HHS Public Access

Author manuscript

Can J Physiol Pharmacol. Author manuscript; available in PMC 2021 September 01.

Published in final edited form as:

Can J Physiol Pharmacol. 2020 September ; 98(9): 587–595. doi:10.1139/cjpp-2019-0630.

Diabetes-related sex differences in the brain endothelin system following ischemia in vivo and in human brain endothelial cells in vitro

Yasir Abdul[1,2], **Weiguo Li**[1,2], **Juan D Vargas**[1,2], **Emily Clark**[1,2], **Lianying He**[1,2], **Sarah Jamil**[1,2], **Adviye Ergul**[1,2]

[1]Department of Pathology and Laboratory Medicine, Medical University of South Carolina

[2]Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC

Abstract

The endothelin (ET) system has been implicated to contribute to the pathophysiology of cognitive impairment and stroke in experimental diabetes. Our goals were to test the hypotheses that 1) circulating and/or peri-infarct ET-1 levels are elevated after stroke in both sexes and this increase is greater in diabetes, 2) ET receptors are differentially regulated in the diabetic brain, 3) brain microvascular endothelial cells (BMVEC) of female and male origin express the ETA receptor subtype, and 4) diabetes and stroke-mimicking conditions increase ET-1 levels in BMVECs of both sexes. Control and diabetic rats were randomized to sham or stroke surgery. BMVECs of male (hBEC5i) and female (hCMEC/D3) origin, cultured under normal and diabetes-mimicking conditions, were exposed to normoxia or hypoxia. Circulating ET-1 levels were higher in diabetic animals and this was more pronounced in the male cohort. Stroke did not further increase plasma ET-1. Tissue ET-1 levels were increased after stroke only in males, whereas peri-infarct ET-1 increased in both control and diabetic females. Male BMVECs secreted more ET-1 than female cells and hypoxia increased ET-1 levels in both cell types. There was sexually dimorphic regulation of ET receptors both in tissue and cell culture samples. There are sex differences in the stroke and diabetes-mediated changes in the brain ET system at endothelial and tissue levels.

Keywords

Diabetes; Stroke; ET-1; Sex; Brain

Introduction

Diabetes is a major risk factor for neurological diseases including acute ischemic stroke and post-stroke cognitive impairment (Flores-Gomez et al. 2019; Li et al. 2018). Individuals with diabetes also experience higher morbidity and mortality associated with these diseases, which collectively are the leading causes of long-term adult disability worldwide (Bertoni et al. 2002). Intriguingly, women suffer disproportionately from both diabetes and ischemic

Corresponding Author: Adviye Ergul, MD, PhD, 171 Ashley Ave. MSC 908, Department of Pathology & Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425, Tel: 1-843-876-5958. ergul@musc.edu.

stroke (Arnetz et al. 2014). From diabetes perspective, women experience more severe diabetes and higher all-cause mortality (Arnetz et al. 2014). From stroke perspective, stroke is the 4th leading cause of death in women but 5th in men (Heron 2019). The cardiovascular protection seen in women before menopause is lost after menopause (Arnetz et al. 2014; Carr 2003; Janghorbani et al. 1993). Unfortunately, women suffer from increased risk, more severe strokes, higher short- term mortality, and a worse quality of life than men (Bushnell et al. 2018; Bushnell et al. 2014; Roy-O'Reilly and McCullough 2018). Lack of understanding of the mechanisms contributing to worsened stroke outcomes in diabetes hamper the development of targeted therapeutic strategies for women.

It is well established that diabetes is a vascular disease. Diabetes-mediated endothelial dysfunction and loss of cerebrovascular integrity can not only contribute to the occurrence of ischemic stroke but also to the dysregulation of cerebral blood flow (CBF) and neurovascular niche, both of which are critical for stroke injury and recovery. We and others have shown that plasma and vascular tissue levels of endothelin-1 (ET-1), the most potent vasoconstrictor with proliferative and inflammatory properties, are increased in diabetes and ET-1 contributes to cerebrovascular dysfunction via the activation of both ETA and ETB receptor subtypes on vascular smooth muscle cells in experimental models of diabetes (Coucha et al. 2018; Li et al. 2018). Several studies reported that blockade of the ETA system after ischemic stroke reduces the severity of injury and improves functional outcomes (Gupta et al. 2005; Matsuo et al. 2001; Zhang et al. 2008). Unfortunately, these preclinical studies in diabetes and ischemic stroke are limited to male animals. Moreover, the impact of ischemic injury on circulating and brain tissue ET-1 and its receptors in both sexes are unknown. While endothelial cells are known to be the major source of ET-1 and mainly possess the ETB receptor subtype, in addition to another early report, we have shown that human brain microvascular endothelial cells (BMVECs) isolated from a male patient uniquely express both ETA and ETB receptors but whether BMVECs from females have the same characteristics is not known (Abdul et al. 2018). Based on these grounds, we postulated that 1) circulating and/or peri-infarct ET-1 levels are elevated after stroke in both sexes and this increase is greater in diabetes, 2) ET receptors are differentially regulated in the diabetic brain, 3) BMVECs of female and male origin express the ETA receptor subtype, and 4) diabetes and stroke-mimicking conditions increase ET-1 levels in BMVECs of both sexes.

Materials and methods

Animals

Animal studies were conducted at the Augusta University in Augusta, GA before the investigators relocated to the Medical University of South Carolina in Charleston, SC. Rats were housed in the animal care facility at Augusta University, which is approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were conducted in accordance with the National Institute of Health (NIH) guidelines for the care and use of animals in research. Furthermore, all protocols were approved by the institutional animal care and use committee.

Diabetes was induced in male and female Wistar rats (Envigo RMS, Inc., Indianapolis, IN) using a high-fat diet/low-dose streptozotocin (HFD/STZ) combination. Diabetic rats were received at 4 weeks of age and immediately started and maintained on a 45% kcal fat diet for the remainder of the study (Research Diets Inc., New Brunswick, NJ). A single dose STZ injection (35 mg/kg; Cayman Chemical, Ann Arbor, MI) was administered intraperitoneally (ip) at 6 weeks of age. If blood glucose was not above 150 mg/dL 5 days post-injection, a second small dose (20 mg/kg) was administered. Control rats were received at 10-11 weeks of age and maintained on regular chow with 4% kcal fat. Body weight and blood glucose were measured twice a week until euthanasia. All female rats underwent surgery during the diestrus phase after careful monitoring of the estrus cycle by vaginal swab.

There were 8 experimental groups: 1) control sham (no intervention) females (n=6), 2) control sham males (n=6), 3) diabetic sham females (n=6), 4) diabetic sham males (n=6), 5) control stroked females (n=5), 6) control stroked males (n=6), 7) diabetic stroked females (n=4), and 8) diabetic stroked males (n=3). While all groups started with 6 animals, 4 diabetic animals (2 male and 2 female) died prior to surgery resulting in lower animal numbers in the diabetic stroke groups. 1 control female and 1 diabetic male died right after surgery. Body weight and blood glucose information at termination are given in Table 1. Since animals were age matched, female animals were smaller than male counterparts. Diabetic animals had higher blood glucose levels.

Middle cerebral artery occlusion (MCAO) surgery

Stroke was induced by transient (60 min) focal cerebral ischemia (MCAO) at 12-15 weeks of age as previously described (Li et al. 2019). In the post-operative period blood glucose was monitored daily.

Euthanasia and specimen collection

Rats were euthanized 3 days post-MCAO using isoflurane overdose and cardiac puncture. Blood (500 μL) was collected via cardiac puncture. Using a brain matrix, the brain was sliced from B, containing the prefrontal cortex, through slice D, containing the hippocampus. Tissue sampled from the peri-infarct area in ipsilateral side of the injury for ELISA and gene expression studies.

BMVEC culture

The male-derived human brain microvascular endothelial cell line HBEC-5i (American Type Culture Collection-ATCC, CRL 3245)and the female-derived human brain microvascular endothelial cell line hCMEC/D3 (kind gift from Dr. J. Zastre at the UGA College of Pharmacy) were cultured in 75 cm^2 culture flasks that were coated with 0.2% w/v gelatin (porcine Type A; Sigma-Aldrich) prior to cell seeding (Eigenmann et al. 2013; Poller et al. 2008; Puech et al. 2018; Wassmer et al. 2006; Weksler et al. 2013). A 1:1 ratio of endothelial growth media (VEC Technologies, Rensselaer, NY, USA) and Medium 199 (Corning, Manassas, VA, USA) was used for cell culture. The VEC media includes serum and antibiotics, while 10% FBS and 1% penicillin-streptomycin were added to the M199. To mimic diabetes and ischemic conditions, male and female cells were seeded on 100 mm plates previously coated with 0.2% w/v gelatin and split into two groups: normal glucose

(NG) and diabetes-mimicking media which contained high glucose (HG, 25 mM) and palmitate (P, 50 μM) in 1:1 ratio in VEC:M199 media. The cells were incubated for two days, with the media being replaced after the first day. On the third day, all cells were starved in Dulbecco's Modification of Eagle's Medium (DMEM; Corning, Manassas, VA, USA) containing 1% penicillin-streptomycin but no serum. After a six-hour starvation period, hypoxia was induced by treatment with 200 μM cobalt(II) chloride hexahydrate for 24 h (Miyamoto et al. 2015; Wu and Yotnda 2011). Cells and supernatants were collected for RT-PCR and ET-1 ELISA, respectively.

Cell Viability Assay

Male and female cells were seeded in a 96-well plate. They were cultured under normoxic and hypoxic conditions in NG and diabetes-mimicking media as described above. Immediately after treatment, a Realtime-Glo MT cell viability substrate (Promega) was added to the cells as per the manufacturer's instructions. A Synergy H1 microplate reader (BioTek) was used to measure luminescence (relative luminescent unit RLU) every two hours for a period of 60 hours and result was presented as area under curve. To determine the effect of ET-1 on cell viability, cells were grown in normal glucose media under normoxic conditions. After serum starvation for 6 h, cells were pretreated with vehicle or ETA receptor antagonist BQ-123 (20 μM) (Tocris Biotech) for 30 minutes period, followed by ET-1 (2 μM). Once treated, luminescence was measured every 2 hours up to 30 hours. Results are given as mean ± SEM of three individual experiments performed in triplicate.

Cell Migration Assay

Male and female cells were seeded in a 12-well plate and cultured as described above. After hypoxia treatment, a scratch was made and photographed using an optical microscope (Olympus IX73). The same location was photographed again 24 hours after treatment. The distance between the cells was measured at 0 and 24 hours (Olympus cellSens) and the percent migration was calculated. Results are given as mean ± SEM of three individual experiments performed in triplicate.

ETA Immunohistochemistry

Male and female cells were grown on chambered slides under normoxic and hypoxic conditions in NG and diabetes-mimicking conditions as described above. After 24 hours of hypoxia cells were fixed in 4% paraformaldehyde for 15 min, and subsequently washed with TBS followed by treatment with 0.2% Triton X-100 for 3 min. After washing, cells were blocked by 5% BSA for 1 h at room temperature. Cells were then incubated with anti-ETA (AER-001, Alomone labs, Israel) antibody at a 1:100 dilution in 0.2% BSA for 3 h at room temperature. Cells were washed and incubated with AlexaFlour 488 conjugated secondary antibody (anti-rabbit; Jackson Immuno Research Laboratories, Inc., West Grove, PA) at a 1:500 dilution at room temperature for 1 h. Negative control slides were incubated with 0.2% BSA in place of the primary antibody (not shown). Slides were imaged on Olympus IX73 microscope (Olympus Corporation, Japan).

ET-1 ELISA

The concentration of ET-1 in the rat plasma, brain homogenates and BMVEC culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) from R&D Systems (QuantiGlo ELISA) following the manufacturer's instructions. The results calculated on triplicate wells for each sample were expressed as pg/mg protein for brain tissue homogenates and pg/ml for plasma samples or cell culture supernatants.

Quantitative real-time PCR (qRT-PCR)

For *in vitro* studies, cells were collected using RNA lysis buffer and RNA was isolated using SV Total RNA isolation system (Promega, USA). Quality and quantity of extracted RNA was assayed using a Nanodrop instrument (NanoDrop Technologies, Wilmington, DE). iScript cDNA synthesis kit (cat #1708891, BioRad, Foster city, CA) was used to reverse transcribe equal quantities of total RNA following the manufacturer's instructions. Primers were custom designed from Invitrogen (Thermo Fisher Scientific). The sequences of primers used in the study are listed in Table 2. qRT-PCR was performed using iScript Reverse Transcription super mix (cat #1708840, Biorad, Foster City, CA) and StepOnePlus Real-Time PCR System (Thermo Fisher Scientific,) as per the manufacturer's protocol. The relative gene expression was analyzed by the delta-delta Ct method using GAPDH as endogenous control gene and normalized to the respective control group.

Statistical analyses

For *in vivo* studies, the main effect of stroke or diabetes as well as the interaction between diabetes and stroke on ET-1 levels and gene expression was determined by two-way ANOVA (Sham vs Stroke) x (Control vs Diabetes) separately for male or female cohorts. For Figure 1 where results are obtained in absolute values for plasma or tissue ET-1 levels, data was also analyzed for sex differences within the diabetic cohorts by two-way ANOVA (Sham vs Stroke) x (Female vs Male). Results (Fig 1-3) are given as dot-plots to show the individual data points in each group. The effect of diabetes-mimicking conditions and hypoxia on cell migration, viability and gene expression was analyzed by two-way ANOVA in a similar manner. Only main effects were given if results were significant. When an interaction was noted, full ANOVA tables were included on each figure. Tukey's multiple comparisons post-hoc test was used to compare means from significant ANOVAs. For Figure 5, change in cell viability with ET-1 or ET-1+BQ-123 was analyzed by one-way ANOVA. Statistical significance was determined at alpha<0.05. GraphPad Prism8 software was used for all analyses.

Results

Diabetes and stroke-mediated increase in circulating and tissue ET-1 levels differs between male and female animals

Stroke did not cause an increase in plasma ET-1 levels in either male or female control rats (Fig. 1A and B). Diabetes elevated ET-1 levels in both female and male animals but the magnitude of this increase was greater in males. Comparison of female and male animals within the diabetic cohorts showed a sex difference in plasma ET-1 levels after stroke (Fig.

1C). There was a trend for interaction (p=0.12) suggesting that stroke decreased ET-1 levels in females but increased it in males. At the tissue level, peri-infarct brain tissue ET-1 level did not change after stroke in control males but increased significantly in diabetic male animals resulting in a trend for interaction (Fig. 1D). In females, there was no difference between the groups (Fig. 1E). Comparison of female and male animals within the diabetic cohorts showed a trend for interaction resulting from increased tissue ET-1 after stroke in males but not in females (Fig. 1F).

Female and male animals display different gene expression patterns for PPET-1 and ET receptors in brain tissue

PPET-1 gene expression was lower after stroke in both control and diabetic male animals (Fig. 2A). In the female cohort, PPET-1 expression appeared to be the highest in sham diabetic animals, but it did not reach significance due to high variability in the diabetic sham animals (Fig. 2D).

Analysis of ETA mRNA expression in male rats suggested an interaction of diabetes and stroke such that stroke lowered ETA mRNA in control but not diabetic animals that already presented with lower ETA expression than controls (Fig. 2B). In the female cohorts, there was no difference between the groups (Fig. 2D).

ETB receptor expression patterns were also different between male and female cohorts. In males, there was both a stroke and diabetes effect resulting in an interaction such that diabetic animals had lower ETB expression than controls and while stroke lowered ETB mRNA in control rats, it increased in the diabetic group (Fig. 2C). In females, stroke caused an increase in expression levels in both control and diabetic rats (Fig. 2F).

There are sex differences in ET-1 production by BMVECs

ET-1 secretion was increased by hypoxia and decreased by diabetes-mimicking conditions in male cells (Fig. 3A). Whereas in female cells hypoxia lowered and diabetic conditions increased ET-1 (Fig. 3D). Similar to plasma and brain tissue, ET-1 levels were lower in female cells. ETA receptor expression was increased by hypoxia under normal or diabetes conditions in male cells (Fig. 3B) (Please note the difference in scale of the Y axes in panels A and D). Interestingly, in female cells, hypoxia had the opposite effect and lowered ETA expression whereas diabetes-mimicking conditions increased ETA mRNA (Fig. 3E). ETB expression was similar under the experimental conditions in both cell lines (Fig. 3C and F).

ETA protein levels were assessed by immunohistochemistry. In male cells, there appeared to be more ETA receptors which increased by hypoxia and high glucose (Fig 4). When hypoxic and diabetic conditions combined, there was pronounced perinuclear staining. In female cells, expression was low and following the mRNA expression pattern, diabetes alone increased ETA receptors but this was suppressed in hypoxia (Fig 4).

There are sex differences in ET-1 effects on BMVEC viability

Viability under hypoxic or diabetes-mimicking conditions also differed for both cell lines (Fig. 5A and B): Male cells exhibited reduced viability with either stimulus and while

female cells showed a similar trend, it did not reach significance. However, when the cells were stimulated with ET-1, cell viability decreased over 24 h in male cells and BQ-123 did not prevent this decrease. In female cells, there was a smaller yet significant decrease in viability and ETA antagonism prevented this.

There are sex differences in migratory properties of BMVECs

Endothelial cell migration responses to hypoxia and/or diabetes-mimicking conditions showed an interaction such that hypoxia lowered cell migration to a greater extent in diabetic conditions with diabetes alone having no effect in male cells (Fig 6A). In female cells, however, diabetes alone increased the migratory response and hypoxia had no effect (Fig 6B).

Discussion

The overall goal of this study was to investigate whether there are sex and disease specific changes in circulating and brain tissue ET-1 levels and its receptors. To achieve this goal, we used female and male control and diabetic rats subjected to sham or stroke surgery as well as brain microvascular endothelial cells of female and male origin. Our novel findings demonstrate that 1) circulating and/or peri-infarct ET-1 levels are elevated after stroke in male but not female diabetic animals, 2) ET receptors are differentially regulated in the diabetic brain, 3) BMVECs of female and male origin express the ETA receptor subtype, 4) hypoxia increases ETA expression in male cells whereas it decreases it in female cells, 5) diabetes and stroke-mimicking conditions increase BMVEC ET-1 secretion to a greater extent in male cells, and 6) ET-1 reduces cell viability in both cell lines and ETA antagonism prevents this response only in female but not male cells.

The study was developed as a result of our long-term interest in exploring the role and mechanisms by which diabetes impacts cerebral complications associated with the disease such as stroke and cognitive impairment. Both of these diseases have a vascular basis (Shi and Vanhoutte 2017; Sorop et al. 2017). Dysregulation of endothelial function and vascular structure can cause long-term reduction in CBF that may lead to vascular cognitive impairment over time or cause acute changes in regulation of CBF in the aftermath of ischemic stroke leading to a greater injury and worse outcomes (Gorelick et al. 2017). ET-1, the most potent vasoconstrictor factor, has long been postulated as a causal factor in diabetic vascular disease. Numerous studies by us and other, in most of which male animals were used, have shown that ET-1 mediates cerebrovascular dysfunction, remodeling and pathological neovascularization in diabetes (Coucha et al. 2018; Li et al. 2018). We have also shown that ischemic stroke overlayed on this pathology by MCA occlusion causes greater vascular injury and worsens functional outcomes without necessarily increasing the infarct size (Ergul et al. 2007; Li et al. 2017). While prevention of vascular remodeling has been shown to reduce vascular injury and improve outcomes after stroke in diabetes, the acute role of endogenous ET-1 in ischemic injury in diabetes remains unknown. To the best of our knowledge, with the exception of one study in which the ETB receptor blockade worsened stroke outcomes (Chuquet et al. 2002), several studies reported reduced neuronal injury and improved short-term functional outcomes with ETA or ETA/ETB receptor

blockade in otherwise healthy control rats (Chen et al. 1999; Gupta et al. 2005; Matsuo et al. 2001; Zhang et al. 2008). Our current results suggesting an increase in peri-infarct ET-1 level in male diabetic animals emphasize the need for studies to determine the short and long-term effects of ET receptor antagonism on functional outcomes after stroke in diabetes in both sexes.

A number of studies reported sex differences in the ET system expression and activation in cardiovascular and renal physiology and diseases (For complete review please see (Gohar et al. 2016; Sasser et al. 2015)). For example, clinical and experimental studies showed that males have higher ET-1 levels than females in hypertension (David et al. 2001; Ergul et al. 1996; Miyauchi et al. 1992). The reactivity to ET-1 also differs between sexes and this may be due to the linked differences in ET receptor expression. In different vascular beds, males have greater ETA receptor expression as compared to females resulting in augmented ETA-mediated vasoconstriction (Ergul et al. 1998; Kittikulsuth et al. 2013; Stauffer et al. 2010; Tostes et al. 2000). Interestingly, human cerebral arteries from women were found to be significantly less responsive to ET-1 as compared to men (Ahnstedt et al. 2013). Whether there are sex differences in the brain ET system that may impact stroke outcomes is not known. Our results showed strong trends for diabetic males having higher circulating and tissue ET-1 after stroke compared with females. Interestingly, we did not see a correlation between PPET-1 mRNA expression and protein levels. An earlier study reported increased neuronal ETA and microglial ETB receptors after stroke in male rats (Loo et al. 2002). In the current study, peri-infarct mRNA expression for ET receptors suggested differential regulation of ETA and ETB receptors, which need to be confirmed at the protein and cellular level with larger sample size. Collectively, these observed differences may have important implications for stroke injury as well as functional and cognitive recovery and highlight the need for preclinical studies with post-stroke ET receptor antagonism in both sexes and in animal models with comorbid diseases like hypertension and diabetes. These findings also call for attention to sex differences in clinical trials. A recent phase II clinical trial (NCT04046484; CTRI/2017/11/010654) provided preliminary evidence that ETB receptor agonism with PMZ-1620 (IRL-1620; INN sovateltide) improves 90-day post stroke outcomes. The impact of sex and comorbidities on outcomes is of great interest and can be further investigated in the ongoing phase III trial (NCT04047563; CTRI/2019/09/021373).

From a preclinical perspective, there is another important consideration of sex differences in the brain ET system and stroke outcomes. Due to its long-lasting vasoconstrictive effects, stereotaxic ET-1 injections, especially into the middle cerebral artery territory, has been used to induce strokes (Fluri et al. 2015; Sommer 2017). Numerous stroke recovery and rehabilitation studies prefer the ET-1-induced stroke model to target the location of ischemic injury so that specific motor and sensory tracks can be investigated (Jones et al. 2009; Karthikeyan et al. 2019). However, an early study reported significant sex differences in ET-1-induced injury across different age groups (Yager et al. 2005). Hence, better understanding of the regulation of brain ET system can have an impact on the design and interpretation of stroke recovery studies that employs ET-1-induced ischemic stroke.

Since endothelial cells are the major source for ET-1 production and our previous study showed that BMVECs of male origin express the ETA receptor, we also investigated sex

differences at the BMVEC level. For these studies, we used well characterized immortalized cells derived from male and female patients (Eigenmann et al. 2013; Poller et al. 2008; Puech et al. 2018; Wassmer et al. 2006; Weksler et al. 2013). Similar to differences in plasma and brain tissue ET-1 levels, BMVECs of male origin showed greater ET-1 secretion. Hypoxia altered ET-1 levels in both cell lines but in opposite direction. In contrast to our expectations, diabetes-mimicking conditions did not further increase ET-1 levels in males but did so in female cells. We focused on the effect of ETA receptor expression and activation on cellular properties since the presence of this receptor on endothelial cells is relatively unusual and emerging to be the case in the brain microvasculature (Abdul et al. 2018; Kawai et al. 1997; Mikhail et al. 2017). In male cells, hypoxia decreased viability and increased ET-1 levels and ETA receptors. We did not test the effect of ETA antagonism on hypoxia-mediated decrease in cell survival. However, exogenous ET-1 treatment reduced cell viability in both cell lines and interestingly, ETA blockade prevented this decrease only in female cells that show relatively less ETA receptors than male cells. Under these stroke and diabetes-mimicking growth conditions, we also observed that female cells displayed strikingly lower migratory properties but the role of ET-1 in this finding is not clear.

There are several limitations that need to be recognized. For *in vitro* studies we used cell lines that had been immortalized by different approaches. There is limited clinical information about the patients these cells were isolated from. Thus, these studies need to be replicated in primary cells. We also employed only ETA antagonism and one ET-1/ antagonist concentration. These findings need to be further confirmed in primary cells over a dose range. For *in vivo* studies, we terminated animals on Day 3 after stroke and may have missed early changes in the ET system. Since our goal was to evaluate the changes in the ET system expression, we used the tissue to measure mRNA and protein levels after stroke surgery and could not assess the infarct size. Greater mortality in the diabetic groups limited sample size. Nevertheless, to the best of our knowledge, this is the first study that suggests potential sex differences in the brain ET system that become more pronounced under diabetic and hypoxic conditions which ultimately may affect stroke recovery in diabetes and emphasizes the need for additional research in this area.

Acknowledgments

Funding This study was supported by Veterans Affairs (VA) Merit Review (BX000347), VA Senior Research Career Scientist Award (IK6 BX004471), National Institute of Health (NIH) R01NS083559 and R01 NS104573 (multi-PI, Susan C. Fagan as co-PI) to Adviye Ergul; and Diabetic Complications Research Consortium DiaComp awards 17AU3831/18AU3903 (DK076169/115255) to Dr. Weiguo Li.

References

Abdul Y, Ward R, Dong G, and Ergul A 2018 Lipopolysaccharide-induced necroptosis of brain microvascular endothelial cells can be prevented by inhibition of endothelin receptors. Physiol Res 67(Suppl 1): S227–S236. doi: 10.33549/physiolres.933842. [PubMed: 29947542]

Ahnstedt H, Cao L, Krause DN, Warfvinge K, Saveland H, Nilsson OG, and Edvinsson L 2013 Male-female differences in upregulation of vasoconstrictor responses in human cerebral arteries. PLoS One 8(4): e62698. doi: 10.1371/journal.pone.0062698. [PubMed: 23658641]

Arnetz L, Ekberg NR, and Alvarsson M 2014 Sex differences in type 2 diabetes: focus on disease course and outcomes. Diabetes Metab Syndr Obes 7: 409–420. doi: 10.2147/DMSO.S51301. [PubMed: 25258546]

Bertoni AG, Krop JS, Anderson GF, and Brancati FL 2002 Diabetes-related morbidity and mortality in a national sample of U.S. elders. Diabetes Care 25(3): 471–475. [PubMed: 11874932]

Bushnell CD, Chaturvedi S, Gage KR, Herson PS, Hurn PD, Jimenez MC, Kittner SJ, Madsen TE, McCullough LD, McDermott M, Reeves MJ, and Rundek T 2018 Sex differences in stroke: Challenges and opportunities. J Cereb Blood Flow Metab 38(12): 2179–2191. doi: 10.1177/0271678X18793324. [PubMed: 30114967]

Bushnell CD, Reeves MJ, Zhao X, Pan W, Prvu-Bettger J, Zimmer L, Olson D, and Peterson E 2014 Sex differences in quality of life after ischemic stroke. Neurology 82(11): 922–931. doi: 10.1212/WNL.0000000000000208. [PubMed: 24510493]

Carr MC 2003 The emergence of the metabolic syndrome with menopause. J Clin Endocrinol Metab 88(6): 2404–2411. doi: 10.1210/jc.2003-030242. [PubMed: 12788835]

Chen Y, McCarron RM, Bembry J, Ruetzler C, Azzam N, Lenz FA, and Spatz M 1999 Nitric oxide modulates endothelin 1-induced Ca2+ mobilization and cytoskeletal F-actin filaments in human cerebromicrovascular endothelial cells. J Cereb Blood Flow Metab 19(2): 133–138. doi: 10.1097/00004647-199902000-00003. [PubMed: 10027767]

Chuquet J, Benchenane K, Toutain J, MacKenzie ET, Roussel S, and Touzani O 2002 Selective blockade of endothelin-B receptors exacerbates ischemic brain damage in the rat. Stroke 33(12): 3019–3025. doi: 10.1161/01.str.0000039401.48915.9f. [PubMed: 12468806]

Coucha M, Abdelsaid M, Ward R, Abdul Y, and Ergul A 2018 Impact of Metabolic Diseases on Cerebral Circulation: Structural and Functional Consequences. Compr Physiol 8(2): 773–799. doi: 10.1002/cphy.c170019. [PubMed: 29687902]

David FL, Carvalho MH, Cobra AL, Nigro D, Fortes ZB, Reboucas NA, and Tostes RC 2001 Ovarian hormones modulate endothelin-1 vascular reactivity and mRNA expression in DOCA-salt hypertensive rats. Hypertension 38(3 Pt 2): 692–696. doi: 10.1161/01.hyp.38.3.692. [PubMed: 11566958]

Eigenmann DE, Xue G, Kim KS, Moses AV, Hamburger M, and Oufir M 2013 Comparative study of four immortalized human brain capillary endothelial cell lines, hCMEC/D3, hBMEC, TY10, and BB19, and optimization of culture conditions, for an in vitro blood-brain barrier model for drug permeability studies. Fluids Barriers CNS 10(1): 33. doi: 10.1186/2045-8118-10-33. [PubMed: 24262108]

Ergul A, Elgebaly MM, Middlemore ML, Li W, Elewa H, Switzer JA, Hall C, Kozak A, and Fagan SC 2007 Increased hemorrhagic transformation and altered infarct size and localization after experimental stroke in a rat model type 2 diabetes. BMC Neurol 7: 33. doi: 10.1186/1471-2377-7-33. [PubMed: 17937795]

Ergul A, Shoemaker K, Puett D, and Tackett RL 1998 Gender differences in the expression of endothelin receptors in human saphenous veins in vitro. J Pharmacol Exp Ther 285(2): 511–517. [PubMed: 9580591]

Ergul S, Parish DC, Puett D, and Ergul A 1996 Racial differences in plasma endothelin-1 concentrations in individuals with essential hypertension. Hypertension 28(4): 652–655. doi: 10.1161/01.hyp.28.4.652. [PubMed: 8843893]

Flores-Gomez AA, de Jesus Gomez-Villalobos M, and Flores G 2019 Consequences of diabetes mellitus on neuronal connectivity in limbic regions. Synapse 73(3): e22082. doi: 10.1002/syn.22082. [PubMed: 30457679]

Fluri F, Schuhmann MK, and Kleinschnitz C 2015 Animal models of ischemic stroke and their application in clinical research. Drug Des Devel Ther 9: 3445–3454. doi: 10.2147/DDDT.S56071.

Gohar EY, Giachini FR, Pollock DM, and Tostes RC 2016 Role of the endothelin system in sexual dimorphism in cardiovascular and renal diseases. Life Sci 159: 20–29. doi: 10.1016/j.lfs.2016.02.093. [PubMed: 26939577]

Gorelick PB, Furie KL, Iadecola C, Smith EE, Waddy SP, Lloyd-Jones DM, Bae HJ, Bauman MA, Dichgans M, Duncan PW, Girgus M, Howard VJ, Lazar RM, Seshadri S, Testai FD, van Gaal S, Yaffe K, Wasiak H, Zerna C, and American Heart Association/American Stroke, A. 2017 Defining Optimal Brain Health in Adults: A Presidential Advisory From the American Heart Association/American Stroke Association. Stroke 48(10): e284–e303. doi: 10.1161/STR.0000000000000148. [PubMed: 28883125]

Gupta YK, Briyal S, Sharma U, Jagannathan NR, and Gulati A 2005 Effect of endothelin antagonist (TAK-044) on cerebral ischemic volume, oxidative stress markers and neurobehavioral parameters in the middle cerebral artery occlusion model of stroke in rats. Life Sci 77(1): 15–27. doi: 10.1016/j.lfs.2004.11.025. [PubMed: 15848215]

Heron M 2019 Deaths: Leading causes for 2017. National Vital Statistics Reports 68(6): 1–77.

Janghorbani M, Hedley AJ, Jones RB, Zhianpour M, and Gilmour WH 1993 Gender differential in all-cause and cardiovascular disease mortality. Int J Epidemiol 22(6): 1056–1063. [PubMed: 8144286]

Jones TA, Allred RP, Adkins DL, Hsu JE, O'Bryant A, and Maldonado MA 2009 Remodeling the brain with behavioral experience after stroke. Stroke 40(3 Suppl): S136–138. doi: 10.1161/STROKEAHA.108.533653. [PubMed: 19064784]

Karthikeyan S, Jeffers MS, Carter A, and Corbett D 2019 Characterizing Spontaneous Motor Recovery Following Cortical and Subcortical Stroke in the Rat. Neurorehabil Neural Repair 33(1): 27–37. doi: 10.1177/1545968318817823. [PubMed: 30526316]

Kawai N, Yamamoto T, Yamamoto H, McCarron RM, and Spatz M 1997 Functional characterization of endothelin receptors on cultured brain capillary endothelial cells of the rat. Neurochem Int 31(4): 597–605. doi: 10.1016/s0197-0186(97)00018-1. [PubMed: 9308010]

Kittikulsuth W, Looney SW, and Pollock DM 2013 Endothelin ET(B) receptors contribute to sex differences in blood pressure elevation in angiotensin II hypertensive rats on a high-salt diet. Clin Exp Pharmacol Physiol 40(6): 362–370. doi: 10.1111/1440-1681.12084. [PubMed: 23713708]

Li W, Abdul Y, Ward R, and Ergul A 2018 Endothelin and diabetic complications: a brain-centric view. Physiol Res 67(Suppl 1): S83–S94. doi: 10.33549/physiolres.933833. [PubMed: 29947530]

Li W, Valenzuela JP, Ward R, Abdelbary M, Dong G, Fagan SC, and Ergul A 2019 Post-stroke neovascularization and functional outcomes differ in diabetes depending on severity of injury and sex: Potential link to hemorrhagic transformation. Exp Neurol 311: 106–114. doi: 10.1016/j.expneurol.2018.09.013. [PubMed: 30243988]

Li W, Ward R, Valenzuela JP, Dong G, Fagan SC, and Ergul A 2017 Diabetes Worsens Functional Outcomes in Young Female Rats: Comparison of Stroke Models, Tissue Plasminogen Activator Effects, and Sexes. Transl Stroke Res. doi: 10.1007/s12975-017-0525-7.

Loo LS, Ng YK, Zhu YZ, Lee HS, and Wong PT 2002 Cortical expression of endothelin receptor subtypes A and B following middle cerebral artery occlusion in rats. Neuroscience 112(4): 993–1000. doi: 10.1016/s0306-4522(02)00043-x. [PubMed: 12088756]

Matsuo Y, Mihara S, Ninomiya M, and Fujimoto M 2001 Protective effect of endothelin type A receptor antagonist on brain edema and injury after transient middle cerebral artery occlusion in rats. Stroke 32(9): 2143–2148. doi: 10.1161/hs0901.94259. [PubMed: 11546909]

Mikhail M, Vachon PH, D'Orleans-Juste P, Jacques D, and Bkaily G 2017 Role of endothelin-1 and its receptors, ETA and ETB, in the survival of human vascular endothelial cells. Can J Physiol Pharmacol 95(10): 1298–1305. doi: 10.1139/cjpp-2017-0412. [PubMed: 28732172]

Miyamoto N, Maki T, Shindo A, Liang AC, Maeda M, Egawa N, Itoh K, Lo EK, Lok J, Ihara M, and Arai K 2015 Astrocytes Promote Oligodendrogenesis after White Matter Damage via Brain-Derived Neurotrophic Factor. J Neurosci 35(41): 14002–14008. doi: 10.1523/JNEUROSCI.1592-15.2015. [PubMed: 26468200]

Miyauchi T, Yanagisawa M, Iida K, Ajisaka R, Suzuki N, Fujino M, Goto K, Masaki T, and Sugishita Y 1992 Age- and sex-related variation of plasma endothelin-1 concentration in normal and hypertensive subjects. Am Heart J 123(4 Pt 1): 1092–1093. doi: 10.1016/0002-8703(92)90734-d. [PubMed: 1549986]

Poller B, Gutmann H, Krahenbuhl S, Weksler B, Romero I, Couraud PO, Tuffin G, Drewe J, and Huwyler J 2008 The human brain endothelial cell line hCMEC/D3 as a human blood-brain barrier model for drug transport studies. J Neurochem 107(5): 1358–1368. doi: 10.1111/j.1471-4159.2008.05730.x. [PubMed: 19013850]

Puech C, Hodin S, Forest V, He Z, Mismetti P, Delavenne X, and Perek N 2018 Assessment of HBEC-5i endothelial cell line cultivated in astrocyte conditioned medium as a human blood-brain barrier model for ABC drug transport studies. Int J Pharm 551(1-2): 281–289. doi: 10.1016/j.ijpharm.2018.09.040. [PubMed: 30240829]

Roy-O'Reilly M, and McCullough LD 2018 Age and Sex Are Critical Factors in Ischemic Stroke Pathology. Endocrinology 159(8): 3120–3131. doi: 10.1210/en.2018-00465. [PubMed: 30010821]

Sasser JM, Brinson KN, Tipton AJ, Crislip GR, and Sullivan JC 2015 Blood pressure, sex, and female sex hormones influence renal inner medullary nitric oxide synthase activity and expression in spontaneously hypertensive rats. J Am Heart Assoc 4(4). doi: 10.1161/JAHA.114.001738.

Shi Y, and Vanhoutte PM 2017 Macro- and microvascular endothelial dysfunction in diabetes. J Diabetes 9(5): 434–449. doi: 10.1111/1753-0407.12521. [PubMed: 28044409]

Sommer CJ 2017 Ischemic stroke: experimental models and reality. Acta Neuropathol 133(2): 245–261. doi: 10.1007/s00401-017-1667-0. [PubMed: 28064357]

Sorop O, Olver TD, van de Wouw J, Heinonen I, van Duin RW, Duncker DJ, and Merkus D 2017 The microcirculation: a key player in obesity-associated cardiovascular disease. Cardiovasc Res 113(9): 1035–1045. doi: 10.1093/cvr/cvx093. [PubMed: 28482008]

Stauffer BL, Westby CM, Greiner JJ, Van Guilder GP, and Desouza CA 2010 Sex differences in endothelin-1-mediated vasoconstrictor tone in middle-aged and older adults. Am J Physiol Regul Integr Comp Physiol 298(2): R261–265. doi: 10.1152/ajpregu.00626.2009. [PubMed: 19939973]

Tostes RC, David FL, Carvalho MH, Nigro D, Scivoletto R, and Fortes ZB 2000 Gender differences in vascular reactivity to endothelin-1 in deoxycorticosterone-salt hypertensive rats. J Cardiovasc Pharmacol 36(5 Suppl 1): S99–101. doi: 10.1097/00005344-200036051-00032. [PubMed: 11078348]

Wassmer SC, de Souza JB, Frere C, Candal FJ, Juhan-Vague I, and Grau GE 2006 TGF-beta1 released from activated platelets can induce TNF-stimulated human brain endothelium apoptosis: a new mechanism for microvascular lesion during cerebral malaria. J Immunol 176(2): 1180–1184. doi: 10.4049/jimmunol.176.2.1180. [PubMed: 16394007]

Weksler B, Romero IA, and Couraud PO 2013 The hCMEC/D3 cell line as a model of the human blood brain barrier. Fluids Barriers CNS 10(1): 16. doi: 10.1186/2045-8118-10-16. [PubMed: 23531482]

Wu D, and Yotnda P 2011 Induction and testing of hypoxia in cell culture. J Vis Exp(54). doi: 10.3791/2899.

Yager JY, Wright S, Armstrong EA, Jahraus CM, and Saucier DM 2005 A new model for determining the influence of age and sex on functional recovery following hypoxic-ischemic brain damage. Dev Neurosci 27(2-4): 112–120. doi: 10.1159/000085982. [PubMed: 16046844]

Zhang RL, Zhang C, Zhang L, Roberts C, Lu M, Kapke A, Cui Y, Ninomiya M, Nagafuji T, Albala B, Zhang ZG, and Chopp M 2008 Synergistic effect of an endothelin type A receptor antagonist, S-0139, with rtPA on the neuroprotection after embolic stroke. Stroke 39(10): 2830–2836. doi: 10.1161/STROKEAHA.108.515684. [PubMed: 18669895]

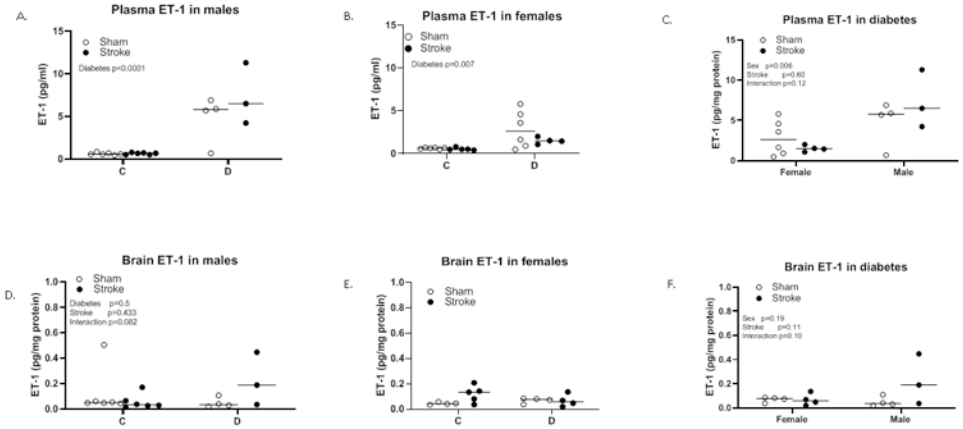

Fig. 1.

Circulating and peri-infarct brain ET-1 levels are higher in diabetic male animals. Effect of stroke and diabetes on male plasma and brain tissue ET-1 levels in male (A and D) and female (B and E) animals are analyzed by two-way ANOVA for each sex. Female and male plasma (C) and brain tissue (F) comparisons were made only in the diabetic cohorts by two-way ANOVA. Significance is indicated on each graph and when there is an interaction or a trend for interaction, full ANOVA tables are shown on the graphs.

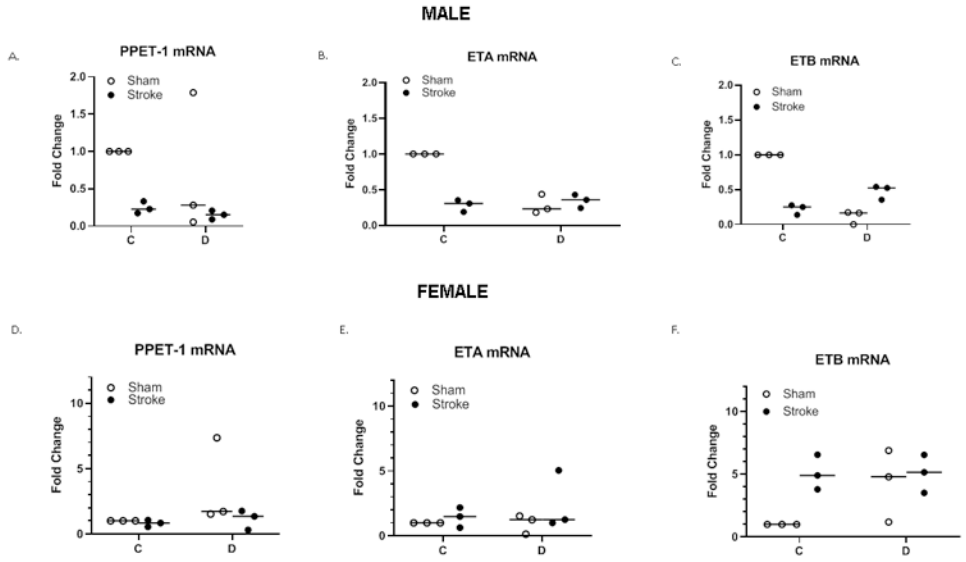

Fig. 2.

Peri-infarct PPET-1 and ET receptor expression is differentially regulated by sex and stroke. Fold changes in PPET-1, ETA and ETB mRNA expression are shown in male (A-C) and female (D-F) animals. Given the large variability and a small sample size, statistical comparisons are not marked.

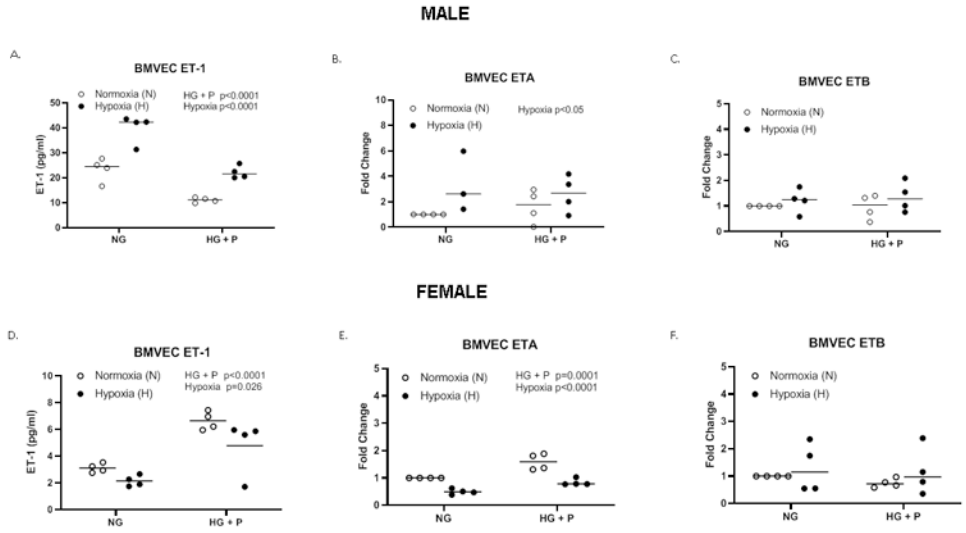

Fig. 3.

Hypoxia increases ET-1 levels to a greater extent in BMVECs of male origin. Diabetes and stroke-mimicking conditions were achieved by culturing cells in high glucose and palmitate (HG+P) containing growth media and exposing to hypoxia induced by $CoCl_2$ treatment. Changes in ET-1 levels and ETA and ETB mRNA expression are shown in male (A-C) and female (D-F) cells. Two-way ANOVA was performed for each sex (normoxia vs hypoxia) x (NG vs HG+P). Significance is indicated on each graph. Results are shown as mean ± SEM of 4 individual experiments performed in multiple replicates. NG: normal glucose, HG+P: high glucose + palmitate

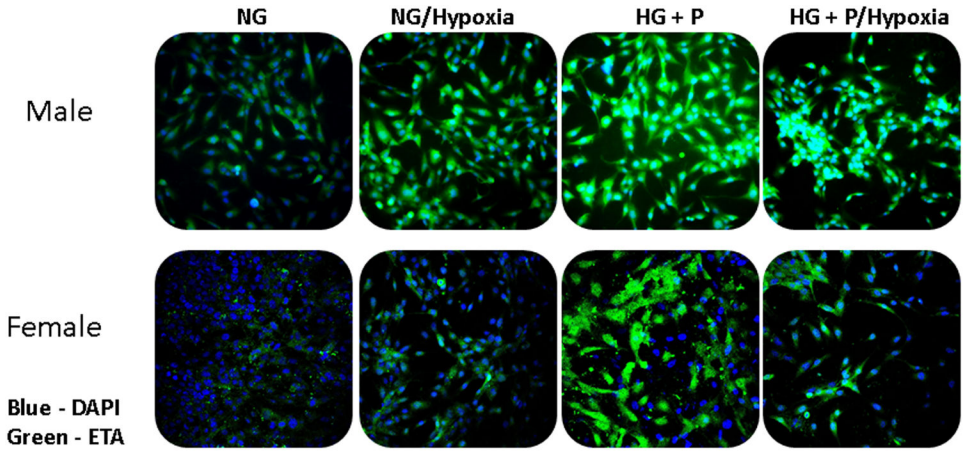

Fig. 4.

BMVECs of male origin exhibit greater signal for ETA receptors as compared to female cells. Representative images from 3 individual experiments are shown. Images were captured at a magnification of 20x. Data was not quantified. NG: normal glucose, HG+P: high glucose + palmitate

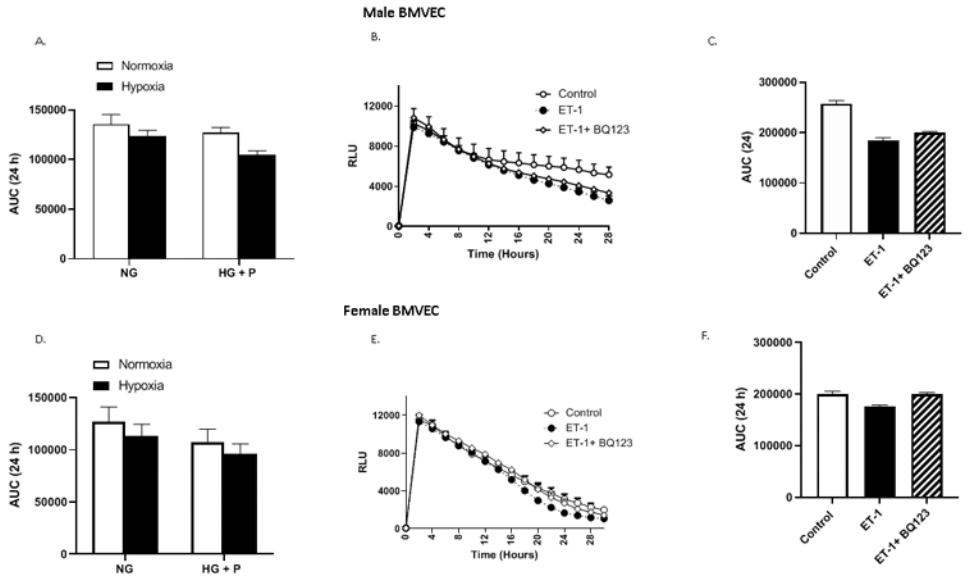

Fig. 5.

ET-1 decreases viability of both cell lines whereas hypoxia attenuates survival only in male BMVECs. Cumulative data for area under the curve (AUC 24 h) for male and female cells is shown in Panels A and D, respectively. ETA receptor blockade with BQ-123 prevents ET-1-mediated decrease in viability in female but not male cells. Representative 28 h tracing of cell viability is shown in Panels B and E and cumulative data is given on Panels C and F. Data was analyzed by one-way ANOVA and results are shown as mean ± SEM of 3 individual experiments performed in triplicate.

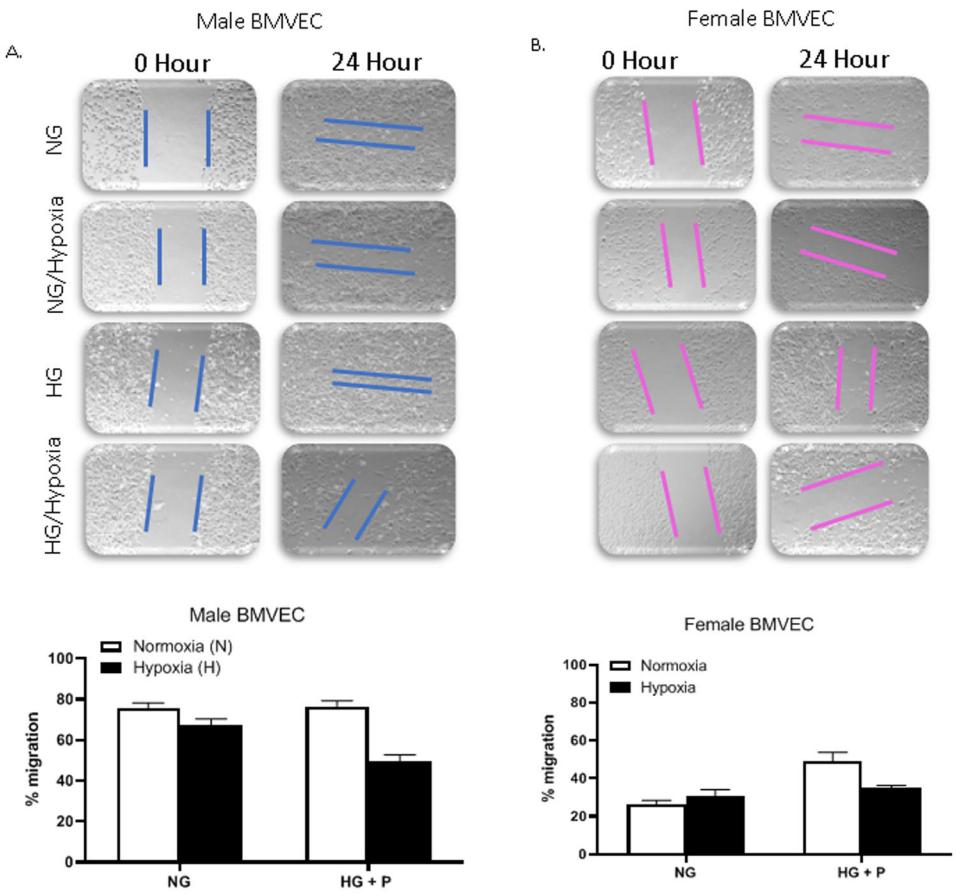

Fig. 6.

Hypoxia decreases migration of male but not female BMVECs. Representative images of for male (A) and female (B) cells at the start (0 h) and end (24 h) of a scratch assay are shown on top and cumulative data expressed as % of the distance covered by the migrating cells at the bottom for each cell line. Scratched areas are indicated by lines. Two-way ANOVA was performed for each sex (normoxia vs hypoxia) x (NG vs HG+P). Full ANOVA table indicating interaction is shown on the graphs. Results are shown as mean ± SEM of 3 individual experiments performed in triplicate.

Table 1.

Experimental groups and metabolic parameters

	Sham				Stroke			
	Male (n=6)		Female (n=6)		Male		Female	
	Control (n=6)	Diabetes (n=4)	Control (n=6)	Diabetes (n=6)	Control (n=6)	Diabetes (n=3)	Control (n=5)	Diabetes (n=4)
Body weight (g)	456±19	397±36	281±13 *	310±35 *	359±6	325±13	288±16 *	304±47
Blood glucose (mg/dl)	82±10	313±48 ^	71±9	326±43 ^	81±7	324±69 ^	81±5	299±51 ^

* p<0.05 vs male

^ p<0.05 vs control

Table 2:

Primer sequence for qRT-PCR

Gene	Forward	Reverse	NCBI Reference
ppET-1	5’-GCTCCTGCTCCTCCTTGATG-3’	5’-TGGTCTGTGGTCTTTGTGGG-3’	NM_012548.2
ETA	5’-TGTGCTGGTTCCCTCTTCAC-3’	5’-TGGTTGCCAGGTTAATGCCA-3’	NM_012550.2
ETB	5’-GCTAGCCATCACTGCGATCT-3’	5’-TGTCTTGGCCACTTCTCGTC-3’	NM_017333.1
GAPDH	5’-GCGAGATCCCGCTAACATCA-3’	5’-CTCGTGGTTCACACCCATCA-3’	NM_017008.4