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Endothelial regulation of calmodulin expression and eNOS–calmodulin interaction in vascular smooth muscle

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Abstract

Calmodulin (CaM) is a Ca^{2+} sensor protein that is required for numerous vascular smooth muscle cell (VSMC) functions. Since CaM is not expressed enough for its many target proteins, factors that modulate its expression and interactions with targets in VSMCs can have extensive effects on vascular functions. VSMCs receive many regulatory inputs from endothelial cells (ECs). However, it is unknown if ECs regulate vascular functions via controlling expression of CaM and its interactions in VSMCs. In this work, we tested the hypothesis that ECs also affect VSMC signaling via regulation of CaM expression and interactions with its target proteins in VSMCs. Using ECs and VSMCs isolated from the same vessels and grown in a co-culture system, we observed that the presence of proliferating ECs significantly upregulates total CaM expression in VSMCs. An imaging module was devised to concurrently measure free Ca^{2+} and CaM levels in VSMCs in co-culture with ECs. Using indo-1/AM and a CaM biosensor built from a modified CaM-binding sequence of endothelial nitric oxide synthase (eNOS), this system revealed that in response to a generic Ca^{2+} signal, free Ca^{2+}-bound CaM level is enhanced ~ threefold in VSMCs in co-culture with proliferating ECs. Interestingly, VSMCs express eNOS and eNOS–CaM association in response to the same Ca^{2+} stimulus is also enhanced ~ threefold in VSMCs co-cultured with ECs. Mechanistically, the endothelium-dependent upregulation of CaM in VSMCs is not affected by inhibition of NO production or endothelin receptors but is prevented by inhibition of vascular endothelial growth factor receptors. Consistently, VEGF-A level is upregulated in VSMCs co-cultured with proliferating ECs. These data indicate a new role of the endothelium in regulating vascular functions via upregulating CaM and its interactions in VSMCs.

Keywords Endothelial cells · Vascular smooth muscle cells · Calmodulin · Nitric oxide synthase · Vascular endothelial growth factor

Introduction

Vascular smooth muscle cells (VSMCs) play crucial roles in vascular functions. Controlled contraction and relaxation of VSMCs are required for the maintenance of vascular tone and blood pressure. Vascular endothelial cells (ECs) secrete many soluble factors that modulate VSMC functions, such as endothelium-derived relaxing factors (EDRFs) such as nitric oxide (NO) and prostacyclin (PGI2), endothelium-derived hyperpolarizing factors, and endothelium-derived constricting factors (EDCFs) [1]. The balance between these factors is essential for vascular homeostasis.

As with many other cell types, many functions of VSMCs are dependent on Ca^{2+} and calmodulin (CaM), a 148-a.a. protein that serves as the ubiquitous Ca^{2+} sensor across tissues [2, 3]. Agonists such as angiotensin II triggers sarcoplasmic reticulum Ca^{2+} release and Ca^{2+} entry, leading to formation of Ca^{2+}–CaM complexes and subsequent activation of numerous CaM target proteins, including myosin light chain kinase, whose activation is needed for VSMC contraction [4, 5]. CaM is estimated to interact with up to 300 cellular proteins [6]. Nevertheless, it is not sufficiently expressed for all its targets. It has long been estimated that up to 60% of the total cellular CaM is engaged in inseparable

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associations that prevent dynamic interactions with target proteins [7]. In smooth muscle cells, it was estimated that only 5% of total CaM is freely available [8]. The shortage of CaM, coupled to its varied affinities for target proteins, leads to dynamic competition in cells as a mode of functional coupling among members of the network of CaM-binding proteins in the vascular endothelium [9, 10]. Limiting CaM conditions have also been observed in cardiomyocytes [11], neurons [12], and HEK 293 cells [13]. Factors that upregulate CaM expression therefore have the potential to alter the activities of CaM-dependent protein network. For example, estrogen activates in the vascular endothelium a feed-forward mechanism at the G protein-coupled estrogen receptor 1 (GPER) that upregulates total and free CaM and prolongs cytoplasmic Ca^{2+} signals [14–16], thereby promoting CaM-dependent activities [14].

Although many VSMC functions are modulated by secretions from the endothelium [1], it is completely unknown if the endothelium can do this via modulation of CaM expression and its interactions with target proteins in VSMCs. In this study, we aimed to test the hypothesis that ECs also regulate VSMC functions by affecting CaM-dependent signaling. We used a cell co-culture system to investigate the effects of primary ECs on CaM expression in VSMCs isolated from the same blood vessels and devised an intracellular imaging module to simultaneously measure free Ca^{2+} and CaM signals in VSMCs co-cultured with ECs. The impact of this effect was examined by assessing CaM interaction with eNOS in VSMCs. Finally, pharmacological studies were carried out to identify the soluble factor secreted from ECs that is responsible for the upregulation in CaM in VSMCs.

Materials and methods

Materials

Newborn calf serum was obtained from Fisher. M199 medium was obtained from Millipore Sigma (St. Louis, MO). Mouse monoclonal anti-CaM antibody was obtained from EMD Millipore (Cat. #05-173MI). Total anti-eNOS antibody was from Cell Signaling (Cat. #9572S). Rabbit anti-β actin antibody was from Thermo Scientific (Cat. #RB9421P1). Rabbit polyclonal anti-VEGF-A antibody was obtained from Proteintech (Cat.# 19003-1-AP). All materials for biosensor and CaM purification were as reported previously [14].

Cell isolation

The protocols and procedures to isolate cells from porcine aortas were approved by Des Moines University Institutional Biosafety Committee. Primary porcine aortic endothelial cells (PAECs) and VSMCs were isolated freshly from the same vessels as described earlier [15–17]. Briefly, for isolation of PAECs, the intima of freshly collected porcine thoracic aortas was gently scraped and resuspended in M-199 medium (Sigma, St. Louis, MO) containing 10% newborn calf serum (Sigma) and 2% penicillin/streptomycin (MP Biomedicals, Solon, OH). Following removal of the intima, a short enzyme incubation with 0.02% collagenase, 0.1% papain, and 4 mM dithiothreitol was done to get rid of residual endothelial cells. The vessels were then rinsed with sterile PBS, and then cut into rectangular strips. The luminal surface of the strips was then incubated with the same enzyme mix for 90 min. The luminal surface was then scraped again, and cells from this enzyme treatment and scraping were collected. Cells were plated in culture dishes and incubated under 5% CO_2 and 90% humidity at 37 °C until subconfluence is reached. Endothelial and smooth muscle morphology and markers were confirmed as previously described [17].

PAECs–VSMCs co-culture

Six-well plates with Transwell permeable membranous inserts (Corning, Corning, NY) were used for co-culturing PAECs and VSMCs. For coimmunoprecipitation experiments that required a large amount of total protein load, monocultures or co-cultures were carried out in 100-mm Transwell dishes with permeable inserts (Corning, Corning, NY). The inserts have pore size of 0.4 µm and suspend ~ 1 mm from the bottom of the well, which allows for passage of soluble constituents. PAECs were seeded on top of the membranous inserts and cultured in a separate outer compartment for several hours to ensure adherence, while VSMCs were cultured on the bottom well of a separate outer compartment in the presence of an empty insert membrane. After several hours, the insert containing the PAECs were moved into the outer chamber containing the VSMCs for co-culture (Fig. 1A). Starting densities for PAECs and VSMCs were determined by cell counting.

Molecular biology

A FRET-based CaM biosensor ($BSCaM_{eNOS}$) was generated by inserting a modified CaM-binding sequence of eNOS (*AA*RKK**A**FKEVANAVKISASLM*AA*) between enhanced versions of cyan and citrine yellow fluorescent proteins (ECFP-EYFPc). The original threonine at position 495 (497 in bovine sequence) was substituted by an alanine to avoid in-cell alteration in phosphorylation at this site, which has been demonstrated to be important for the affinity of CaM and eNOS interaction in both endogenous and heterologous expression systems [9, 10, 18]. Two alanine residues were incorporated to the ends of the modified CaM-binding

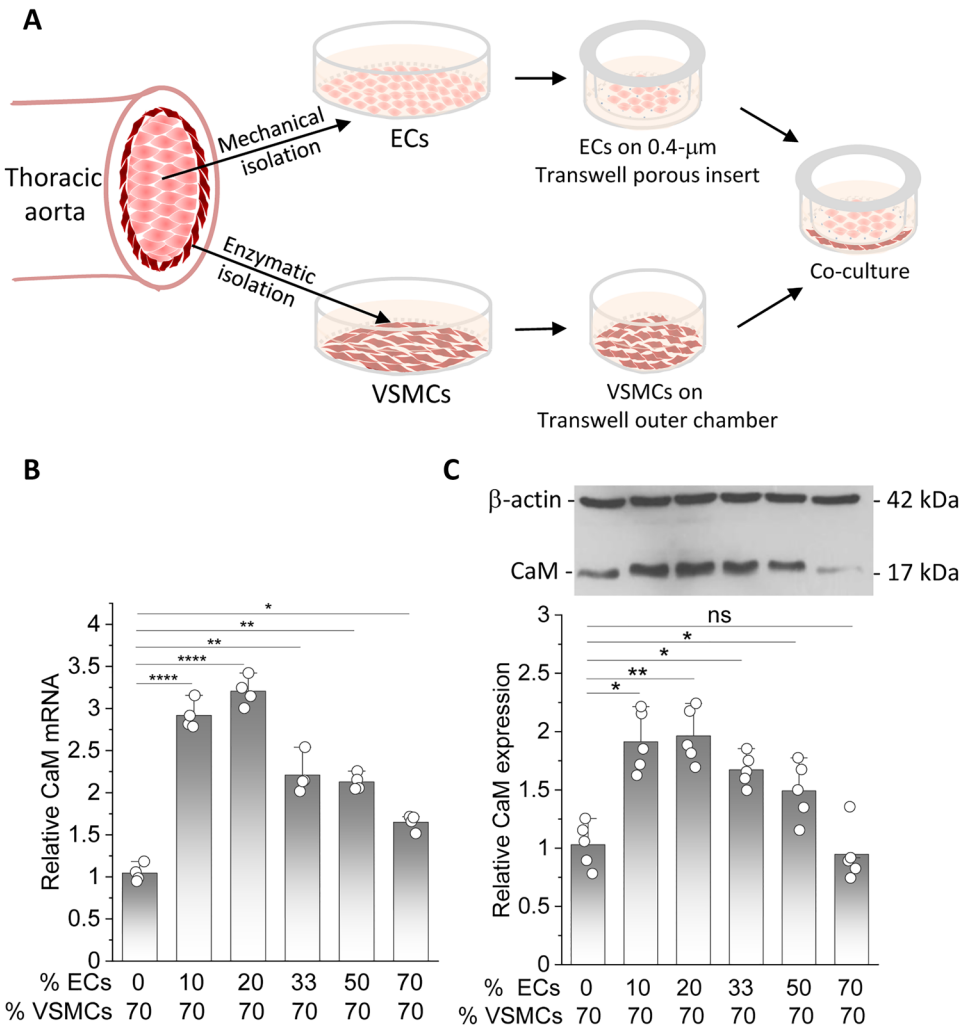

Fig. 1 CaM mRNA and protein expression in VSMCs in monoculture or co-culture with PAECs. **A** Procedure of isolation and co-culture of ECs and VSMCs isolated from the same vessels. See text for details. **B** Relative CaM mRNA from VSMCs co-cultured with PAECs at specified starting density ratios. **C** Total CaM expression in VSMCs in monoculture or co-culture with ECs in specified starting densities. β-actin and CaM were simultaneously probed on the same SDS-PAGE membranes. Histogram, relative CaM expression. *, **, ***, and ****p values < 0.05, 0.01, 0.001 and 0.0001 vs monoculture, respectively, in one-way ANOVA and Bonferroni post hoc test; $n = 4$–5 separate experiments

sequence to minimize any restraint the fluorescent proteins may impose upon the interaction between CaM and the CaM-binding sequence. Two restriction sites KpnI and AgeI then connected the N and C terminal ends of the sequence with ECFP and EYFPc, respectively. The modified insert was PCR-generated from a bovine eNOS template [19]. with CACTATGGTACCGCCGCTAGGAAGAAGG and GAT TACACCGGTAGCAGCCATGAGTGAGG as forward and reverse primers, respectively. The construct was expressed in a pET bacterial vector and a pCDNA3.1 mammalian vector, as described previously for other FRET-based biosensors [14, 15]. Total mRNA isolation from VSMCs and RT-PCR for *CALM1* were carried out as described previously in detail for PAECs [14].

CaM, CaM sensor and biosensor-CaM titration

$BSCaM_{eNOS}$ and CaM were expressed and purified as described previously [14, 20]. Titrations between $BSCaM_{eNOS}$ and CaM were carried out as described in detail previously, with modification in CaM concentrations [15]. $BSCaM_{eNOS}$ fractional saturation and K_d value for CaM were determined as described previously [14, 15].

Simultaneous measurement of free $Ca^{2+}$and Ca^{2+}–CaM in VSMCs co-cultured with ECs

A ~ 15-mm circle was thermally bored out from the bottom center of each well in the 6-Transwell plate and a 25-mm #1.5 glass coverslip was attached from the outer surface (see Fig. 2A), followed by UV sterilization. VSMCs were transfected with a pcDNA3.1 mammalian plasmid encoding $BSCaM_{eNOS}$ using a Nucleofector II-S transfection system (Lonza Inc., Walkersville, MD) prior to being seeded on the coverslip for monoculture or co-culture with PAECs. After 48 h, cells were loaded with 5 µM indo-1/AM for 30 min at 37 °C. The plate was then fit onto the microscope stage for simultaneous imaging of indo-1 and $BSCaM_{eNOS}$ signals in the VSMCs while in co-culture with the ECs. The insert possesses openings near the brim, through which a gel loading

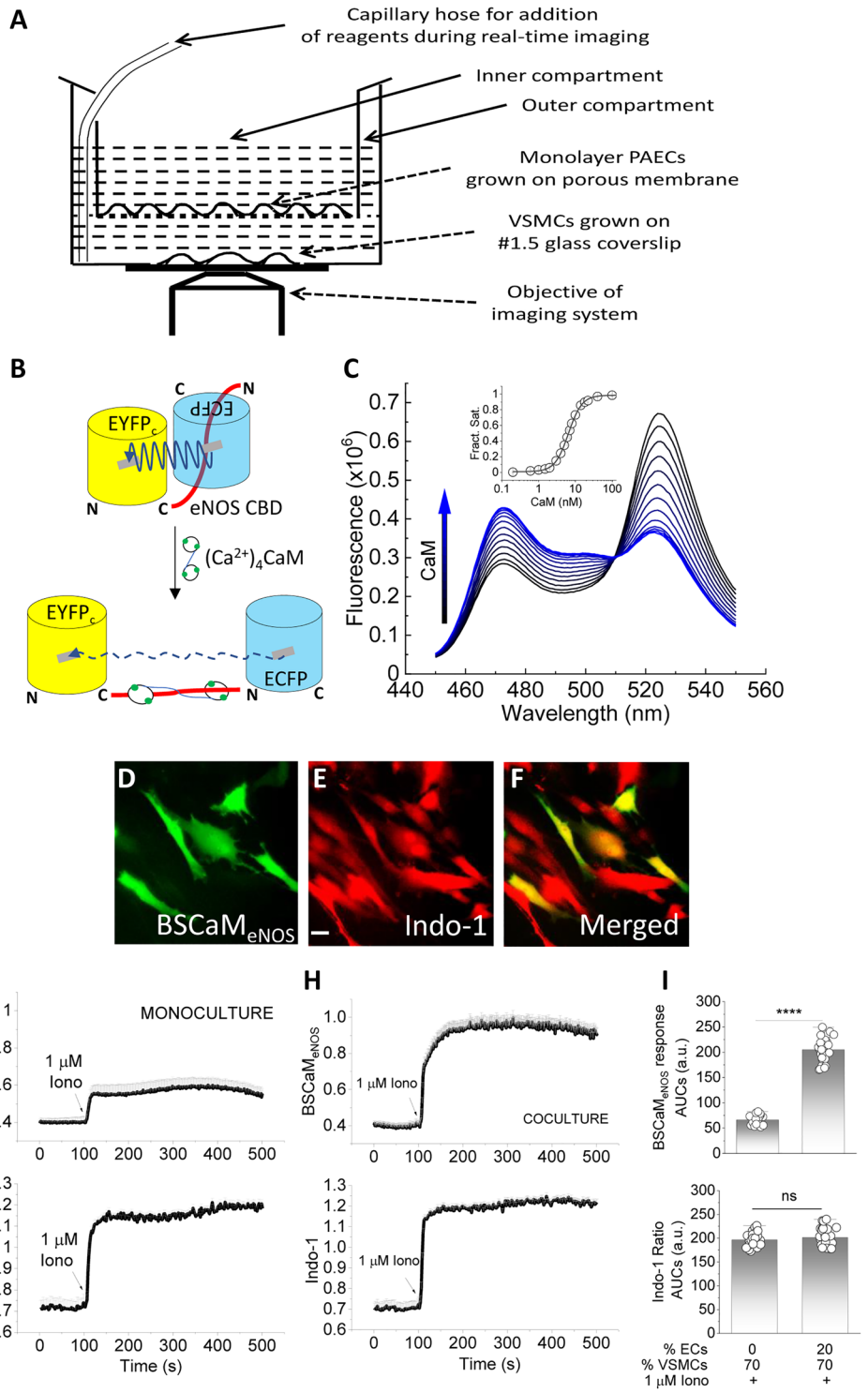

Fig. 2 Free CaM levels in VSMCs in monoculture or co-culture with PAECs. **A** Diagram depicting the modification of the Transwell co-culture module for imaging of Ca^{2+} and Ca^{2+}–CaM in the underlying VSMCs. See text for description. **B** $BSCaM_{eNOS}$ configuration in the unbound (upper panel) or bound state with Ca^{2+}-saturated CaM. See text for description. **C** Emission spectra of $BSCaM_{eNOS}$ in response to CaM titration in saturating Ca^{2+} concentration. *Inset* plot of $BSCaM_{eNOS}$ average fractional saturation as a function of CaM. **D** Fluorescence of $BSCaM_{eNOS}$ in VSMCs. **E** Pseudocolor indo-1 fluorescence from cells in the same microscopic field as in **D**. **F** Merged image of **D** and **E** Elongated cells expressing $BSCaM_{eNOS}$ and loaded with indo-1/AM were chosen for imaging. Cells with equal starting $BSCaM_{eNOS}$ fluorescence were selected. **G** Free CaM (upper panel) measured simultaneously with Ca^{2+} (lower panel) in monocultured VSMCs in response to 1 µM ionomycin. **H** Free CaM (upper panel) measured simultaneously with Ca^{2+} in VSMCs co-culture with PAECs at 70%:20% VSMCs:PAECs starting density ratio. $n = 25–30$ cells for each condition from 6 to 8 separate experiments. **I** Average areas under the curve (AUCs) values from BSCaMeNOS responses in G and H. $***p < 0.001$, two-sample *t* test

pipette tip was used to deliver reagents during imaging. $BSCaM_{eNOS}$ and indo-1 excitation was alternated between $\lambda 430$ nm and $\lambda 340$ nm for 100 ms each per 1-s cycle using a Lamda-DG4 illuminator (Sutter Instruments) with a 1-ms switching interval. Emission lights were passed through a custom 410/30 M/460LP polychroic (Chroma Technology Inc., VT) and selected through sputtered bandpass filters of $\lambda 480$ and $\lambda 535$ nm for $BSCaM_{eNOS}$, and $\lambda 405$ and $\lambda 485$ nm for indo-1, in a Lambda-10 filter switcher (Sutter Instruments) with switching 40-ms intervals between wavelengths. Filtered emission lights were then collected via an Andor DU-885 EMCCD camera (Oxford Instruments – ANDOR).

Data were processed by the Imaging Workbench 6.1 software (INDEC Biosystems, CA). After background subtraction of individual intensities, ratios between emission intensities at λ405 nm and λ480 nm for indo-1 (λ340-nm excitation) and λ480 nm and λ535 nm for $BSCaM_{eNOS}$ (λ430-nm excitation) were obtained to indicate changes in free Ca^{2+} and Ca^{2+}-bound CaM, respectively.

Coimmunoprecipitation and Western blotting

Following treatment and cell lysis, the protein content was measured using BCA assay (Pierce) in triplicate and the protein content for each sample was determined on the average of the triplicate. For coimmunoprecipitation, 3 mg total cellular proteins were rocked with primary antibodies overnight at 4 °C, followed by mixing with equal amounts of protein A/G beads (Fisher) for 2 h at 4 °C. After centrifugation, removal of supernatant and washes, antibody-protein complexes were eluted and subjected to SDS-PAGE. For non-IP Western blotting, all lanes in each gel were loaded with equal amounts of proteins, adjusted for detectability with each antibody. Following SDS-PAGE and transfer, membranes from co-IP samples were separated between the levels of the prey and the bait proteins prior to incubation of fragments with specific antibodies. For immunoblots that assessed protein expression levels, whenever possible, primary antibodies recognizing the protein of interest and a house-keeping protein were simultaneously incubated with the same membrane in a "double primary" approach to show both proteins on the same development. Membrane fragments were developed using enhanced chemiluminescence. Signals were detected in a ChemiDoc™ XRS + system (Bio-Rad). For co-IP data, densitometric values of the baits were corrected for those of the preys, determined using the volume tool in the Image Lab 6.0 software (Bio-Rad).

Statistical analysis

Statistical analysis was carried out using Student's *t* test or one-way ANOVA. Bonferroni post-hoc tests were subsequently applied where appropriate for group comparisons. Statistical significance was set at $p < 0.05$. Analysis was carried out using OriginPro 2020 software (OriginLab, Corp., Northampton MA).

Results

Proliferating PAECs promotes CaM expression in VSMCs

To initially examine the effect of the endothelium on CaM-dependent signaling in VSMCs, we measured the effects of co-culturing with PAECs on CaM mRNA and total CaM expression in VSMCs. PAECs and VSMCs were freshly isolated from the same vessels [14, 15, 17]. Since ECs directly interact with VSMCs via myoendothelial junctions (MEJs) [21–28] and since the MEJs can originate from VSMCs or ECs [29, 30], co-culture models that permit formation of MEJs such as culturing the two cell types on opposing sides of a porous membrane present difficulty in assessing the effect of ECs on CaM expression and CaM-dependent signaling in VSMCs. We therefore used the Transwell co-culture apparatus, in which PAECs were cultured on the inner luminal insert surface, while VSMCs were cultured on the bottom surface of the outer compartment. The porous membrane with 0.4-µm pore size is ~ 1 mm above the bottom surface, permitting secretions from ECs to reach the VSMCs while avoiding formation of MEJs. PAECs and were cultured separately on the insert membrane in a separate chamber while the VSMCs were cultured on the bottom surface for 3 h to ensure adherence of both cell types before the insert containing adherent PAECs was moved into the chamber housing the VSMCs (Fig. 1A). Control experiments with only PAECs cultured on the insert for 48 h showed no PAECs in the outer compartment after 48 h (data not shown).

When cultured separately, PAECs exhibited higher proliferation rates than the VSMCs. Therefore, we tested the effects of various initial densities of PAECs on potential effects on CaM expression in VSMCs. VSMCs were cultured at 70% initial confluence (1.8×10^6 cells/plate) with varied starting densities of PAECs (10, 20, 33, 50 and 70%, corresponding to 1.4×10^5, 2.8×10^5, 4.62×10^5, 7.0×10^5, and 9.8×10^5 cells/plate, respectively) for 48 h. CaM mRNA was significantly increased ~ 3.2-fold in VSMCs co-cultured with 20% starting confluence of PAECs. The increase in CaM mRNA was progressively reduced with increasing starting densities of the PAECs (Fig. 1B). Consistently, immunoblotting for CaM from VSMC lysate showed that compared to VSMCs in monoculture, total CaM was doubled in VSMCs co-cultured with low (10–20%) starting PAECs density. This effect was reduced with increasing starting densities of PAECs and was abolished as PAECs were started at similar density with the VSMCs (Fig. 1C).

PAECs promotes increases in free CaM levels in VSMCs

Most CaM is caught in inseparable interactions [7], and the fraction of free CaM for dynamic interactions with its targets is relatively low in most cell types studied [8–11, 13]. The increase in total CaM expression as assessed by immunoblotting may therefore not reflect the actual changes in available CaM for interactions with target proteins. It was thus important to assess the effects of ECs on free CaM in

VSMCs in co-culture. As Ca^{2+}–CaM complex formation in cells depends on both the Ca^{2+} signals and level of expression of CaM, it was necessary to measure both free Ca^{2+} and CaM levels in VSMCs in monoculture or co-culture with PAECs. To do this, we modified the Transwell chamber to allow for intracellular imaging of VSMCs while in co-culture with the PAECs (Fig. 2A and "Materials and methods"). Cell viability and proliferation of the VSMCs were not affected when cultured in this module (not shown). To measure both free CaM levels, we used $BSCaM_{eNOS}$, a FRET CaM biosensor generated based on a modified CaM-binding domain of eNOS (see "Materials and methods"). This sequence was chosen to corroborate subsequent studies examining the presence of eNOS in VSMCs and its association with CaM in VSMCs co-cultured with ECs. As with previous CaM sensors, $BSCaM_{eNOS}$ consists of two fluorescent proteins, ECFP and citrine EYFP (EYFPc), the latter containing the V69L and Q70M substitutions that renders the EYFP insensitive to changes in cytoplasmic pH [10, 31]. The CaM-binding sequence of eNOS was incorporated between ECFP and EYFPc (Fig. 2B, upper panel). Upon CaM binding, FRET between ECFP and EYFPc is disrupted (Fig. 2B, lower panel). Monitoring the FRET changes allows for assessment of the free Ca^{2+}-bound CaM concentration in the cells. Figure 2C shows $BSCaM_{eNOS}$ spectral changes upon titration of purified CaM in the presence of saturating Ca^{2+} concentration. Fits of average biosensor fractional saturation as a function of free CaM (inset) yielded an apparent K_d value of 4.5 ± 0.8 nM.

VSMCs were transfected with $BSCaM_{eNOS}$ prior to co-culture with ECs. After 2 days of monoculture or co-culture of VSMCs and PAECs with a 70%:20% starting densities, the cells were loaded with indo-1/AM, and free Ca^{2+} and CaM levels were simultaneously measured in the VSMCs. Figure 2E–F show representative $BSCaM_{eNOS}$, indo-1, and merged fluorescence images, respectively. For consistency, only elongated VSMCs that expressed both $BSCaM_{eNOS}$ and indo-1 signals were selected for imaging. Care was taken to select cells with similar levels of $BSCaM_{eNOS}$ expression for comparison, using initial fluorescence intensity at baseline condition. To avoid potential changes in surface receptors and receptor-mediated Ca^{2+} entry in VSMCs due to co-culture with PAECs, Ca^{2+} signals in VSMCs were produced by treatment with the Ca^{2+} ionophore ionomycin (1 μM). This treatment produced rapid rises in intracellular Ca^{2+} concentration that was sustained for several minutes and were of similar amplitude in monocultured VSMCs (Fig. 2G and I, lower panels) and VSMCs co-cultured with PAECs (Fig. 2H and I, lower panels). However, $BSCaM_{eNOS}$ signals measured simultaneously in the same cells demonstrated that free Ca^{2+}-bound CaM levels in VSMCs co-cultured with ECs (Fig. 2H and I, upper panels) were ~ threefold higher than in VSMCs in monoculture (Fig. 2G and I, upper panels).

CaM–eNOS interaction in VSMCs

To explore the impact of the increases in total and free CaM in VSMCs, we examined the expression of eNOS and its association with CaM in VSMCs in monoculture and co-culture with ECs. Expression of eNOS has been demonstrated in vascular smooth muscle from pigs, humans, and rats [32–34]. Messenger RNA of eNOS has also been detected in intestinal smooth muscle cells [35]. More recently, eNOS has been confirmed in pulmonary arterial smooth muscle cells [34, 36]. Binding of CaM is an absolute requirement for eNOS activity [37], and factors that upregulate total and free CaM in the endothelium have been shown to promote eNOS–CaM interaction and activity [14]. Little is known regarding any role of the vascular endothelium in regulating eNOS in VSMCs, much less eNOS–CaM interaction. We aimed to confirm eNOS expression in VSMCs and examine if the increases in CaM expression by co-culturing with PAECs would promote eNOS–CaM interaction in VSMCs. VSMCs were plated in monoculture at 70% starting density or in co-culture with PAECs at a 70%:20% starting densities in 100-mm Transwell co-culture plates. After 48 h, VSMCs were treated with 1 μM ionomycin for 3 min prior to lysis. A fraction of the total lysate was used to compare eNOS expression levels. No difference was observed in eNOS expression while there was significantly higher CaM level in VSMCs co-cultured with ECs (Fig. 3, right panel). The remaining lysate was immunoprecipitated with an anti-eNOS antibody, followed by immunoblotting of the pull-down fractions for CaM and eNOS. A threefold increase was observed in eNOS–CaM interaction in VSMCs co-cultured with the PAECs (Fig. 3, left panel).

Mechanism of the CaM-elevating effect of ECs on VSMCs

The data above indicate that a factor secreted from ECs upregulates CaM expression in VSMCs. To explore the potential mechanism of this effect, we co-cultured PAECs and VSMCs with a 20%:70% starting densities in the presence or absence of several inhibitors of known factors secreted from ECs. Nitric oxide was an obvious initial target. Cells were co-cultured in the presence or absence of 100 μM L-NAME, a concentration previously shown to sufficiently inhibit NO production in ECs [9]. No effect of inhibiting eNOS was observed on the upregulation of CaM expression in VSMCs co-cultured with PAECs (Fig. 4A). The endothelium also secretes endothelin-1 (ET-1), a potent vasoactive substance that is known to upregulate a number of genes in vascular smooth muscle, such as gp91phox [38], NF-κB [39], and TNF-α [40]. To test the potential role of ET-1 in the observed endothelium-mediated upregulation of CaM in VSMCs, VSMCs were cultured alone or with PAECs for

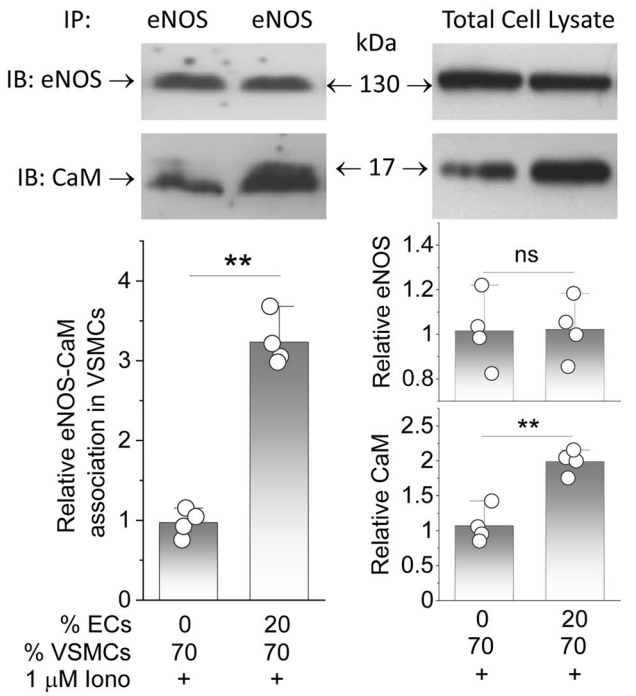

Fig. 3 Expression of eNOS and eNOS–CaM interactions in VSMCs mono-cultured or co-cultured with PAECs. VSMCs in monoculture or co-culture with PAECs at 70%:20% SMC:EC starting confluence ratio were treated acutely with 1 μM ionomycin for 3 min prior to lysis. Equal total protein loads from lysates were immunoprecipitated for eNOS, followed by immunoblotting of the pulldown fractions for eNOS (left panel, upper immunoblot) and CaM (left panel, lower immunoblot). Left panel histogram, relative CaM in eNOS pulldown fractions. Right panel shows expression in corresponding total cell lysate for eNOS (upper immunoblot) and CaM (lower immunoblot). Histograms, relative densitometric values of eNOS (upper) and CaM (lower). $^{**}p < 0.01$, two-sample paired t test, from $n = 4$ separate experiments

48 h in the absence or presence of various doses of the ET_A/ET_B dual inhibitor bosentan (K_i values of 4.7 and 95 nM for ET_A and ET_B, respectively). No effect was observed with up to 10 μM bosentan on the increases in total CaM expression in VSMCs caused by coculturing with PAECs (Fig. 4B). We next tested the possibility that vascular endothelial growth factor (VEGF) could be responsible for the effect of PAECs to upregulate CaM in VSMCs. VEGF receptors have been shown to be present and functional in VSMCs [41, 42]. VSMCs were cultured alone or with PAECs for 48 h prior to lysis. Cells were treated with vehicle or various concentration of CBO-P11, an antagonist of VEGFR1 and VEGFR2 (K_i values of 0.7 and 1.3 μM, respectively). Interestingly, CBO-P11 dose-dependently prevented the effect of PAECs to upregulate CaM in VSMCs (Fig. 4C). To further assess the role of VEGF in the effect of the PAECs to promote CaM expression in VSMCs, VSMCs were cultured at 70% starting density with or without co-culture with various densities of PAECs as described in Fig. 1B–C for 48 h, followed by immunoblotting for VEGF-A in the VSMCs. VEGF-A levels were significantly upregulated in VSMCs co-cultured with the PAECs at 20% and 33% and not at other densities (Fig. 4D).

Discussion

This study describes a new role of the endothelium: promoting CaM expression in VSMCs. With an estimated over 300 target proteins and 5% of total CaM being freely available in VSMCs [6, 8], an increase in CaM expression is predicted to have extensive effects in this tissue. Our results showed that both CaM mRNA and total protein expression were upregulated in VSMCs co-cultured with low starting density of ECs isolated from the same vessels. The impact on intracellular available CaM was confirmed by the simultaneous measurement of Ca^{2+} and free Ca^{2+}-bound CaM in VSMCs in co-culture with ECs, which demonstrated a ~ threefold higher free CaM in response to a Ca^{2+} signal of similar magnitude as in monocultured VSMCs. As most interactions between CaM and its target proteins are Ca^{2+}-dependent, the concentrations of Ca^{2+} also play an important role in dictating the outcomes. In this context, previous works have shown that direct interaction between coronary ECs and VSMCs does not affect cytoplasmic or nuclear Ca^{2+} concentrations in the VSMCs, but only increases the nuclear Ca^{2+} concentration in the ECs [43]. Thus, in our co-culture system, the functional impact on the CaM-dependent proteome promoted by the ECs is predicted to result from the increase in free CaM level and not from changes in free Ca^{2+} concentrations. Importantly, the finding that low starting densities of ECs produce much more CaM-upregulating effect in VSMCs indicates that this effect is produced by proliferating ECs. This suggests that this effect could be important in conditions that involve rapid endothelial proliferation and regeneration, such as neovascularization and post-stenting intimal injuries.

The functional outcome of the upregulation in CaM level in VSMCs is predicted to be widespread, considering the limiting CaM equilibrium in this tissue [8]. VSMC functions may be affected by endothelium-induced increases in the associations between CaM and myosin light chain kinase and critical VSMC surface receptors that are direct CaM targets [4, 17, 44–46]. Interestingly, a direct outcome of the endothelium-induced upregulation in CaM expression is the increased eNOS–CaM association in VSMCs. Due to differences in DNA methylation, the expression of eNOS is more selective for ECs [47], and until recently the role of eNOS in VSMCs received little attention [34, 36]. Our results confirmed that eNOS is expressed in VSMCs and demonstrated that eNOS–CaM interaction in VSMCs is promoted by the endothelium. The latter is evidenced by

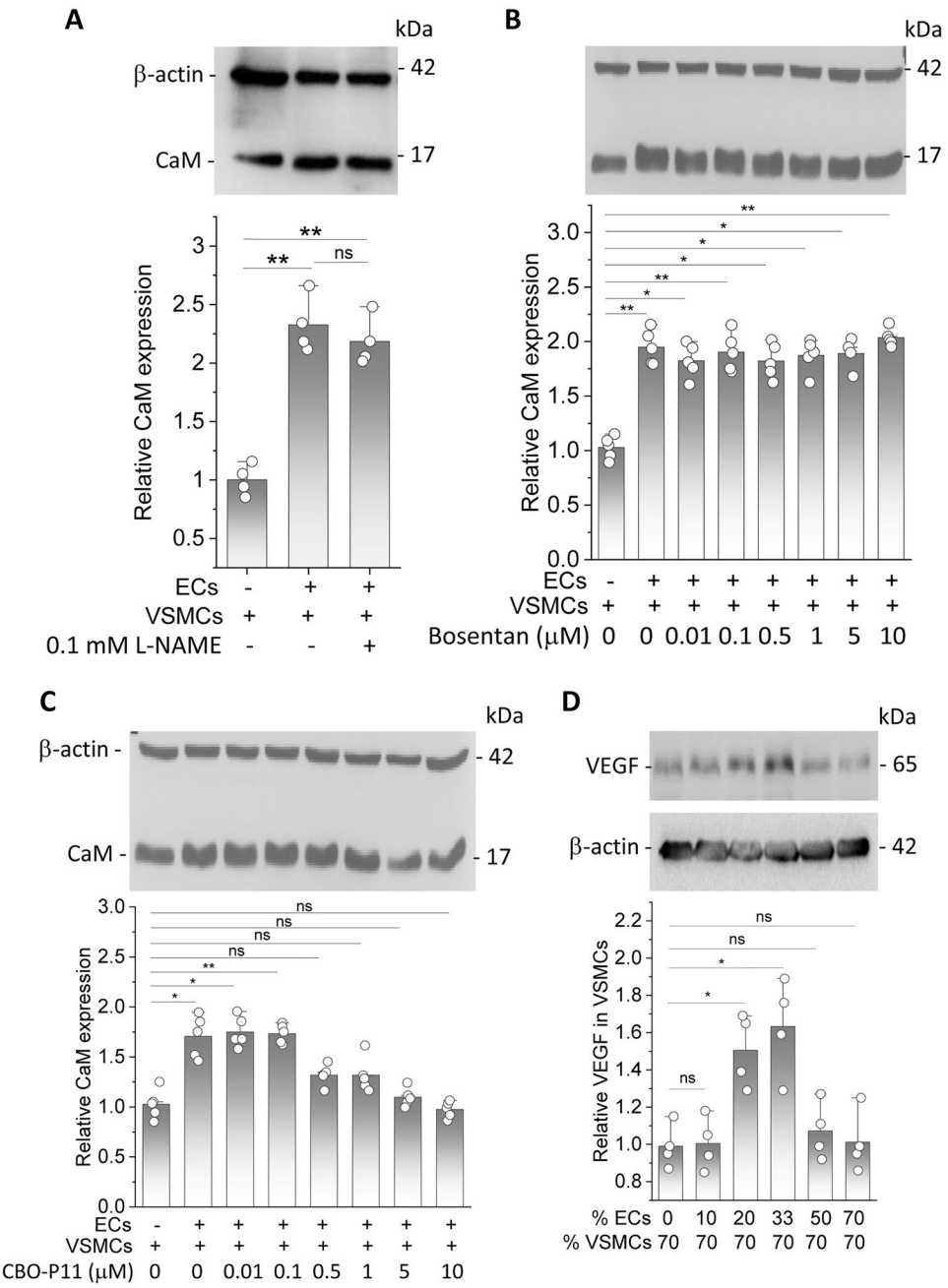

Fig. 4 Effects of inhibiting eNOS, ET_A/ET_B, and VEGFR1/2 on EC-induced upregulation of CaM in VSMCs. **A–C** VSMCs (70% starting density) were cultured with or without 20% starting PAECs density for 48 h in the presence or absence of 100 μM L-NAME (**A**), specified doses of bosentan (**B**) or CBO-P11 (**C**) prior to lysis for SDS-PAGE. Upper immunoblots, β-actin and CaM were simultaneously probed on SDS-PAGE membranes. Histograms, relative CaM/β-actin densitometric ratios. **D** VSMCs and PAECs were co-cultured at specified starting density ratios for 48 h prior to lysis of VSMCs, followed by SDS-PAGE. Upper immunoblot, VEGF-A level in VSMCs, lower immunoblot, β-actin. Histogram, relative VEGF-A level in VSMCs. * and ** $p < 0.05$ and 0.01 from monoculture values, one-way ANOVA and Bonferroni post hoc test; $n = 4–5$ separate experiments

the co-IP data showing increased eNOS–CaM association in VSMCs co-cultured with ECs and is corroborated by the finding that in response to similar Ca^{2+} signals, CaM binding to the biosensor built on eNOS–CaM-binding domain in VSMCs is increased ~ threefold in the presence of ECs. A predicted functional outcome of this effect is an increase in nitric oxide produced by eNOS from VSMCs. Speculatively, during EC regeneration and neovasculogenesis, the insufficient production of nitric oxide from the ECs would be partly compensated for by that from the VSMCs, thus balancing the role of the endothelium and vascular smooth muscle in regulating vascular tone (Fig. 5).

As inhibitors of VEGFR1/2 are now widely used in cancer therapy, the findings here warrant some attention to effects that may come from elimination of the endothelium-dependent CaM-elevating effects in VSMCs.

Limitations of the study

Our studies have provided evidence that VEGF is upregulated in VSMCs co-cultured with proliferating PAECs and that pharmacological inhibition of VEGF abolishes the effect of the PAECs to upregulate CaM expression. We do not know the mechanism whereby VEGF level in

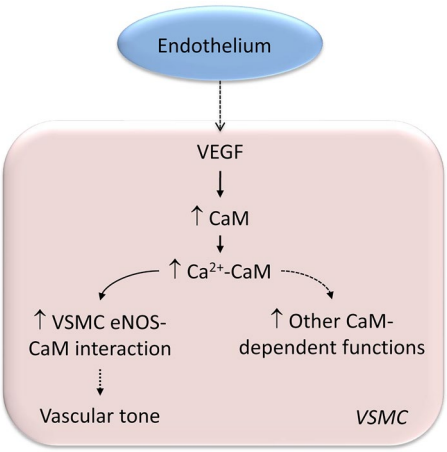

Fig. 5 Proposed effects and mechanism of the vascular endothelium in regulating CaM expression and target interactions in vascular smooth muscle. See text for details. *VEGF* vascular endothelial growth factor; *Ca^{2+}–CaM* Ca^{2+}-saturated calmodulin; *eNOS* endothelial nitric oxide synthase; *VSMCs* vascular smooth muscle cells

VSMCs is upregulated by the proliferating PAECs. Nevertheless, an effect of VEGF in promoting CaM expression and CaM-dependent activities in VSMCs is consistent with observations that subjects treated with VEGF inhibitors exhibit increased mean arterial blood pressure [48]. Studies using ECs and VSMCs isolated from VEGFR knockout animals would further confirm these observations.

In conclusion, we have identified a novel role of the vascular endothelium in promoting total and free CaM expression and CaM-dependent interactions in VSMCs, presumably via the effect of enhanced VEGF production from ECs. The physiological implications from this effect are predicted to be widespread given the limiting CaM equilibrium in VSMCs.

Author contributions QT-T conceived the study and designed experiments. MGS, MVM, and JG carried out the experiments and data analysis. QK-T wrote the paper. All authors reviewed the manuscript.

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Data availability The data in this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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