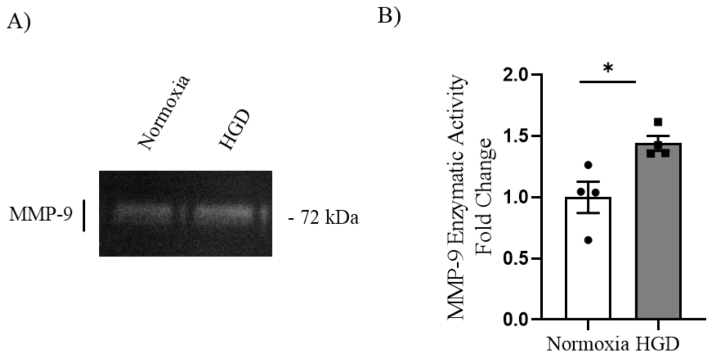

Figure 10. A) Representative zymography gel of active matrix metalloproteinase 9 (MMP-9) in primary human brain microvascular endothelial cells (HBMECs) media treated with either normoxic or hypoxia plus glucose deprivation (HGD) conditions for 3 hours. B) Summary of enzymatic activity for MMP-9 expressed as a fold changed to normoxia. *P=0.0323; Mean ± SEM. n=4/group**.**

Figure 11. Extracellular MMP-9 activity was detected in HBVSMC cell lysate and JNJ0966 treatment appeared to attenuate this response.

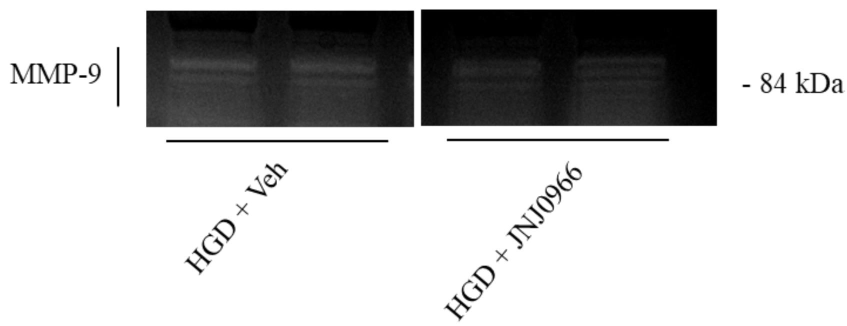

Figure 11. Representative zymography gel of active matrix metalloproteinase 9 (MMP-9) in primary human brain vascular smooth muscle cell (HBVSMC) cell lysate treated with either hypoxia plus glucose deprivation (HGD) or normoxia ± JNJ0966 (0.5 μM) conditions for 3 hours.

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