Endothelin A (ETA) Receptors Contribute to Senescence of Brain Microvascular Endothelial Cells

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Abstract

Cellular senescence plays a pivotal role in the aging and progression of neurodegenerative diseases including vascular cognitive impairment and dementia (VCID). In postmortem brains from individuals with VCID, endothelin -1 (ET-1) levels closely correlate with blood barrier breakdown and cerebral hypoperfusion. Brain microvascular endothelial cells (BMVECs), previously thought to have exclusively ETB receptors, also possess ETA receptors however, the functional significance of this receptor in BMVECs is not known. We hypothesize that ETA receptors mediate BMVECs senescence. Serum starved human BMVECs (HBEC5i) were incubated with ET-1 (1μM) in the presence/absence of ETA receptor antagonist BQ123 (20 μM). Cells were collected for Western blot and RT-qPCR analyses. Treatment of ET-1 increased protein expression of ETA receptor, while was prevented by the ETA receptor antagonist. ET-1 increased p21, p16, p53, and LIF1, cyclin D1 protein levels, and β-galactosidase accumulation, which were prevented in the presence of ETA blockade. While there was no change in tight junction proteins, ET-1 decreased adherent junction protein VE-cadherin levels. In conclusion, ET-1 upregulates ETA receptors in BMVECs in an autocrine manner and triggers the activation of senescence. These *in vitro* findings need to be further studied *in vivo* to establish the role of ETA receptors in the progression of endothelial senescence in VCID.

Keywords

Endothelial cells; Endothelin-1; Senescence; Brain

INTRODUCTION

Brain health depends upon cardiovascular health. Endothelial dysfunction leads to improper regulation of cerebral blood flow (CBF) and disruption of blood-brain barrier (BBB) integrity. As such, it is not surprising that cerebrovascular dysfunction, which can occur with age and under comorbid conditions like diabetes and hypertension, contributes to neurodegenerative diseases like Alzheimer's disease and related dementias (ADRD)

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including Vascular Contributions to Cognitive Impairment and Dementia (VCID) (Gorelick et al. 2017; Iadecola 2017). Cellular senescence is one mechanism that is also involved in age-related pathologies (Chandrasekaran et al. 2017; Sikora et al. 2021a; Sikora et al. 2021b; van Deursen 2014). Senescence represents a stress response (aging/pathological conditions) in which cells withdraw from the cell cycle and lose the capability to replicate. It is characterized as an irreversible cell cycle arrest (Kuilman et al. 2010; Pawlikowski et al. 2013). Senescent cells show very distinctive changes in morphology as well as molecular functions. While senescence-associated secretory phenotype (SASP) with increased secretion of inflammatory modulators and increased senescence-associated β-galactosidase (SA-β-gal) activity are hallmarks of senescence marker (Coppe et al. 2010), it is increasingly recognized by the scientific community that the senescent phenotype is heterogeneous (Gorgoulis et al. 2019; Sikora et al. 2021b). SASP can secrete various cytokines and matrix metalloproteinases (MMPs) (Sikora et al. 2021b). Accumulation of senescent cells in various tissues is believed to contribute to progressive functional impairment (Blasiak 2020; He and Sharpless 2017). Initially, senescence was considered an irreversible process of growth arrest occurring in response to cellular aging but with the advancement in the field it is well known that other cellular stimulations, including oxidative stress, cytokines, activated oncogenes and DNA damage, etc. can also induce senescence (Bernadotte et al. 2016; Chandrasekaran et al. 2017; Sikora et al. 2021b). This stress-induced premature senescence has broad implications for health and disease and can be reversible (Kritsilis et al. 2018; Xiong and Zhou 2019). Endothelial senescence is associated with vascular dysfunction, BBB disruption, and vascular stiffness leading to impaired neurovascular coupling (Blair et al. 2015; Bryant et al. 2020; Tian and Li 2014; Yamazaki et al. 2016). While the etiology of senescence is multifactorial, the identification of factors that promote endothelial senescence in the brain may link mechanisms that are involved in the development and progression of neurovascular pathologies.

The family of endothelins, consisting of three related vasoactive peptides, ET-1, ET-2, and ET-3, plays important roles in cardiovascular (patho)physiology and embryonic development (Davenport et al. 2016). While ET-1 was the first isoform to be isolated as the most potent vasoconstrictor peptide, ETs mediate diverse effects via two distinct G protein-coupled receptor subtypes, ETA and ETB (D'Orleans-Juste et al. 2019). All components of the endothelin system including ET peptides and both ETA and ETB receptors are expressed in all cell types of the neurovascular unit (NVU) (Davenport et al. 2016; Fouda et al. 2019). In the vasculature, the ETA receptor is localized mainly on vascular smooth muscle cells (VSMCs) of arteries and arterioles as well as on pericytes surrounding capillaries and mediates contractile and proliferative responses to ET-1. Whereas the ETB receptor subtype is mainly found on endothelial cells and mediates vasodilation via the release of relaxing factors such as NO (Davenport et al. 2016; Fouda et al. 2019). In addition to another early report, we have shown that human brain microvascular endothelial cells (BMVECs) uniquely express both ETA and ETB receptors (Abdul et al. 2020b; Abdul et al. 2018). The functional significance of the ETA receptor subtype in BMVEC is not fully known. In light of accumulating evidence that ET-1-mediated cerebrovascular dysfunction contributes to the dysregulation of CBF and ensuing cognitive deficits in patients and experimental models. Thus, elucidating the role of ETA receptor activation in the senescence of BMVECs

could uncover multiple mechanisms by which ET-1 mediates neurovascular pathologies. We hypothesized that the ETA receptor stimulation activates the senescence machinery and dysregulates barrier function proteins in BMVECs.

MATERIALS AND METHODS

BMVECs Culture and Treatments:

Experiments were performed in male-derived human brain microvascular endothelial cell line HBEC-5i (American Type Culture Collection-ATCC, CRL 3245). Cells were cultured in 75 cm^2 culture flasks that were coated with 0.2% w/v gelatin (porcine Type A; Sigma-Aldrich) before cell seeding. 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. Cells were incubated with ET-1 (1μM) in the presence/absence of ETA receptor antagonist BQ123 (20 μM; cells were treated 30 minutes before ET-1 treatment) for 16 hours. Experimental design is depicted in Figure 1. Dose of ET-1 and BQ123 were selected based on our previous reports (Abdul et al. 2020a; Abdul et al. 2020b). BQ123 was dissolved in distilled water and further diluted with working media. Thus, the control group did not receive vehicle treatment. The cell lysate was collected for Western blot analysis and RT-qPCR.

Senescence Associated β-galactosidase (SA-β-gal) Detection:

Activation of SA-β-gal is commonly used as a marker of senescence. The CellEvent Senescence green detection kit (Invitrogen; C10850) was used to detect the senescence in BMVECs after stimulation with H_2O_2 (100μM) or TNFα (20ng/ml) or Aβ-40 (5μM) for 3–5 days or ET-1(1μM) for 16 hours. Briefly, cells were grown to confluency on chambered slides followed by treatment with the above-mentioned agents. Cells were washed with PBS and fixed with 0.4% paraformaldehyde for 10 minutes at room temperature. Cells were again washed with 1%BSA in PBS and then a 200μl working solution of fluorescent substrate for β-galactosidase was added and incubated at 37 °C for 2 hours. Cells were washed three times with PBS and a coverslip was placed. Slides were imaged on an Olympus IX73 microscope (Olympus Corporation, Japan) for the detection of green fluorescence using Alexa flour 488/FITC filter set. Immunofluorescence intensities were quantified using Image- J software (1.52a version).

Quantitative RealTime PCR (RT-qPCR):

For *in vitro* studies, cells were collected using RNA lysis buffer, and RNA was isolated using SV Total RNA isolation system (Promega, USA). The quality and quantity of extracted RNA were 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 1. RT-qPCR 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 as fold change was analyzed by the delta-delta CT method using RPS13 as an endogenous control gene.

Western Blot Analysis:

Equivalent amounts of cell lysates (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 overnight incubation at 4°C with respective ET receptor antibodies (anti-ETA receptor; ab85163, Abcam or anti-ETB receptor; AER002, Alomone Labs) or senescence marker proteins p16INK4a (PA1–30670, Fisher scientific), p21 (AHZ0422, Invitrogen), cyclin D1 (92G2, Cell Signaling), lamin A/C (2032, Cell Signaling) or membrane integrity marker proteins occludin1 (OC-3F10, Invitrogen), claudin5 (35–2500, Invitrogen), p53 (DO-7, Invitrogen) VE-cadherin (2158, Cell Signaling) at 1:1000 dilution or anti-β-actin at 1:20000 dilutions. After washing, membranes were incubated for 1 hour at 20°C with appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated; dilution 1:5000). 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 Amersham imager 680 (GE Healthcare Bio-Sciences Corp., Marlborough, MA). Relative band intensity was determined by densitometry on Image-J and normalized with β-actin protein.

Data Analysis:

Data presented here are from three separate experiments (n=3) in duplicates or triplicates. One-way ANOVA was used to analyze data with multiple groups and followed by a Tukey's post-hoc comparison. Data are expressed as mean ± SEM. $p < 0.05$ was considered significant.

RESULTS

Stress induces senescence in BMVECs

Incubation of BMVECs with a low dose of H_2O_2 (100μM) for 3 days as a precursor of oxidative stress increased the staining of SA-β gal immunofluorescence (Fig. 2). Treatment with TNF-α (20ng/ml media) for 3 days as an inflammatory cytokine also increased the SA-β gal activity (Fig. 2). Incubation with Aβ-40 (5μM) for 5 days as an age-related factor also led to augmented SA-β gal staining (Fig. 2).

ET-1 increases the ETA receptor expression in BMVECs

Incubation of BMVECs with ET-1 for 16 hours increase the mRNA levels of ETA (Fig. 3A) but not ETB (Fig. 3B) receptors. At the protein level, ET-1 treatment increased ETA receptors, and blockade of ETA receptors prevented this change (Fig. 3C). However, ETB protein levels were not different (Fig. 3D).

The ETA receptor contributes to ET-1-mediated Senescence

Incubation of BMVECs with ET-1 increased the SA-β gal staining, while the presence of the ETA receptor antagonist, BQ123, prevented this increase (Fig. 4). There appeared to be an increase in mRNA levels of leukocyte inhibitory factor-1 (LIF1), LIF receptor, and p21 levels with ET-1 treatment but this did not reach significance, and ETA receptor inhibition had no effect (Fig. 5A–C). On the other hand, at the protein level, LIF1, p21, and cyclin D1 levels were increased with ET-1 treatment and again blockade of the ETA receptor prevented this response (Fig. 5D, E, and G). There was no change in p16 levels (Fig. 5F). ET-1 treatment lowered lamin A/C, and nuclear envelope proteins but receptor blockade had no effect (Fig. 5H). Expression of p53 protein level was increased with ET-1 treatment while, presence of ETA antagonist prevented the increase (Fig.5I).

ET-1 treatment alters the expression of adherent junction protein VE-cadherin in BMVECs

To identify a possible link between ET-1-mediated senescence and barrier function in BMVECs, we measured the mRNA as well as protein levels of tight and adherent junction proteins. We observed an inconclusive trend in mRNA levels of zo-1 and VE-cadherin (Fig. 6A and B). Interestingly, ET-1 increased claudin 5 mRNA, and ETA blockade prevented this increase (Fig. 6C). At the protein level, there was a trend for decreased occludin-1 (p=0.08) (Fig. 6D). VE-cadherin was decreased with ET-1 treatment, but ETA antagonism improved it (Fig. 6E). There was no difference in claudin 5 protein levels (Fig. 6F).

DISCUSSION

The presence of ETA receptors in endothelial cells is unique to the brain. We previously reported that ET-1 decreased the viability of BMVECs but ETA blockade did not prevent this effect (Abdul et al. 2020b). The overall goal of this study was to further investigate the role of these receptors in cell senescence since emerging evidence suggests that endothelial cell senescence in the brain may be a trigger for neurovascular pathologies in cognitive impairment, and ET-1 has been reported to induce senescence in other cell types (Alcalde-Estevez et al. 2020; Au et al. 2020). To achieve this goal, we used a reductionist approach and stimulated human BMVECs with ET-1 as well as other well-known inducers of senescence including amyloidβ, TNF,α and hydrogen peroxide, and examined a panel of senescence markers at the mRNA as well as protein levels. Our findings demonstrate that 1) ET-1 treatment increases the expression of ETA receptors in a feed-forward manner; 2) ET-1 mediates a senescence-like state; 3) blockade of ETA receptors prevents ET-1-mediated changes in senescence-associated markers, and 3) ET-1 does not affect the expression of tight junction proteins but lowers adherent junction protein VE-cadherin within the time frame of our experimental paradigm.

Senescence, originally described by Hayflick and Moorhead (Hayflick and Moorhead 1961) as a state in which human fibroblasts stop replicating and responding to growth factors after a certain number of passages in vitro, arises from telomere shortening and was immediately associated with aging (Chandrasekaran et al. 2017; van Deursen 2014). While there is a link, aging and senescence are not synonymous. Senescence is a stress response and can be induced by many factors such as hypertension and diabetes (He and Sharpless

2017). Senescence within the NVU, which is comprised of BMVECs, pericytes, smooth muscle cells, glial cells (astrocytes, microglia, and oligodendrocytes), and neurons, can lead to dysregulation of neurovascular function altogether (Schaeffer and Iadecola 2021). Recent studies showing that therapies targeting senescence with senolytic or xenomorphic agents can prevent the development of neurodegenerative diseases provide support for this concept (Kim and Kim 2019). Since endothelial dysfunction and associated dysregulation of CBF and BBB integrity are the earliest biomarkers that can be detected before neuronal pathologies like neurofibrillary tangles and cognitive deficits, it is reasonable to speculate that endothelial senescence can propagate senescence within the NVU. Indeed, recent studies have shown that senescence is associated with vascular dysfunction, BBB disruption, and vascular stiffness leading to impaired neurovascular coupling (Blair et al. 2015; Bryant et al. 2020; Graves and Baker 2020; Tian and Li 2014; Yamazaki et al. 2016). A senescent endothelial cell undergoes oxidative stress, dysregulation of calcium signaling, impairment in mitochondrial biogenesis along with cell cycle arrest (Korolchuk et al. 2017; Kumari and Jat 2021; Liu et al. 2014). A recent study showed the presence of senescent endothelial cells in the aging brain using a single-cell RNA sequencing (Kiss et al. 2020). Since increased brain ET-1 levels are associated with hypoperfusion in age-related neurodegenerative diseases and the presence of ETA receptors on endothelial cells is unique to the brain, we were interested in the role of this receptor in BMVEC senescence. Recognition of ET-1 as a senescence inducer is recent. ET-1 induces cellular senescence and fibrosis in cultured myoblasts leading to aging-related sarcopenia (Alcalde-Estevez et al. 2020). It is also reported that ET-1 induces chondrocyte senescence and cartilage damage via endothelin receptor type B in a post-traumatic osteoarthritis mouse model (Au et al. 2020). In human umbilical vein endothelial cells, senescence was positively associated with increased ET-1 production (Olmos et al. 2017). The current study provides new evidence that ETA receptor activation by ET-1 alters the expression of senescence-associated markers in BMVECs.

Senescent cells acquire a so-called SASP that is critical for the propagation of senescence and chronic low-grade inflammation (Khan et al. 2017; Kumari and Jat 2021). Increased expression and secretion of proinflammatory cytokines like TNF-α, IL-8, and IL-6 can alter tissue microenvironment and function while amplifying the senescence phenotype (Csiszar et al. 2003). Another well-established factor that brings senescence is the oxidative stress (Salvador et al. 2021). In the current study, in addition to ET-1, BMVECs were treated with a SASP factor-like TNF-α, a low dose of H_2O_2, and amyloid-β (Aβ−40) that is closely linked with the aging brain. All these mediators increased the SA-β gal activity, which is the prototypical marker of senescence. In the case of ET-1 stimulation, blockade of the ETA receptor completely prevented this increase suggesting that this receptor subtype is responsible for mediating this effect. As briefly mentioned above and elegantly discussed in these review papers, the senescent phenotype is heterogeneous and should be investigated with multiple markers (Gorgoulis et al. 2019; Sikora et al. 2021b). Indeed, the International Cell Senescence Association provided recommendations on how to use molecular and cellular features of senescence as biomarkers (Gorgoulis et al. 2019). It is recommended to begin with SA-β gal staining and follow with additional markers. Senescent cells acquire a typical flat and enlarged shape with increased expression of senescence biomarkers like p53, p21, and p16 and cell cycle regulators such as Cyclin D1,

a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6 (Blasiak 2020; Campisi and Robert 2014; Coppe et al. 2010; Kumari and Jat 2021). Crosstalk among the cellular pathways defines the fate of cells to maintain tissue and organ homeostasis. The p53 gene plays a pivotal role in determining the fate of a cell and is involved in the regulation of metabolism, autophagy, DNA damage repair, cell cycle arrest, quiescence, senescence, and apoptosis. Chen and Li et al. demonstrated that a sub-lethal dose of H_2O_2 was able to induce senescence in fibroblast, while higher doses induced apoptosis. They also reported that p53 levels were two times higher in apoptotic cells compared to p53 levels in growth arrest conditions indicating the level of p53 can shift the cells to either side (Chen et al. 2000). In the current study, we also studied the effector proteins of the p53 pathway as senescence markers. LIF1 is a multifunctional protein, plays different roles in a highly context-dependent manner, and can negatively regulate tumor suppressor p53 in colorectal cancer and promotes the chemo-resistance (Yu et al. 2014). Based on our observation, an increase in LIF and LIFR with ET-1 treatment could be responsible for shifting the cells towards growth arrest/senescence instead of apoptosis. An increase in expression of other senescence markers p21 and cyclin D1 as well as a decrease in lamin A/C, nuclear scaffold proteins, support the opinion. More interestingly, the lack of an increase in these senescence markers in the presence of ETA antagonist identifies ET-1 and ETA receptors as a mediator of BMVECs senescence. In myoblast cells, ET-1 increases p16 expression and SA-β gal activity without a change in the p53 protein expression through the ETA receptor (Alcalde-Estevez et al. 2020). In the current study, we observed an ET-1-mediated increase in p53 protein, which was prevented in the presence of an ETA antagonist. In chondrocytes ET-1 and H_2O_2 both induced senescence via the ETB receptor (Au et al. 2020). In this study, our focus was to establish the role of the ETA and did not use dual or ETB selective receptor antagonist. It seems that both ETA and ETB receptors can contribute to senescence in a cell-type-dependent manner. It is one of the limitations of the current study that we used only ETA blockade.

Considering the tight and adherent junction proteins as major determinants of BBB integrity, we also looked at the expression of several barrier function proteins in response to ET-1 treatment. Adaptor proteins acting as cytoskeletal linkers such as zonula occludens-1 (zo-1), VE-cadherin, and claudin-5 control the endothelial survival and stabilization of the blood vessel assembly (Crosby et al. 2005; Jia et al. 2014; Lochhead et al. 2020; Zihni et al. 2016). Salvador *et al.* reported the changes in endothelial tight junction proteins in response to H_2O_2-induced senescence and observed changes in the expression of VE cadherin, zo-1, and claudin-5 (Salvador et al. 2021). In the current study, we observed a different pattern in mRNA and protein expression of these tight junction proteins. We detected a significant decrease in VE-cadherin that was partially prevented by BQ-123. These findings need to be tested further to determine whether barrier function is compromised.

It is established that endothelial cells undergo senescence, however, this is the first study to demonstrate brain endothelial cells also undergo senescence in response to ET-1, and the ETA receptor contributes to the process. The senescent cells loose functional properties and could contribute to dysregulation of NVU. Neurodegenerative diseases and aging increase the brain and circulating ET-1 level, thus the ET-1 could be a key regulator of senescence and contribute to VCID. Nevertheless, these claims need to be tested in in-vivo models of

neurodegenerative diseases. We used a simple approach to start testing the diverse roles ETA receptors can play in the biology of BMVECs. We used one dose of ET-1 and investigated one-time points. We also used a human BMVEC line established from a male patient. We have previously reported that ET-1 does not affect survival in these cells but it does decrease survival in a human BMVEC line established from a female patient, which can be prevented by ETA blockade (Abdul et al. 2020b). It would be interesting to replicate this study in female cells. The impact of endothelial senescence on neighboring cells cannot be evaluated with our model. As recognized above, we used only the ETA blockade. Nevertheless, these findings are important to implicate the ET system in diseases associated with neurovascular degeneration and provide evidence that this system can play roles beyond the regulation of cerebrovascular reactivity. We propose that as studies are designed to investigate the role of the ET system in ADRD using genetic and pharmacological approaches, endothelial senescence should be evaluated as an endpoint.

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DATA AVAILABILITY STATEMENT

Data generated or analyzed during the study are available from the corresponding author upon reasonable request.

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Study-1

Study-2
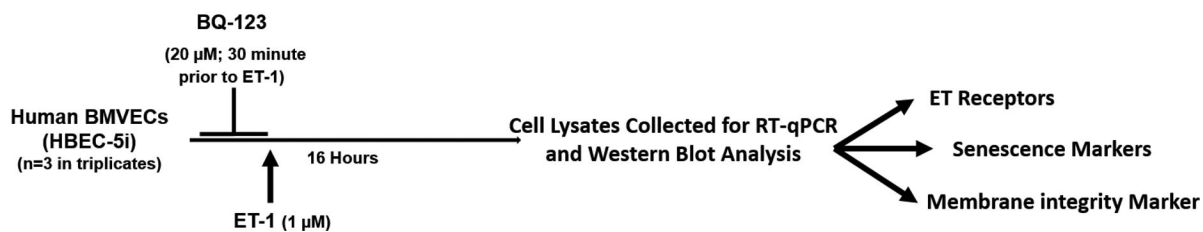
Fig. 1.
Schematic representation of cell culture experiments.
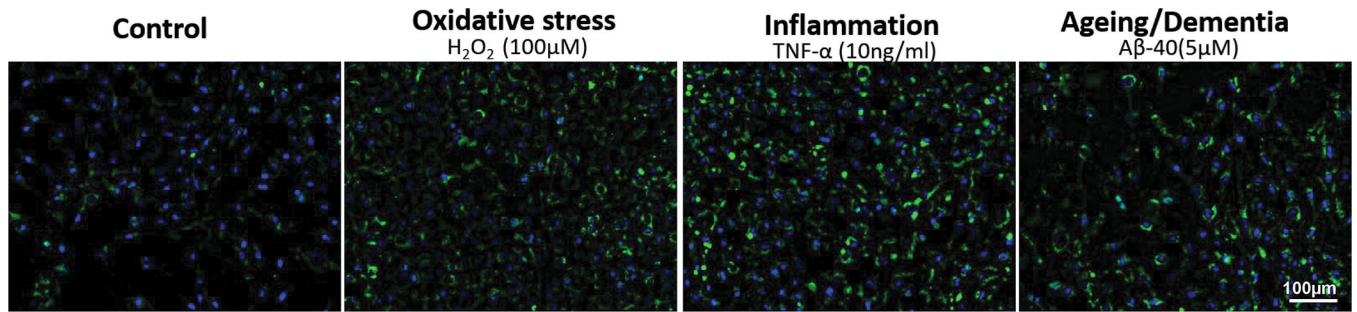
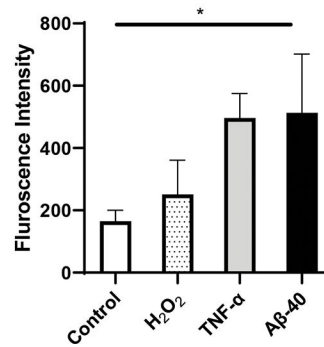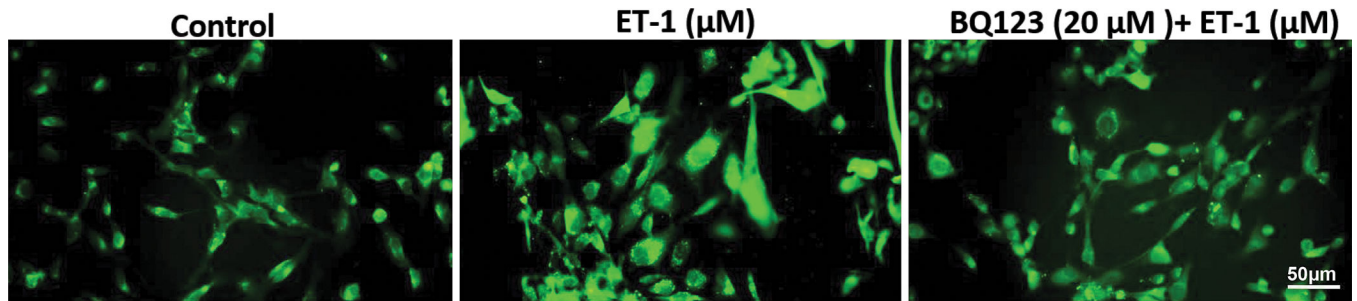Green: Senescence associated β-galactosidase staining, Blue: DAPI, nuclear staining

Fig. 2.
Treatment of BMVECs with an oxidative stress inducer, an inflammatory cytokine, and age-related factor increases senescence associated (SA)- β gal activity. Incubation of BMVECs with a low dose of H_2O_2 (100µM; 3 days), TNF-α (20ng/ml media for 3 days), and Aβ–40 (5µM; for 5 days) increased the SA-β gal staining. Green immunofluorescence indicates the increase in SA-β gal activity. Images were captured at a 20x magnification scale bar is 100 µm (n=3). Data were analyzed with one-way ANOVA, *p,0.05.
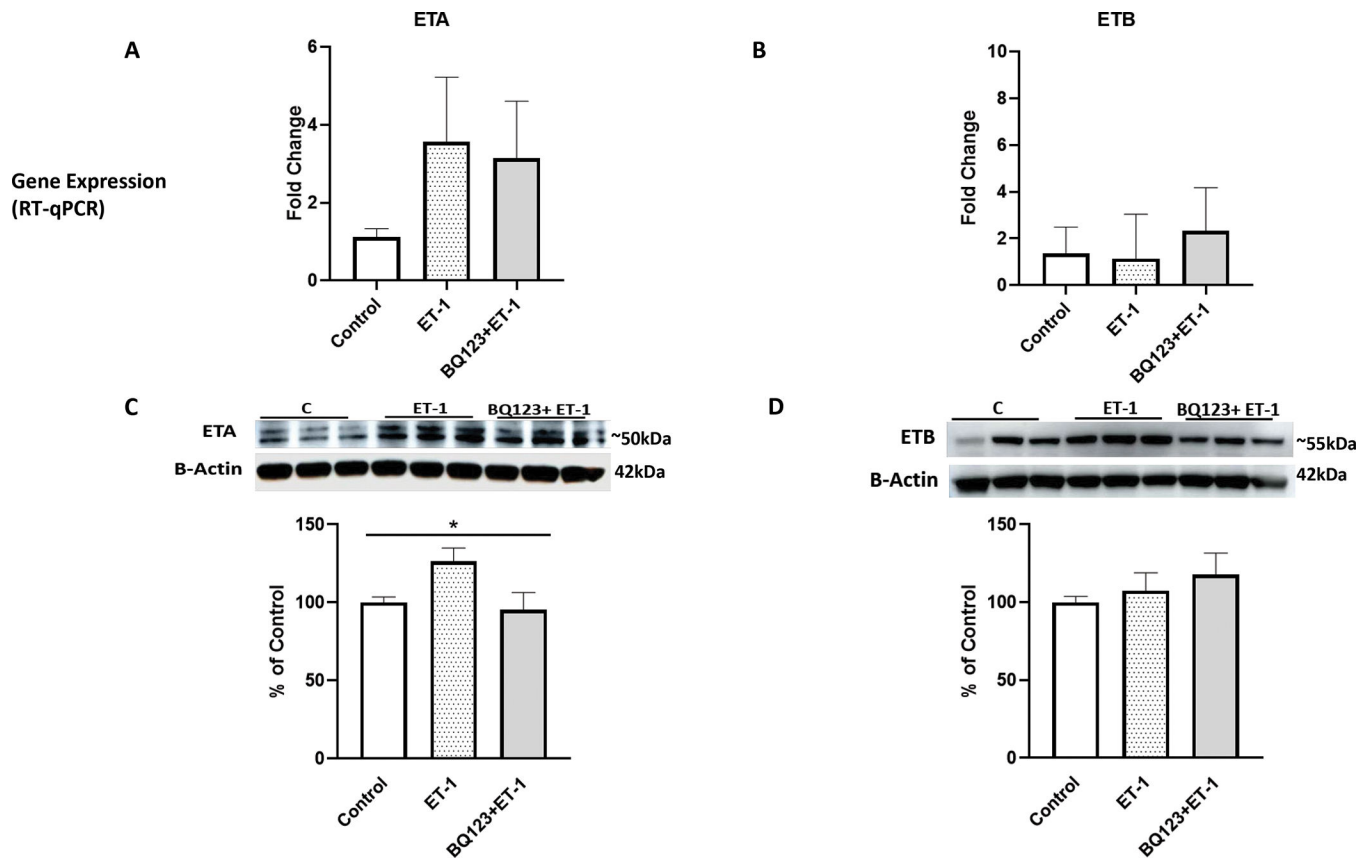
Fig. 3.
ET-1 increases the ETA receptor protein in BMVECs. ETA and ETB receptor expression levels were measured by RT-qPCR (A&B) and Western blotting (C&D). (A&C) Incubation of BMVECs with ET-1 (1μM) for 16 hours increased protein but not the mRNA level of the ETA receptor and pre-incubation with ETA antagonist BQ-123 prevented this increase. ET-1 treatment did not affect mRNA (B) or protein levels of the ETB receptor. RT-qPCR data are presented fold change while Western blot data are normalized with β-actin and presented as mean ± SEM of percent of control (n=3; in triplicates). Data were analyzed with one-way ANOVA, *p<0.05.
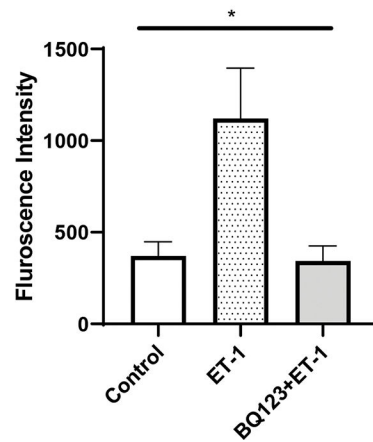
Green: Senescence associated β-galactosidase staining

Fig. 4.
ET-1 treatment increased the SA-β gal staining which was prevented by the presence
of ETA receptor antagonist BQ-123. Cells were treated with ET-1 (1μM) for 16 hours
and ETA receptor antagonist (BQ123; 20 μM; treated 30 minutes before ET-1 treatment).
Green immunofluorescence indicates the SA-β gal activity. Images were captured at 20x
magnification with 2x zoom, scale bar is 50μm (n=3). Data were analyzed with one-way
ANOVA, *p<0.05.
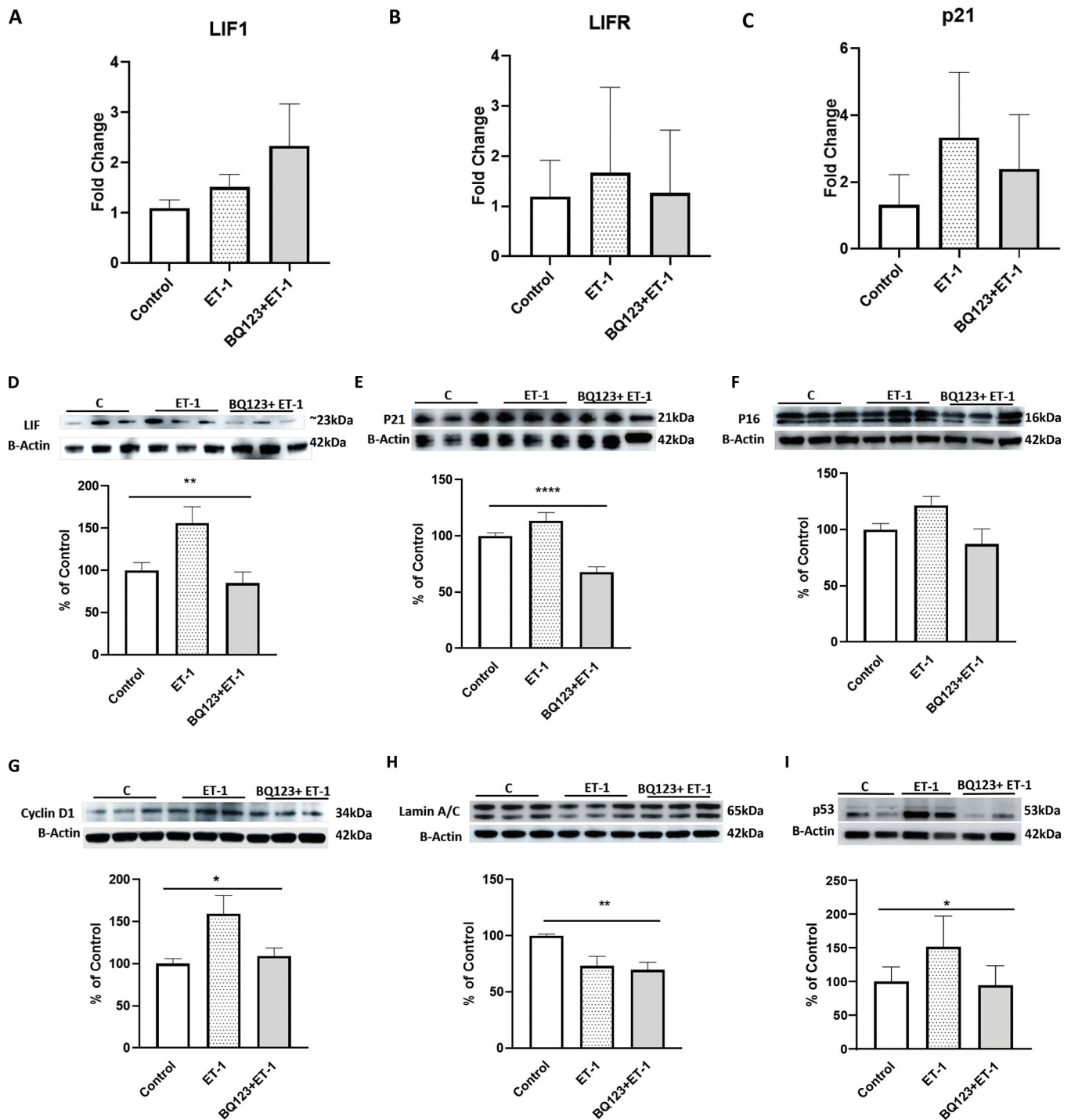
Gene Expression (RT-qPCR)

Fig. 5.

ET-1 treatment increased senescence marker proteins. ETA receptor antagonism reversed this phenomenon. mRNA levels of LIF1 (A), LIF receptor (B), and p21 (C) levels were not affected by ET-1 treatment or ETA receptor inhibition. Senescence marker proteins LIF1 (D), p21 (E), and cyclin D1 (G) but not p16 (F) were increased with ET-1 treatment which was prevented by the ETA receptor blockade. (H) Lamin A/C was significantly decreased with ET-1 treatment and ETA inhibition did not change it. Cells were treated with ET-1 (1μM) for 16 hours and ETA receptor antagonist (BQ123; 20 μM; treated 30 minutes before ET-1 treatment). RT-qPCR data are presented fold change values while Western blot data are normalized with β-actin and presented as percent of control (n=3; in duplicate or triplicates). Data are plotted as mean ± SEM (n=3; in triplicates). Data was analyzed with one way ANOVA, *p<0.05, **p<0.01, ****p<0.0001.
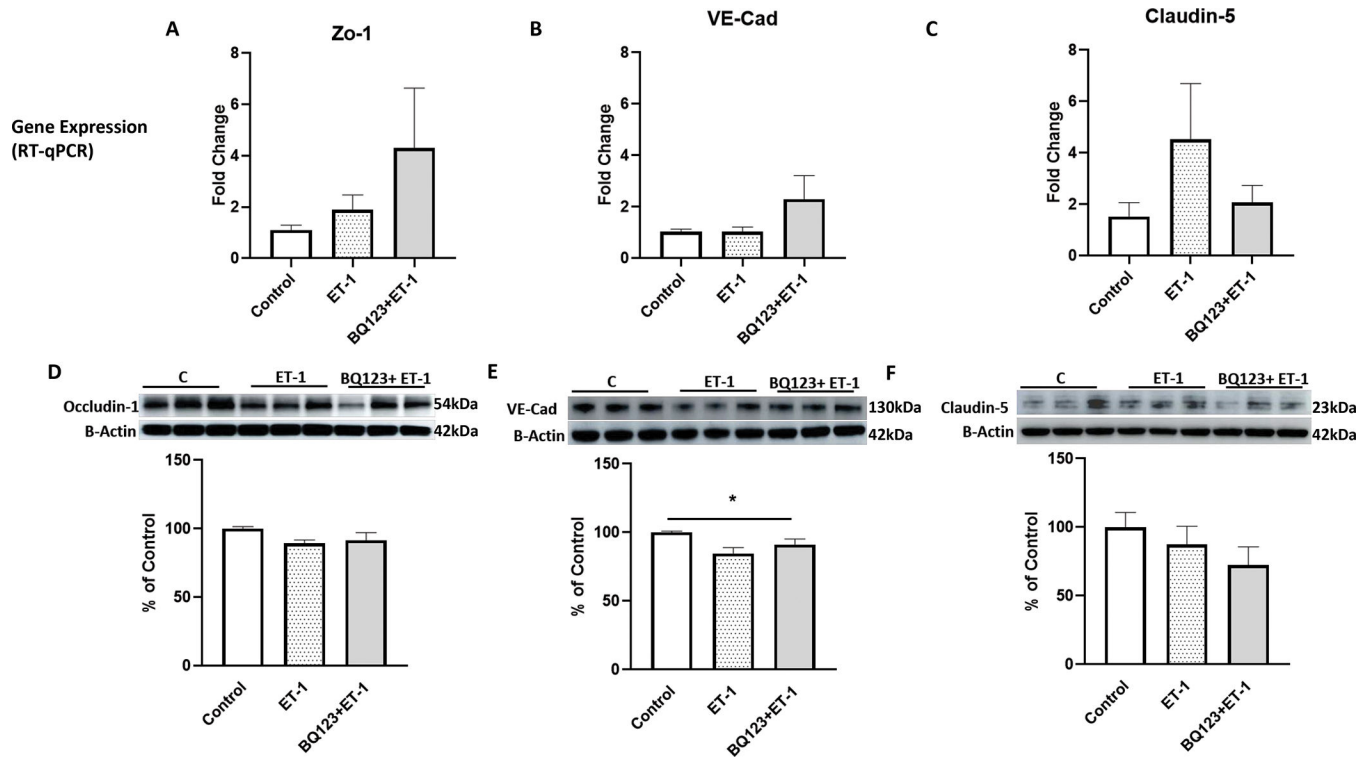
Fig. 6.
ET-1 decreased the expression of tight junction proteins in BMVECs. (A) mRNA expression of zo-1, VE-cadherin, and claudin-5 were differently regulated in response to ET-1 and ETA antagonism. (B) Protein expression of occludin-1, VE-cadherin, and claudin-5 was decreased with ET-1, while ETA antagonism did not improve it. Cells were treated with ET-1 (1μM) for 16 hours and ETA receptor antagonist (BQ123; 20 μM; treated 30 minutes before ET-1 treatment). RT-qPCR data are presented as fold change values, while Western blot data are normalized with β-actin and presented as percent of control (n=3; in duplicate or triplicates). Data are plotted as mean ± SEM (n=3; in triplicates). Data were analyzed with one-way ANOVA, *p<0.05.

Table. 1

List of primer sequences used for RT-qPCR analysis of genes.

Gene	Forward	Reverse	NCBI Reference
ETA	5'-ACCTGTATGCTCAATGCCAC-3'	5'-CAGTGCACACCAAGGGCATA-3'	NM_001957.4
ETB	5'-GCTGTCCCTGAAGCCATAGG-3'	5'-GGCCAATGGCAAGCAGAAAT-3'	NM_000115.4
LIF	5'-AGGGCATCGCTAAACCCAAA-3'	5'-TCAGCTTCATCACAGCCCAG-3'	NM_002309.4
LIFR	5'-AGCCTCAAGCAAAACCAGAA-3'	5'-TTGGCCTGAGGTCTGTAACC-3'	NM_001127671.2
p21	5'-AGGTGGACCTGGAGACTCTCAG-3'	5'-TCCTCTTGGAGAAGATCAGCCG-3'	NM_000389.5
Zo-1	5'-CGCTCAAGAGGAAGCTGTGG-3'	5'-GAGGGTTTTCCTTGGCTGAC-3'	NM_001301025.3
CLDN5	5'-GGGTTTGTGTCCCTGCCTAA-3'	5'-CCAGTGCAAGATCCCAGAGG-3'	NM_001363066.2
CDH5	5'-AGTGTGTGAGAACGCTGTCC-3'	5'-CACGTTTCGTGGTGTTATGTCC-3'	NM_001795.5
RPS-13	5'-GACGACGTGAAGGAGCAGAT-3'	5'-GGAAGATCAGGAGCAAGTCCC-3'	NM_001017.3

ETA, Endothelin receptor type A; ETB, Endothelin receptor type B; LIF, Leukemia inhibitory factor; LIFR, Leukemia inhibitory factor receptor; p21, Cyclin-dependent kinase inhibitor 1; Zo-1, tight junction protein-1; CLDN5, Claudin-5; CDH5, Vascular Endothelial Cadherin or VE-Cadherin; RPS-13, Ribosomal protein S-13.