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Paper

The synthetic cannabinoid 5F-MDMB-PICA enhances the metabolic activity and angiogenesis in human brain microvascular endothelial cells by upregulation of VEGF, ANG-1, and ANG-2

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Brain angiogenesis, the formation of new blood vessels from existing brain vasculature, has been previously associated with neural plasticity and addictive behaviors related to substances. Synthetic cannabinoids (SCs) have become increasingly popular due to their ability to mimic the effects of cannabis, offering high potency and easy accessibility. In the current study, we reveal that the SC 5F-MDMB-PICA, the most common SC in the United States in 2019, increases cell metabolic activity and promotes angiogenesis in human brain microvascular endothelial cells (HBMECs). First, we performed an MTT assay to evaluate the effects of 5F-MDMB-PICA treatment at various concentrations (0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM) on HBMECs metabolic activity. The results demonstrated higher concentrations of the SC improved cell metabolic activity. Furthermore, 5F-MDMB-PICA treatment enhanced tube formation and migration of HBMECs in a dosage-dependent manner. Additionally, the mRNA, secreted protein, and intracellular protein levels of vascular endothelial growth factor, angiopoietin-1, and angiopoietin-2, which are involved in the regulation of angiogenesis, as well as the protein levels of cannabinoid receptor type-1, were all increased following treatment with 5F-MDMB-PICA. Notably, the phosphorylation levels at Serine 9 residue of glycogen synthase kinase-3β were also increased in the 5F-MDMB-PICA treated HBMECs. Collectively, our findings demonstrate that 5F-MDMB-PICA can enhance angiogenesis in HBMECs, suggesting the significant role of angiogenesis in the response to SCs. Manipulating this interaction may pave the way for innovative treatments targeting SC addiction and angiogenesis-related conditions.

Key words: 5F-MDMB-PICA; angiogenesis; ANG-1 and -2; cannabinoid receptors; synthetic cannabinoids; VEGF.

1. Introduction

Angiogenesis is described as the process of new blood vessel generation from pre-existing vessels. It is an important process involved in nutrient supply, immune functions, and tissue regeneration and growth.[1] Angiogenesis is regulated by multiple factors that work cooperatively to finely control the process. Endothelial cells express surface oxygen sensors and hypoxia-inducible factors (HIF-1 and HIF-2α), which enable them to sense the hypoxic gradient during embryogenesis and tissue growth, facilitating accurate direction and adjustment of vessel formation to optimize blood flow.[1,2] HIFs control vascular endothelial growth factor (VEGF) secretion, often described as the main regulatory molecule in angiogenesis, along with other regulatory factors including angiopoietin-1 (ANG-1) and angiopoietin-2 (ANG-2).[2,3] These secreted factors, in conjunction with matrix metalloproteinases, transform a quiescent endothelial cell into a tip cell that guides vessel growth through adhesion and deadhesion, leading to cell migration. Moreover, stalk cells, which surround the tip cells, are important for the formation of the endothelial lumen where they constantly divide and extend the stalk.[1] Brain vascular formation begins in embryogenesis, primitive angioblasts form the perineural vascular plexus through invasion of the head region, eventually covering the entire neural tube.[4] Like other vasculature, angiogenesis in the brain begins with the formation of the blood vessels, followed by stabilizing, pruning, and specialization. Angiogenic factors play key roles in these 4 steps. For instance, ANG-1 interacts with Tie receptors (1 and 2) during the maturation and stabilization of the new vessels.[2,5]

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase that was initially identified and studied for its role in glycogenesis. It was later discovered to be a regulator of various cellular functions including, proliferation, metabolism, embryonic development, and brain functions.[6] Moreover, GSK-3β has been implicated in regulation of behavioral patterns induced by drug abuse and addiction by modulating neural plasticity and memory formation.[7,8] GSK-3β activity is controlled by the phosphorylation of specific target residues. Phosphorylation of Tyr216 renders GSK-3β constitutively active, while phosphorylation of Ser9 leads to its inactivation.[7,9,10] Furthermore, GSK-3β has been identified as an important regulator of angiogenesis. By mediating signalling pathways in endothelial cells, GSK-3β enables these

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cells to adjust vessel development by regulating both migration and survival.[9] Additionally, GSK-3β inhibition leads to a decrease in the degradation of HIF-1α, where the accumulation of HIF-1α stimulates the activation of Wnt/β-catenin signaling cascade through the secretion of VEGF, which leads to the induction of angiogenesis.[2,3,11]

Synthetic cannabinoids (SCs) are recently developed psychoactive substances developed to mimic the effects of the phytocannabinoid (−)-trans-D9-tetrahydrocannabinol, a naturally occurring compound found in the cannabis plant, in pharmacological studies.[12,13] Through the investigation of SC use, researchers have characterized the endocannabinoid system, which includes two receptors: cannabinoid receptor type-1 (CB1R), highly abundant in the nervous system, and cannabinoid receptor type-2 (CB2R), primarily expressed by immune system cells.[14] However, malpractice in certain laboratories led to the misuse of SCs for recreational usage.[15] SCs are highly addictive substances, as they exhibit a greater affinity to the CB1R and CB2R compared to Tetrahydrocannabinol (THC).[16] For instance, SCs interact with CB1R to demonstrate its addictive properties on its users by directly affecting the reward system and increasing tonic dopamine levels through firing rate elevation of A10 dopamine neurons, as well as phasic dopamine events in the ventral tegmental area.[17] This interaction also inhibits the activity of adenylyl cyclase, leading to decreased levels of cAMP production, which is a known regulator of neural functions such as neurotransmission, development, and plasticity.[13] Moreover, the use of SCs poses significantly higher health risks than compared to their natural counterpart. These risks most probably stem from the higher potency of these drugs, along with insufficient or sometimes absent safety regulations.[18] Consequently, the occurrence of adverse and sometimes life-threatening pharmacological and psychological side effects is common. These side effects include increased blood pressure, tremors, nausea, vomiting, fine motor skill impairment, kidney injury, anxiety, agitation, psychosis, hallucinations, and violent behavior along with an elevated mortality rate.[12,13,19,20] The SC 5F-MDMB-PICA [methyl-(S)-2-[1-(5-fluoropentyl)-1H-indole-3-carboxamido]-3,3-dimethylbutanoate], also known as MDMB-2201 or 5-fluoro MDMB-2201, belongs to the indole group of SCs. It was initially discovered in the United States and Europe in 2016 (Fig. 1; $C_{21}H_{29}FN_2O_3$).[21] By 2019, it had become the most common SC in the United States[22] This SC highly interacts with cannabinoid receptors in the body, particularly CB1R receptors, and shares compositional similarities with 5F-MDMB-PINACA, where the shared fluoropentyl side chain indicates its high affinity to CB1R.[23,24] The physiological effects of 5F-MDMB-PICA are not fully characterized. Nevertheless, emerging evidence suggests that 5F-MDMB-PICA can affect cellular cAMP levels, with results similar to previous studies on the general effects of SCs.[25] One study found that when human embryonic kidney cells were exposed to 10 μM of 5F-MDMB-PICA, the cell's ability to stimulate or inhibit cAMP levels based on whether they have been treated with pertussis toxin was enhanced.[26]

In the present study, our hypothesis was that the SC 5F-MDMB-PICA could influence the viability and angiogenesis in human brain microvascular endothelial cells (HBMECs) in vitro. Additionally, we investigated the involvement of cellular pathways by targeting GSK-3β, CB1R, and various pro-angiogenic factors, such as VEGF, ANG-1, and ANG-2, to elucidate the relationship between the receptors of the endocannabinoid system and brain angiogenesis.

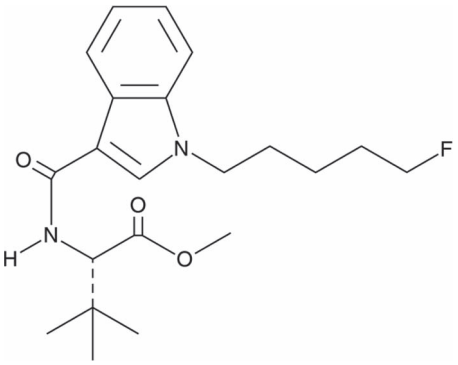

Fig. 1. The chemical structure of 5F-MDMB-PICA.

2. Materials and methods

2.1 Cell line

HBMECs (CRL-3245) acquired from American Type Culture Collection (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle Serum mixed with F-12 (1:1) (DMEM/F12) (Euroclone S.p.A, Pero, Italy), complemented with fetal bovine serum (10%), penicillin and streptomycin (1%), and a Microvascular Endothelial Cell Growth Kit-BBE (hydrocortisone, bovine brain extract, L-glutamine, recombinant human epidermal growth factor (rhEGF), heparin sulfate, fetal bovine serum, and ascorbic acid) (PCS-110-040) (ATCC, Manassas, VA). Cultured cells were grown in 5% CO_2 at 37 °C. Cell passage was conducted in a 1:4 ratio when cell confluence reached 80%.

2.2 Synthetic cannabinoids treatment

5F-MDMB-PICA was obtained from Cayman Chemical (Ann Arbor, Michigan) and solubilized in dimethyl sulfoxide (DMSO) to produce a stock solution (2 mg/ml). Afterward, 6 concentrations were then prepared from the stock solution through serial dilution (0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM). The control group functions as a baseline for assessing the impact of 5F-MDMB-PICA on brain endothelial cells. The control group was established using serum-free media and DMSO.

2.3 MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is a colorimetric method used to assess the metabolic activity of cells by measuring the conversion of the yellow tetrazolium salt into purple formazan crystals. A total of 5 x 10^3 HBMECs were seeded into each well of a 96-well plate and incubated for 24 hours. After that, five concentrations of 5F-MDMB-PICA (ranging from 0.0001 μM to 1 μM) were added into cells in triplicates for 24 hours. Thereafter, the medium was exchanged for serum-free media (SFM) containing the MTT solution (5 mg/ml) (ATCC, Manassas, VA) for 4 hours. Finally, DMSO was added to the cells followed by shaking for 10 minutes to solubilize the formazan crystals. An ELISA reader measuring at an absorbance of 570 nm was used to quantify the cell viability per well. MTT assay was repeated 3 times in triplicates.

2.4 Scratch healing assay

An in-vitro scratch-healing assay was performed to measure the migration rates of the HBMECs. HBMECs were initially cultured onto a 12-well plate and allowed to grow for 24 hours until reaching an adequate level of confluence. Once the desired confluence was achieved, the cell monolayer was "scratched" using a 1 ml micro-pipette tip, followed by gentle washing with phosphate buffer saline (PBS) for complete removal of detached cells. After

Table 1. Primer sequences used in RT-qPCR.

Primer	Forward sequence	Reverse sequence
β-actin	5′-GGAGATTACTGCCCTGGCTCCTA-3′	5′-GACTCATCGTACTCCTGCTTGCTG-3′
VEGF	5′-GCACGTTGGCTCACTTCCAG-3′	5′-TGGTCGGAACCAGAATCTTTATCTC-3′
ANG-1	5′-ACCGTGAGGATGGAAGCCTAGA-3′	5′-AATGAACTCGTTCCCAAGCCAATA-3′
ANG-2	5′-CTTCAAGTCAGGACTCACCACCA-3′	5′-CCACCCATGTCCATGTCACAG-3′

that, SFM containing 5F-MDMB-PICA (0.0001 μM–1 μM) was added to the cells in triplicates. Scratch recovery was measured by capturing microscopic images at the moment of wounding (baseline) and after 24 hours. Measurements were performed using the ImageJ software. The wound recovery percentage was calculated according to the formula: (total distance of wound – average uncovered distance)/(total distance of wound) * 100%.[27] Scratch healing assay was repeated three times in triplicates.

2.5 Tube formation assay

An in-vitro tube formation assay was performed to measure the angiogenic capacity of HBMECs. First, basement membrane extract (BME) (Trevigen, Gaithersburg, MD) was thawed at 4 °C overnight. Then, 50 μl of BME was added to a pre-cooled 96-well plate and allowed to polymerize at 37 °C for 30 minutes. Subsequently, 2 x 10^4 HBMECs suspended in SFM mixed with 3 different doses of 5F-MDMB-PICA (0.0001 μM, 0.01 μM, and 1 μM) were cultured on the BME-coated wells as well as the untreated (control) cells for 24 hours. Microscopic images of closed tubes originating from the cells were used to assess the tube-like structure formation capacity of the cells by direct measurement of the number of these structures, along with counting the number of loop structures, total tube length, and number of branching points. Tube formation assay was repeated 3 times in triplicates.

2.6 RNA purification and RT-qPCR analysis

Total RNA was purified from HBMECs using the RNA purification kit (PP-210 L) (Jena Bioscience, Munich, Germany). The procedure was employed following the manufacturer's recommendations. Following RNA extraction, a gDNA removal kit (PP-219) (Jena Bioscience, Jena, Germany) was used for further purification. Following purification, RNA purity and concentration were measured by a Nano-drop device ND-1000 (Bio Drop, UK). The purified RNA was then reverse-transcribed into cDNA and quantified by one-step RT-qPCR using the kit SOLIscript 1-step SolisGreen (08-63-00250) (Solis BioDyne, Tartu, Estonia). The primer sequences for the 3 studied genes (VEGF, ANG-1, and ANG-2), as well as the reference control gene (β-actin) are listed in Table 1. The RT-qPCR protocol used is provided in section Appendix as Supplementary Table S1. RT-qPCR was repeated 3 times in triplicates.

2.7 Western blot

Western blotting was used to measure the protein expression level of VEGF, ANG-1, ANG-2, and the phosphorylation of GSK-3β at Ser9 in HBMECs. Briefly, cell lysis was performed using Radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with phosphate protease inhibitors. Next, the protein concentration was quantified by a protein assay kit (Bio-Rad, Hercules, CA, USA). The β-actin protein was used as a baseline for the comparison of protein levels of proangiogenic factors VEGF, ANG-1, and ANG-2 (provided as a ratio of expressed protein/β-Actin). About 20 μg of protein from each sample were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane. Membrane blocking was performed using 2% bovine serum albumin (BSA). Then, the primary antibodies anti-phospho-Ser9-GSK3β (9336S; 1:500; Cell Signaling Technology), anti-Total-GSK3β (PA5-95845; 1:1,000, ThermoFisher), anti-VEGF (ab46154; 1:500, Abcam), anti-ANG-1 (ab94684; 1:500, Abcam), anti-ANG-2 (ab153934; 1:500, Abcam), anti-cannabinoid receptor type 1 (ab259323; 1:1,000, Abcam) and anti-β-Actin (4967S; 1:1,000; Cell Signaling Technology) were incubated with the membrane overnight at 4 °C. Finally, secondary antibodies were incubated, and the fluorescent signal was detected with enhanced chemiluminescence. The Western blot was repeated 2 times.

2.8 ELISA

An ELISA technique was performed to measure the concentration of proteins secreted into the media. Conditioned media used in the analysis was collected 24 hours after treatment with 5F-MDMB-PICA and centrifuged at 10,000 RPM for 10 minutes. The supernatant was kept at −80 °C until the analysis was performed. All analysis was performed in 3 experiments with 3 triplicate wells per concertation. The concentration of VEGF (ab100662), ANG-1 (ab99972), and ANG-2 (ab99971) were measured using commercially available kits acquired from Abcam (Abcam; Cambridge, MA), and procedures were done in concordance with the manufacturer's recommendations.

2.9 Statistical analysis

We assessed the distribution of our data for normality, which indicated that it followed a normal distribution. The assay results were analyzed using a one-way ANOVA followed by a Turkey post-hoc test using GraphPad Prism (version 9..0 GraphPad Software, La Jolla, CA). Results with a *P*-value lower than 0.05 were considered statistically significant.

3. Results

3.1 Cannabinoid receptor type 1 expression is regulated during the process of angiogenesis

In order to evaluate the modulation of CB1R expression during angiogenesis in HBMECs, the study was initiated by examining quiescent HBMECs cells at time 0. Subsequently, cell proliferation was induced by treating the cells with proangiogenic media supplemented with fibroblast growth factors (bFGF; 10 ng/ml). CB1R protein levels were evaluated at various time intervals, specifically at 24 hours, 48 hours, and 72 hours. Notably, the expression of CB1R receptor protein demonstrated a gradual increase over time, with significant differences observed at 24 hours ($P = 0.021$), 48 hours ($P = 0.0003$), and 72 hours ($P < 0.0001$) (Fig. 2a and b). These findings indicate a correlation between the CB1R receptor and angiogenesis.

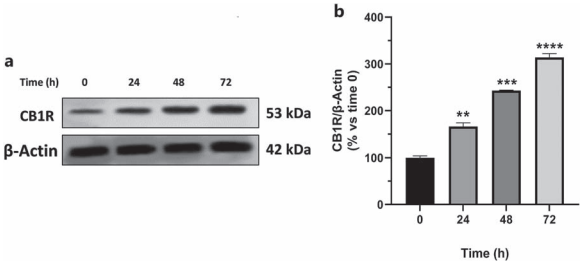

Fig. 2. The expression of CB1R has been observed to rise during angiogenesis stimulation. HBMECs were cultured in proangiogenic media supplemented with bFGF (10 ng/ml) for 24, 48, and 72 hours. Subsequently, (a) western blot images were acquired, and CB1R bands were analyzed using ImageJ software. The findings are presented in (b) with a quantitative analysis of the CB1R expression rates. Data were quantified and presented as mean ± SEM ($n = 3$). (**) means $P > 0.01$, (***) means $P > 0.0001$, and (****) means $P > 0.0001$.

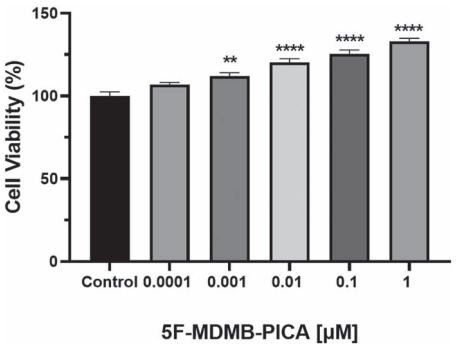

Fig. 3. 5F-MDMB-PICA treatment improves HBMECs metabolic activity of HBMECs. Cell metabolic activity rate was analyzed using an MTT assay after treatment with 5F-MDMB-PICA. First, 5×10^3 HBMECs were seeded in a 96-well plate for 24 hours. Five different concentrations of 5F-MDMB-PICA (0.0001 μM–1 μM) were incubated for 24 hours. After that, the media containing 5F-MDMB-PICA was discarded and replaced with MTT (5 mg/ml) and further incubated for 4 hours at 37 °C and 5% CO_2. Finally, the formazan crystals were dissolved using DMSO and the absorbance was measured at 570 nm in an ELISA reader. The treatment significantly enhanced cell viability in the concentrations 0.001 μM–1 μM when compared to the control. Data were quantified and presented as mean ± SEM ($n = 3$). (**) means $P > 0.01$ and (****) means $P > 0.0001$.

3.2 5F-MDMB-PICA treatment enhances HBMEC metabolic activity

After performing the MTT assay on HBMECs treated with 5 different 5F-MDMB-PICA doses (0.0001 μM–1 μM), it was observed that concentrations ranging between 0.001 μM and 1 μM significantly increased cell metabolism rate compared to the untreated group ($P = 0.0045$ for 0.001 μM, $P < 0.0001$ for 0.01, 0.1, and 1 μM). The increase in cell metabolism rate was positively correlated with higher concentrations of 5F-MDMB-PICA. The highest increase, reaching 1.3-fold, was observed with the 1 μM treatment (Fig. 3).

3.3 Treatment with 5F-MDMB-PICA promotes the migration rates of HBMECs

Endothelial cell migration rates of HBMECs, a fundamental process of angiogenesis, were exposed to 5 different 5F-MDMB-PICA concentrations that were assayed using an in-vitro scratch-healing assay. The results showed that cells treated with 5F-MDMB-PICA exhibited significantly higher migration rates compared to the control group within the treatment range of 0.001 μM to 1 μM (Fig. 4a) ($P = 0.0392$ for 0.001 μM, $P = 0.0002$ for 0.01 μM, $P < 0.0001$ for 0.1 and 1 μM). A maximal increase was observed in the 1 μM treatment group (Fig. 4b).

3.4 5F-MDMB-PICA treatment increases the tubulogenic activity of HBMECs

An in-vitro tube formation assay was performed to assess the angiogenic capacity of 5F-MDMB-PICA-treated HBMECs. The 5F-MDMB-PICA treatments at 0.01 μM and 1 μM demonstrated significantly higher angiogenic parameters. The tube numbers ($P < 0.0001$ for 0.01 μM and 1 μM), total tube length ($P < 0.0001$ for 0.01 μM and 1 μM), number of loops ($P < 0.0001$ for 0.01 μM and 1 μM), and number of branch points ($P = 0.0008$ for 0.01 μM and $P < 0.0001$ for 1 μM) were all increased after 5F-MDMB-PICA treatment (Fig. 5a–e).

3.5 5F-MDMB-PICA treatment upregulates mRNA levels of proangiogenic factors VEGF, ANG-1, and ANG-2

RT-qPCR was used to investigate the potential impact of 5F-MDMB-PICA treatment on the mRNA expression of proangiogenic factors in HBMECs. The results revealed a significant upregulation of VEGF expression in the 0.01 μM, 0.1 μM, and 1 μM groups ($P < 0.0001$) (Fig. 6a). Moreover, ANG-1 was significantly upregulated in the 0.001 μM ($P = 0.0433$), 0.01 μM ($P = 0.0031$), 0.1 μM, and 1 μM ($P < 0.0001$) groups compared to the untreated group (Fig. 6b). mRNA levels of ANG-2 were also elevated at 0.01 μM ($P = 0.0014$), 0.1 μM, and 1 μM ($P < 0.0001$) (Fig. 6c).

3.6 Treatment with 5F-MDMB-PICA upregulates the intracellular protein expression of VEGF, ANG-1, and ANG-2, while also increasing the phosphorylation levels of GSK-3β at the Ser9 residue

The intracellular protein expression levels of the VEGF, ANG-1, ANG-2, and phospho-Ser9-GSK-3β in the HBMECs cultured with 5F-MDMB-PICA. Specific bands of VEGF were detected at ~27 kDa in 5F-MDMB-PICA-treated HBMECs as well as the control. The VEGF protein was significantly expressed at 0.0001 μM, 0.01 μM, and 1 μM ($P < 0.0001$) (Fig. 7a and b). Both ANG-1 and ANG-2 bands were detected at ~57 kDa in 5F-MDMB-PICA-treated HBMECs at different concentrations. ANG-1 protein was significantly upregulated at 0.0001 μM ($P = 0.0011$), 0.01 μM, and 1 μM ($P < 0.0001$), whereas ANG-2 was significantly upregulated at 0.01 μM and 1 μM ($P < 0.0001$) (Fig. 7c and d). Furthermore, phospho-Ser9-GSK-3β bands were observed at ~46 kDa and detected at 0.0001 μM, 0.01 μM, and 1 μM ($P < 0.0001$) in the cells treated with 5F-MDMB-PICA compared to control (Fig. 7e).

3.7 5F-MDMB-PICA treatment elevates levels of secreted VEGF, ANG-1, and ANG-2

Serum levels of proangiogenic factors VEGF, ANG-1, and ANG-2 secreted by cells treated with 5F-MDMB-PICA were measured using ELISA. We detected a significant increase in the VEGF and ANG-1 levels after treatment with 5F-MDMB-PICA at 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM ($P < 0.0001$) (Fig. 8a and b). Furthermore, the secretion of ANG-2 has increased at 0.01 μM, 0.1 μM, and 1 μM ($P < 0.0001$) (Fig. 8c).

4. Discussion

Cannabinoid receptors have been recognized as potential therapeutic targets for the treatment of various diseases, including those related to angiogenesis. The endocannabinoid system,

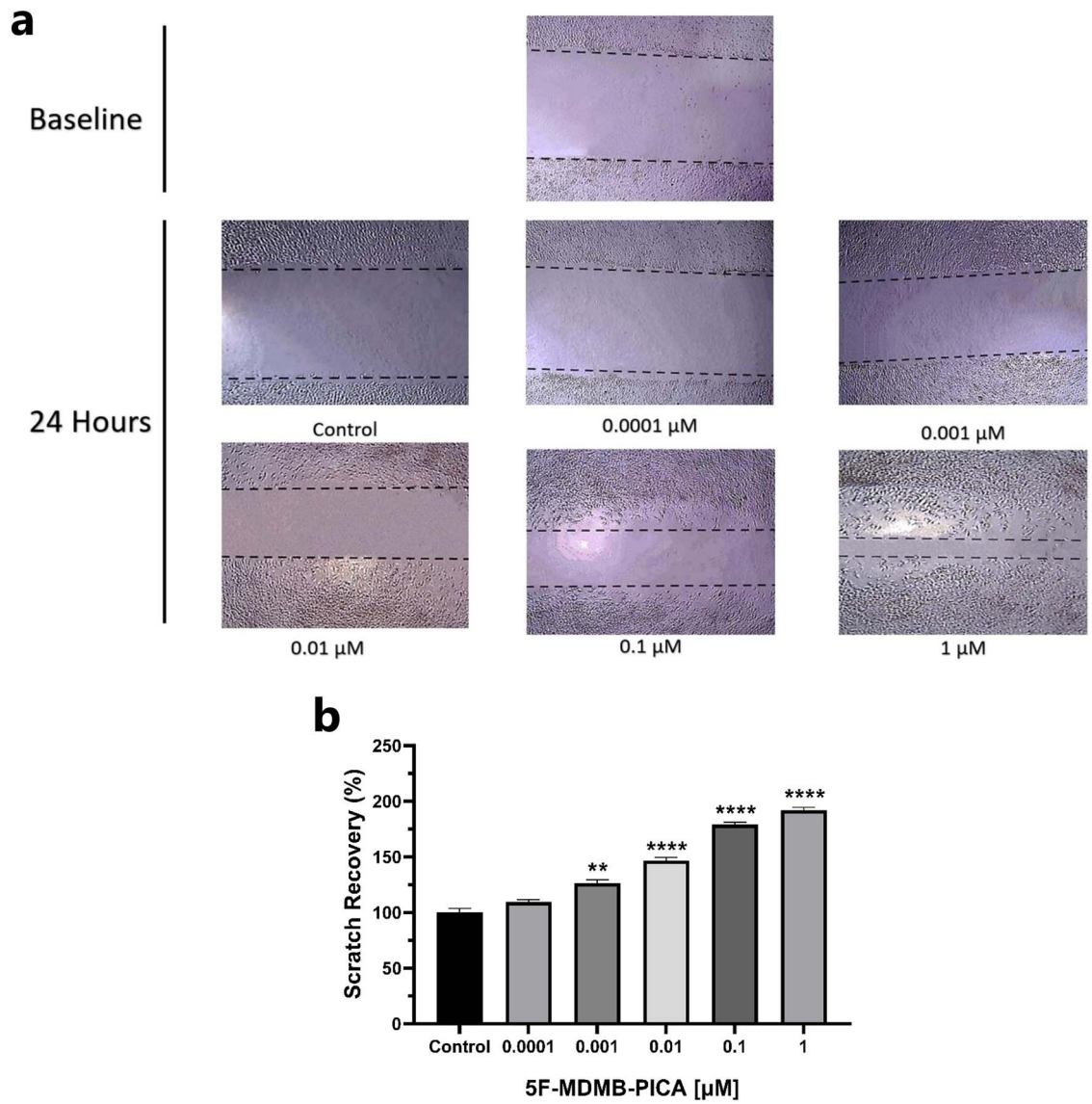

Fig. 4. 5F-MDMB-PICA treatment enhances cell migration in HBMECs. HBMECs were grown in a 12-well plate for 24 hours. Next, the cell monolayer was scratched using a 1,000 μl pipette tip after the cells reached the appropriate confluency. (a) Microscopic images of HBMEC migration at baseline (zero time) and at 24 hours after treatment with different doses of 5F-MDMB-PICA. (b) Quantitative analysis of the migration data. 5F-MDMB-PICA doses (0.001 μM–1 μM demonstrated significantly higher migration rates compared to the control. Data were quantified from 3 experiments, where each was done in duplicates and presented as mean ± SEM ($n = 3$). (**) means $P > 0.01$ and (****) means $P > 0.0001$.

which includes cannabinoid receptors, plays a crucial role in regulating several physiological processes, including immune responses, inflammation, and vascular function. As a result, modulating cannabinoid receptors has shown promise in the context of angiogenesis-related diseases. Previous research on SCs has highlighted their potential as treatments for various conditions, including cancer, neurodegenerative diseases, and Retinal diseases.[28–30] However, it is crucial to acknowledge that SCs, including 5F-MDMB-PICA, have not been approved for medical use and are classified as illicit drugs in many countries due to their potential for abuse and harmful health effects.[29] Despite the high affinity of 5F-MDMB-PICA to CB1R and its potential addictive properties,[23] to the best of our knowledge, this is the only study aimed at investigating the effects of 5F-MDMB-PICA on brain endothelial cells.

Recent studies have delved into the association between the endocannabinoid system (ECS), specifically CB1R receptors, and angiogenesis. Endocannabinoids like anandamide and 2-arachidonoylglycerol (2-AG) activate CB1R, stimulating various intracellular signaling cascades involved in angiogenesis. One critical pathway influenced by CB1R activation is the mitogen-activated protein kinase (MAPK) pathway, including the signaling of extracellular signal-regulated kinase (ERK). It has been demonstrated that CB1R-mediated activation of ERK signaling promotes angiogenesis in endothelial cells.[31] The current investigation was initiated by observing that the expression of CB1R is relatively low in quiescent endothelial cells but becomes induced during the angiogenic process at the protein level. These findings are consistent with previous studies demonstrating the upregulation of CB1R expression during the angiogenic process in human umbilical vein endothelial cells.[32]

It is essential to recognize that the effects of cannabinoid receptor activation on cell viability can vary depending on factors such as cell type, concentration, and timing of

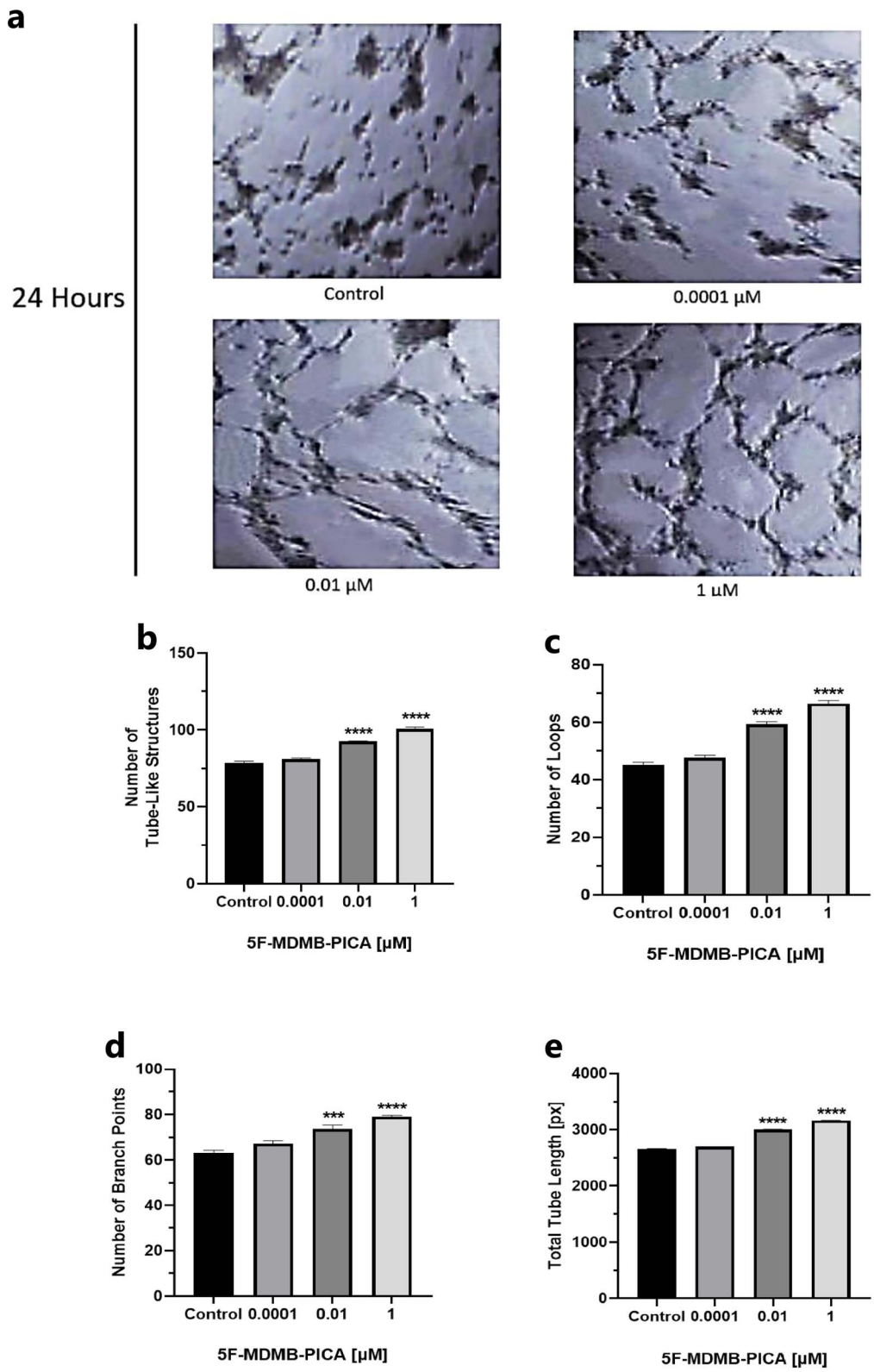

Fig. 5. The tubulogenic activity of HBMECs is increased by 5F-MDMB-PICA treatment. A tube-formation assay was performed to measure the effects of 5F-MDMB-PICA on the angiogenic capacity. BME-coated plates were seeded with 2 × 104 HBMECs suspended in SFM treated with 5F-MDMB-PICA (0.0001 μM, 0.01 μM, and 1 μM). (a) Microscopic images of tubular structures formed by HBMECs treated with 5F-MDMB-PICA after 24 hours of treatment. Significant increases have been observed in all angiogenic measurements including (b) the number of tube-like structures, (c) loops, (d) branch points, and (e) total tube lengths. Data presented as mean ± SEM ($n = 3$). (***) means $P > 0.001$ and (****) means $P > 0.0001$.

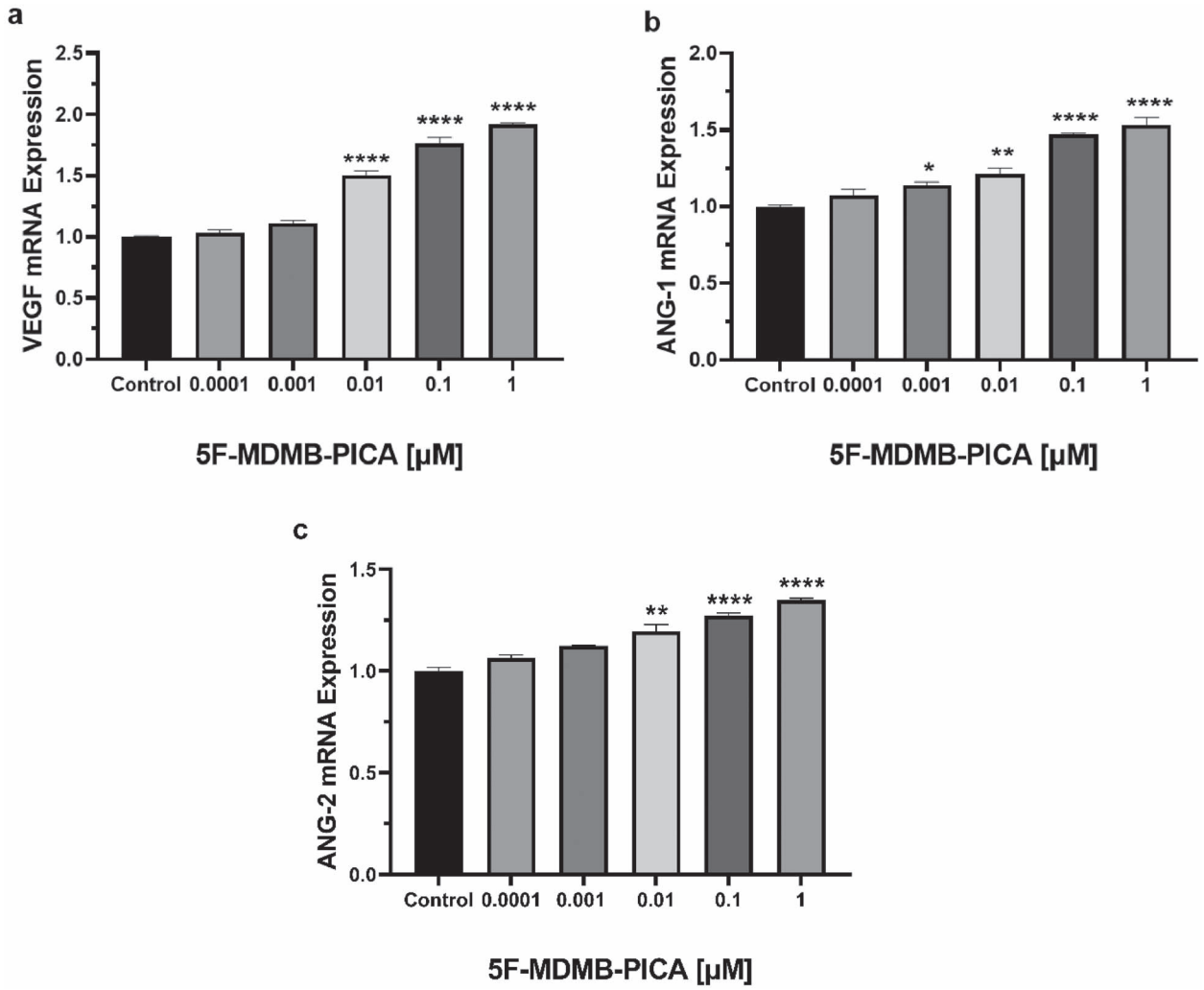

Fig. 6. 5F-MDMB-PICA upregulates mRNA levels of proangiogenic factors VEGF, ANG-1, and ANG-2. RT-qPCR was performed on 5F-MDMB-PICA treated cells (0.0001 μM–1 μM) to quantify the expression level of (a) VEGF, (b) ANG-1, and (c) ANG-2 on the mRNA level. Data presented as mean ± SEM ($n = 3$). (*) means $P < 0.05$, (**) means $P < 0.01$, (***) means $P < 0.001$, and (****) means $P < 0.0001$.

cannabinoid exposure. While some studies indicate pro-survival effects of cannabinoid receptor activation, others show pro-apoptotic effects. Additionally, the activation of these receptors may have different outcomes depending on the presence of specific ligands, agonists, or antagonists. For instance, the cannabinoid WIN 55,212-2 has been shown to enhance cell viability and promote an anti-inflammatory response in cultured astrocytes. WIN 55,212-2 increases the expression of the antioxidant Cu/Zn SOD and can prevent inflammation induced by Aβ1–42 in cultured astrocytes.[33] Conditioned media derived from breast cancer cells (MDA-MB-231) significantly reduced the viability of osteoblast-like UMR-106 cells. Conversely, media from MDA-MB-231 cells pre-treated with GW405833, a SC, improved UMR-106 cell viability.[34] In the brain, activation of CB1R receptors has been linked to neuroprotection by promoting cell survival and inhibiting apoptosis. Studies suggest that CB1R receptor activation can reduce neuronal damage in various neurodegenerative conditions, such as Alzheimer's disease and ischemic stroke.[35,36] We performed an MTT assay on HBMECs treated with 5F-MDMB-PICA to determine its effect on cell metabolic assay. The results suggest a low cytotoxic effect of the drug on HBMECs. These findings are consistent with a previous study that demonstrated the stimulatory effect of XLR-11, a SC, on metabolic rate while exhibiting a decreased cytotoxic effect on HBMECs.[27]

Activation of cannabinoid receptors has been shown to promote cell migration and tube formation in endothelial cells. Interestingly, The inactivation of CB1R receptors led to the inhibition of bFGF-induced endothelial migration and capillary-like tube formation, affecting pro-survival and migratory pathways involving ERK, Akt, FAK, JNK, Rho, and MMP-2.[32] Moreover, the migration rate and angiogenic capacity significantly increased in the presence of various concentrations of XLR-11 compared to the control.[27] Our results suggest that 5F-MDMB-PICA treatment increases the migration of cells and enhances the tube formation capacity of HBMECs in vitro. VEGF is a key proangiogenic factor and the most extensively studied growth factor in angiogenesis. It is primarily secreted by cells experiencing low oxygen levels (hypoxia) and activates signaling pathways in endothelial cells to stimulate angiogenesis.[37] VEGF induces endothelial cell migration, proliferation, and tube formation, crucial steps in the formation of new blood vessels. It also increases vascular permeability, allowing the leakage of plasma proteins and immune cells to the site of angiogenesis, which facilitates vessel growth.[37] Angiopoietins (ANG-1 and ANG-2) are growth factors that interact with

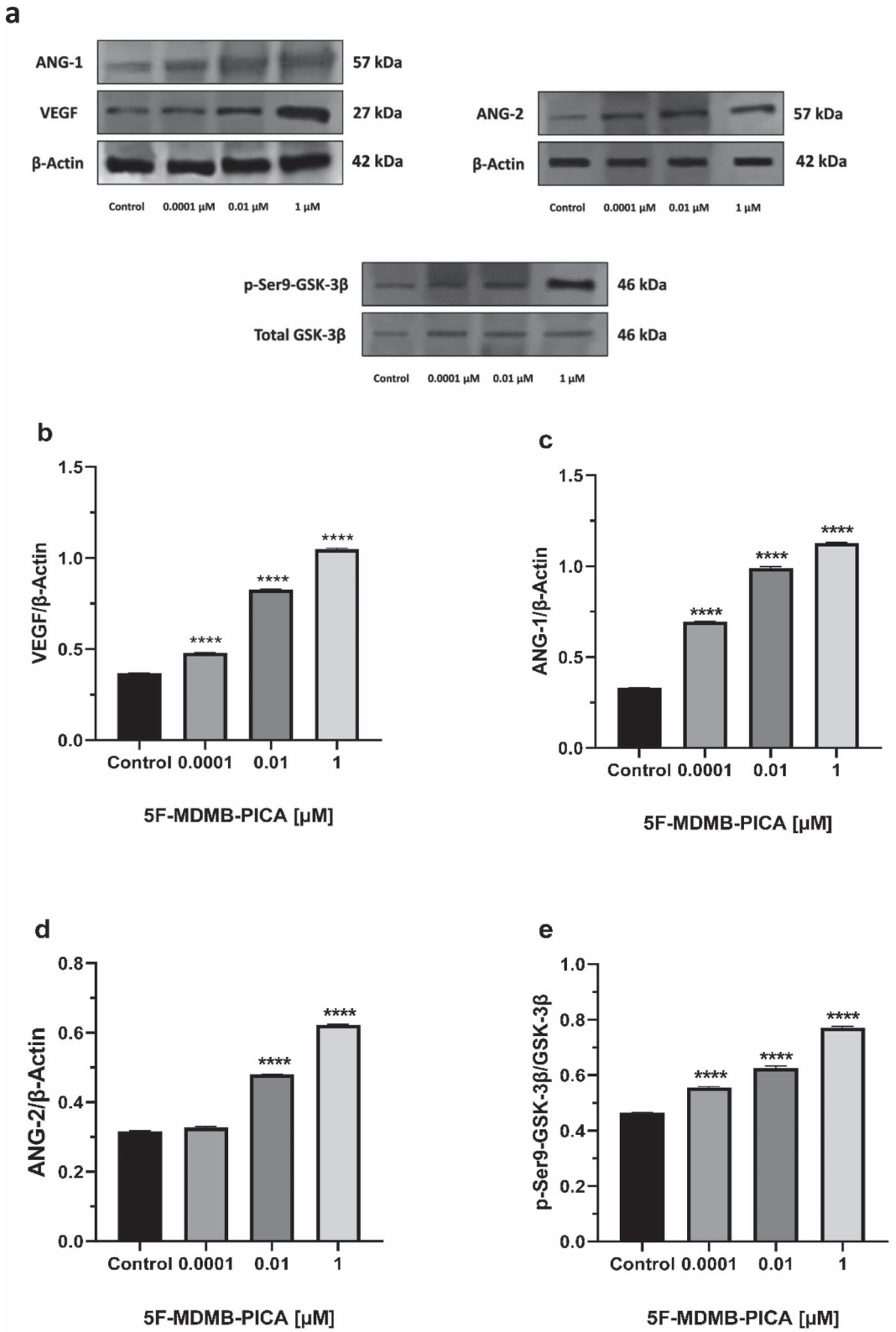

Fig. 7. 5F-MDMB-PICA treatment increases proangiogenic factors VEGF, ANG-1, ANG-2, and p-Ser9-GSK-3β protein levels in HBMECs. (a) Western blot bands images of GSK-3β, p-GSK-3β, VEGF, ANG-1, ANG-2, and β-actin isolated from HBMECs treated with 5F-MDMB-PICA. Western blot was performed on 5F-MDMB-PICA treated HBMECs (0.0001 μM, 0.01 μM, and 1 μM) to measure protein levels of VEGF, ANG-1, ANG-2, GSK-3β, and p-GSK-3β in the cells, while the reference protein used for the measurements was β-actin. Briefly, proteins were extracted using RIPA lysis buffer combined with phosphate-protease inhibitors followed by measuring the protein concentration of each sample. Then, SDS-PAGE was used to load 20 μg of proteins from each sample followed by blotting onto a polyvinylidene fluoride membrane. Blocking with 2% bovine serum albumin was performed before the addition of primary antibodies against the target proteins and overnight incubation. Finally, HRP-conjugate secondary antibodies were incubated with the membrane, and the signals were detected using enhanced chemiluminescence. Quantification of the expression rates of (b) VEGF, (c) ANG-1, (d) ANG-2, and (e) phospho-Ser9-GSK-3β. Data presented as mean ± SEM ($n = 2$). (**) means $P < 0.01$ and (****) means $P < 0.0001$.

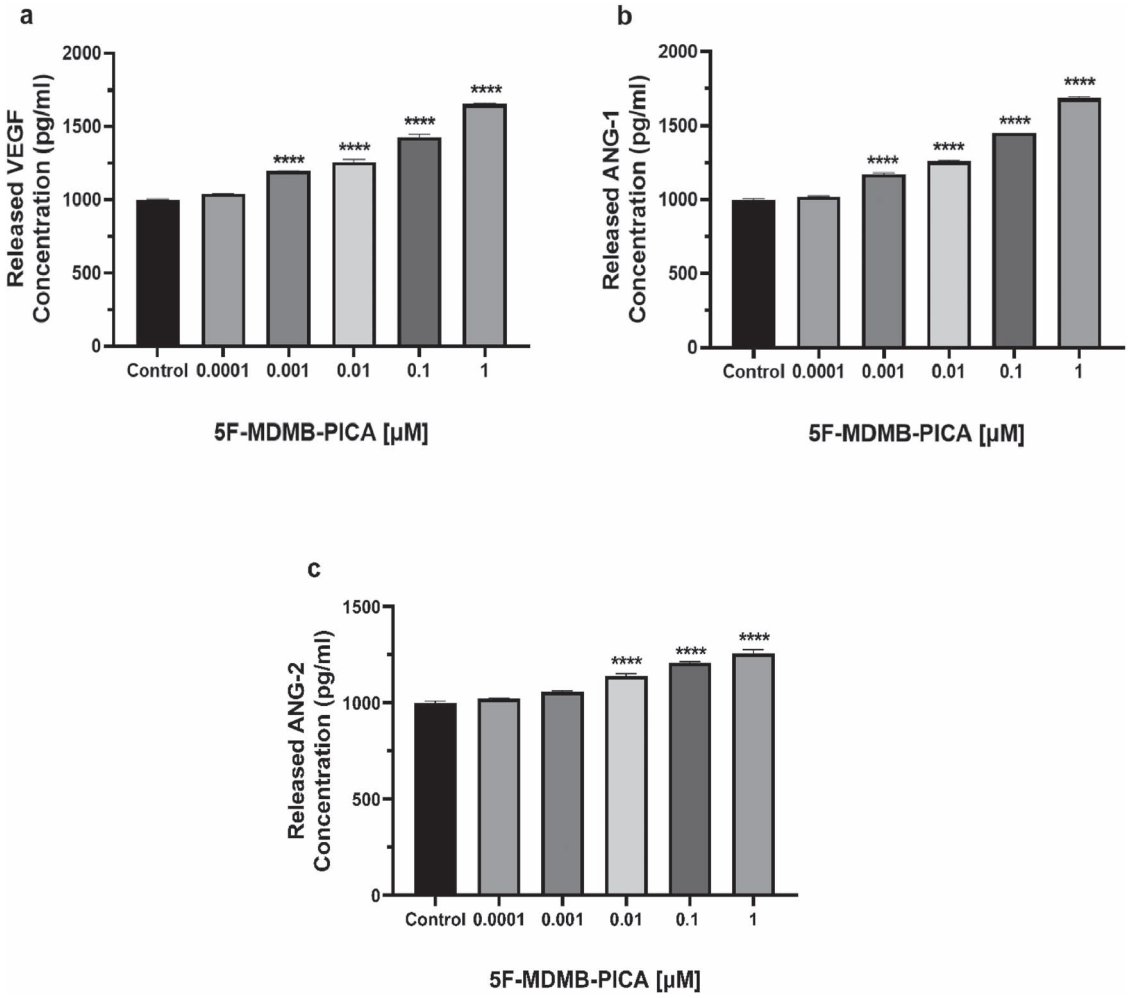

Fig. 8. 5F-MDMB-PICA treatment increases serum levels of VEGF, ANG-1, and ANG-2. ELISA was performed on 5F-MDMB-PICA treated HBMECs (0.0001 μM–1 μM) to measure secretion levels of proangiogenic factors VEGF, ANG-1, and ANG-2 in the media. Conditioned media from wells treated with concentrations had significantly higher secreted angiogenic factor levels compared to the control (a-c). Data presented as mean ± SEM ($n = 3$). (****) means $P < 0.0001$.

endothelial cells through the Tie-2 receptor, which is expressed on endothelial cell surfaces. ANG-1 is primarily responsible for vessel maturation and stability. It promotes the recruitment of pericytes and smooth muscle cells to the developing blood vessels, providing structural support and preventing vessel regression. ANG-1/Tie-2 signaling also promotes the formation of tight and stable cell-to-cell junctions in endothelial cells, essential for maintaining the integrity of blood vessels.[38] In contrast, ANG-2 functions as a context-dependent antagonist or agonist of Tie-2 signaling. It is produced in quiescent or destabilized vessels and can weaken endothelial cell–cell junctions. ANG-2 allows for the loosening of cell-to-cell connections, promoting endothelial cell responsiveness to other proangiogenic signals like VEGF. This loosening of cell junctions is essential for the initiation of sprouting and new vessel formation in response to proangiogenic signals.[38] XLR-11 has been observed to increase the expression of VEGF, ANG-1, and ANG-2 at the mRNA level, along with their release into the media and intracellular expression in HBMECs.[39] In the current investigation, we discovered that 5F-MDMB-PICA administration substantially increased the expression of VEGF, ANG-1, and ANG-2 in HBMECs following cannabinoid receptor activation. These findings imply that angiogenic-associated proteins, such as VEGF, ANG-1, and ANG-2, could have a significant role in promoting the angiogenesis associated with cannabinoid receptor activation in the brain.

Activation of CB1R by cannabinoids, whether endocannabinoids or exogenous cannabinoids, leads to downstream signaling events that can result in the inactivation of GSK-3β through Ser9 phosphorylation. Inactivating the IGF-1R/AKT/GSK-3β axis through the knockdown of CB1R and CB2 receptors inhibits migration and invasion in MCF-7 cells and T47D cells under chronic intermittent hypoxia conditions.[40] Moreover, electroacupuncture pretreatment provides protection against cerebral ischemia/reperfusion injury through CB1R-mediated phosphorylation of GSK-3β.[41] Arachidonyl-2-chloroethylamide induces mitochondrial biogenesis and improves mitochondrial function at the onset of cerebral ischemia, leading to the alleviation of cerebral ischemia injury. The phosphorylation of GSK-3β may play a role in the regulation of mitochondrial biogenesis induced by arachidonyl-2-chloroethylamide.[42] Furthermore, XLR-11 elevated the phosphorylation of GSK-3β at Ser9 in HBMECs.[39] Our findings indicate that the treatment with 5F-MDMB-PICA resulted in an increase in the phosphorylation of GSK-3β at Ser9 in HBMECs. Understanding the intricate roles of GSK-3β in angiogenesis may offer valuable insights into the development of novel therapeutic approaches for angiogenesis-related disorders.

5. Conclusion

Although there are many types of SCs, they are usually grouped based on their chemical structure, and 5F-MDMB-PICA falls under the indole group of SCs. Therefore, the results of this study could apply to the broader indole group of SCs. To the best of our knowledge, this is the first research conducted on 5F-MDMB-PICA and its physiological effects. Treatment of HBMECs with the SC 5F-MDMB-PICA increases the metabolic activity and angiogenic capacity of the HBMECs in vitro. These findings provide valuable insights into the effects of SCs on brain endothelial cells, which could contribute to the development of new drugs and the understanding of angiogenesis-related diseases. It is important to acknowledge that angiogenesis is a complex process involving numerous essential and regulatory molecules, some of which were not examined in this study, such as platelet-derived growth factors, FGF, and the NOTCH and WNT signaling pathways. The use of cannabinoid receptor antagonists will indeed help advance our understanding of the specific role these receptors play in angiogenesis. Future investigations focusing on the effects of other SCs in the indole group, as well as other groups, on various physiological and pharmacological parameters, are also recommended.

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Supplementary material

Supplementary material is available at *TOXRES Journal* online.

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Ethical approval

Not applicable.

Data availability

All data from this study are included in the published article and the Supplementary Information Files.

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