TRPV1 mediates cellular uptake of anandamide and thus promotes endothelial cell proliferation and network-formation

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ABSTRACT

Anandamide (N-arachidonyl ethanolamide, AEA) is an endogenous cannabinoid that is involved in various pathological conditions, including cardiovascular diseases and tumor-angiogenesis. Herein, we tested the involvement of classical cannabinoid receptors (CBRs) and the Ca²⁺-channel transient receptor potential vanilloid 1 (TRPV1) on cellular AEA uptake and its effect on endothelial cell proliferation and network-formation. Uptake of the fluorescence-labeled anandamide (SKM4-45-1) was monitored in human endothelial colony-forming cells (ECFCs) and a human endothelial-vein cell line (EA.hy926). Involvement of the receptors during AEA translocation was determined by selective pharmacological inhibition (AM251, SR144528, CID16020046, SB366791) and molecular interference by TRPV1-selective siRNA-mediated knock-down and TRPV1 overexpression. We show that exclusively TRPV1 contributes essentially to AEA transport into endothelial cells in a Ca²⁺-independent manner. This TRPV1 function is a prerequisite for AEA-induced endothelial cell proliferation and network-formation. Our findings point to a so far unknown moonlighting function of TRPV1 as Ca²⁺-independent contributor/regulator of AEA uptake. We propose TRPV1 as representing a promising target for development of pharmacological therapies against AEA-triggered endothelial cell functions, including their stimulatory effect on tumor-angiogenesis.

KEY WORDS: transient receptor potential vanilloid 1, TRPV1, anandamide, AEA, endothelial colony-forming cells, ECFC, anandamide transport, proliferation, network-formation, angiogenesis

INTRODUCTION

Anandamide (AEA; N-arachidonyl ethanolamide Sanskrit for bliss) is the most prominent and most extensively studied endocannabinoid. AEA activates distinct G-protein coupled receptors (GPR), known as cannabinoid receptors (CBRs), including CB₁R, CB₂R and GPR55 as well as the Ca²⁺-channel transient receptor potential vanilloid 1 (TRPV1) causing multiple biological effects on different tissues (Howlett et al., 2002; Pertwee et al., 2010; Galve-Roperh et al., 2013). Exemplarily, AEA mediates neuronal regulation, inflammatory response (Howlett et al., 2002; Pertwee et al., 2010) and cardiovascular effects including the dilatation of blood vessels, cardio protection after cardiac ischemia/infarction and tumor-angiogenesis (Deutsch et al., 1997; Wagner et al., 1997; Pisanti et al., 2011). Importantly, because these receptors have been recently found to be functionally localized intracellularly (Rozenfeld and Devi, 2008; Brailoiu et al., 2011; den Boon et al., 2012; Fowler, 2013), the cellular uptake mechanisms of AEA obviously gained importance for the physiological function of this endocannabinoid. Since essential cellular mechanisms comprising how endocannabinoids bypass the plasma membrane remain unresolved the development of pharmacological therapies is hampered (McFarland and Barker, 2004; Fowler, 2013).

Evidence for different hypothetic AEA translocation mechanisms have been reported ranging from involvement of a putative transporting protein called fatty acid amid hydrolase (FAAH) to FAAH-independent facilitated or even passive diffusion (Hillard and Campbell, 1997; Glaser et al., 2003; Fegley et al., 2004; McFarland and Barker, 2004; Fowler, 2013; Björklund et al., 2014). In these studies, a compound called AM404 was originally described to be an endogenous cannabinoid reuptake inhibitor (Costa et al., 2006). However, subsequent data have been inconclusive and rose doubts whether an AEA transporter even existed (Glaser et al., 2003; Fegley et al., 2004). Not the least these doubts arose because the AM404 effect could not uniquely be assigned to FAAH inactivation, but inhibition of cyclooxygenase (Fowler, 2013; Björklund et al., 2014) and TRPV1 Ca²⁺-channeling function (Höggestätt et al., 2005).

TRPV1 is a tetramer protein each subunit composed of six transmembrane spanning domains and is known to contribute to acute and persistent pain (Caterina et al., 1997; Starowicz et al., 2007; Basbaum et al., 2009). Up to now it is assumed that AEA binds to the intracellular face of the capsaicin receptor TRPV1 leading to opening of the Ca²⁺- permeable channel pore (De Petrocellis et al., 2001; van der Stelt et al., 2005). Therefore, TRPV1 has been used as a tool to indirectly monitor intracellular AEA and its uptake based on increasing cytoplasmic Ca²⁺-levels (De Petrocellis et al., 2001; Ligresti et al., 2010). However, this notion has been recently challenged by evidence showing that TRPV1 could be activated at the outer pore by a bivalent tarantula toxin (Bohlen et al., 2010). Thrillingly, two reports published back to back have subsequently refined structural analysis of TRPV1 using electron cryo-microscopy revealing a hydrophobic binding pocket for capsaicin and AEA that is accessible from the extracellular side (Cao et al., 2013; Liao et al., 2013), thus indicating that these compounds access TRPV1 from the outside.
Based on the intracellular location of the endocannabinoid receptors (Rozenfeld and Devi, 2008; Brailoiu et al., 2011; den Boon et al., 2012; Fowler, 2013), the AEA transporter might represent a bottleneck for AEA action and, therefore, provides a promising target for the development of pharmacological therapies for various AEA-related function in the progression of diseases. It has been reported that AEA is involved in tumor-angiogenesis and can be produced in different sources of endothelial progenitor cells (EPCs) including human peripheral blood, umbilical cord and aortic derived endothelial cells (Optiz et al., 2007; Pisanti et al., 2007; Pisanti et al., 2011). The so called vessel wall-derived endothelial colony-forming cells (ECFCs) are a subtype of EPCs that have a high clonogenic and proliferation potential and show a robust vessel-forming capacity in vivo (Ingram et al., 2005; Yoder et al., 2007; Reinisch et al., 2009). These characteristics make ECFCs a favorable cellular tool to study the potential influence of AEA on cell behavior and yield a promising target for pro- and anti-angiogenic therapies.

In the present study a fluorescence-labeled analogue of AEA (SKM4-45-1) (Muthian et al., 2000) was used to monitor the AEA uptake into ECFCs and the immortalized human endothelial vein cell line (EA.hy926). The involvement of CB1R, CB2R, GPR55 and TRPV1 during AEA translocation was determined by using pharmacological and genetic inhibition and overexpression, respectively. We describe herein that exclusively TRPV1 but not GPR55 and TRPV1 during AEA translocation was determined by

**RESULTS**

**Revealing the involved receptor for AEA uptake in endothelial cells**

To allow a quantifiable visualization of the cellular uptake of AEA in real time, the cellular accumulation of SKM4-45-1, a labeled non-fluorescent analogue of AEA that is cleaved by intracellular esterases and becomes fluorescent (Muthian et al., 2000) (supplementary material Fig. S1A) was measured fluorometrically in human ECFCs and the human umbilical vein endothelial cell-derived cell line EA.hy926. ECFCs accumulate SKM4-45-1 in a time- and concentration-dependent manner (Fig. 1A,B) and concentration- (Fig. 1C) dependent manner. This findings highlight the importance of TRPV1 for AEA uptake and its subsequent cellular function and presents TRPV1 as essential regulator of the AEA-induced pro-angiogenic effects. Therefore, our study suggests TRPV1 to be a promising therapeutic target for various AEA-related pathologies such as cardiovascular diseases and tumor-angiogenesis.

**TRPV1 drives AEA uptake in endothelial cells**

Next, we evaluated whether the observed TRPV1-mediated AEA uptake is genuinely affected by capsaicin binding to the recently

**Fig. 1.** Endothelial colony-forming cells (ECFCs) accumulate the anandamide (AEA) analog SKM4-45-1 in a time and concentration dependent manner. (A) Representative images of phase contrast (upper images) and SKM4-45-1-derived fluorescence in ECFCs (green, lower images) at incubation time-points 0, 10, 20 and 30 min (at 20× magnification). (B) Kinetics of SKM-45-1 uptake in ECFCs. Increase in fluorescence was documented every 10 sec throughout 30 min. (n=8; ± SEM). (C) Concentration response of SKM4-45-1 uptake in ECFCs. Increase in fluorescence intensity with various concentrations of SKM4-45-1 (0.1, 0.3, 1, 3, 10, 30 μM) after 30 min (± SEM) of incubation in % of the maximal fluorescence intensity is blotted (n=3).
described extracellular hydrophobic binding site (Cao et al., 2013; Liao et al., 2013). Upon simultaneous application of SKM4-45-1 (1 μM) with capsaicin (0.1 μM) the accumulation of SKM4-45-1 in ECFCs and EA.hy926 was significantly reduced by 66.8 ± 3.6% and 61.2 ± 4.6%, respectively (Fig. 3A; supplementary material Fig. S2A). To confirm the data obtained with the TRPV1 antagonist SB366791, TRPV1 expression was diminished by transient transfection with respective siRNA. Efficiency of siRNA against TRPV1 expression was depicted by quantitative real time (RT)-PCR (Fig. 4A) and western blot (Fig. 4B). In fact, TRPV1-RFP transfected cells showed a strong increase in SKM4-45-1 uptake by 219.4 ± 26.9% as compared to empty vector controls (Fig. 4C,D). A similar effect was also observed with TRPV1 overexpression in the human cancer cell lines, HeLa and Hek293 (supplementary material Fig. S3A). However, the TRPV1 inhibitor SB366791 prevented TRPV1-RFP transfected EA.hy926 to perform a significant increase of SKM4-45-1 uptake as compared to controls (Fig. 4E). In order to determine the influence of Ca²⁺ that is carried by TRPV1 on the contribution of TRPV1 to AEA uptake, SKM4-45-1 internalization was measured in nominal Ca²⁺-free (i.e. EGTA buffered) medium. Removal of extracellular Ca²⁺ did not affect SKM4-45-1 uptake, thus, pointing to a capsaicin-sensitive but Ca²⁺-independent engagement of TRPV1 to AEA uptake (supplementary material Fig. S3B).

**TRPV1-mediated AEA uptake promotes endothelial cell proliferation and network-formation**

To explore whether or not the observed contribution of TRPV1 for AEA uptake is of physiological relevance, we investigated whether the observed TRPV1-mediated uptake of AEA is involved in the two major vascular functions of AEA, proliferation and network-formation. Therefore, ECFC proliferation and network-formation were tested and the contribution of TRPV1 was determined. Both, AEA (1 μM) as well as SKM4-45-1 (1 μM) were equipotent in promoting ECFC proliferation within 48 h by 69.7 ± 4.7% and 48.3 ± 4.3%, respectively (Fig. 5A). AEA stimulated ECFC proliferation in a concentration-dependent manner with an EC₅₀ of 0.05 (0.04 to 0.08) μM (Fig. 5B). Notably, at a concentration of ≥10 μM AEA ECFCs did not proliferate within 48 h. To evaluate the involvement of TRPV1 during the AEA-induced (1 μM) proliferation increase, the TRPV1 inhibitor SB366791 (10 μM) was applied and proliferation. Within 48 h inhibition of TRPV1 counteracted the stimulatory effect of AEA (1 μM) on ECFC proliferation by 87.1 ± 13.2% (Fig. 5C). Genetic knock-down of TRPV1 in ECFC by a transient transfection with the respective siRNA showed a similar inhibitory effect on proliferation (Fig. 5D) and reversed the effect of AEA on ECFC proliferation (Fig. 5D). The TRPV1 agonist capsaicin (0.1 μM) increased ECFC proliferation by itself (app. 56.1 ± 5.6% of the effect of 1 μM AEA) while it prevented AEA-induced ECFC proliferation (supplementary material Fig. S4A).

Moreover, ECFC network-formation was tested on growth factor-reduced matrigel (Reinsch et al., 2009; Hofmann et al., 2012) under the influence of the TRPV1 antagonists SB366791. Within 12 h AEA (1 μM) significantly increased branch-point formation of ECFCs by 57.9 ± 1.5% as compared to the vehicle controls (Fig. 6A,B). Simultaneous treatment of ECFCs with AEA (1 μM) and the TRPV1 antagonist SB366791 (10 μM) prevented the stimulatory effect of AEA on ECFC network-formation (Fig. 6B), whereby SB366791 alone had no effect on network-formation.

**DISCUSSION**

Moonlighting of a protein describes the performance of an additional, unrelated function apart from its established one. It has long been known that the endocannabinoid AEA activates the capsaicin receptor TRPV1 and leads to an increase of intracellular Ca²⁺-levels (Ralevic et al., 2001; van der Stelt et al., 2005;...
debate whether the observed pathological effects of AEA are ischemia/infarction) or tumor-angiogenesis. It is a matter of vessel formation (e.g. pulmonary hypertension, cardiac conditions including cardiovascular diseases requiring new derived angiogenesis.

AEA was the first endocannabinoid to be discovered (Devane et al., 1992; Liao et al., 2013) extracellular hydrophobic pocket of that both substances compete for the same recently described (Cao et al., 2008; Ligresti et al., 2010) extracellular hydrophobic pocket of internalization of SKM4-45-1 in our experiments, thus, indicating uptake in endothelial cells and their progenitors. Consistently, shown that AEA and SKM4-45-1 competed for the same binding effect detected at 1 μM AEA, verifying the functional equality of SKM4-45-1 with AEA. Other studies (Muthian et al., 2000) have putative endocannabinoid orphan receptor GPR55 and TRPV1. The individual role of these receptors in AEA-mediated vascular function of ECFCs like cell proliferation and network-formation has not been investigated so far but might be of particular importance for the exploration of the therapeutic potential of the endocannabinoid system in regard of endothelial cell-vascular repair and neovascularization.

AEA has been shown to activate TRPV1 leading to an elevation of cytoplasmic Ca2+-levels (De Petrocellis et al., 2001; Ralevic et al., 2001; van der Stelt et al., 2005; Starowicz et al., 2007; Ligresti et al., 2010; Blumberg et al., 2011; Liao et al., 2013). Hence, it has been reported that depending on the integrin clustering status AEA-mediates different endothelial responses (Waldeck-Weiermair et al., 2008) while AEA failed to trigger cellular Ca2+ responses in the presence of extracellular Ca2+ in the EA.hy926 cells although this cell type expresses large amount of TRPV1 (Fig. 2A). In our present study we observed a concentration-dependent uptake of the AEA-analogue SKM4-45-1 in human endothelial colony-forming cells (ECFCs) and in the human umbilical vein endothelial cell-derived cell line EA.hy926 cells. Apparently, both AEA as well as SKM4-45-1 increase ECFC proliferation with a maximum pro-proliferative function of ECFCs like cell proliferation and network-formation has been investigated so far but might be of particular importance for the exploration of the therapeutic potential of the endocannabinoid system in regard of endothelial cell-vascular repair and neovascularization.

Quality of images: Yes

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Quality of interpretation: Yes

Quality of conclusions: Yes
TRPV1. An additional explanation could be a desensitization of TRPV1 during capsaicin treatment leading to a reduced SKM4-45-1 uptake, however, the genuine mechanism must further be evaluated. Nevertheless, our data point to capsaicin as a potent inhibitor of AEA uptake, a phenomenon that might explain the inhibitory effect of capsaicin on tumor angiogenesis and growth (Min et al., 2004). Blocking of the classical cannabinoid receptors CB1R and CB2R or orphan receptor GPR55 with respective selective inhibitors (AM251 (Gatley et al., 1996; Pertwee et al., 2010), SR144528 (Rinaldi-Carmona et al., 1998) and CID16020046 (Kargl et al., 2013), respectively) had no significant effect on SKM4-45-1 uptake, however, the genuine mechanism must further be evaluated. Nevertheless, our data point to capsaicin as a potent inhibitor of AEA uptake, a phenomenon that might explain the inhibitory effect of capsaicin on tumor angiogenesis and growth (Min et al., 2004).

Recently, by describing an inhibitory effect of the fatty acid amide hydrolase (FAAH) inhibitor methyl arachidonyl fluorophosphates (MAFP) (De Petrocellis et al., 1997; Martin et al., 2000; Glaser et al., 2003) on the AEA uptake of neuroblastoma and astrocytoma cells, the importance of FAAH activity for AEA uptake was demonstrated. However, in accordance to other reports (Högstätt et al., 2005; Costa et al., 2006), in the present work the FAAH inhibitor MAFP had no effect on SKM4-45-1 uptake, thus accumulation of SKM4-45-1 in ECFCs and EA.hy926 cells appears independent of FAAH activity. In contrast, cellular accumulation of SKM4-45-1 could be circumvented when applying the pan transport inhibitor AM404 that has been proposed to effect FAAH activity (Högstätt et al., 2005; Costa et al., 2006). However, AM404 was also described as TRPV1 agonist, like capsaicin (Högstätt et al., 2005). In view of this report and our presented findings that capsaicin prevented SKM4-45-1 uptake, we speculate that AM404 inhibits SKM4-45-1 uptake either by a competitive mechanism (AEA-like action) and/or as a TRPV1 agonist (capsaicin-like action).

The crucial contribution of TRPV1 to AEA/SKM4-45-1 uptake into endothelial cells was further supported by our findings on the reduced SKM4-45-1 uptake in cells with diminuend TRPV1 expression while in cells overexpressing TRPV1 SKM4-45-1 uptake was boosted. Since removal of extracellular Ca2+ did not affect SKM4-45-1 uptake, the contribution of TRPV1 to AEA uptake appears independent from Ca2+. This is in contrast to our previous findings on the interplay between CB1R and GPR55 in the endothelial cell line EA.hy926 where removal of extracellular Ca2+ uncovers GPR55-dependent Ca2+ signaling upon AEA due to clustering of integrins that relieve the GPR55 from the inhibitory effect of the CB1R-signaling cascade (Waldeck-Weiermair et al., 2008). In addition to the interaction between two other AEA surface receptors, the present study reveals a

**Fig. 4.** Overexpression of TRPV1 leads to an increase in SKM4-45-1 uptake in human endothelial cells. (A) Verification of TRPV1 mRNA overexpression in EA.hy926 cells. Cells were transfected with a TRPV1 encoding plasmid conjugated to a red fluorescence protein (TRPV1-RFP) or an empty vector control. Effectiveness of overexpression was verified by RT-PCR (left panel) and quantitative real time (q) PCR (right panel). Values show % TRPV1 corrected for GAPDH as compared to empty vector (vector control) (n=3; ± SEM); *** indicates p < 0.001 significance as compared to control. (B) Verification of TRPV1 protein overexpression in EA.hy926 cells. Effectiveness of TRPV1-RFP overexpression was verified by western blot using antibodies against TRPV1 and β-Actin, as indicated, and quantified by ImageJ (right panel). Values show % TRPV1 corrected for β-Actin as compared to untreated and empty vector controls (n=3; ± SEM); *** indicates p < 0.001 significance as compared to control. (C) Single cell fluorescence images of SKM4-45-1 accumulation in control EA.hy926 cells and cells overexpressing TRPV1-RFP (at 100× magnification). RFP expression (left panel; red signal) and SKM4-45-1 uptake (middle panel; green signal) was measured after 30 minutes incubation and single channel pictures where overlaid (right panel). (D) Columns represent SKM4-45-1 accumulation in untransfected EA.hy926 cells, vector control cells and cells overexpressing TRPV1-RFP. SKM4-45-1 uptake was measured after 30 minutes incubation. Values show % fluorescence as compared to untransfected controls (n=3; ± SEM). *** indicates p < 0.001 significance as compared to untransfected control. (E) Columns represent SKM4-45-1 accumulation in control EA.hy926 cells, cells overexpressing TRPV1 in the presence of SB366791. SKM4-45-1 uptake in untransfected, empty vector control transfected and TRPV1-RFP transfected EA.hy926 was measured after 30 minutes simultaneous incubation of 1 mM SKM4-45-1 with 10 μM SB366791. Values show % fluorescence as compared to untransfected controls (n=3; ± SEM). There is no significant difference to untransfected controls. # indicates p ≤ 0.01 significant decrease as compared to TRPV1-RFP transfected cells without SB366791 treatment.

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The involvement of the endocannabinoid system during cancer formation and tumor-induced angiogenesis is jet unresolved moonlighting function of TRPV1 as contributor/regulator to/of AEA transport into endothelial cells.

Our present findings, along with the report of J. M. Rehman team (Opitz et al., 2007), who described that ECFCs release substantial amounts of AEA, allow us to hypothesize on a key function of AEA in the control of endothelial cells. At concentrations larger than 10 μM, AEA exhibited anti-proliferative and anti-angiogenic effects in human umbilical vein endothelial cells (HUVECs) and inhibited tumor-induced angiogenesis in a 3D tumor-angiogenesis model in vitro (Pisanti et al., 2007; Guindon and Höhmann, 2011). In accordance with these reports, in the present study, AEA at concentrations ≥10 μM failed to trigger ECFC proliferation and the cells subsequently detached. In contrast, it has been reported that AEA at concentrations 1 μM increases HUVECs proliferation and, via activation of CB1R initiates neovascularization in a mouse model of oxygen-induced retinopathy (Pisanti et al., 2011). Consistent with these reports, we see that low AEA concentrations (0.01–1 μM) resemble levels of endogenously produced endocannabinoids, also increased ECFC proliferation and network-formation. Hence, at least to some reports in the literature, the contradicting data arguing between a pro- (Pisanti et al., 2011) and anti-angiogenic (Pisanti et al., 2007) effect of AEA could in part result from cell specific differences in the expression levels/patterns of TRPV1 and the CBRs between ECFCs. Alternatively, our data suggest that the discrepancy to the previously described effects of AEA on angiogenesis could be a consequence of different AEA concentrations. In our studies we see a significant pro-proliferative and pro-angiogenic effect of AEA in concentrations 1 μM in ECFCs. These findings highlight the potential importance of the endocannabinoid system in activation of ECFCs to establish network/angiogenesis.

The data presented herein show that the effect of AEA on ECFC proliferation and network-formation essentially depends on TRPV1. Notably, the AEA-induced increase of ECFC proliferation correlated with active TRPV1 and its effect could be reversed in TRPV1 knock-down ECFCs. Further, inactivation of TRPV1 also prevented the AEA-induced branch point formation. In both assays ECFCs where strongly effected by inhibition of TRPV1 activity. Taken together our data strongly support the new paradigm that AEA might promote endothelial
cell-established angiogenesis in vivo and therefore could participate in tumor-angiogenesis (Folkman, 1995; Pisanti et al., 2011).

Notably, elevated levels of AEA have been found in human glioblastomas and meningiomas (Petersen et al., 2005) and blocking of CB$_2$R has been shown to inhibit tumor growth and glioblastomas and meningiomas (Petersen et al., 2005) and therefore could participate in tumor-angiogenesis (Folkman, 1995; Pisanti et al., 2007; Pisanti et al., 2011).

Accordingly, it is tempting to speculate that (certain) tumor cells might produce and release high levels of AEA to attract endothelial cells and subsequently lead to tumor-angiogenesis. Our present data suggest that the angiogenic effect of tumor-derived AEA on endothelial cells essentially involves TRPV1-mediated uptake of AEA, subsequently followed by activation of intracellular AEA receptors (Rozenfeld and Devi, 2008; Brailoiu et al., 2013) including the gels were taken with a gel documentation system (GenoSmart, VWR). PCR-primers were chosen to span at least one exon/intron boundary to minimize amplification of contaminating genomic DNA. Primer sequences were as follows: TRPV1, forward 5'-GGATCGATATGAAGAAATGGAGCAGCAC-3' and reverse 5'-GAAGCGG-3', which adds a ClaI restriction site and TRPV1_rev 5'-AAAAGCCTTCCTTCCTCCCGGAAAGG3'-3', which adds a Stopcodon and a HindIII restriction site. To obtain an N-terminal tagged TRPV1, the PCR product was subcloned into a RFP containing pcDNA3.1+ vector. EA.hy926 cells were transfected with protein constructs as described earlier. The efficiency of overexpression and of an appropriate negative control was approved by real-time, quantitative PCR and western blot.

**Isolation of total RNA and cDNA synthesis**

Total cellular RNA was extracted using the PegGOLD Total RNA Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and quantified spectrophotometrically at 260/280 nm. For cDNA preparation, 2 μg of total RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Gibco, Invitrogen, Vienna, Austria) including 1 μl RNasin® Plus RNase Inhibitor (Promega) following the manufacturer’s instructions.

**Conventional RT-PCR**

Conventional PCR was carried out in a personal Thermocycler (Primus 25 advanced, PEQLAB) using GoTaq® G2 Hot Start Green Master Mix (Promega, no. M7423) following the manufacturer’s instructions. PCR profiles were as follows: an initial denaturation step at 95°C for 2.5 min, followed by 27 cycles (β-actin) or 30 cycles (TRPV1) with 1 min denaturation (95°C), 30 s annealing (60°C for β-actin; 58°C for TRPV1) and 30 s elongation (72°C), and a final extension of 7 min (72°C). PCR products (20 μl) were separated by standard gel electrophoresis on 1.5% agarose gels at 70 V for 60 min in 1x TAE buffer containing 5 μl/ml Roti®-GelStain (Carl ROTH GmbH, no. 3865.1). Digitized UV-images of the gels were taken with a gel documentation system (GenoSmart, VWR). PCR-primers were chosen to span at least one exon/intron boundary to minimize amplification of contaminating genomic DNA. Primer sequences were as follows: TRPV1, forward 5’-CACGCGGAGGACATTTCCACATCTAC-3’ and reverse 5’-GCTGTCCCAACAAAAACGGGGTCT-3’ (amplicon 322 bp); β-actin, forward 5’-TCTACCACCTTCTCCGCAG3’ and reverse 5’-CACGCGGATACCAATGGCTA-3’.
(amplicon 294 bp); CB2, forward 5'-CCGCGATGAATCATCACC-3' and reverse 5'-TGCCCAAGCTTATCACGAC-3' (amplicon 354 bp); GPR55, forward 5'-GTCGCCACCTCTACATCATC-3' and reverse 5'-CGTCCAGGGTATCATCAGG-3' (amplicon 367 bp); and β-Actin (as housekeeping gene), forward 5'-GCAAGAGATGCTCACC-3' and reverse 5'-GCACAGCTTGATGACACG-3' (amplicon 240 bp). Band intensities were compared by ImageJ (National Institutes of Health, Bethesda, MD).

**Quantitative RealTime-PCR (qRT-PCR)**

The efficiency of siRNA knock-down as well as TRPV1 over-expression was quantified by real-time PCR. qRT-PCR was carried out in a Light Cycler 480 System (Roche, Basel, Switzerland) using the GoTaq® qPCR Master Mix (Promega; no. A6002) following the manufacturer’s instructions. Gene-specific primers used for qRT-PCR were as follows: TRPV1, forward 5'-GACCACCTGGAACACCAACG-3' (amplicon 177 bp), and QuantiTect® Primer Assay (Qiagen, Hilden, Germany) for human GAPDH (no. QT01192646) as housekeeping gene. Real-time amplification data were analyzed using the REST-MCS beta software version 2 [August 2006]. PCR reactions were examined visually for primer-dimers and other non-specific amplifications on 2% agarose gels.

**Western blot analysis**

Western blots were performed according to standard protocols. Cells were directly lyzed with 1× Laemmli sample buffer, heat denatured and resolved in a 4–20% SDS-PAGE (Bio-Rad) in parallel to full-range rainbow® molecular weight marker (GE Healthcare, Munich, Germany) and transferred onto a PVDF-membrane (Millipore). Specific proteins were detected using antibodies against CB1R (Abcam), CB2R (Thermo Scientific), GPR55 (Thermo Scientific) and TRPV1 (Cell Signaling) antibodies compared to housekeeping protein β-actin (Santa Cruz), followed by anti-mouse (Cell Signaling) or anti-rabbit (Abcam) HRP antibody and visualized with ‘super signal west pico lumino/ enhancer developing solution’ (Thermo Scientific).

**Anandamide uptake studies**

Three different ECFC donors and EA.hy926 cells were grown to 70% confluence in either 8-well Ibi碟 treated slides (Ibi, Planegg, Germany) or on glass cover slips (Ø = 30 mm), placed in a 6-well plate (Nunc). The fluorescent AEA analogue SKM-4-45-1 (Cayman Chemical Europe, Tallinn, Estonia) was applied to ECFCs and EA.hy926 in concentrations as indicated in conventional or Ca2+-free EGTA buffered medium. Fluorescence increase was documented with a Zeiss LSM 410 microscope (Zeiss, Jena, Germany) on 10× magnification by obtaining serial pictures every 10 seconds throughout 30 minutes. Single cell fluorescence increase was analyzed by VisiView® (Visiion Systems, Puchheim, Germany). Changes in SKM-4-45-1 uptake was detected similarly in EFCCs and EA.hy926 in the presence of anandamide (0.001–30 M), CID1602046 (20 μM), SB366791 (0.1 M), and Capsaicin (0.1 M) (all obtained from Tocris Bioscience, Bristol, UK). AM404 (10 μM), AM251 (0.1 μM), SR144528 (1 μM), Methyl Arachidonyl Fluorophosphonate (MAFP, 0.1 μM) (all obtained from Cayman Chemical Europe, Tallinn, Estonia) as well as after treatment with siRNA against TRPV1 and RFP-TRPV1. Changes in Fluo-4/AM (Life Technologies) fluorescence intensity were observed as described with and without SB366791 pre-treatment. High resolution imaging of 100× magnification of TRPV1 overexpressing EA.hy926 cells was performed using the same confocal microscope.

**Proliferation assay**

Three different ECFC donors were seeded in 6-well plates (Nalge Nunc, Rochester, NY) in EGM-2 at a density of 3,000/cm2 and allowed to adhere for 24 hours. Subsequently, cells were subjected to growth factor reduced medium with or without AEA and different endocannabinoid receptor agonists and antagonists as indicated. The substances anandamide, CID1602046, SB366791 and Capsaicin were obtained by Tocris Bioscience (Northpoint, Avonmouth, Bristol, UK). SKM4-45-1, AM251, SR144528 (Cayman Chemical Europe, Tallinn, Estonia). After 48 hours treatment cells were harvested and cell numbers analyzed by a Casy cell counter (Roche, Mannheim, Germany).

**In vitro angiogenesis assay**

Capillary-like network formation of three different ECFC donors on growth factor reduced Matrigel® (BD, Biosciences, San Jose, CA, USA) was performed according to instruction manual. Influence of different endocannabinoid receptor agonists and antagonists was tested as indicated. Network formation (12–14 hours) was documented with a Nikon Coolpix 4500 camera on a Nikon TMS-F microscope (Nikon, Amsterdam, Netherlands). Branch-points were counted by ImageJ (National Institutes of Health, Bethesda, MD).

**Statistics**

All experiments were performed with three donors of ECFCs and the EA.hy926 cells in at least triplicate. n values refer to number of experimental days. Data were compared using ANOVA and subsequent Bonferroni post hoc test or two-tailed Student’s t-test assuming unequal variances. Statistical significance was assumed when p<0.05. EC50 values are given as the mean and the 95% confidential interval in parenthesis.

**List of abbreviations**

AEA, anandamide; CBRs, cannabinoid receptors; TRPV1, transient receptor potential vanilloid 1; EA.hy926, human endothelial vein cell line; ECFCs, endothelial colony-forming cells; FAAH, fatty acid amide hydrolase; GPR, G-protein coupled receptors; MAFP, methyl arachidonyl fluorophosphonate.

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**Competing interests**

The authors have no competing interests to declare.

**Author contributions**

N.A.H. performed experiments on SKM4-45-1 uptake, cell proliferation and network-formation, analyzed, designed the study and wrote the manuscript; S.B. and M.W.-W. performed and analyzed the PCR experiments; M.W.-W. together with C.K. designed the respective primers. D.S. provided the ECFCs; R.M. analyzed the experiments and W.F.G. designed the study and wrote the manuscript.

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