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Lipopolysaccharide-Induced Necroptosis of Brain Microvascular Endothelial Cells Can Be Prevented by Inhibition of Endothelin Receptors

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Summary

Over activation of the endothelin-1 (ET-1) system in disease states contributes to endothelial dysfunction. On the other hand, ET-1 promotes proliferation and survival of endothelial cells. Regulation of programmed cell death (PCD) pathways is critical for cell survival. Recently discovered necroptosis (regulated necrosis) is a pathological PCD mechanism mediated by the activation of toll like receptor 4 (TLR4), which also happens to stimulate ET-1 production in dendritic cells. To establish the effect of ET-1 on PCD and survival of human brain microvascular endothelial cells (BMVECs) under control and inflammatory conditions, BMVECs were treated with ET-1 (10 nM, 100 nM and 1 μM) or lipopolysaccharide (LPS, 100 ng/ml). ET receptors were blocked with bosentan (10 μM). Under normal growth conditions, exogenous ET-1 reduced BMVEC viability and migration at a relatively high concentration (1 μM). This was accompanied with activation of necroptosis and apoptosis marker genes. LPS decreased endogenous ET-1 secretion, increased ET_B receptor expression and activated necroptosis. Even though ET-1 levels were low (less than 10 nM levels used under normal growth conditions), blocking of ET receptors with bosentan inhibited the necroptosis pathway and improved the cell migration ability of BMVECs, suggesting that under inflammatory conditions, ET-1 activates PCD pathways in BMVECs even at physiological levels.

Key words

LPS • Necroptosis • Apoptosis • Ferroptosis • Endothelin

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Introduction

Brain is a unique organ, which has no energy reserves and requires constant blood supply for proper function (Iadecola 2017). To meet this high demand, it is a highly vascularized organ with a capillary bed that runs approximately 400-miles long (Begley *et al.* 2003, Cipolla 2009). Thus, regulation of cerebrovascular structure, integrity and function is of utmost importance for cerebral perfusion. In this regard, brain microvascular endothelial cells (BMVECs) are fundamentally important as they participate in the formation of 1) the neurovascular unit, which provides direct communication between neurons, glial cells and the vasculature, and 2) blood brain barrier, which separates the brain from circulating blood. In many disease states, especially under inflammatory conditions, BMVECs are an early target thus; better understanding of the cell survival/death properties can identify mechanisms to improve endothelial function and integrity. Recent advances in the field of programmed cell death (PCD) studies showed that in addition to apoptosis, caspase-independent pathways such as necroptosis and ferroptosis are also involved in cell death processes in different cell types (Dixon *et al.* 2012, Linkermann *et al.* 2014, Xie *et al.* 2016, Yu *et al.* 2017). However, whether these PCD pathways occur in BMVECs remained unknown.

It is well established that ET-1 contributes to cerebrovascular dysfunction and remodeling in different disease states. Experimental data suggests that endothelial ET-1 secretion occurs mainly toward the medial layer

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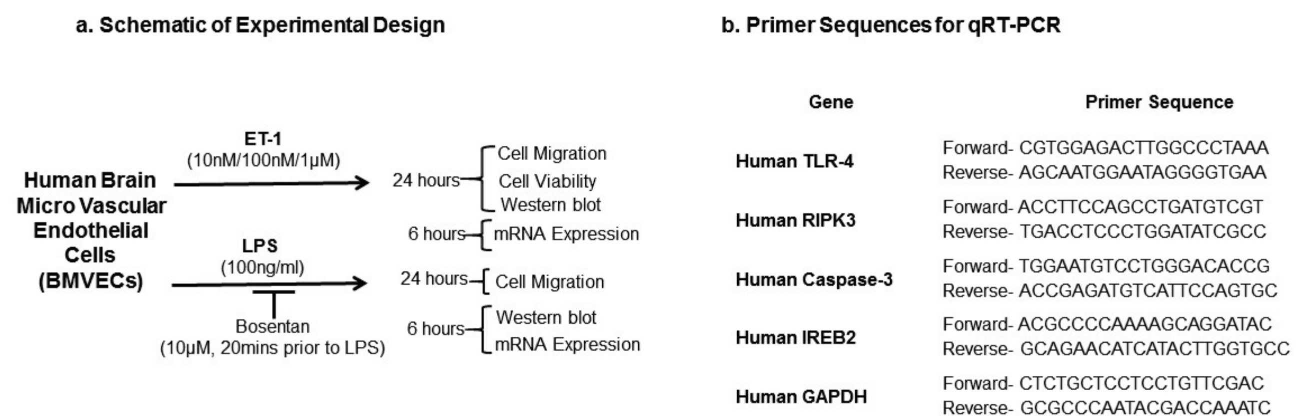
(Wagner *et al.* 1992) and the vascular effects observed could be due to mostly paracrine and autocrine functions, rather than endocrine effects. ET-1 promotes endothelial cell survival and prevents apoptosis in an autocrine manner (Cifarelli *et al.* 2012). Anti-apoptotic action of ET-1 is also observed in cardiac myocytes (Ogata *et al.* 2003) and human umbilical vein endothelial cells (Dong *et al.* 2005). Furthermore, ET-1 induces angiogenic responses in cultured endothelial cells through ET_B receptors and stimulates neovascularization *in vivo* in concert with VEGF (Morbidelli *et al.* 1995, Salani *et al.* 2000). We have shown that dual blockade of ET_A and ET_B receptors with bosentan reverses diabetes-mediated pathological neovascularization in the brain (Abdelsaid *et al.* 2014). On the other hand, ET-1 can cause apoptosis of endothelial progenitor cells in a hypertension model (Chen *et al.* 2012). However, the effect of ET-1 on different cell death pathways in BMVECs, especially under inflammatory conditions is not studied. Thus, the objective of current study was to investigate the role of ET-1/ET receptors on survival and PCD pathways in BMVECs.

Materials and Methods

BMVEC culture and treatments

Experiments were performed using immortalized human brain microvascular endothelial cells known as HBEC-5i (ATCC-CRL3245). Cells were gown in endothelial growth media (VEC technologies, Rensselaer, NY, USA) and plated on flasks coated with 0.1 % gelatin. VEC is a complete media containing FBS, antibiotics and growth factors (concentration details are not provided by manufacturer on the data sheets). Cells were grown to 80-90 % confluency and passage 5-8 were used in current experiments. Cells were serum starved in Dulbecco's Modified Eagle's medium (DMEM) for 2 h and treated with drugs in 2 % FBS containing DMEM. Cells were treated with various concentrations of ET-1 (0, 10 nM, 100 nM or 1 µM) or lipopolysaccharide (LPS, 100 ng/ml) for 24 h and viability and cell death pathways were assessed as described below and on Figure 1a. To determine the role of ET-1 in LPS-induced cell death, cells were preincubated with bosentan (10 µM) 20 min before LPS challenge. For gene expression studies, cells were harvested 6 h after stimulation.



Gene	Primer Sequence
Human TLR-4	Forward- CGTGGAGACTTGGCCCTAAA Reverse- AGCAATGGAATAGGGGTGAA
Human RIPK3	Forward- ACCTTCCAGCCTGATGTCGT Reverse- TGACCTCCCTGGATATCGCC
Human Caspase-3	Forward- TGGAATGTCCTGGGACACCG Reverse- ACCGAGATGTCATTCCAGTGC
Human IREB2	Forward- ACGCCCCAAAAGCAGGATAC Reverse- GCAGAACATCATACTTGGTGCC
Human GAPDH	Forward- CTCTGCTCCTCCTGTTCGAC Reverse- GCGCCCAATACGACCAAATC

Fig. 1. Schematic representation of experimental design. **a**) Primer sequences used in qRT-PCR and **b**) endpoints measured after ET-1 and LPS treatment.

Cell viability assay

Cell viability was measured by RealTime Glo-MT Viability Assay kit (Promega, Madison, WI, USA). Briefly, BMVECs were grown to 80-90 % confluency in a 96-well plate. Media was replaced with low FBS (2 % FBS) containing DMEM and allowed to stabilize for 4 h. Reagents in kit were prepared according to manufacturer's instructions. MT cell viability assay substrate and NanoLuc enzyme were diluted with test compounds (10 nM, 100 nM, 1 µM ET-1 and 100 ng/ml LPS) in media and cells were treated for 24 h. Luminescence was measured at 1, 2, 3, 6, 9, 12 and 24 h after treatment. Relative luminescence unit (RLU) was plotted against time in hours. Time at 50 % of the cells were viable (t50) was determined.

Cell migration assay

Cell migration assay (wound healing) was performed as described before (Abdelsaid *et al.* 2013). Briefly, BMVECs were grown to confluence on a 12-well

plate. Monolayer was wounded with a single sterile cell scraper of fixed diameter. Images of wounded areas were taken before treatment with test compounds and after 24 h. Cell migration was calculated by measuring migration distance normalized to initial distance of the wound using AxioObserver Zeiss Microscope software and data is expressed as the percentage of untreated control cells.

Western blot analysis

PCD pathways were assessed by measuring protein levels of phospho(p)RIP3, IREB-2, and caspase-3 as markers for necroptosis, ferroptosis and apoptosis, respectively. Briefly, equivalent amounts of cell lysates of BMVECs (20 µg protein/lane) were loaded onto 10 % SDS-PAGE, proteins separated, and proteins transferred to nitrocellulose membranes. The membranes were blocked with 5 % bovine serum albumin followed by incubation for 12 h at 4 ºC with primary antibody anti-toll like receptor-4 (TLR-4, ab22048, Abcam, Cambridge, MA, USA), anti-phosho(p)RIP3 (ab209384, Abcam), anti-MyD88 (4283, Cell Signaling Tech., Danvers, MA, USA), anti-IREB2 (ab106296, Abcam), anti-caspase-3 (9662, Cell Signaling Tech.), anti-ET_A receptor (ab85163, Abcam) or anti-ET_B receptor (AER002, Alomone labs, Jerusalem, Israel) at 1:1,000 dilution or anti β-actin at 1:3,000 dilution. After washing, membranes were incubated for 1 h at 20 ºC with appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated; dilution 1:3,000). Pre-stained molecular weight markers were run in parallel to identify the molecular weight of proteins of interest. For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent reagent and the signals were monitored on Alpha Imager (Alpha Innotech, San Leandro, CA, USA). Relative band intensity was determined by densitometry software (Alpha Innotech, ProteinSimple, San Jose, CA, USA) and normalized with β-actin protein.

Quantitative real time PCR (qRT-PCR)

BMVECs were lysed in 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, USA). iScript cDNA synthesis kit (cat #1708891, BioRad, Foster City, CA, USA) was used to reverse transcribe equal quantities of total RNA following the manufacturer's instructions. Primers were custom designed from Invitrogen (Thermo Fisher Scientific, Grand Island, NY, USA). The sequences of primers used in the study are listed in Figure 1b. qRT-PCR was performed using iScript Reverse Transcription super mix (cat #1708840, Biorad, Foster City, CA, USA) 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.

ET-1 ELISA

BMVECs were incubated with LPS (100 ng/ml) for 6 h. Media was collected and ET-1 was measured using commercially available ELISA kit (ab10072, Abcam) following the manufacturer's instruction. Results were normalized with total protein in cell lysate and expressed as pg/mg protein.

Data analysis

One-way ANOVA was used to analyze data with multiple groups and followed by a Tukey's *post hoc* comparison. Student t-test was used to compare data with only two groups. Data was expressed as mean ± SEM. $p<0.05$ was considered significant.

Results

ET-1 lowers cell survival at high but not low concentrations

BMVECs were incubated with ET-1 (10 nM, 100 nM or 1 µM) for 24 h and cell viability was measured as relative luminescence unit (RLU) at different time points (Fig. 2a). Time required for 50 % reduction in viability (t50) was different among the groups (Fig. 2b, $p=0.0025$). *Post hoc* analysis indicated that 1 µM ET-1 significantly reduced the t50. Cell migration, an assay to assess the angiogenic properties of BMVECs, showed similar results. 1 µM ET-1 significantly reduced the cell migration of BMVECs (Fig. 2d).

PCD markers including pRIPK3 (necroptosis), cleaved caspase-3 (apoptosis) and IREB2 (ferroptosis) were assessed in cell lysates after 24 h of ET-1 treatment. None of the marker proteins was different between the groups (Figs 3a-3c). However, mRNA expression after 6 h of ET-1 treatment showed differences (Fig. 4). *Post hoc* analysis indicated that TLR-4 mRNA was significantly higher in cells treated with 1 µM ET-1 (Fig. 4a). Expression of RIPK3 decreased with 10 and 100 nM ET-1 but increased with 1 µM ET-1 treatment

(Fig. 4b). Apoptotic marker caspase-3 was reduced with the low and moderate dose of ET-1 (Fig. 4c). In contrast, 1 µM ET-1 upregulated the caspase-3 gene expression. We did not observe any changes in mRNA expression of IREB2 (Fig. 4d).

ET receptor inhibition reduced the LPS-mediated PCD in BMVECs

LPS (100 ng/ml) treatment for 6 h increased the mRNA levels of TLR-4 ($p<0.05$) (Fig. 5a). While it was not significant, LPS had the most effect on RIPK3, mRNA expression (Fig. 5b).

LPS treatment reduced ET-1 secretion (Fig. 6). BMVECs expressed the ET_A receptor and its expression did not change with LPS treatment, whereas ET_B receptor expression was increased significantly (Figs 6b and 6c). LPS treatment also increased TLR-4 protein as measured by immunoblotting (Fig. 7a). Preincubation of BMVECs with ET receptor blocker bosentan reduced the expression of TLR-4 as well as downstream signaling partners Myd88 and phosphoRIPK3 proteins (Figs 7b and 7c). Additionally, inhibition of ET receptors improved the migration of BMVECs after LPS treatment (Fig. 7d, $p<0.001$).

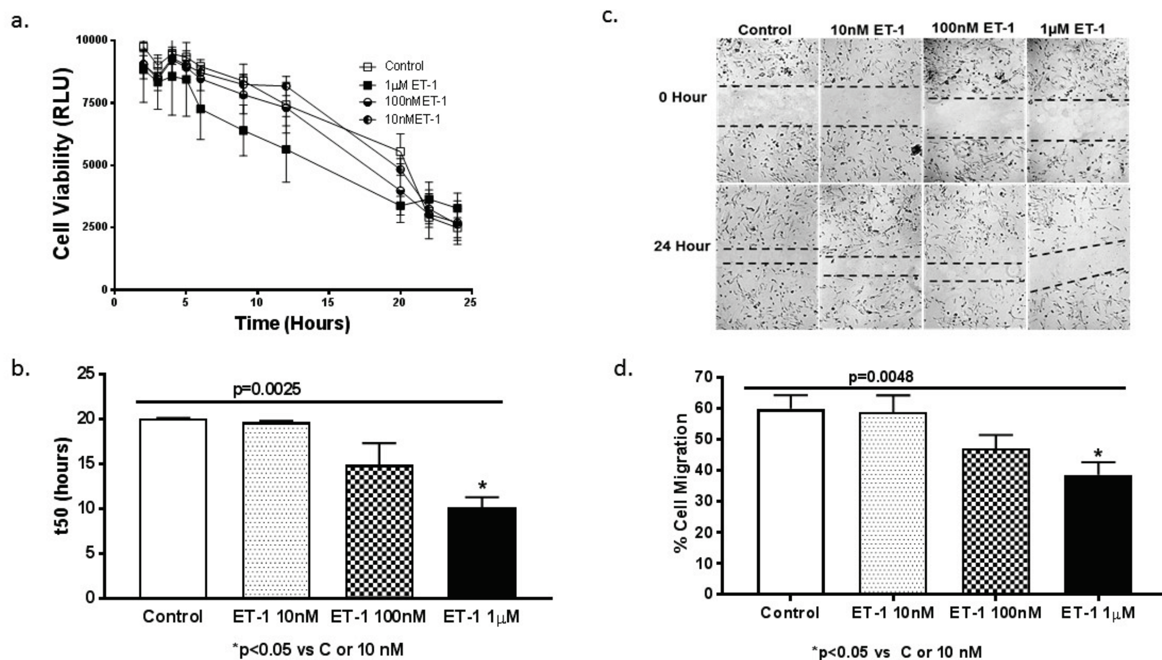


Fig. 2. Exogenous ET-1 stimulation lowers BMVEC viability and cell migration at high concentrations. BMVECs were treated with 10 nM, 100 nM or 1 µM ET-1 for 24 h and **a**) cell viability was measured as relative luminescence (RLU) for 24 h and **b**) time at 50 % viable cells (t50) was calculated 1 µM dose of ET-1 have significant reduction in t50 (* $p<0.05$ vs. control or 10 nM ET-1). Cell migration was measured by scratch assay **c**) and **d**) it was significantly reduced with 1 µM dose of ET-1 (* $p<0.05$ vs. control or 10 nM ET-1, n=4-6).

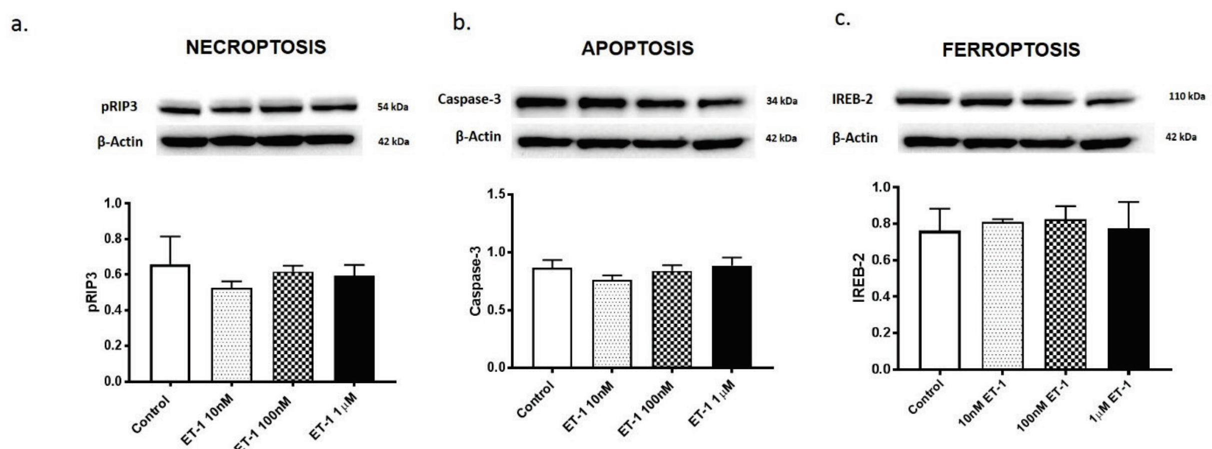


Fig. 3. Programmed cell death (PCD) marker proteins were not different after 24 h of ET-1 treatment. **a**) Necroptosis (pRIPK3), **b**) apoptosis (caspase-3) and **c**) ferroptosis (IREB2) proteins were measured in cell lysate after 24 h of ET-1 treatment. There was no significant difference in any of these marker proteins (n=3-4).

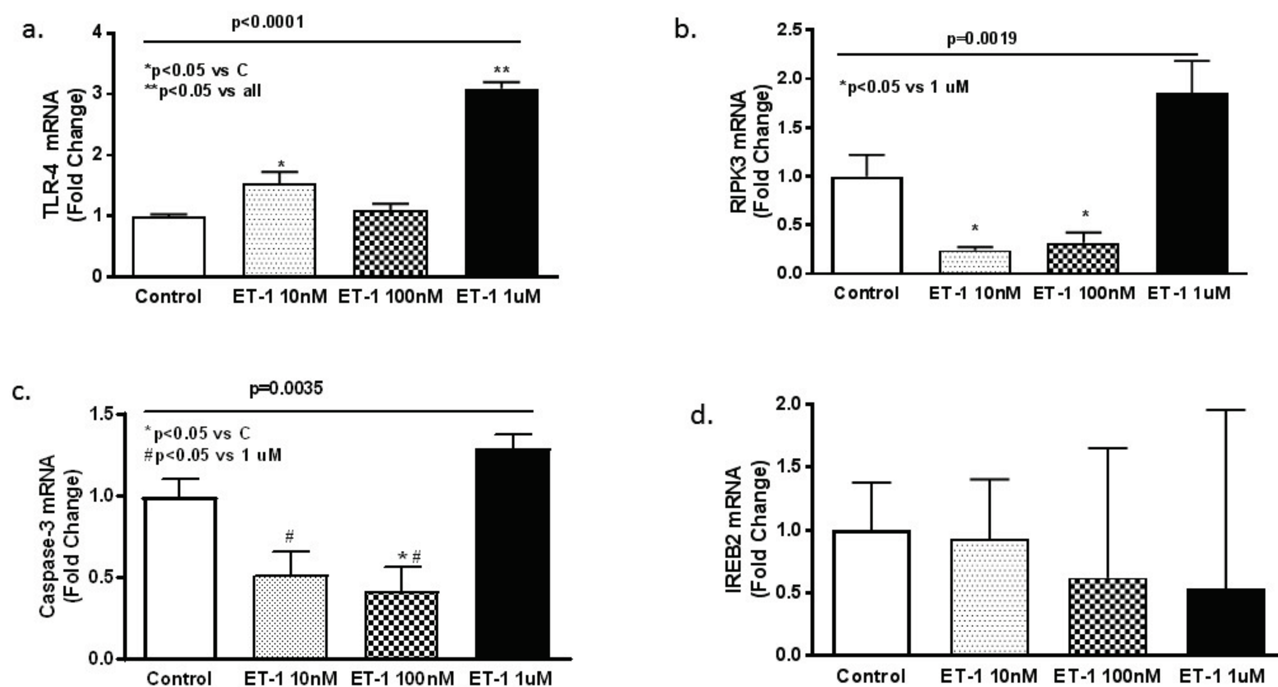


Fig. 4. ET-1 has dose dependent regulation of programmed cell death (PCD) marker genes on BMVECs measured after 6 h. Necroptosis marker gene **a**) TLR-4 was upregulated with 10 nM ET-1 (*p<0.05 vs. control) and 1 μM ET-1 (** p<0.05 vs. all groups, n=3-4), while **b**) RIPK3 was significantly upregulated with 1 μM dose of ET-1 (* p<0.05 vs. control). Apoptosis marker gene **c**) caspase-3 was down regulated with 10 nM and 100 nM ET-1 (* p<0.05 vs. control); however, it was upregulated with the 1 μM ET-1 (# p<0.05 vs. 10 nM or 100 nM ET-1, n=3). Ferroptosis marker gene **d**) IREB2 expression was not different between the groups (n=3).

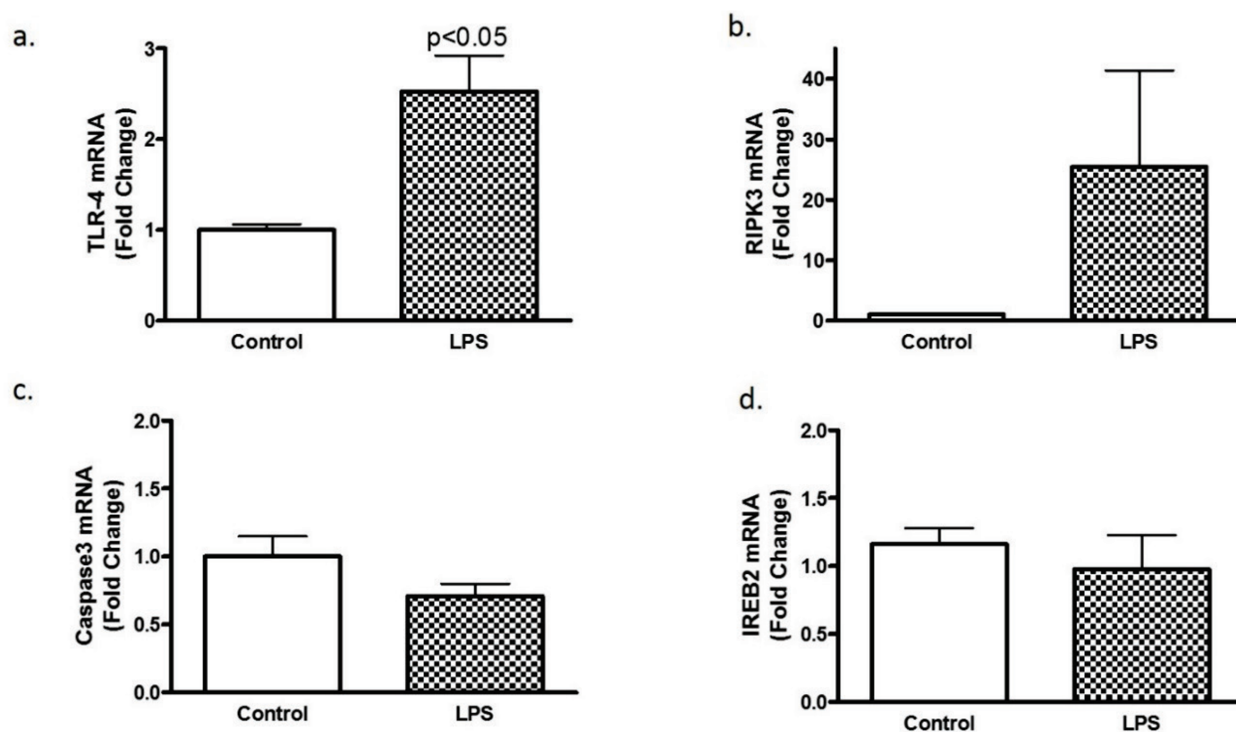


Fig. 5. LPS treatment for 6 h upregulate the necroptosis marker genes. **a**) TLR-4 gene was upregulated with the LPS (100 ng/ml) treatment (* p<0.05 vs. control). **b**) RIPK3 gene was also upregulated with LPS treatment. **c**) Apoptosis (caspase-3) and **d**) ferroptosis (IREB2) marker genes were not different between LPS and control group (n=4-6).

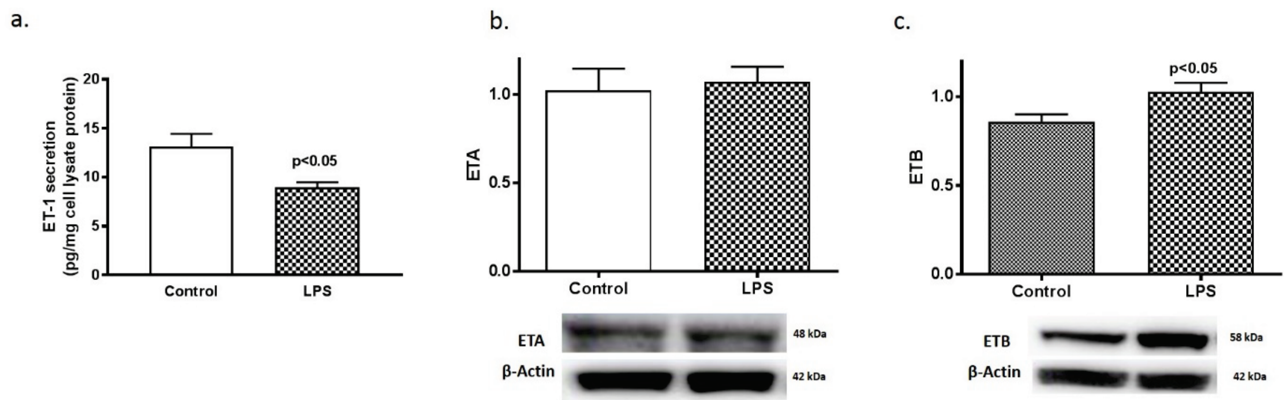


Fig. 6. LPS treatment can modulate the ET system in BMVECs. **a**) Secreted ET-1 measured in media was significantly reduced with LPS (100 ng/ml) treatment (* p<0.05 vs. control, n=3). **b**) ET_A receptor expression was measured in cell lysate and it was not different between the groups, while expression of **c**) ET_B receptor was significantly increased with the LPS treatment (* p<0.05 vs. control, n=4-6).

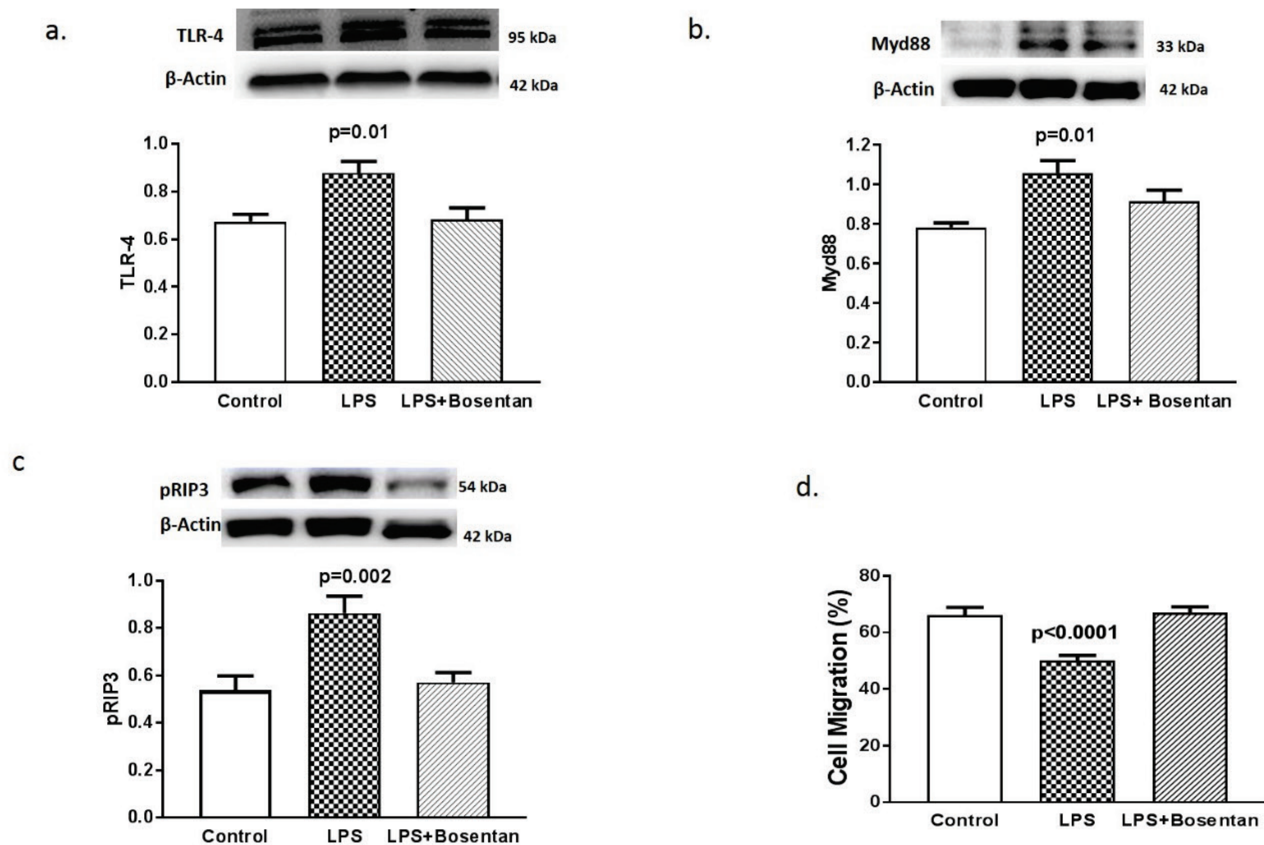


Fig. 7. ET receptor blockade with bosentan inhibit the LPS mediated necroptosis pathway in BMVECs. Expression of **a**) TLR-4 protein expression was upregulated with LPS (100 ng/ml), while pre incubation of cell with bosentan (10 μM; 20 min prior to LPS addition) inhibited the LPS mediated upregulation of TLR-4 (* p=0.01 vs. control, n=4-6). Downstream proteins in TLR-4 pathways, **b**) Myd88 and **c**) phospho-RIPK3 were also upregulated with the LPS treatment whereas, inhibition of ET receptor with bosentan mitigated it (* p=0.01 and 0.002, respectively, vs. control, n=4-6). **d**) LPS treatment reduced the cell migration property of BMVECs, while inhibition of ET receptor with bosentan improved it (* p<0.0001, n=4-6).

Discussion

Vascular endothelial cells are important for maintenance of structural and functional homeostasis of the neurovascular unit and BBB. While ET-1 has been shown to mediate pathological remodeling and neovascularization in the cerebrovasculature, its effect on cell survival and death may vary depending on cell type, vascular bed and disease conditions. Thus, it becomes relevant to determine the impact of ET-1 on BMVEC

survival and death. In the current study, we observed that under normal growth conditions, exogenous ET-1 stimulation shows a dose dependent effect on BMVECs: 1) Low (10 nM) and moderate (100 nM) concentrations of ET-1 retain cell viability while a pharmacological high dose of 1 μM ET-1 reduces it. 2) Angiogenic property as measured by cell migration is also maintained with low and moderate dose of ET-1, while high dose inhibits the cell migration. 3) These changes are concomitant with the inhibition and activation of necroptotic and apoptotic gene expression at physiological lower doses and high dose, respectively. 4) On the other hand, in inflammatory conditions mimicked by LPS, even when endogenous ET-1 production is low, blockade of ET receptors with bosentan improves the cell viability and migration while reducing necroptosis suggesting that under disease microenvironment ET-1 can exert differential effects.

PCD is fundamental feature of cells devoted to maintenance of cellular structures and homeostasis of body. Most extensively studied form of PCD is apoptosis. It is characterized by cell shrinkage, plasma membrane blebbing, nuclear condensation and DNA fragmentation. It is associated with minimal inflammation and mainly facilitate cell elimination and cell-corpus removal (Danial *et al.* 2004). Apoptosis is mediated by a group of specialized protein cleaving enzymes caspases (Thornberry 1998). Apoptotic caspases are broadly divided in two groups, first initiator caspases (caspase 8-9) and second effector caspases (caspase 3-7) (Salvesen *et al.* 2011). A second prominent form of PCD is necroptosis. Like necrosis, necroptosis is characterized by cell and its organelles swelling and by consequent cell implosion. Unlike necrosis, necroptosis is triggered by programmed upstream molecular pathways (Christofferson *et al.* 2010, Tait *et al.* 2008). The signaling pathway that initiates necroptosis is well defined with TNFα and TNFR1. In addition, damage associated molecular patterns (DAMPs) like LPS and viral nucleic acid products and their receptors like toll like receptors, NOD like receptors and viral DNA receptors can also trigger the necroptotic signaling (Kaiser *et al.* 2013, Vanlangenakker *et al.* 2012). Another iron dependent form of programmed regulated cell death is called ferroptosis (Dixon *et al.* 2012). It involves iron catalyzed lipid damage. Although, the PCD mechanisms are well studied in immune and cancer cells, its regulation in BMVECs especially under inflammatory conditions are not explored. Our results show that necroptosis but no other PCD pathways are activated in these cells with concomitant activation of TLR4.

ET-1 has the ability to modulate PCD. It has anti-apoptotic actions on cardiac myocytes, renal carcinoma, ovarian carcinoma and prostrate epithelial cells (DeBosch *et al.* 2006, Del Bufalo *et al.* 2002, Pflug *et al.* 2007). ET-1 upregulation increases hepatocellular carcinoma cell proliferation, migration, progression and inhibit apoptosis (Lu *et al.* 2012). Incubation of serum starved fibroblast or aortic endothelial cells with ET-1 prevents apoptosis (Shichiri *et al.* 1998a,b). Within the brain, reduced ET-1 level was associated with increased NLRP3 inflammasome formation in hippocampal neurons (Ward *et al.* 2016). On the other hand, ET-1 mediates glutamate induced retinal cell death (Kobayashi *et al.* 2005) and ET_B receptor inhibition prevents neuronal apoptotic cell death (Siren *et al.* 2002, Yagami *et al.* 2002), while dual inhibition of ET receptors inhibits the ischemia induced necrosis (Hvaal *et al.* 1999). However, regulation of PCD by ET system in BMVECs is not established. Based on these evidences, our first objective was to determine the effect of exogenous ET-1 treatment on BMVEC survival under normal growth conditions. We observed that mild to moderate levels of ET-1 was not detrimental for cell viability and cell migration of BMVECs and in fact, inhibited the necroptosis (RIPK-3) and apoptosis (caspase-3) marker genes. Whereas a high concentration of ET-1 that is more representative of a pharmacological dose seems to have detrimental effect on BMVECs. We observed that high ET-1 decreases the cell viability and migration and it triggered the activation of necroptotic and apoptotic gene expression. Previous findings by Shichiri *et al.* (1998a,b), in serum starved aortic endothelial cells also support the observation that ET-1 has pro-survival effects. They demonstrated that ET-1 act as an autocrine/paracrine survival factor for endothelial cells (Shichiri *et al.* 1998a,b). While, another study reported incubation of HUVECs with ET-1 for 24 h activated apoptosis through ET_B receptor mediated ROS generation (Dong *et al.* 2005). Observed activation of necroptosis not apoptosis in 6 h suggests ET-1 has time dependent regulation of RCD in BMVECs.

Under inflammatory and endotoxemic stress conditions, ET-1 mediated effects are disrupted and shifted towards a potent and longer lasting vasoconstriction (Baveja *et al.* 2002a,b). Studies have shown that LPS induces apoptosis in different types of endothelial cells including HUVEC and lung-derived normal human microvascular endothelial cells (Choi *et al.* 1998, Frey *et al.* 1998, Haimovitz-Friedman *et al.*

1997, Koide *et al.* 1996, Yokochi *et al.* 1998). LPS release into the circulation induces endothelial apoptosis *in vivo* and thus causes microvascular injury (Hotchkiss *et al.* 1999). It is also reported that LPS pretreatment significantly inhibit ET-1 mediated eNOS activation. These evidences tempted us to use LPS to mimic inflammatory conditions and investigate the involvement of the ET system in RCD in this setting. We observed the activation of necroptosis marker genes as well as proteins after LPS treatment. However, we did not observe a difference in apoptotic marker gene after 6 h of LPS treatment. ET-1 measured in media was significantly reduced and it was accompanied with increase in expression of ET_B receptor proteins in BMVECs cell lysate. While, blocking of ET receptors with bosentan inhibited the necroptosis pathway and improved the cell migration ability.

There are several limitations that must be discussed. In the current study only LPS was used to trigger PCD. Thus, it becomes important to test the response of BMVECs with other apoptotic, necroptosis activators and even use the BMVECs from disease models. Another limitation of current study is the use of single PCD marker gene. Screening of a number of apoptotic, necroptosis and ferroptosis marker genes would have strengthen the findings. Typically, only the ET_B receptor has been reported on endothelial cells. However, an early study demonstrated the presence of the ET_A subtype in brain endothelial cells (Kawai *et al.* 1997). In this study, we also detected this receptor subtype in BMVECs. Since, both ET_A and ET_B receptors are found on BMVECs, selective blocking of each receptor in future studies can provide further information on ET-1-mediated regulation of PCD mechanisms.

In conclusion, our findings suggest that ET-1 mediated cell survival and cell migration responses depend on the microenvironment. While ET-1 reduces viability only at high pharmacological conditions under normal growth conditions, even low levels of ET-1 can promote necroptosis under inflammatory conditions. Thus, we conclude that BMVECs are an early target in disease states and better understanding of the cell survival/death properties can identify mechanisms to improve endothelial function and integrity. The current study is first one to address the role of the ET system on PCD in BMVECs setting the platform for future studies in the area of PCD regulation by ET-1 in the microvasculature.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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