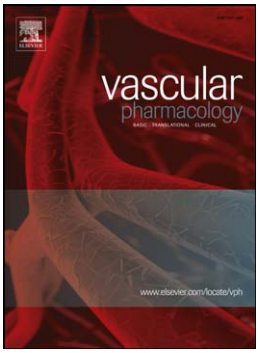

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Arachidonic acid-evoked Ca^{2+} signals promote nitric oxide release and proliferation in human endothelial colony forming cells

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ABSTRACT

Arachidonic acid (AA) stimulates endothelial cell (EC) proliferation through an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), that, in turn, promotes nitric oxide (NO) release. AA-evoked Ca^{2+} signals are mainly mediated by Transient Receptor Potential Vanilloid 4 (TRPV4) channels. Circulating endothelial colony forming cells (ECFCs) represent the only established precursors of ECs. In the present study, we therefore, sought to elucidate whether AA promotes human ECFC (hECFC) proliferation through an increase in $[Ca^{2+}]_i$ and the following activation of the endothelial NO synthase (eNOS). AA induced a dose-dependent $[Ca^{2+}]_i$ raise that was mimicked by its non-metabolizable analogue eicosatetraynoic acid. AA-evoked Ca^{2+} signals required both intracellular Ca^{2+} release and external Ca^{2+} inflow. AA-induced Ca^{2+} release was mediated by inositol-1,4,5-trisphosphate receptors from the endoplasmic reticulum and by two pore channel 1 from the acidic stores of the endolysosomal system. AA-evoked Ca^{2+} entry was, in turn, mediated by TRPV4, while it did not involve store-operated Ca^{2+} entry. Moreover, AA caused an increase in NO levels which was blocked by preventing the concomitant increase in $[Ca^{2+}]_i$ and by inhibiting eNOS activity with NG-nitro-L-arginine methyl ester (L-NAME). Finally, AA per se did not stimulate hECFC growth, but potentiated growth factors-induced hECFC proliferation in a Ca^{2+}- and NO-dependent manner. Therefore, AA-evoked Ca^{2+} signals emerge as an additional target to prevent cancer vascularisation, which may be sustained by ECFC recruitment.

1. INTRODUCTION

Arachidonic acid (AA) is a 20-carbon omega-6 polyunsaturated fatty acid (PUFA) which is stored in the plasma membrane esterified to glycerol in phospholipids at the sn-2 position and carries out many structural, signalling and homeostatic functions. Upon cell stimulation, AA is released from the membrane pool by the activation of three different phospholipases, A2, C and D, to exert either an autocrine and/or a paracrine effect on neighbouring cells. AA may further be converted by the catalytic activities of three enzyme families: cyclooxygenases (COX), which produce prostaglandins, prostacyclins and thromboxanes; lipoxygenases (LOX), that generate leukotrienes, and cytocrome P450 monooxygenases (CYP450), yielding multiple epoxyeicosatrienoic (EETs) and hydroperoxyeicosatetraenoic acids [1, 2]. AA and its metabolic derivates, collectively known as eicosanoids, have long been known to promote endothelial cell (EC) proliferation, migration, and *in vitro* tubulogenesis [1-5]. AA stimulates angiogenesis by causing an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular ECs, the ensuing Ca^{2+} signal being then decoded by a number of Ca^{2+}-dependent effectors, pivotal among which is the Ca^{2+}/calmodulin (Ca/CaM)-dependent endothelial nitric oxide (NO) synthase (eNOS) [2, 6, 7]. AA-induced Ca^{2+} signals in ECs are mainly mediated by Transient Receptor Potential Vanilloid 4 (TRPV4) channel [8-13], a polymodal Ca^{2+}-permeable pathway that mediates a pro-angiogenic influx of Ca^{2+} in ECs from several vascular trees [14, 15]. Likewise, endothelial TRPV4 channels may be gated by several CYP450A-derived AA metabolites, such as 5,6-EET and 8,9-EET [16-19]. The Ca^{2+} response to AA in other cellular models is more complex and involves additional pathways, including: inositol-1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) and ryanodine receptors (RyRs) [20-23], that release Ca^{2+} from the endoplasmic reticulum (ER), the most abundant endogenous Ca^{2+} reservoir; the acidic Ca^{2+} stores of the endolysosomal (EL) system [22]; AA-regulated Ca^{2+} (ARC) channels and leukotriene C4 (LTC4)-regulated Ca^{2+} (LRC) channels, that are contributed from heteromers of the novel Ca^{2+}-permeable channels Orai1 and Orai3 and are gated, respectively, by AA itself and its LOX-dependent metabolite LTC4 [24, 25]. A recent study showed that Orai3 may mediate AA- and LTC4-dependent Ca^{2+} inflow also in human vascular ECs [26], albeit it is not constitutively expressed on the plasma membrane.

Unlike fully differentiated ECs, the pro-angiogenic effect exerted by AA on their more immature precursors is unclear. Endothelial progenitor cells (EPCs) are mobilized either from the bone marrow (BM) or the arterial wall to replace senescent/damaged ECs and rescue local blood perfusion in ischemic tissues [27-29]. Furthermore, EPCs play a key role in carcinogenesis by sustaining the early

phases of the angiogenic switch in a growing number of solid tumors [30-32]. Several approaches have been developed to select and expand circulating EPCs e*x vivo* from peripheral and umbilical cord blood. These strategies combined proper cell culture protocols, clonogenic and functional assays, and the careful examination of endothelial/hematopoietic surface antigens to identify three distinct EPC subsets: colony forming units-ECs (CFU-ECs), circulating angiogenic cells (CACs) and endothelial colony forming cells (ECFCs) [27-29]. However, it has now been established that CFU-ECs and CACs belong to the hematopoietic lineage, are unable to acquire an endothelial phenotype, do not show any appreciable clonogenic potential and stimulate angiogenesis *in vivo* in a paracrine manner. Conversely, there is now general agreement that ECFCs represent true endothelial progenitors by differentiating into mature ECs and originating patent vessels *in vivo*. Moreover, ECFCs display a great proliferative potential and replate into secondary and tertiary colonies [33]. Finally, ECFCs may reside within the intima of human umbilical vein, pulmonary artery and aortic vessel walls, thus contributing to preserve vascular integrity [28]. Recent work from our group has unveiled a central role for Ca^{2+} signalling in the intricate network of pathways that drive ECFC activity [29, 34]. In particular, we found that store-operated Ca^{2+} entry (SOCE) controls proliferation and *in vitro* tubulogenesis in circulating ECFCs by recruiting either the Ca^{2+}-dependent transcription factor NF-κB [35] or the Ca^{2+}/calmodulin (CaM)-dependent eNOS [36]. However, ECFCs also express TRPV4 which promotes the proliferation process when acting in concert with extracellular growth factors [37]. Intriguingly, AA may be released by BM stromal cells to regulate proliferation, survival and differentiation in hematopoietic cells [38-40]. This leaves open the possibility that AA influences other cell constituents of BM microenvironment, such as EPCs [39]. Moreover, vascular wall-resident hECFCs could be activated by free AA contained in human plasma [41], whose concentration (5.3-13.1 μM) may increase up to the mid-micromolar range in response to platelet activation [42], physical exercise [43], inflammation [44], and tissue ischemia [45, 46].

Therefore, we endeavoured the present investigation to assess whether AA elicits pro-angiogenic Ca^{2+} and NO signals in human ECFCs (hECFCs). To achieve these aims, we exploited Ca^{2+} and NO live cell imaging, real-time reverse transcriptase polymerase chain reaction (qRT-PCR), and proliferation assays. We found that AA elicits a dose-dependent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in hECFCs that impinges on the concerted recruitment of ER-embedded $InsP_3Rs$, nicotinic acid adenine dinucleotide phosphate (NAADP)-sensitive two pore channel 1 (TPC1) on the EL stores and TRPV4 channels on the plasma membrane. The elevation in $[Ca^{2+}]_i$ evoked by AA, in turn, led to a robust NO production, which seems to require the concomitant activation of all the

Ca^{2+}-permeable routes involved in the signal. Finally, we showed that AA induces hECFC proliferation in a Ca^{2+}- and NO-dependent manner. These findings support an important role for AA in stimulating hECFC growth and hit at this lipid mediator as an alternative tool to improve the outcome of therapeutic angiogenesis and as an alternative target for anti-cancer treatments.

2. Material and methods

2.1 Isolation and cultivation of endothelial colony forming cells

Blood samples (40 ml) collected in EDTA (ethylenediaminetetraacetic acid)-containing tubes were obtained from healthy human volunteers aged from 22 to 28 years old. The Institutional Review Board at "Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo Foundation" in Pavia approved all protocols and specifically approved this study. Informed written consent was obtained according to the Declaration of Helsinki of 1975 as revised in 2008. We focussed on the so-called endothelial colony forming cells (ECFCs), a subgroup of EPCs which are found in the $CD34^+$ $CD45^-$ fraction of circulating mononuclear cells, exhibit robust proliferative potential and form capillary-like structures *in vitro* [33]. To isolate ECFCs, mononuclear cells (MNCs) were separated from peripheral blood by density gradient centrifugation on lymphocyte separation medium for 30 min at 400g and washed twice in EBM-2 with 2% FCS. A median of 36 x 10^6 MNCs (range 18-66) was plated on collagen-coated culture dishes (BD Biosciences) in the presence of the endothelial cell growth medium EGM-2 MV Bullet Kit (Lonza) containing endothelial basal medium (EBM-2), 5% foetal bovine serum (FBS), recombinant human (rh) EGF, rhVEGF, rhFGF-B, rhIGF-1, ascorbic acid and heparin, and maintained at 37°C in 5% CO_2 and humidified atmosphere. Non-adherent cells were discarded after 2 days and thereafter, medium was changed three times a week. The outgrowth of ECs from adherent MNCs was characterized by the formation of a cluster of cobblestone-shaped cells. That ECFC-derived colonies belonged to endothelial lineage was confirmed as described in [47] and [48]. In more detail, EPC-derived colonies were characterized by staining them with anti-CD31, anti-CD105, anti-CD144, anti-CD146, anti-vWf, anti-CD45, and anti-CD14 monoclonal antibodies and by assessment of capillary-like network formation in the *in vitro* Matrigel assay.

For our experiments, we have mainly used endothelial cells obtained from early passage ECFC (P1-3, which roughly encompasses a 15-18 day period) with the purpose to avoid (or maximally reduce) any potential bias due to cell differentiation. However, in order to make sure that the phenotype of the cells did not change throughout the experiments, in preliminary experiments we tested the immunophenotype of ECFCs at different passages and we found no differences [48]. We also tested whether functional differences occurred when early (P2) and late (P6) passage-ECFCs were used by testing the in vitro capacity of capillary network formation in a Matrigel assay and found no differences between early and late passage ECFC-derived cells.

2.2 Solutions

Physiological salt solution (PSS) had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, 10 Hepes. In Ca^{2+}-free solution ($0Ca^{2+}$), Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Solutions were titrated to pH 7.4 with NaOH. The osmolality of PSS as measured with an osmometer (Wescor 5500, Logan, UT) was 338 mmol/kg.

2.3 $[Ca^{2+}]_i$ and NO measurements

hECFCs were loaded with 4 μM fura-2 acetoxymethyl ester (fura-2/AM; 1 mM stock in dimethyl sulfoxide) in PSS for 1 hour at room temperature. After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and the cells observed by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany), usually equipped with a Zeiss ×40 Achroplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). hECFCs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light and a second neutral density filter (optical density=0.3) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. A round diaphragm was used to increase the contrast. The excitation filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot on-line the fluorescence from 10 up to100 rectangular "regions of interest" (ROI). Each ROI was identified by a number. Since cell borders were not clearly identifiable, a ROI may not include the whole cell or may include part of an adjacent cell. Adjacent ROIs never superimposed. $[Ca^{2+}]_i$ was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed "ratio"). An increase in $[Ca^{2+}]_i$ causes an increase in the ratio [48, 49]. Ratio measurements were performed and plotted on-line every 3 s. The experiments were performed at room temperature (22°C). We calibrated the resting $[Ca^{2+}]_i$ by using the Grynkiewicz method, as shown in [50]. Its average value was equal to 82.2±11.0 nM (n=97).

NO was measured as described in [51] and [36]. Briefly, hECFCs were loaded with the membrane permeable NO-sensitive dye 4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate (10 μM) for 60 min at room temperature and washed in PSS for one further hour. DAF-FM fluorescence was measured by using the same equipment described for Ca^{2+} recordings but with a different filter set, i.e. excitation at 480 nm and emission at 535 nm wavelength (emission intensity was

shortly termed "NO_i"). NO measurements were performed and plotted on-line every 5 s. Again, off-line analysis was performed by using custom-made macros developed by Microsoft Office Excel software. The experiments were performed at room temperature. DAF-FM is a single-wavelength dye and, therefore, the baseline NO signal was measured by simply recording the baseline fluorescence of DAF-FM after at least 15 min of stabilization of the tracing. At this point, the recording was initiated and the basal NO levels were measured by averaging the value of DAF-FM fluorescence at 300±50 sec from the beginning of the experiment (0.379±0.0062, n=3), as shown in [36, 52, 53]. The cells were then exposed to AA, cyclopiazonic acid (CPA) or GSK1016790A (GSK).

Data relative to both Ca^{2+} and NO signals are presented as mean±SE, while the number of cells analyzed is indicated between parentheses. Each data set refers to measurements conducted on hECFCs isolated from at least three independent donors.

2.4 RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

For the analysis of the expression of ryanodine receptors (RyRs), total RNA was extracted with the miRNeasy Mini Kit (Qiagen, Germany) and further DNA purified by on-column digestion with the RNase-free DNase Set (Qiagen), according to the manufacturer's instructions [54]. cDNA synthesis was carried out using the iScript kit (Bio-Rad, California, USA). In brief, 500 ng of total RNA sample was reverse transcribed using a blend of oligo-dT and random primers, subsequently diluted with nuclease-free water to 3 ng/μL (total RNA equivalent) and stored at -80°C. The gene expression assays for *RYR1* (Cat. # hs00166991_m1), *RYR2* (Cat. # hs00181461_m1) and *RYR3* (Cat. # hs00168821_m1). Quantification of transcripts was carried out in a 20 μL reaction mix containing 1X SsoAdvanced Universal Probes Supermix and 1X of each assay. The PCR conditions were 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec and 60°C for 15 sec .Three reference genes (*GAPDH, YWHAZ, TBP*) were analysed according to method previously decribed [54]. For each experiment, 5 μL of cDNA (corresponding to 15 ng of total RNA) were used. The PCR data were collected using the CFX96 Real-Time System (Bio-Rad). Each sample was tested in duplicate.

For the analysis of two-pore channel (TPC) expression, total RNA was extracted from the EPCs and RCCs using the QIAzol Lysis Reagent (QIAGEN, Italy). The first cDNA copy was synthesized from 1 µg total RNA using random hexamers and M-MLV Reverse Transcriptase (Invitrogen S.R.L., Milan, Italy). Real-time PCRwas performed using GoTaq qPCR master mix according to manufacturer's instructions (Promega, Milan, Italy) on a SFX96 Real-time system (Biorad, Segrate, Italy). Oligonucleotide primers were obtained from Sigma: TPC1: forward=

GAGTTTGGATGACGACGTGC, reverse= GAGTCGTGGATGGCATAGCT (NM_001143819.1); TPC2: forward= CTTACCGCAGCATCCAAGTC, reverse= GTAAAGCCACATCGAGCTGG (NM_139075.3). Ribosomal S18 subunit was used as housekeeping gene: forward= TGCGAGTACTCAACACCAACA, reverse= CTGCTTTCCTCAACACCACA (NM_213557).

2.5 ***Proliferation assays***

As described elsewhere [36, 48, 49], growth kinetics were evaluated by plating a total of 1 x 10^5 ECFCs-derived cells (first passage) in 30-mm collagen-treated dishes in the presence of: 1) EBM-2+5% FBS; 2) EBM-2+5% FBS+ 20 μM AA; 3) EGM-2; 4) EGM-2+20 μM AA. Cultures were incubated at 37°C (in 5% CO2 and humidified atmosphere) and cell growth assessed every day until confluence was reached in control cultures. At this point, cells were recovered by trypsinization from all dishes and the cell number assessed by counting in a haemocytometer. In order to assess the impact of Ca^{2+} signalling and NO on AA-promoted ECFC proliferation, cells were seeded in the presence of EGM-2+20 μM AA supplemented with 1) 2-Aminoethoxydiphenyl borate (2-APB; 50 μM); 2) Ned-19 (100 μM); 3) GPN (200 μM); 4) RN-1734 (10 μM); 5) 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; 30 μM); and 6) N^G-nitro-l-arginine methyl ester (L-NAME; 100 μM). Preliminary experiments showed no unspecific or toxic effect for each agent when used at these concentrations. Each assay was repeated in triplicate.

2.6 Cell cultures

HELA, UKE-1, DAMI and K562 cell lines were routinely cultured from laboratory stocks in the appropriate medium (IMDM or RPMI) supplemented with 10% fetal bovine serum, 2% glutamine and 1% penicillin/streptomycin, at 37°C in a fully humidified incubator in the presence of 5% CO2. Human umbilical vein endothelial cells (Cat. # C2517AS) were purchased from Lonza (Lonza Walkersville Inc., Maryland, USA) and directly used for RNA extraction without being cultured. Mesenchimal stem cells (MSCs) were obtained from healthy subjects' spleen samples according to the previously described isolation method [55].

3. RESULTS

3.1 Arachidonic acid-induced Ca^{2+} signals in hECFCs require intracellular Ca^{2+} mobilization and extracellular Ca^{2+} entry in human endothelial colony forming cells

As widely reviewed in [41], while the serum concentration of AA may raise up to 600 μM, the physiological levels of free AA in tissues and cells falls within the low-to-mid micromolar range (1-150 μM) due to the presence of the extracellular fatty acid binding protein albumin [56]. Consequently, most studies investigated the outcome of AA on intracellular Ca^{2+} signalling and its downstream Ca^{2+}-dependent pathways by administrating AA in a concentration range spanning from 1 up to 45 μM [8, 20, 26, 57-60]. Previous results from our group showed that 1-2 μM AA is not sufficient to elevate intracellular Ca^{2+} levels in hECFCs [37]. Herein, we found that 20 μM AA caused an immediate rise in $[Ca^{2+}]_i$ that rapidly returned to the baseline upon agonist washout and then resumed upon agonist re-addition to the perfusate (Fig. 1A). When AA was maintained over time in the recording solution, the Ca^{2+} response consisted in an initial Ca^{2+} peak that was followed by a plateau level of intermediate amplitude (Fig. 1B). Fig. 1B depicts representative Ca^{2+} traces of the dose-response relationship of AA-induced Ca^{2+} signals in hECFCs. Statistical analysis revealed that the amplitude of the intracellular Ca^{2+} elevation progressively augmented by raising AA concentration ([AA]) from 5 μM up to 50 μM (Fig. 1D). Conversely, while the percentage of responding cells significantly ($p<0.05$) increased by bringing up [AA] from 5 μM to 20 μM, it did not further change when [AA] was enhanced up to 50 μM (Fig. 1C). The biphasic kinetics of AA-evoked increase in $[Ca^{2+}]_i$ was constant across the concentration range probed (Fig. 1B). In order to assess which Ca^{2+} sources underlie these Ca^{2+} signals, in the subsequent set of experiments, AA was applied in the absence of external Ca^{2+} ($0Ca^{2+}$), after which Ca^{2+} was reintroduced in the perfusate in the continued presence of the agonist. At all the concentrations tested (5-50 μM), AA caused a transient increase in $[Ca^{2+}]$ under $0Ca^{2+}$ conditions, which reflects Ca^{2+} release from the intracellular reservoir (Fig. 2A-C). The subsequent restitution of Ca^{2+} to the bath induced a second elevation in $[Ca^{2+}]_i$ which depends on extracellular Ca^{2+} entry (Fig. 2A-C). Notably, both phases of the Ca^{2+} response to AA, i.e. Ca^{2+} mobilization and Ca^{2+} inflow, increased by enhancing [AA] from 5 μM up to 50 μM. Taken together, these experiments indicate that AA increases $[Ca^{2+}]_i$ in hECFCs by mobilizing intracellularly stored Ca^{2+} and inducing Ca^{2+} inflow. To assess whether AA increases $[Ca^{2+}]_i$ through one of its metabolites, we probed the effect of eicosatetraynoic acid (ETYA), a non-metabolizable analog of AA that blocks lipoxygenase,

cycloxygenase and CYP450 by acting as a false substrate [61]. ETYA (20 μM) elicited a Ca^{2+} signal whose amplitude (Fig. 3A and 3C) and kinetics (Fig. 3A) were similar to that evoked by AA (20 μ). Moreover, there was no significant difference in the fraction of responding cells between ETYA and AA. Therefore, AA-induced intracellular Ca^{2+} waves do not require AA metabolism.

3.2 Arachidonic acid induced Ca^{2+} release from $InsP_3Rs$ and TPCs in hECFCs in human endothelial colony forming cells

In order to unravel the components of the Ca^{2+} toolkit that underpins the Ca^{2+} response to AA in hECFCs, we first focussed on intracellular Ca^{2+} mobilization. All these experiments were carried out under $0Ca^{2+}$ conditions to prevent any confounding interference with extracellular Ca^{2+} influx. In our previous investigations, $InsP_3$ emerged as the most powerful Ca^{2+} releasing second messenger in these cells [34]. Therefore, we first inhibited the $InsP_3$-dependent signalling pathway by pre-treating the cells with either 2-aminoethoxydiphenyl borate (2-APB; 50 μM, 20 min) or U73122 (10 μM, 10 min), a potent inhibitor of phospholipase C (PLC), the enzyme which cleaves $InsP_3$ from phosphatidylinositol 4,5-bisphosphate (PIP_2) [35, 62]. Neither of these drugs, however, fully suppressed the Ca^{2+} response to AA (Fig. 4A and Fig. 4E), albeit its amplitude was significantly ($p<0.05$) reduced as compared to control cells (Fig. 4F). AA could recruit the PLCβ/$InsP_3$ signalling pathway by activating the G-protein coupled receptor 40 (GPR40) [63]. However, palmitoleic acid (20 μM), a more potent GRP40 agonist than AA, failed to evoke any detectable increase in $[Ca^{2+}]_i$ in hECFCs (n=120; not shown). AA has previously been shown to trigger Ca^{2+} release by directly stimulating RyRs [21]. We, therefore, used qRT-PCR to evaluate the expression of the three RyR isoforms in hECFCs. As shown in Table 1, RyR transcripts were absent in these cells, despite the fact that our primers were able to detect RyR1, RyR2 and RyR3 in other cell types. Finally, we depleted the ER Ca^{2+} reservoir with CPA (10 μM), a selective inhibitor of the Sarco-Endoplasmic Reticulum Ca^{2+} ATPase (SERCA), in the absence of external Ca^{2+}. CPA prevents SERCA from capturing Ca^{2+} constitutively leaking from the ER, thereby causing a transient increase in $[Ca^{2+}]_i$ which is due to passive store emptying [47]. As shown in Fig. 4C, in addition to suppressing the Ca^{2+} response to the $InsP_3$-producing autacoid ATP (100 μM), CPA inhibited AA-induced Ca^{2+} release. The statistical analysis of the effects exerted by CPA is reported in Figures 4E and Fig. 4F. A recent study further demonstrated that EL acidic Ca^{2+} store may support $InsP_3$-dependent Ca^{2+} mobilization evoked by AA in rat pancreatic β cells [22]. A sizeable EL acidic Ca^{2+} store is also present in hECFCs [64]. TPC1 and TPC2 have recently been shown to mediate

NAADP-dependent Ca^{2+} release from the EL store in a multitude of cell types [65-67]. Our qRT-PCR analysis revealed that TPC1 is the most abundant TPC isoform expressed in hECFCs (Fig.4B). Ned-19 (100 μM, 1 hour), a specific inhibitor of NAADP-induced Ca^{2+} release from TPCs, abrogated AA-evoked Ca^{2+} signals (Fig. 4A, Fig. 4E and Fig. 4F). Disrupting the acidic Ca^{2+} store of the EL system with glycyl-L-phenylalanine 2-naphthylamide (GPN; 200 μM) evoked a robust Ca^{2+} mobilization and prevented the Ca^{2+} response to AA (Fig. 4C, Fig. 4E and Fig. 4F). Collectively, these findings show that AA mobilizes ER and EL Ca^{2+} stores by, respectively, recruiting $InsP_3Rs$ and TPCs. In support of this evidence, 2-APB (50 μM, 20 min) and Ned-19 (100 μM, 1 hour) prevented ETYA-evoked endogenous Ca^{2+} release (Supplementary Figure 1).

3.2 Arachidonic acid stimulates Ca^{2+} inflow through TRPV4 channels and inhibits store-operated Ca^{2+} entry in human endothelial colony forming cells

The next step was to unravel the Ca^{2+}-permeable pathway(s) underlying AA-evoked Ca^{2+} inflow in hECFCs. As mentioned above, TRPV4 channels are the most likely candidates to mediate AA-evoked Ca^{2+} entry in vascular endothelium [8-13]. We have previously shown that RN-1734 and ruthenium red (RR) selectively block TRPV4-dependent Ca^{2+} influx in hECFCs [37]. We found that both RN-1734 (20 μM, 30 min) and RR (10 μM, 30 min) reduced the amplitude and curtailed the duration of the Ca^{2+} response to AA (20 μM) (Fig. 5A and Fig. 5C). Conversely, the fraction of responding cells was affected by neither of these treatments (Fig. 5B). The statistical analysis of the effects exerted by RN-1734 and RR on AA-elicited Ca^{2+} signals is reported in Figures 5B and Fig. 5C. In the same way, RN-1734 (20 μM, 30 min) significantly ($p<0.05$) reduced the amplitude of ETYA-evoked Ca^{2+} signals without affecting the fraction of responding cells (Supplementary Figure 1). As depicted in Fig. 2, AA heavily mobilizes the ER Ca^{2+} pool in hECFCs. As observed for other $InsP_3$-producing stimuli, such as ATP [47] and VEGF [35], the following drop in ER Ca^{2+} levels should lead to SOCE activation. However, it has long been known that AA inhibits store-dependent Ca^{2+} influx in many cell types [9, 68, 69]. In order to assess this issue in hECFCs, we applied CPA in the presence of external Ca^{2+} to deplete the endogenous Ca^{2+} reservoir and fully gate SOCE [62]. Under these conditions, the Ca^{2+} response to CPA consisted in an initial increase in $[Ca^{2+}]_i$ due to passive Ca^{2+} mobilization from the ER followed by a decline to a steady-state level which is due to the development of SOCE [47, 62]. When added at 30 min after the beginning of the stimulation with CPA, AA caused a significant reduction in SOCE (Fig. 5D). This relatively rapid decrease to resting Ca^{2+} levels was followed by a persistent increase in $[Ca^{2+}]_i$ that disappeared in the presence of RN-1734 (Fig. 5D).

Therefore, these data strongly indicate that SOCE is blocked, rather than activated, by AA and that TRPV4 is the main mediator of AA-induced Ca^{2+} influx in hECFCs.

3.3 Arachidonic acid-induced Ca^{2+} signals determine NO production in hECFCs in human endothelial colony forming cells

Having dissected the signalling machinery shaping the Ca^{2+} response to AA, we turned to investigate whether AA-evoked Ca^{2+} signals drive NO synthesis in hECFCs, as reported in mature ECs [2, 6, 7]. To this aim, we exploited the NO-sensitive fluorochrome, DAF-FM, which is commonly used to monitor intracellular NO levels both in vascular ECs and in hECFCs [36, 70-72]. AA (20 μM) caused a gradual, but persistent increase in DAF-FM fluorescence which arose with a considerable delay as compared to Ca^{2+} signals (Fig. 6A; please, compare with Fig. 1A). AA-evoked NO production was prevented by pre-treating the cells with L-NAME (100 μM, 75 min), a selective inhibitor of all NOS isoforms, and BAPTA (30 μM, 2 h), a membrane permeable buffer of intracellular Ca^{2+} levels. These findings, therefore, show that AA-dependent NO synthesis impinges on the accompanying increase in $[Ca^{2+}]_i$. In order to decipher the Ca^{2+} sources for activation of endogenous NO production, we challenged the cells with AA in the absence of external Ca^{2+}. Under these conditions, AA (20 μM) did not cause any evident increase in NO levels. Surprisingly, we could not detect any elevation in DAF-FM fluorescence even upon Ca^{2+} restitution to the bathing medium (Fig. 6B). In other words, Ca^{2+} release and Ca^{2+} entry alone were not sufficient to trigger NO synthesis in hECFCs challenged with AA. Conversely, control cells displayed a robust production of NO in response to AA (20 μM) (not shown). Consistent with these data, AA did not elicit any increase in DAF-FM fluorescence in cells pre-treated with either 2-APB (50 μM, 20 min) or Ned-19 (100 μM, 1 hour) to fully abrogate ER- and EL-dependent Ca^{2+} release (Fig. 6C), respectively, or with RN-1434 (20 μM, 30 min) to inhibit TRPV4-dependent Ca^{2+} inflow (Fig. 6D). Similar to AA, ETYA triggered a NO signal very similar to that evoked by AA (Fig. 6E). The statistical analysis of the effects exerted by all these reagents on NO levels is summarized in Fig. 6F. Interestingly, when CPA (10 μM) was added in the presence of external Ca^{2+} to fully activate SOCE, it failed to generate any detectable increase in NO levels (Fig. 7A). Conversely, the pharmacological activation of TRPV4 channels with GSK (10 nM) [37] caused a delayed, but persistent elevation in NO that was prevented by RN-1434 (20 μM, 30 min) (Fig. 7B and Fig. 7C). However, the percentage of hECFCs generating a robust NO signal in response to GSK was low when compared to AA (Fig. 7B). Therefore, TRPV4-mediated Ca^{2+} inflow may *per se* result in NO

production, albeit only in a minor fraction of cells. Altogether, these results clearly show that the Ca^{2+} response to AA leads to a subsequent elevation in intracellular NO concentration ($[NO]_i$) in hECFCs. Notably, full activation of eNOS by AA requires both endogenous Ca^{2+} mobilization and extracellular Ca^{2+} inflow in these cells.

3.4 Arachidonic acid utilizes intracellular Ca^{2+} and NO signals to promote human endothelial colony forming cell proliferation

The last step was to assess whether and how AA instigates hECFC proliferation. Cells were plated in the presence of: 1) EBM-2 supplemented with 5%FBS; 2) EBM-2 supplemented with 5%FBS and 20 μM AA; 3) EGM-2, which is an endothelial differentiation medium enriched with growth factors and routinely employed to expand ECFCs *in vitro*; 4) EGM-2 supplemented with 20 μM AA. As shown in Fig. 8A, after three days in culture, AA alone (i.e. co-applied with EBM-2+5% FBS) was not able to stimulate cell growth, while hECFCs seeded with EGM-2 reached confluence after five days in culture, as expected. Nevertheless, when AA was added along with EGM-2, hECFCs exhibited a significant ($p<0.05$) increase in their proliferation rate. These results imply that AA supports hECFC growth, albeit only in conjunction with other mitogens (i.e. those contained in EGM-2). However, AA (20 μM) failed to promote proliferation when the concomitant increases in $[Ca^{2+}]_i$ and $[NO]_i$ were prevented with BAPTA (30 μM) and L-NAME (100 μM), respectively. The same results were obtained when AA was applied in the presence of either 2-APB (50 μM), Ned-19 (100 μM); GPN (200 μM), or RN-1734 (20 μM). Taken together, these results provide the evidence that AA promotes hECFC proliferation by utilizing an elevation in $[Ca^{2+}]_i$ to recruit eNOS and trigger NO production.

4. DISCUSSION

AA or its metabolites have long been known to stimulate angiogenesis through an increase in $[Ca^{2+}]_i$ and the consequent engagement of the Ca^{2+}/CaM-sensitive decoder eNOS [2, 6-9]. Their effect on circulating EPCs is, however, less clear. Intracellular Ca^{2+} signals play a central role in the intricate network of signalling pathways that control the fate of hECFCs, one of the most studied EPC subsets which may reside either in the BM or the vascular wall [32, 34, 73]. Herein, we demonstrate that AA induces hECFC proliferation through an elevation in $[Ca^{2+}]_i$, which in turn results to the generation of the pro-angiogenic gasotransmitter NO [2, 7].

AA caused a dose-dependent increase in $[Ca^{2+}]_i$ in hECFCs starting at 5 μM. This signal (as well as the consequent NO release, see below) did not depend on AA metabolism as ETYA, which is not a substrate of AA metabolizing enzymes, generated a $[Ca^{2+}]_i$ rise whose magnitude, kinetics and pharmacological profile were similar to those elicited by AA. The Ca^{2+} response to AA at each concentration tested (5 μM, 20 μM, 50 μM) consisted both in intracellular Ca^{2+} mobilization and extracellular Ca^{2+} entry. We previously showed that AA is unable to augment intracellular Ca^{2+} levels when applied at 1-2 μM [37]. These results are partially different from those obtained in mature ECs, such as human coronary arterioles ECs [13] and breast tumor-derived ECs (BTECs) [9], in which AA induces Ca^{2+} influx, but not Ca^{2+} release, at concentrations ranging between 0.5-10 μM and 1 nM-1 μM, respectively. These discrepancies could reflect the well known heterogeneity in terms of Ca^{2+} signalling between hECFCs and mature endothelium [74]. However, AA has recently been shown to induce a robust Ca^{2+} release in human umbilical vein ECs (HUVECs) when applied at >10 μM [26]. Likewise, in ECs from non-human species, AA mobilized ER stored Ca^{2+} at doses ≥10 μM [3, 23]. In order to appreciate the physiological relevance of our observations, we must recall that hECFCs may be found in both the BM and arterial walls. Now, the concentration range of AA that causes intracellular Ca^{2+} signals in hECFCs is the same as that reported to control hematopoietic cell proliferation, differentiation and survival [39, 75, 76]. It is, therefore, feasible that AA levels locally rise to the low-to-mid micromolar range in BM microenvironment and influence BM-derived stem cells, including hECFCs. Free AA concentration has indeed been reported to occur between 0.5 and 100 μM [41]. Extracellular albumin prevents free AA from raising up to the high micromolar range [56], however, the physiological concentration of AA most frequently measured in local tissues and cells ranges in the

tens of micromolar [41, 60]. Moreover, AA levels may increase up to hundreds of micromolar in response to physical exercise, tissue ischemia or inflammation. This feature gains particular relevance when recalling that AA is usually unable to exert any significant biological effect at doses lower than 1-10 μM [41]. For instance, the concentration of free AA in human plasma is equal to 5.1-13.1 μM [60] and may locally increase upon platelet release [41], thereby activating vascular wall-resident cells [58], including hECFCs. In addition, AA may increase up to ≈500 μM upon intense physical exercise [43], which has long been known to stimulate EPC mobilization [77]. Furthermore, several pathological conditions that involve an increase in EPC frequency and biological activity, such as cardiac ischemia [29] and brain injury [78], cause the accumulation of massive amounts (up to 10-to-13 fold) of AA (see also below) [45, 46]. Likewise, the inflammatory state causes an increase in the local levels of AA that may rapidly attain the micromolar range [41]. For instance, free AA rises up to 100 μM in inflamed skin [44]. Therefore, hECFCs are sensitive to the range of AA concentrations that is predicted to exert a physiological role in both health and disease. In particular, as more widely illustrated below, the liberation of high amounts of AA under pathological conditions could play an important role in hECFC activation.

The fact that the endogenous Ca^{2+} pool is mobilized already at the lowest active dose (i.e. 5 μM) indicates that intracellular Ca^{2+} release plays an important role in the overall Ca^{2+} response to AA in hECFCs. We have previously shown that the ER represents the main intracellular Ca^{2+} reservoir in these cells [47]. Consistently, depleting the ER Ca^{2+} content with CPA prevented AA-induced intracellular Ca^{2+} mobilization. Likewise, blocking the PLC/$InsP_3$ signalling pathway with either U73122 or 2-APB significantly reduced AA-evoked Ca^{2+} discharge. The most likely interpretation of these results is that AA activates a Gq-protein coupled receptor, thereby leading to PLC stimulation, $InsP_3$ synthesis and $InsP_3$-dependent Ca^{2+} release [79]. It has been shown that free fatty acids, including AA, control insulin secretion from rat pancreas β-cells by activating the rat orphan GPR40 [63]. However, palmitoleic acid failed to elevate intracellular Ca^{2+} levels under our conditions, thereby ruling out such possibility in hECFCs. However, it has long been known that AA stimulates the phosphoinositide cycle and releases Ca^{2+} in non-neural tissues by promoting the association between a 700 kDa protein named AHNKAK and PLCγ-1 [80-82]. It is, therefore, conceivable that the same mechanism underlies AA-evoked $InsP_3$ production and ER-dependent Ca^{2+} mobilization. Yet, as aforementioned, blocking the PLC/$InsP_3$ signalling pathway did not fully abrogate the Ca^{2+} response to AA. This observation suggests that an additional source of Ca^{2+} contributes to endogenous Ca^{2+}

mobilization. AA directly triggers Ca^{2+} release from RyRs in many cell types [21, 83], but RyRs are absent in hECFCs. A recent investigation unveiled that AA liberates Ca^{2+} stored within the acidic stores of the EL system through a still unknown mechanism [22]. The newly discovered Ca^{2+}-releasing messenger NAADP gates the EL Ca^{2+} channels, TPC1 and TPC2, to increase $[Ca^{2+}]_i$ in a multitude of cell types across the phylogenetic tree [65, 66]. NAADP very likely does so through the intermediation of a 22- and 23-kilodalton pair of proteins [84]. We showed that TPC1 is abundantly expressed in hECFCs, while TPC2 levels are low. Moreover, the Ca^{2+} response to AA was nearly abolished by a selective TPC antagonist, i.e. Ned-19, and by the depletion of EL Ca^{2+} stores with GPN. The mechanism by which AA targets TPC1 is yet to be elucidated, but it could require the interposition of an auxiliary/ancillary subunit, as required for NAADP [84], or an alteration in membrane curvature, as suggested for RyRs [22]. Alternatively, AA-evoked $InsP_3$-dependent Ca^{2+} discharge could signal back to the acidic Ca^{2+} stores by promoting the Ca^{2+}-dependent synthesis of NAADP or by directly stimulating TPC1 [85]. Collectively, therefore, these data indicate that AA-evoked intracellular Ca^{2+} release in hECFCs is shaped by two independent routes, namely $InsP_3$Rs and TPC1, which mediate Ca^{2+} efflux from the ER and EL Ca^{2+} pools, respectively.

AA-dependent Ca^{2+} inflow in vascular endothelium has long been ascribed to TRPV4 channels [8, 10-14]. We have recently shown that a functional TRPV4 channel is expressed in hECFCs, in which it is sensitive to the two widely employed antagonists, RN-1734 and RR [37]. Consistent with these studies, both these drugs profoundly reduced the amplitude of the Ca^{2+} response to AA and curtailed its duration. These findings strongly indicate that TRPV4 is the main mediator of AA-elicited Ca^{2+} entry in hECFCs. Previous studies demonstrated that endothelial TRPV4 channels may be gated by lower doses of AA [8, 13]. However, we could not find any detectable Ca^{2+} response to 1-2 μM AA [37]. These discrepancies could be easily reconciled when recalling that the opening of a few TRPV4 channels per EC originates low amplitude, local Ca^{2+} entry events that have been termed sparklets and cannot be visualized by conventional Fura-2- or Fluo-4-based epifluorescence imaging [86, 87]. Thus, it is feasible that AA triggers Ca^{2+} entry already at <5 μM also in hECFCs, but we cannot detect it. Two further pieces of evidence indicate that AA-dependent Ca^{2+} inflow is almost entirely carried by TRPV4 channels. First, SOCE, a major Ca^{2+} influx pathway in hECFCs, is inhibited by AA. Therefore, AA depletes the ER Ca^{2+} pool, but this does not lead to Orai1 and TRPC1 activation on the plasma membrane. This result fully concurs with other reports, which showed AA-induced SOCE inhibition in several cell models [57, 68, 69], including human ECs [9]. Second, AA-evoked Ca^{2+} plateau at regular

1.8 mM extracellular Ca^{2+} concentration was totally abolished in the presence of either RN-1734 or RR. Likewise, the delayed increase in $[Ca^{2+}]_i$ evoked by AA after SOCE inhibition was fully suppressed by RN-1734. These data strongly suggest that other Ca^{2+}-permeable conductances are unlikely to contribute to AA-dependent Ca^{2+} influx. Once again, we cannot rule out the possibility that other AA-sensitive pathways, such as Orai3, mediate a localized influx of Ca^{2+} that falls below the resolution limit of our imaging system [88]. We should, however, recall that in human umbilical vein ECs (HUVECs), presently the sole EC model where Orai3 has been shown to bring about AA-evoked Ca^{2+} inflow, Orai3 translocates to the plasma membrane only upon AA conversion into LTC4 [26]. Conversely, AA metabolites do not seem to be involved in the Ca^{2+} response described in our investigation, which makes Orai3 an unlikely actor in AA signalling in hECFCs. The molecular bases of TRPV4 activation by AA are still unclear. While several excellent review have recently addressed the molecular bases of TRPV4 gating by several stimuli [89, 90], including 4α-phorbol 12,13-didecanoate (4α-PDD), phorbol 12-myristate 13-acetate (PMA) and cell swelling, the molecular interaction between AA and TRPV4 is unknown. As for RyRs [22], AA could alter the local lipid microenvironment in the plasma membrane, particularly its microviscosity or stiffness, thereby activating the channel or rendering it sensitive to an intracellular modulator (for instance Ca^{2+}, that can stimulate TRPV4). Alternatively, AA could directly bind the channel protein, as reported for the voltage-gated Na^{+} channel NaV1.5 and the voltage-gated Ca^{2+} channel CaV3 [91, 92]. No specific AA-binding domain has been yet identified [92]. However, the electron cryo-microscopy structure of TRPV1 revealed the presence of several lipid-binding sites, such as those located in the hydrophobic portals linking the membrane and the central pore cavity and intersubunit segment between S5 from one subunit and S4 from the adjacent subunit [93]. These sites have been proposed to mediate lipid gating also in other TRP channels, such as TRPV4.

AA-evoked Ca^{2+} signals result in NO production in mature ECs [2, 6, 12]. Likewise, several lines of evidence indicate that AA increases intracellular NO levels in hECFCs in a Ca^{2+}-dependent manner. First, AA-dependent NO synthesis significantly lags behind the increase in $[Ca^{2+}]_i$, which suggest that it cannot trigger it, as reported in other cell types [20]. Second, buffering intracellular Ca^{2+} levels with BAPTA at a dose known to inhibit Ca^{2+}-dependent processes in hECFCs abolished AA-induced NO generation. Third, any pharmacological manipulation of the Ca^{2+} response to AA severely compromised the accompanying elevation in NO concentration. It is particularly remarkable that both components of AA-evoked Ca^{2+} signals, i.e. intracellular Ca^{2+} release and extracellular Ca^{2+} inflow,

were required to recruit eNOS. This feature is evident in cells which were exposed to AA first in the absence and then in the presence of Ca^{2+} in the recording solution (see Fig. 6B). In neither of such conditions AA was able to produce a detectable increase in DAF-FM fluorescence. This result was mimicked by inhibiting either ER-dependent Ca^{2+} release, EL-dependent Ca^{2+} mobilization or TRPV4-dependent Ca^{2+} influx alone and strongly suggests that eNOS engagement requires a global, rather than a local [94], elevation in $[Ca^{2+}]_i$ in hECFCs. In this view, stimulating TRPV4 with GSK independently on $InsP_3Rs$ and TPC1 produced a robust NO signal only in a minor fraction of cells as compared to AA. These data further suggest that the co-activation of ER-, EL- and plasma membrane-resident channels is necessary for AA to bring about a measurable elevation in DAF-FM fluorescence. It is conceivable that eNOS lies in close proximity with different Ca^{2+}-permeable conductances, i.e. $InsP_3Rs$, TPC1 and TRPV4, and that their combined recruitment is needed to elevate NO levels in hECFCs. The finding that SOCE does not stimulate a detectable NO release is not at odd with our recent findings on infantile hemangioma-derived ECFCs (IH-ECFCs). Accordingly, this study demonstrated that a constitutive SOCE triggers a massive NO production in IH-ECFCs, while its effect is much weaker in N-ECFCs [36].

In addition to triggering vasodilation [13], exogenous AA uses NO to deliver a potent angiogenic signal to vascular ECs [2, 9]. Moreover, several growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), use AA and NO as intracellular messengers to stimulate EC proliferation [6, 95]. Consistently, AA promoted hECFC proliferation, albeit in conjunction with a cocktail of growth factors (i.e. the EGM-2 BulletKit) which is commercially available to promote hECFC expansion *in vitro*. This finding strongly suggests that AA *per se* is not capable of stimulating hECFC growth, but it may enhance this process in the presence of other mitogens: in this view, the BM niche, which is enriched with VEGF and bFGF [39, 96], represents a suitable microenvironment for stromal cells-released AA to promote hECFC proliferation. In addition, hECFCs are also resident within the vascular endothelial intima, from which they are rapidly recruited for vascular repair [28, 97]. This issue gains physiological relevance as free AA accumulates in ischemic tissues which, at the same time, promote hECFC activation by releasing numerous growth factors and cytokines [29, 46, 77, 98]. Therefore, AA might boost growth factors-induced hECFC proliferation, thereby contributing to accelerate vascular repair *in situ*. Likewise, the massive release of AA observed upon an intense physical exercise [43] could further enhance the local rate of hECFC proliferation, which would facilitate the expansion of the vascular network sustaining

skeletal muscle activity. The pro-angiogenic effect of AA was impaired by inhibiting either the concomitant increase in $[Ca^{2+}]_i$ or the subsequent production of NO. These results concur with the established role for Ca^{2+} signalling in determining hECFC fate [34, 73]. This latter feature could also explain why targeting the Ca^{2+} machinery reduced hECFC growth significantly below the control (i.e. EGM-2 BulletKit alone) values. Our previous work has indeed shown that such medium stimulates hECFC expansion in a Ca^{2+}- dependent manner [36, 47, 48]. Thus, the Ca^{2+}/CaM-dependent enzyme eNOS represents the most likely mechanistic link between AA-evoked Ca^{2+} signals and growth also in hECFCs. It should, however, be pointed out that AA alone is able to induce proliferation in fully differentiated ECs, such as breast tumor-derived ECs [3] and bovine aortic endothelial cells [3]. This discrepancy reflects the well known heterogeneity in the signalling pathways endowed to mature ECs and their precursors [34, 74]. AA-dependent EC proliferation is tightly mediated by an intracellular Ca^{2+} signal [2, 3, 95]. It is, therefore, conceivable that the Ca^{2+}-dependent NO release induced by AA in hECFCs is not sufficient to trigger the process of replication and that is must be aided by mitogens-induced NO production. Alternatively, AA-evoked NO release may support the action of additional proliferative pathways recruited by extracellular growth factors, such as mitogen-activated protein kinases and extracellular-signal-regulated kinases [99].

5. CONCLUSION

This study demonstrated that AA, a potent mitogen in mature ECs, stimulates cell growth also in human endothelial precursors in a Ca^{2+}- and NO-dependent manner. This observation gains further relevance when considering that hECFCs, i.e. the EPC population herein employed, is the only truly endothelial progenitor so far identified.. The Ca^{2+} machinery recruited by AA in hECFCs is subtly more complex as compared to mature ECs, since, in addition to TRPV4 channels, it also involves $InsP_3Rs$ and TPC1, although the molecular bases of their activation remain to be elucidated. Future experiments will have to solve this issue. hECFCs act as a double-edged sword in the organism. While they are necessary to restore local blood perfusion upon an ischemic insult, they may sustain the highly detrimental angiogenic switch in solid tumors. It turns out that future studies will have to assess whether AA may serve as a useful strategy to accelerate *in vitro* hECFC expansion for autologous cell therapy. Moreover, it will be important to assess whether AA influence on hECFCs is altered in tumor-derived cells. Pioneering work by Fiorio Pla and Munaron has unveiled that AA-elicited TRPV4-mediated Ca^{2+} inflow in dramatically larger in breast tumoder-derived ECs (BTECs) as compared to normal ECs [8]. This is reflected in an enhanced stimulation of cell migration in tumor-derived ECs [8], which renders AA and TRPV4 two important actors in the process of tumor vascularisation [8, 100]. Remodelling of the intracellular Ca^{2+} toolkit is an established hallmark of tumor-associated hECFCs [32, 48]. It will be, therefore, important to assess whether AA-evoked Ca^{2+} and NO signals are deranged in these cells and whether such dysfunction results in abnormal cancer vascularisation.

References

[1] L. Munaron, Shuffling the cards in signal transduction: Calcium, arachidonic acid and mechanosensitivity, World J Biol Chem, 2 (2011) 59-66.
[2] L. Munaron, Intracellular calcium, endothelial cells and angiogenesis, Recent Pat Anticancer Drug Discov, 1 (2006) 105-119.
[3] A. Fiorio Pla, L. Munaron, Calcium influx, arachidonic acid,and control of endothelial cell proliferation, Cell Calcium, 30 (2001) 235-244.
[4] I. Fleming, The cytochrome P450 pathway in angiogenesis and endothelial cell biology, Cancer Metastasis Rev, 30 (2011) 541-555.
[5] N.V. Bogatcheva, M.G. Sergeeva, S.M. Dudek, A.D. Verin, Arachidonic acid cascade in endothelial pathobiology, Microvasc Res, 69 (2005) 107-127.
[6] A. Mottola, S. Antoniotti, D. Lovisolo, L. Munaron, Regulation of noncapacitative calcium entry by arachidonic acid and nitric oxide in endothelial cells, FASEB J, 19 (2005) 2075-2077.
[7] D. Mancardi, A.F. Pla, F. Moccia, F. Tanzi, L. Munaron, Old and new gasotransmitters in the cardiovascular system: focus on the role of nitric oxide and hydrogen sulfide in endothelial cells and cardiomyocytes, Curr Pharm Biotechnol, 12 (2011) 1406-1415.
[8] A.F. Pla, H.L. Ong, K.T. Cheng, A. Brossa, B. Bussolati, T. Lockwich, B. Paria, L. Munaron, I.S. Ambudkar, TRPV4 mediates tumor-derived endothelial cell migration via arachidonic acid-activated actin remodeling, Oncogene, 31 (2012) 200-212.
[9] A.F. Pla, C. Grange, S. Antoniotti, C. Tomatis, A. Merlino, B. Bussolati, L. Munaron, Arachidonic acid-induced Ca2+ entry is involved in early steps of tumor angiogenesis, Mol Cancer Res, 6 (2008) 535-545.
[10] S.P. Marrelli, R.G. O'neil, R.C. Brown, R.M. Bryan, PLA2 and TRPV4 channels regulate endothelial calcium in cerebral arteries, Am J Physiol Heart Circ Physiol, 292 (2007) H1390-1397.
[11] V. Hartmannsgruber, W.-T. Heyken, M. Kacik, A. Kaistha, I. Grgic, C. Harteneck, W. Liedtke, J. Hoyer, R. Koehler, Arterial Response to Shear Stress Critically Depends on Endothelial TRPV4 Expression, Plos One, 2 (2007).
[12] R. Köhler, W.T. Heyken, P. Heinau, R. Schubert, H. Si, M. Kacik, C. Busch, I. Grgic, T. Maier, J. Hoyer, Evidence for a functional role of endothelial transient receptor potential V4 in shear stress-induced vasodilatation, Arterioscler Thromb Vasc Biol, 26 (2006) 1495-1502.
[13] X. Zheng, N.S. Zinkevich, D. Gebremedhin, K.M. Gauthier, Y. Nishijima, J. Fang, D.A. Wilcox, W.B. Campbell, D.D. Gutterman, D.X. Zhang, Arachidonic acid-induced dilation in human coronary arterioles: convergence of signaling mechanisms on endothelial TRPV4-mediated Ca2+ entry, J Am Heart Assoc, 2 (2013) e000080.
[14] A. Fiorio Pla, D. Gkika, Emerging role of TRP channels in cell migration: from tumor vascularization to metastasis, Front Physiol, 4 (2013) 311.
[15] F. Moccia, R. Berra-Romani, F. Tanzi, Update on vascular endothelial Ca2+ signalling: A tale of ion channels, pumps and transporters, World J Biol Chem, 3 (2012) 127-158.
[16] H. Watanabe, J. Vriens, J. Prenen, G. Droogmans, T. Voets, B. Nilius, Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels, Nature, 424 (2003) 434-438.
[17] J. Vriens, G. Owsianik, B. Fisslthaler, M. Suzuki, A. Janssens, T. Voets, C. Morisseau, B.D. Hammock, I. Fleming, R. Busse, B. Nilius, Modulation of the Ca2+ permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium, Circ Res, 97 (2005) 908-915.

[18] A.E. Loot, R. Popp, B. Fisslthaler, J. Vriens, B. Nilius, I. Fleming, Role of cytochrome P450-dependent transient receptor potential V4 activation in flow-induced vasodilatation, Cardiovasc Res, 80 (2008) 445-452.
[19] M.Y. Jian, J.A. King, A.B. Al-Mehdi, W. Liedtke, M.I. Townsley, High vascular pressure-induced lung injury requires P450 epoxygenase-dependent activation of TRPV4, Am J Respir Cell Mol Biol, 38 (2008) 386-392.
[20] E.L. Watson, K.L. Jacobson, J.C. Singh, D.H. DiJulio, Arachidonic acid regulates two Ca2+ entry pathways via nitric oxide, Cell Signal, 16 (2004) 157-165.
[21] O.O. Woolcott, A.J. Gustafsson, M. Dzabic, C. Pierro, P. Tedeschi, J. Sandgren, M.R. Bari, K.H. Nguyen, M. Bianchi, M. Rakonjac, O. Rådmark, C.G. Ostenson, M.S. Islam, Arachidonic acid is a physiological activator of the ryanodine receptor in pancreatic beta-cells, Cell Calcium, 39 (2006) 529-537.
[22] V. Yeung-Yam-Wah, A.K. Lee, A. Tse, Arachidonic acid mobilizes Ca2+ from the endoplasmic reticulum and an acidic store in rat pancreatic β cells, Cell Calcium, 51 (2012) 140-148.
[23] J. Evans, Y. Ko, W. Mata, M. Saquib, J. Eldridge, A. Cohen-Gadol, H.A. Leaver, S. Wang, M.T. Rizzo, Arachidonic acid induces brain endothelial cell apoptosis via p38-MAPK and intracellular calcium signaling, Microvasc Res, 98 (2015) 145-158.
[24] J.C. González-Cobos, X. Zhang, W. Zhang, B. Ruhle, R.K. Motiani, R. Schindl, M. Muik, A.M. Spinelli, J.M. Bisaillon, A.V. Shinde, M. Fahrner, H.A. Singer, K. Matrougui, M. Barroso, C. Romanin, M. Trebak, Store-independent Orai1/3 channels activated by intracrine leukotriene C4: role in neointimal hyperplasia, Circ Res, 112 (2013) 1013-1025.
[25] T.J. Shuttleworth, Orai3--the 'exceptional' Orai?, J Physiol, 590 (2012) 241-257.
[26] J. Li, A.F. Bruns, B. Hou, B. Rode, P.J. Webster, M.A. Bailey, H.L. Appleby, N.K. Moss, J.E. Ritchie, N.Y. Yuldasheva, S. Tumova, M. Quinney, L. McKeown, H. Taylor, K.R. Prasad, D. Burke, D. O'Regan, K.E. Porter, R. Foster, M.T. Kearney, D.J. Beech, Orai3 Surface Accumulation and Calcium Entry Evoked by Vascular Endothelial Growth Factor, Arterioscler Thromb Vasc Biol, 35 (2015) 1987-1994.
[27] R. Madonna, R. De Caterina, Circulating endothelial progenitor cells: Do they live up to their name?, Vascul Pharmacol, 67-69 (2015) 2-5.
[28] D.P. Basile, M.C. Yoder, Circulating and tissue resident endothelial progenitor cells, J Cell Physiol, 229 (2014) 10-16.
[29] F. Moccia, F.A. Ruffinatti, E. Zuccolo, Intracellular Ca(2)(+) Signals to Reconstruct A Broken Heart: Still A Theoretical Approach?, Curr Drug Targets, 16 (2015) 793-815.
[30] D.C. Gao, D. Nolan, K. McDonnell, L. Vahdat, R. Benezra, N. Altorki, V. Mittal, Bone marrow-derived endothelial progenitor cells contribute to the angiogenic switch in tumor growth and metastatic progression, Biochim Biophys Acta, 1796 (2009) 33-40.
[31] F. Moccia, E. Zuccolo, V. Poletto, M. Cinelli, E. Bonetti, G. Guerra, V. Rosti, Endothelial progenitor cells support tumour growth and metastatisation: implications for the resistance to anti-angiogenic therapy, Tumour Biol, (2015).
[32] F. Moccia, V. Poletto, May the remodeling of the Ca(2+) toolkit in endothelial progenitor cells derived from cancer patients suggest alternative targets for anti-angiogenic treatment?, Biochim Biophys Acta, 1853 (2015) 1958-1973.
[33] D.A. Ingram, L.E. Mead, H. Tanaka, V. Meade, A. Fenoglio, K. Mortell, K. Pollok, M.J. Ferkowicz, D. Gilley, M.C. Yoder, Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood, Blood, 104 (2004) 2752-2760.
[34] F. Moccia, G. Guerra, Ca(2+) Signalling in Endothelial Progenitor Cells: Friend or Foe?, J Cell Physiol, 231 (2016) 314-327.

[35] S. Dragoni, U. Laforenza, E. Bonetti, F. Lodola, C. Bottino, R. Berra-Romani, G. Carlo Bongio, M.P. Cinelli, G. Guerra, P. Pedrazzoli, V. Rosti, F. Tanzi, F. Moccia, Vascular endothelial growth factor stimulates endothelial colony forming cells proliferation and tubulogenesis by inducing oscillations in intracellular Ca2+ concentration, Stem Cells, 29 (2011) 1898-1907.
[36] E. Zuccolo, C. Bottino, F. Diofano, V. Poletto, A.C. Codazzi, S. Mannarino, R. Campanelli, G. Fois, G.L. Marseglia, G. Guerra, D. Montagna, U. Laforenza, V. Rosti, M. Massa, F. Moccia, Constitutive store-operated Ca2+ entry leads to enhanced nitric oxide production and proliferation in infantile hemangioma-derived endothelial colony forming cells, Stem Cells Dev, (2015).
[37] S. Dragoni, G. Guerra, A. Fiorio Pla, G. Bertoni, A. Rappa, V. Poletto, C. Bottino, A. Aronica, F. Lodola, M.P. Cinelli, U. Laforenza, V. Rosti, F. Tanzi, L. Munaron, F. Moccia, A functional Transient Receptor Potential Vanilloid 4 (TRPV4) channel is expressed in human endothelial progenitor cells, J Cell Physiol, 230 (2015) 95-104.
[38] Y. Denizot, C. Dulery, F. Trimoreau, V. Desplat, V. Praloran, Arachidonic acid and human bone marrow stromal cells, Biochim Biophys Acta, 1402 (1998) 209-215.
[39] M.T. Rizzo, The role of arachidonic acid in normal and malignant hematopoiesis, Prostaglandins Leukot Essent Fatty Acids, 66 (2002) 57-69.
[40] R.S. Richmond, E.A. Tallant, P.E. Gallagher, C.M. Ferrario, W.B. Strawn, Angiotensin II stimulates arachidonic acid release from bone marrow stromal cells, J Renin Angiotensin Aldosterone Syst, 5 (2004) 176-182.
[41] A.R. Brash, Arachidonic acid as a bioactive molecule, J Clin Invest, 107 (2001) 1339-1345.
[42] E.J. Neufeld, P.W. Majerus, Arachidonate release and phosphatidic acid turnover in stimulated human platelets, J Biol Chem, 258 (1983) 2461-2467.
[43] R.M. Giordano, J.W. Newman, T.L. Pedersen, M.I. Ramos, C.L. Stebbins, Effects of dynamic exercise on plasma arachidonic acid epoxides and diols in human volunteers, Int J Sport Nutr Exerc Metab, 21 (2011) 471-479.
[44] S. Hammarstrom, M. Hamberg, B. Samuelsson, E.A. Duell, M. Stawiski, J.J. Voorhees, Increased concentrations of nonesterified arachidonic acid, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid, prostaglandin E2, and prostaglandin F2alpha in epidermis of psoriasis, Proc Natl Acad Sci U S A, 72 (1975) 5130-5134.
[45] V. Allaj, C. Guo, D. Nie, Non-steroid anti-inflammatory drugs, prostaglandins, and cancer, Cell Biosci, 3 (2013) 8.
[46] C.M. Jenkins, A. Cedars, R.W. Gross, Eicosanoid signalling pathways in the heart, Cardiovasc Res, 82 (2009) 240-249.
[47] Y. Sánchez-Hernández, U. Laforenza, E. Bonetti, J. Fontana, S. Dragoni, M. Russo, J.E. Avelino-Cruz, S. Schinelli, D. Testa, G. Guerra, V. Rosti, F. Tanzi, F. Moccia, Store-operated Ca(2+) entry is expressed in human endothelial progenitor cells, Stem Cells Dev, 19 (2010) 1967-1981.
[48] F. Lodola, U. Laforenza, E. Bonetti, D. Lim, S. Dragoni, C. Bottino, H.L. Ong, G. Guerra, C. Ganini, M. Massa, M. Manzoni, I.S. Ambudkar, A.A. Genazzani, V. Rosti, P. Pedrazzoli, F. Tanzi, F. Moccia, C. Porta, Store-operated Ca^{2+} entry is remodelled and controls in vitro angiogenesis in endothelial progenitor cells isolated from tumoral patients, PloS One, 7 (2012) e42541.
[49] Y. Sanchez-Hernandez, U. Laforenza, E. Bonetti, J. Fontana, S. Dragoni, M. Russo, J.E. Avelino-Cruz, S. Schinelli, D. Testa, G. Guerra, V. Rosti, F. Tanzi, F. Moccia, Store-Operated Ca2+ Entry Is Expressed in Human Endothelial Progenitor Cells, Stem Cells Dev, 19 (2010) 1967-1981.
[50] F. Moccia, R. Berra-Romani, S. Baruffi, S. Spaggiari, S. Signorelli, L. Castelli, J. Magistretti, V. Taglietti, F. Tanzi, Ca2+ uptake by the endoplasmic reticulum Ca2+-ATPase in rat microvascular endothelial cells, Biochem J, 364 (2002) 235-244.
[51] R. Berra-Romani, J.E. Avelino-Cruz, A. Raqeeb, A. Della Corte, M. Cinelli, S. Montagnani, G. Guerra, F. Moccia, F. Tanzi, Ca2+-dependent nitric oxide release in the injured endothelium of excised

rat aorta: a promising mechanism applying in vascular prosthetic devices in aging patients, BMC Surg, 13 Suppl 2 (2013) S40.
[52] B. Selvakumar, D.T. Hess, P.J. Goldschmidt-Clermont, J.S. Stamler, Co-regulation of constitutive nitric oxide synthases and NADPH oxidase by the small GTPase Rac, FEBS Lett, 582 (2008) 2195-2202.
[53] T. Pearson, A. McArdle, M.J. Jackson, Nitric oxide availability is increased in contracting skeletal muscle from aged mice, but does not differentially decrease muscle superoxide, Free Radic Biol Med, 78 (2015) 82-88.
[54] P. Catarsi, V. Rosti, G. Morreale, V. Poletto, L. Villani, R. Bertorelli, M. Pedrazzini, M. Zorzetto, G. Barosi, JAK2 exon 14 skipping in patients with primary myelofibrosis: a minor splice variant modulated by the JAK2-V617F allele burden, PLoS One, 10 (2015) e0116636.
[55] M.E. Bernardo, N. Zaffaroni, F. Novara, A.M. Cometa, M.A. Avanzini, A. Moretta, D. Montagna, R. Maccario, R. Villa, M.G. Daidone, O. Zuffardi, F. Locatelli, Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms, Cancer Res, 67 (2007) 9142-9149.
[56] M.J. McArthur, B.P. Atshaves, A. Frolov, W.D. Foxworth, A.B. Kier, F. Schroeder, Cellular uptake and intracellular trafficking of long chain fatty acids, J Lipid Res, 40 (1999) 1371-1383.
[57] C. Dubois, F. Vanden Abeele, V. Lehen'kyi, D. Gkika, B. Guarmit, G. Lepage, C. Slomianny, A.S. Borowiec, G. Bidaux, M. Benahmed, Y. Shuba, N. Prevarskaya, Remodeling of channel-forming ORAI proteins determines an oncogenic switch in prostate cancer, Cancer Cell, 26 (2014) 19-32.
[58] M.A. Thompson, Y.S. Prakash, C.M. Pabelick, Arachidonate-regulated Ca(2+) influx in human airway smooth muscle, Am J Respir Cell Mol Biol, 51 (2014) 68-76.
[59] J.L. Thompson, T.J. Shuttleworth, Molecular basis of activation of the arachidonate-regulated Ca2+ (ARC) channel, a store-independent Orai channel, by plasma membrane STIM1, J Physiol, 591 (2013) 3507-3523.
[60] H. Meves, Arachidonic acid and ion channels: an update, Br J Pharmacol, 155 (2008) 4-16.
[61] L. Munaron, S. Antoniotti, C. Distasi, D. Lovisolo, Arachidonic acid mediates calcium influx induced by basic fibroblast growth factor in Balb-c 3T3 fibroblasts, Cell Calcium, 22 (1997) 179-188.
[62] S. Dragoni, U. Laforenza, E. Bonetti, F. Lodola, C. Bottino, G. Guerra, A. Borghesi, M. Stronati, V. Rosti, F. Tanzi, F. Moccia, Canonical transient receptor potential 3 channel triggers vascular endothelial growth factor-induced intracellular Ca2+ oscillations in endothelial progenitor cells isolated from umbilical cord blood, Stem Cells Dev, 22 (2013) 2561-2580.
[63] Y. Itoh, Y. Kawamata, M. Harada, M. Kobayashi, R. Fujii, S. Fukusumi, K. Ogi, M. Hosoya, Y. Tanaka, H. Uejima, H. Tanaka, M. Maruyama, R. Satoh, S. Okubo, H. Kizawa, H. Komatsu, F. Matsumura, Y. Noguchi, T. Shinohara, S. Hinuma, Y. Fujisawa, M. Fujino, Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40, Nature, 422 (2003) 173-176.
[64] E. Zuccolo, D. Lim, V. Poletto, G. Guerra, F. Tanzi, V. Rosti, F. Moccia, Acidic Ca^{2+} stores interact with the endoplasmic reticulum to shape intracellular Ca2 + signals in human endothelial progenitor cells, Vascular Pharmacology, 2015, pp. 70-71.
[65] A. Galione, A.J. Morgan, A. Arredouani, L.C. Davis, K. Rietdorf, M. Ruas, J. Parrington, NAADP as an intracellular messenger regulating lysosomal calcium-release channels, Biochem Soc Trans, 38 (2010) 1424-1431.
[66] A.J. Morgan, F.M. Platt, E. Lloyd-Evans, A. Galione, Molecular mechanisms of endolysosomal Ca2+ signalling in health and disease, Biochem J, 439 (2011) 349-374.
[67] V. Ronco, D.M. Potenza, F. Denti, S. Vullo, G. Gagliano, M. Tognolina, G. Guerra, P. Pinton, A.A. Genazzani, L. Mapelli, D. Lim, F. Moccia, A novel Ca^{2+}-mediated cross-talk between

endoplasmic reticulum and acidic organelles: implications for NAADP-dependent Ca^{2+} signalling, Cell Calcium, 57 (2015) 89-100.
[68] A.M. Holmes, H.L. Roderick, F. McDonald, M.D. Bootman, Interaction between store-operated and arachidonate-activated calcium entry, Cell Calcium, 41 (2007) 1-12.
[69] D. Luo, L.M. Broad, G.S. Bird, J.W. Putney, Mutual antagonism of calcium entry by capacitative and arachidonic acid-mediated calcium entry pathways, J Biol Chem, 276 (2001) 20186-20189.
[70] E.N. Dedkova, L.A. Blatter, Nitric oxide inhibits capacitative Ca2+ entry and enhances endoplasmic reticulum Ca2+ uptake in bovine vascular endothelial cells, J Physiol, 539 (2002) 77-91.
[71] A. Raqeeb, J. Sheng, N. Ao, A.P. Braun, Purinergic P2Y2 receptors mediate rapid Ca(2+) mobilization, membrane hyperpolarization and nitric oxide production in human vascular endothelial cells, Cell Calcium, 49 (2011) 240-248.
[72] J.Z. Sheng, S. Ella, M.J. Davis, M.A. Hill, A.P. Braun, Openers of SKCa and IKCa channels enhance agonist-evoked endothelial nitric oxide synthesis and arteriolar vasodilation, FASEB J, 23 (2009) 1138-1145.
[73] F. Moccia, F. Tanzi, L. Munaron, Endothelial remodelling and intracellular calcium machinery, Curr Mol Med, 14 (2014) 457-480.
[74] F. Moccia, S. Dragoni, F. Lodola, E. Bonetti, C. Bottino, G. Guerra, U. Laforenza, V. Rosti, F. Tanzi, Store-dependent Ca(2+) entry in endothelial progenitor cells as a perspective tool to enhance cell-based therapy and adverse tumour vascularization, Curr Med Chem, 19 (2012) 5802-5818.
[75] M.E. Surette, J.D. Winkler, A.N. Fonteh, F.H. Chilton, Relationship between arachidonate--phospholipid remodeling and apoptosis, Biochemistry, 35 (1996) 9187-9196.
[76] F.H. Chilton, M.E. Surette, J.D. Winkler, Arachidonate-phospholipid remodeling and cell proliferation, Adv Exp Med Biol, 416 (1996) 169-172.
[77] J. Ben-Shoshan, J. George, Endothelial progenitor cells as therapeutic vectors in cardiovascular disorders: from experimental models to human trials, Pharmacol Ther, 115 (2007) 25-36.
[78] S. Li, Y. Tian, X. Huang, Y. Zhang, D. Wang, H. Wei, J. Dong, R. Jiang, J. Zhang, Intravenous transfusion of endothelial colony-forming cells attenuates vascular degeneration after cerebral aneurysm induction, Brain Res, 1593 (2014) 65-75.
[79] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: Dynamics, homeostasis and remodelling, Nat Rev Mol Cell Biol, 4 (2003) 517-529.
[80] F. Sekiya, Y.S. Bae, D.Y. Jhon, S.C. Hwang, S.G. Rhee, AHNAK, a protein that binds and activates phospholipase C-gamma1 in the presence of arachidonic acid, J Biol Chem, 274 (1999) 13900-13907.
[81] I.H. Lee, J.O. You, K.S. Ha, D.S. Bae, P.G. Suh, S.G. Rhee, Y.S. Bae, AHNAK-mediated activation of phospholipase C-gamma1 through protein kinase C, J Biol Chem, 279 (2004) 26645-26653.
[82] P. Zeitler, S. Handwerger, Arachidonic acid stimulates phosphoinositide hydrolysis and human placental lactogen release in an enriched fraction of placental cells, Mol Pharmacol, 28 (1985) 549-554.
[83] E.R. Muslikhov, I.F. Sukhanova, P.V. Avdonin, Arachidonic acid activates release of calcium ions from reticulum via ryanodine receptor channels in C2C12 skeletal myotubes, Biochemistry (Mosc), 79 (2014) 435-439.
[84] A.H. Guse, Linking NAADP to ion channel activity: a unifying hypothesis, Sci Signal, 5 (2012) pe18.
[85] A.J. Morgan, L.C. Davis, S.K. Wagner, A.M. Lewis, J. Parrington, G.C. Churchill, A. Galione, Bidirectional Ca^{2+} signaling occurs between the endoplasmic reticulum and acidic organelles, J Cell Biol, 200 (2013) 789-805.

[86] M.N. Sullivan, S. Earley, TRP channel Ca(2+) sparklets: fundamental signals underlying endothelium-dependent hyperpolarization, Am J Physiol Cell Physiol, 305 (2013) C999-C1008.
[87] S.K. Sonkusare, A.D. Bonev, J. Ledoux, W. Liedtke, M.I. Kotlikoff, T.J. Heppner, D.C. Hill-Eubanks, M.T. Nelson, Elementary Ca2+ signals through endothelial TRPV4 channels regulate vascular function, Science, 336 (2012) 597-601.
[88] M. Isshiki, Y.S. Ying, T. Fujita, R.G. Anderson, A molecular sensor detects signal transduction from caveolae in living cells, J Biol Chem, 277 (2002) 43389-43398.
[89] W. Everaerts, B. Nilius, G. Owsianik, The vanilloid transient receptor potential channel TRPV4: from structure to disease, Prog Biophys Mol Biol, 103 (2010) 2-17.
[90] S. Heller, R.G. O'Neil, Molecular Mechanisms of TRPV4 Gating, (2007).
[91] J. Chemin, M. Cazade, P. Lory, Modulation of T-type calcium channels by bioactive lipids, Pflugers Arch, 466 (2014) 689-700.
[92] M.L. Roberts-Crowley, T. Mitra-Ganguli, L. Liu, A.R. Rittenhouse, Regulation of voltage-gated Ca2+ channels by lipids, Cell Calcium, 45 (2009) 589-601.
[93] F.J. Taberner, G. Fernandez-Ballester, A. Fernandez-Carvajal, A. Ferrer-Montiel, TRP channels interaction with lipids and its implications in disease, Biochim Biophys Acta, 1848 (2015) 1818-1827.
[94] R.D. Minshall, W.C. Sessa, R.V. Stan, R.G. Anderson, A.B. Malik, Caveolin regulation of endothelial function, Am J Physiol Lung Cell Mol Physiol, 285 (2003) L1179-1183.
[95] S. Antoniotti, A.F. Pla, S. Pregnolato, A. Mottola, D. Lovisolo, L. Munaron, Control of endothelial cell proliferation by calcium influx and arachidonic acid metabolism: A pharmacological approach, J Cell Physiol, 197 (2003) 370-378.
[96] B. Torok-Storb, M. Iwata, L. Graf, J. Gianotti, H. Horton, M.C. Byrne, Dissecting the marrow microenvironment, Ann N Y Acad Sci, 872 (1999) 164-170.
[97] M.C. Yoder, Human endothelial progenitor cells, Cold Spring Harb Perspect Med, 2 (2012) a006692.
[98] H. Katsuki, S. Okuda, Arachidonic acid as a neurotoxic and neurotrophic substance, Prog Neurobiol, 46 (1995) 607-636.
[99] S. Koch, L. Claesson-Welsh, Signal transduction by vascular endothelial growth factor receptors, Cold Spring Harb Perspect Med, 2 (2012) a006502.
[100] C.A. Hyde, S. Missailidis, Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis, Int Immunopharmacol, 9 (2009) 701-715.

FIGURE LEGENDS

Figure 1. Arachidonic acid elicits a dose-dependent increase in $[Ca^{2+}]_i$ in human endothelial colony forming cells. A, 20 μM AA induced a rapid elevation in $[Ca^{2+}]_i$ that reversed when the agonist was removed from the bath. The re-addition of AA at the same dose evoked a second $[Ca^{2+}]_i$ rise that reached the same peak as the first one. In this and the following figures, agonists and drugs were administered at the time indicated by the horizontal bars. B, representative Ca^{2+} traces of the dose-response relationship of AA-induced Ca^{2+} signals in hECFCs. C, mean±SE of the percentage of responding cells recorded at various AA concentrations. D, mean±SE of the amplitude of the initial Ca^{2+} peak induced by various AA concentrations. The asterisk indicates $p<0.05$.

Figure 2. Arachidonic acid elicits both Ca^{2+} release and Ca^{2+} entry in human endothelial colony forming cells. HECFCs have been stimulated with different doses of AA (A, 5 μM; B, 20 μM; C, 50 μM) in the absence of external Ca^{2+} ($0Ca^{2+}$) to exclusively monitor intracellular Ca^{2+} mobilization. When $[Ca^{2+}]_i$ decayed to resting levels, extracellular Ca^{2+} was restored to exclusively measure external Ca^{2+} influx. D, mean±SE of the amplitude of Ca^{2+} release and Ca^{2+} entry induced by AA at the difference concentrations tested. The asterisk indicates $p<0.05$.

Figure 3. Arachidonic acid-evoked increase in $[Ca^{2+}]_i$ in human endothelial colony forming cells does not require AA metabolism. A, 20 μM eicosatetraynoic acid (ETYA), a non-metabolizable analogue of AA, causes a similar increase in $[Ca^{2+}]_I$ to that induced by 20 μM AA. B, mean±SE of the percentage of responding cells recorded in the presence of ETYA (20 μM) and AA (20 μM). C, mean±SE of the magnitude of ETYA- and AA-evoked $[Ca^{2+}]_i$ elevations.

Figure 4. $InsP_3Rs$ and TPC1 mediate AA-evoked intracellular Ca^{2+} release. A, representative traces of the Ca^{2+} signals evoked by AA (20 μM) in the absence and in the presence of either 2-APB (50 μM, 20 min), a specific $InsP_3R$ inhibitor, U73122 (10 μM, 10 min), which selectively blocks PLC activity, or Ned-19 (100 μM), a selective TPC antagonist. These recordings were carried out in the absence of extracellular Ca^{2+} ($0Ca^{2+}$) in order to selectively focus on intracellular Ca^{2+} mobilization. B, mRNA levels of TPC1 and TPC2 in hECFCs. Data are expressed as mean ± SE of three qPCR runs performed in triplicate using samples prepared from three healthy subjects. Negative

controls were conducted by omitting reverse transcriptase reaction (not shown). The asterisk indicates $p<0.05$. C, depleting the ER Ca^{2+} pool by administrating the selective SERCA inhibitor, CPA (10 μM) upon removal of external Ca^{2+} ($0Ca^{2+}$) prevented the Ca^{2+} response both to the $InsP_3$-producing agonist, ATP (100 μM), and to AA (20 μM). D, GPN (200 μM), a freely diffusible dipeptide that causes the osmotic lysis of cathepsin C-positive lysosomes, caused an increase in $[Ca^{2+}]_i$. due to the liberation of the lysosomal Ca^{2+} content. The subsequent addition of AA (20 μM) failed to generate any detectable Ca^{2+} signal. E, mean±SE of the percentage of responding cells in the presence of the designated treatments. The asterisk indicates $p<0.05$. F, mean±SE of the magnitude of AA-evoked $[Ca^{2+}]_i$ elevations under the designated treatments. The asterisk indicates $p<0.05$.

Figure 5. Arachidonic acid activates TRPV4 and inhibits SOCE in human endothelial colony forming cells. A, representative traces of the Ca^{2+} signals elicited by AA (20 μM) in the absence and in the presence of RN-1734 (20 μM, 30 min) or ruthenium red (RR; 10 μM, 30 min), two specific TRPV4 blockers. B, mean±SE of the percentage of responding cells in the presence of the designated treatments. The asterisk indicates $p<0.05$. C, mean±SE of the magnitude of AA-evoked $[Ca^{2+}]_i$ elevations under the designated treatments. The asterisk indicates $p<0.05$. D, the application of AA (20 μM) upon CPA-evoked Ca^{2+} plateau caused a rapid decrease in Ca^{2+} entry which was due to SOCE inhibition. The decrease in Ca^{2+} inflow was followed by a sustained Ca^{2+} signal that disappeared when hECFCs were pre-treated in the presence of RN-1734 (20 μM, 30 min).

Figure 6. Arachidonic acid stimulates Ca^{2+}-dependent NO production in human endothelial colony forming cells. A, representative tracings of AA-evoked NO synthesis in the absence and in the presence of either L-NAME (100 μM, 75 min) or BAPTA (30 μM, 2 h). As evident from the trace, there is no detectable increase in NO release under such conditions. C, representative tracings of AA-induced NO production in the absence and in the presence of 2-APB (50 μM, 20 min) or Ned-19 (100 μM, 1 hour). D, representative tracings of AA-evoked NO signals in the absence and in the presence of RN-1734 (20 μM, 30 min). In all these experiments, AA was administrated at 20 μM. E, the elevations in NO levels triggered by AA (20 μM) and ETYA (20 μM) displayed the same amplitude and kinetics. F, mean±SE of the magnitude of AA-evoked NO synthesis under the designated treatments. The asterisk indicated $p<0.05$. The amplitude of ETYA-evoked NO release was similar to that stimulated by AA.

Figure 7. TRPV4, but not SOCE, stimulates NO production in human endothelial colony forming cells. A, the activation of SOCE with CPA (10 μM) did not elicit any detectable elevation in NO levels in hECFCs. B, GSK (10 nM), a selective TRPV4 agonist, could trigger a sizeable NO signal in hECFCs. C, mean±SE of the percentage of hECFCs showing NO production in response to either CPA or GSK. The asterisk indicates $p<0.05$.

Figure 8. Arachidonic acid promotes growth factors-induced proliferation in human endothelial colony forming cells. A, mean±SE of the fold change in hECFC proliferation under the designated treatments. Please, note that AA (20 μM) per se was not able to induce cell replication. The asterisk indicates $p<0.05$. B and C, mean±SE of the fold change in hECFC proliferation under the designated treatments. AA does not increase the rate of cell proliferation upon inhibition of the concomitant Ca^{2+} and NO signals.

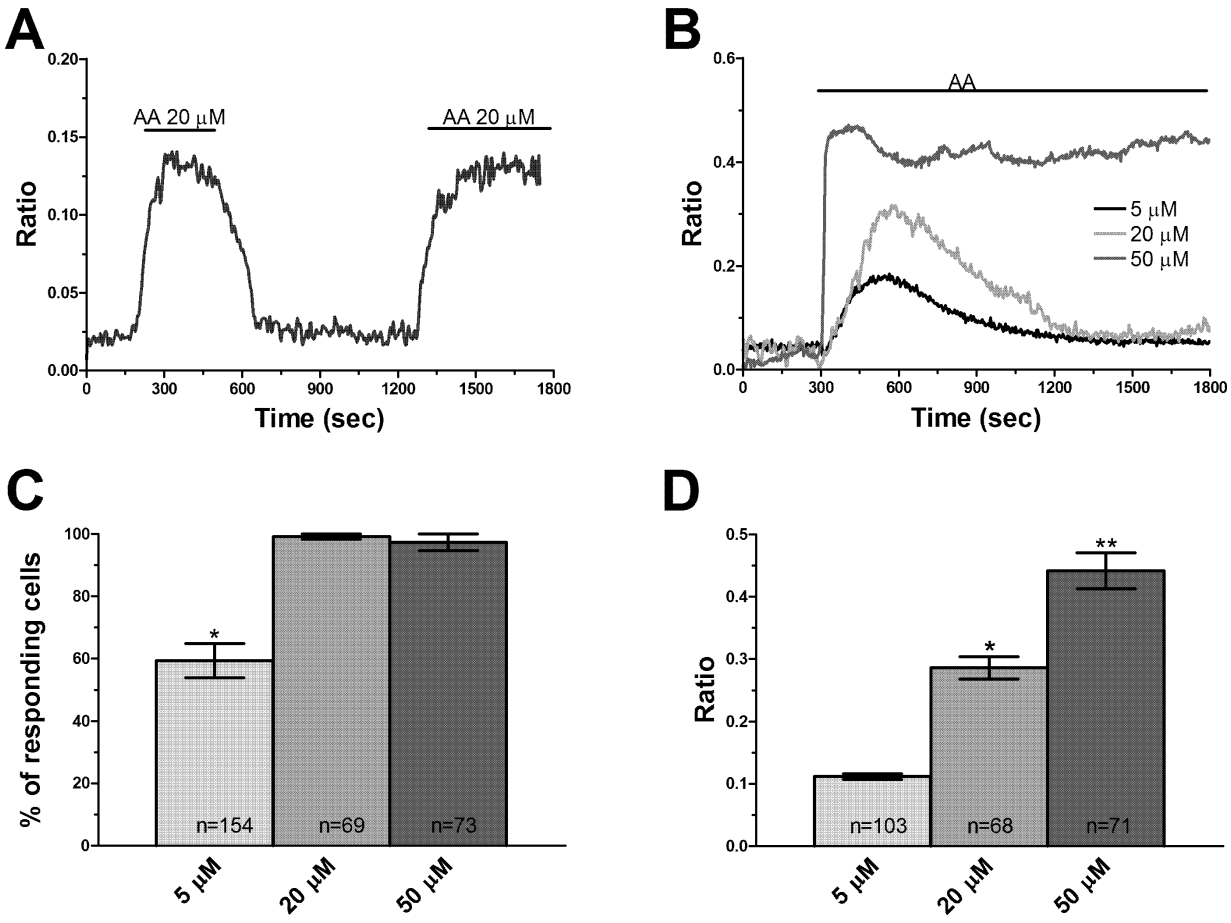


Figure 1

Arachidonic acid signaling in human ECFCs

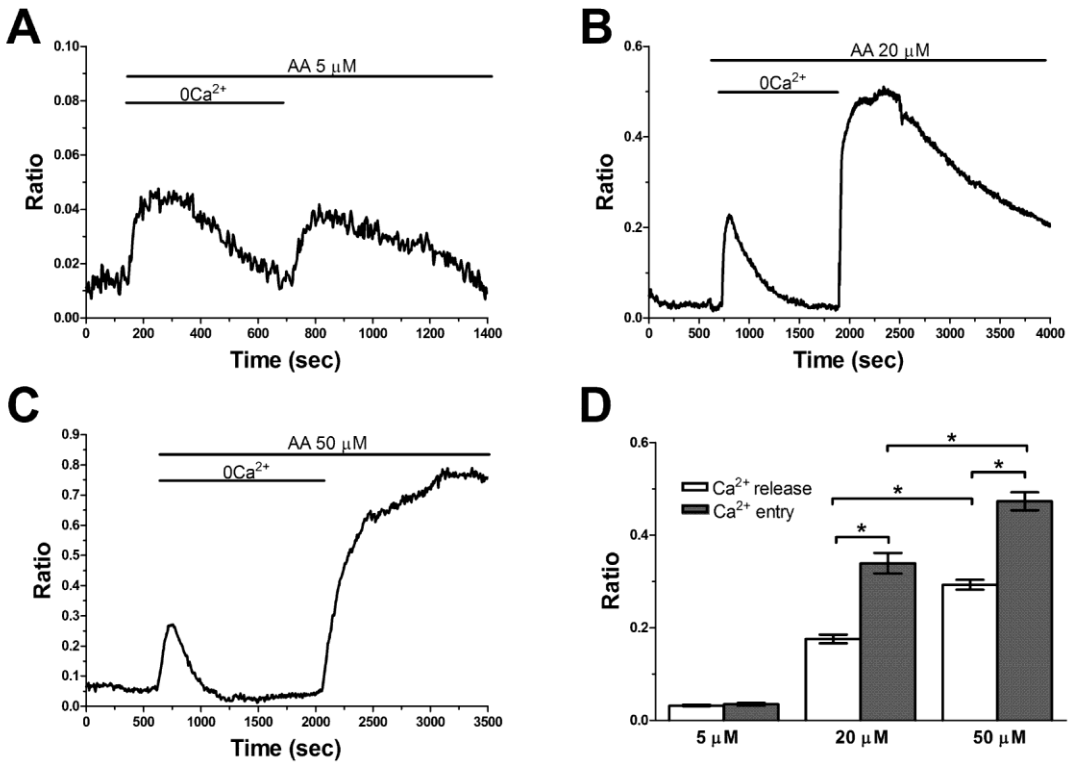


Figure 2

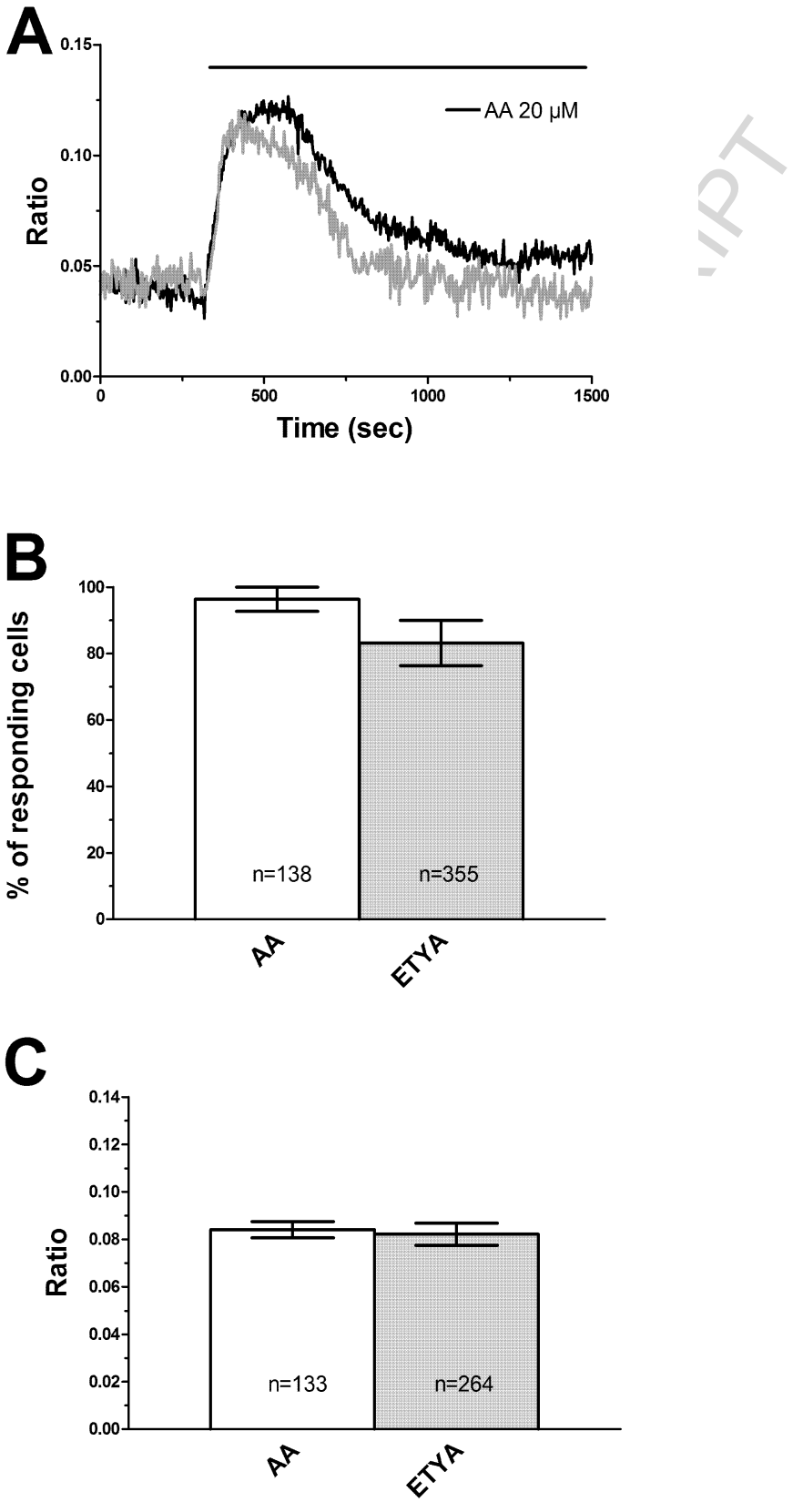


Figure 3

Arachidonic acid signaling in human ECFCs

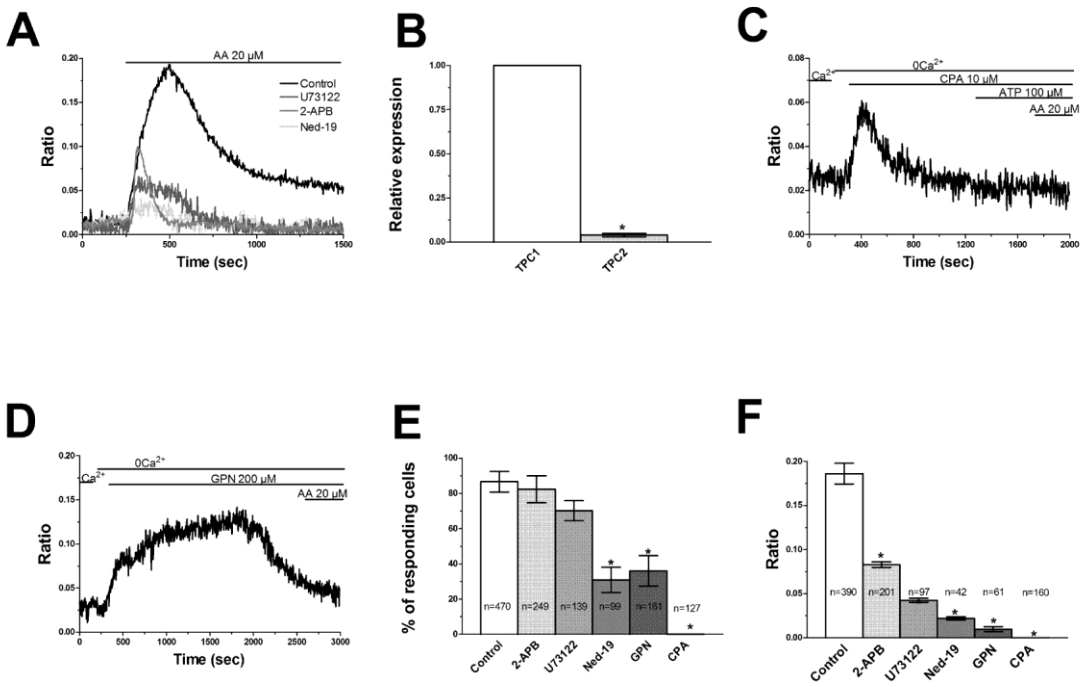


Figure 4

Arachidonic acid signaling in human ECFCs

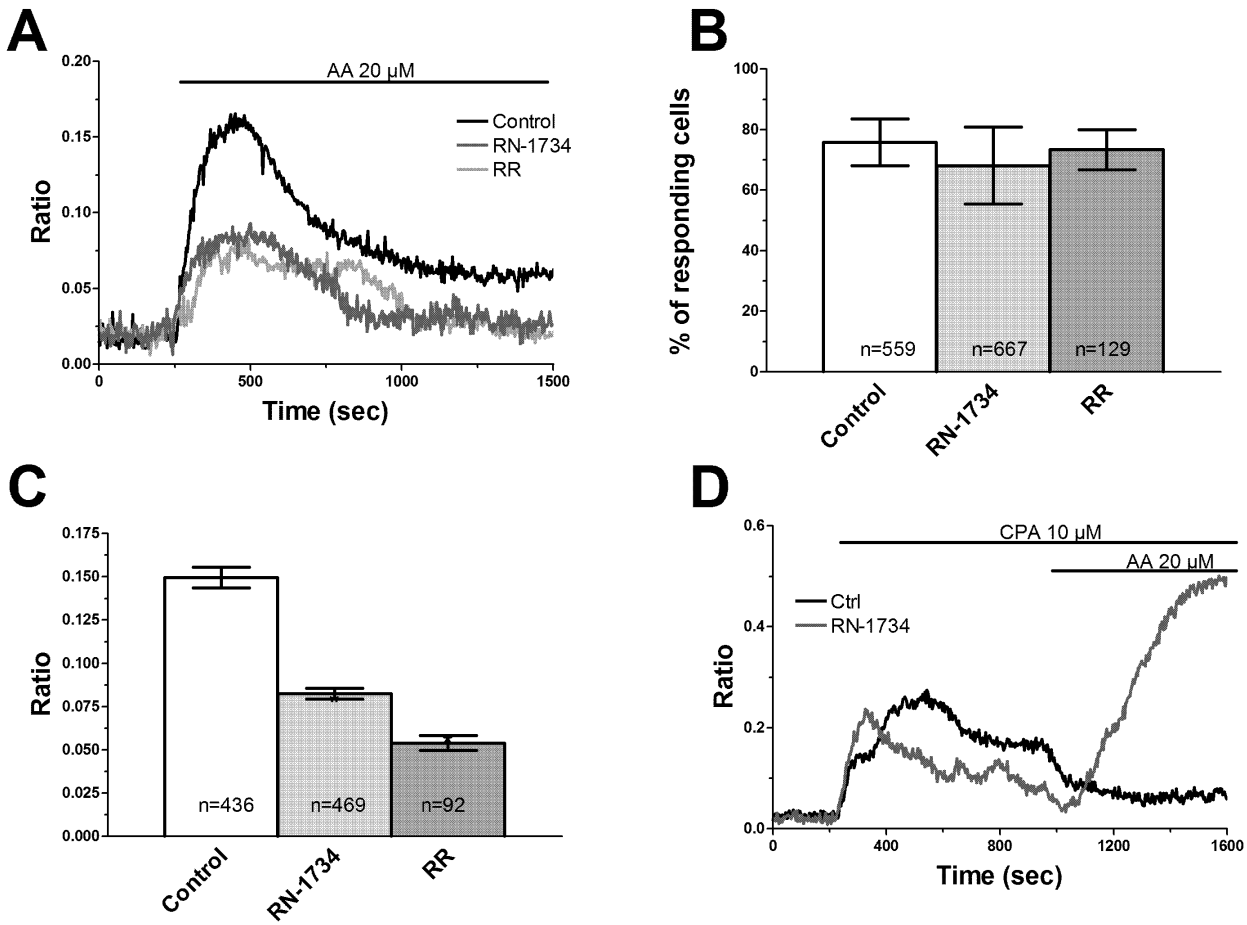


Figure 5

Arachidonic acid signaling in human ECFCs

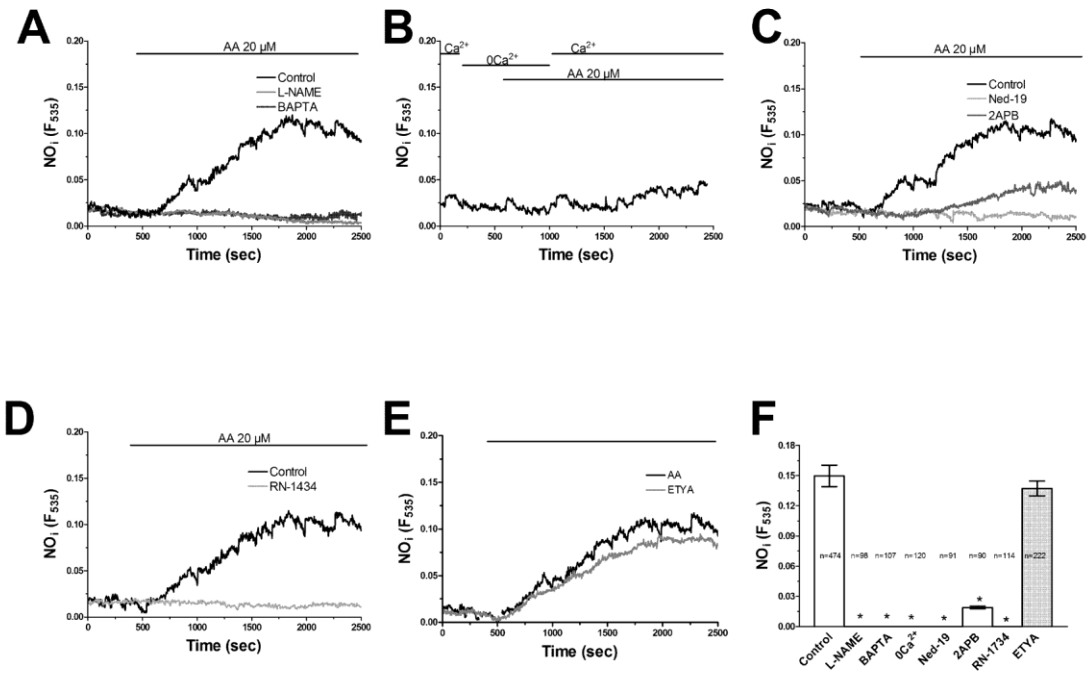


Figure 6

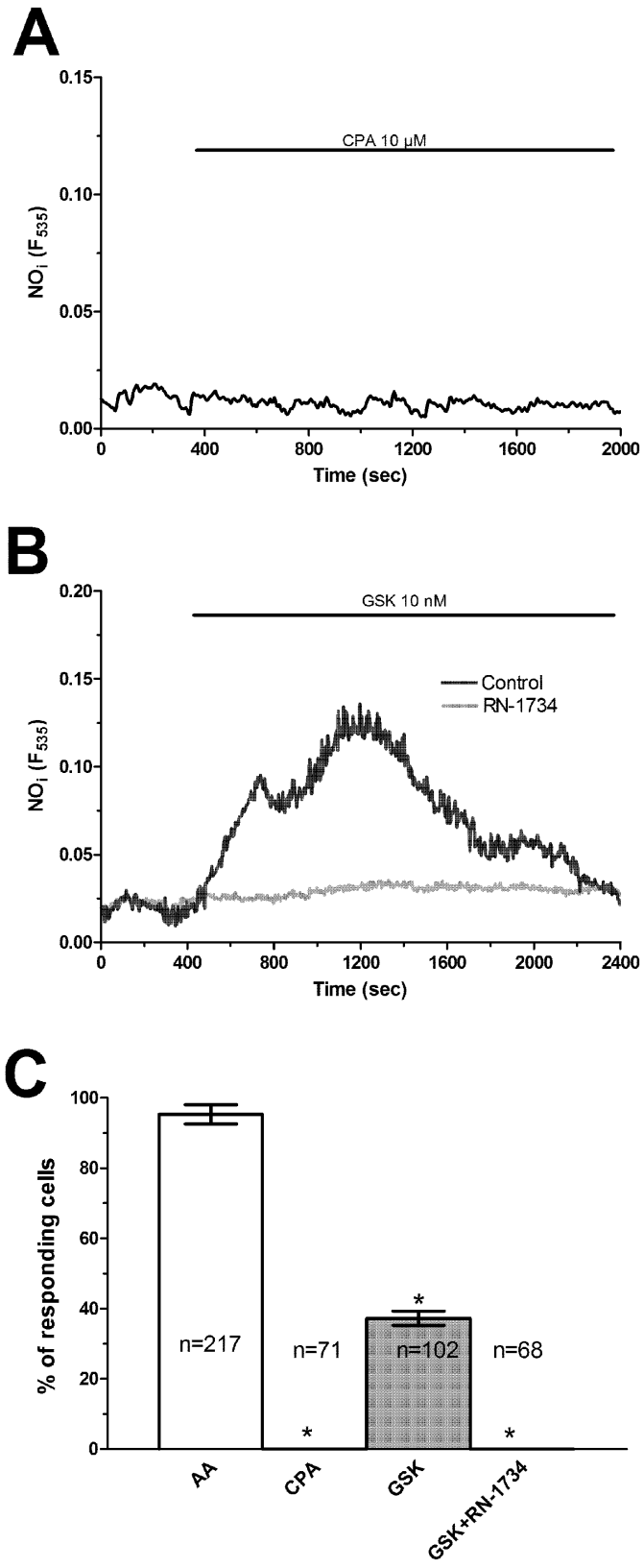


Figure 7

Arachidonic acid signaling in human ECFCs

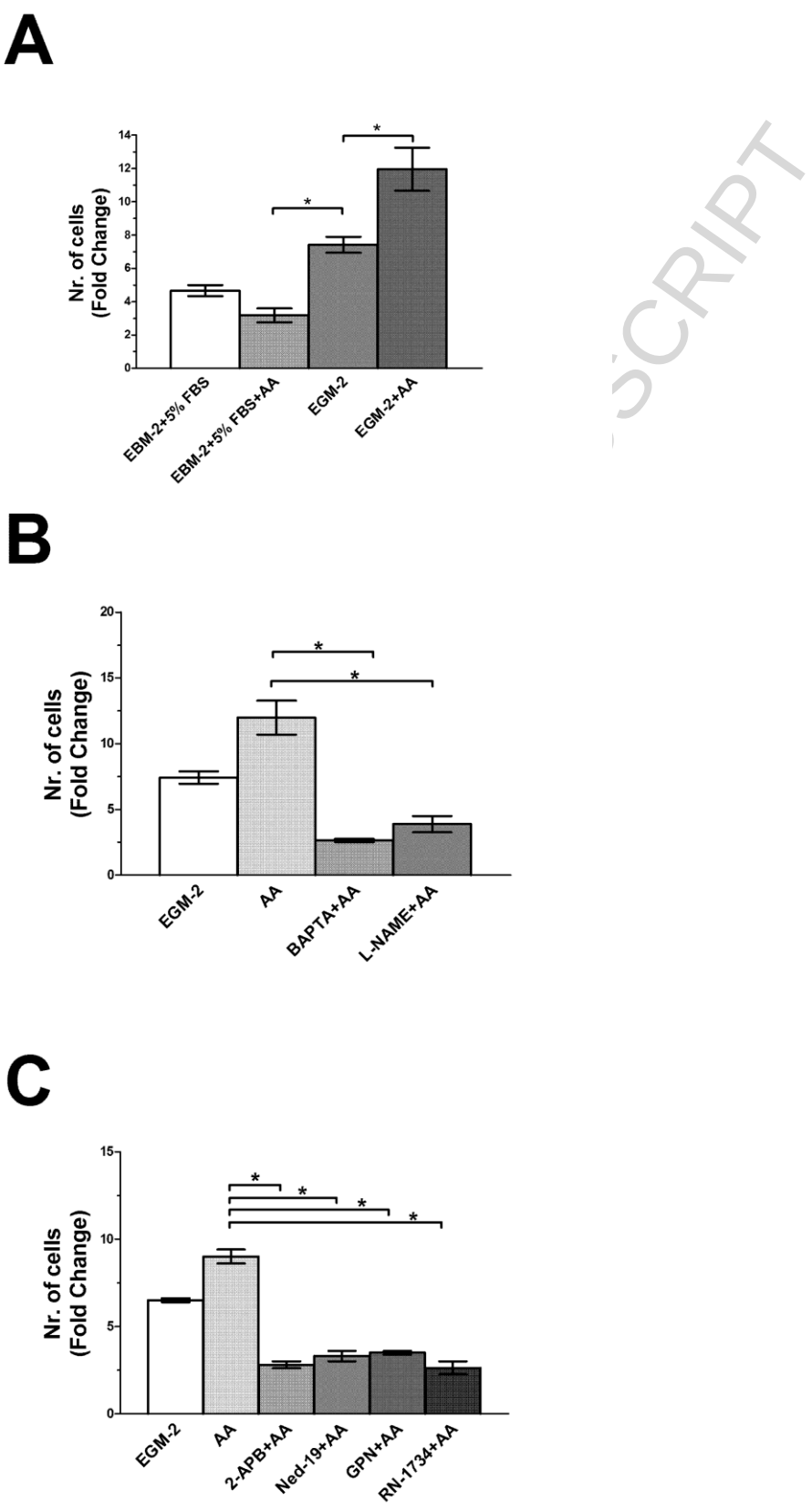
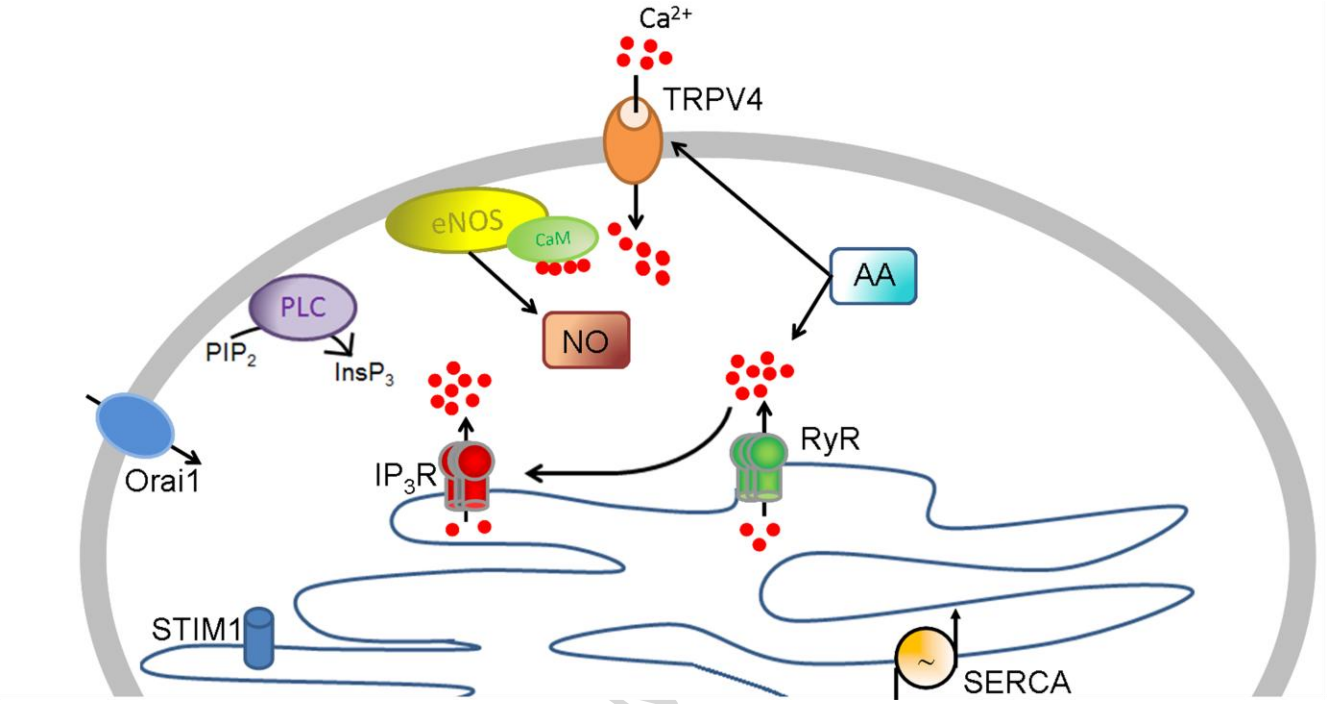


Figure 8

Table 1. Ryanodine receptor transcripts are absent in human endothelial colony forming cells

Sample	Cq (SD) GAPDH	Cq (SD) YWHAZ	Cq (SD) TBP	Cq (SD) RYR1	Cq (SD) RYR2	Cq (SD) RYR3	ΔCq (GAPDH)
hECFC 1	17.68 (0.03)	20.15 (0.08)	25.49 (0.11)	-	-	-	-
hECFC 2	18.09 (0.06)	19.96 (0.10)	24.86 (0.12)	-	-	-	-
hECFC 3	17.61 (0.09)	19.25 (0.10)	24.47 (0.09)	-	-	-	-
K562	17.15 (0.01)	19.14 (0.03)	22.21 (0.08)	32.10 (0.14)	-	-	14.95
UKE1	17.14 (0.02)	19.13 (0.001)	23.02 (0.04)	-	-	-	-
DAMI	17.26 (0.13)	21.86 (0.28)	24.61 (0.07)	38.82 (0.55)	-	-	21.56
HELA	18.28 (0.06)	22.20 (0.06)	26.32 (0.05)	-	-	-	-
Mesenchymal	18.27 (0.08)	20.09 (0.18)	24.53 (0.05)	-	34.11 (0.07)	-	15.84
HUVEC	17.81 (0.03)	19.47 (0.02)	24.62 (0.01)	-	-	39.18 (0.12)	21.37

Arachidonic acid signaling in human ECFCs



Graphical abstract