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Research Article

Soluble fms-like tyrosine kinase-1 and angiotensin2 target calcitonin gene-related peptide family peptides in maternal vascular smooth muscle cells in pregnancy†

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Abstract

Calcitonin gene-related peptide (CALCB), adrenomedullin (ADM), and adrenomedullin2 (ADM2) are hypotensive peptides that belong to CALCB family of peptides. Goal of this study was to identify the effect of fms-like tyrosine kinase (sFLT-1) and angiotensin2 (Ang2) on the function of these peptides in OA smooth muscle cells (OASMC) and assess the sensitivity of OA for these peptides in preeclampsia (PE) and normotensive pregnancy. Methods: Peptide function was assessed by Cyclic adenosine monophosphate (cAMP) assays and wire myograph; mRNA expression by Polymerase chain reaction (PCR) and protein-protein interaction by proximity ligation assay and co-immunoprecipitation. Findings: All three peptides increased cAMP synthesis in the order of efficacy CALCB > ADM = ADM2 and vascular endothelial growth factor (VEGF) mRNA in OASMC ($P < 0.05$); sFLT-1 mediated decrease in cAMP synthesis ($P < 0.05$) is differentially rescued by all three CALCB family peptides in OASMC ($P < 0.005$); sFLT-1 decreased receptor activity-modifying protein (RAMP)1 and RAMP2 mRNA expression ($P < 0.05$); Ang2 decreased the expression of calcitonin-receptor-like receptor and RAMP1 mRNA and desensitized CALCB and ADM2 receptors in OASMC ($P < 0.05$); sFLT-1 increased RAMP1and Ang2 type 1 receptor (AT1R) interaction in OASMC which is inhibited in presence of all three peptides; and all three peptides relax OA in PE with enhanced ADM2 response ($P < 0.05$). Conclusion: sFLT-1 and Ang2 impair OASMC mediated functional responses of CALCB family peptides which can be inhibited by respective peptide treatment. The sensitivity of OA for CALCB, ADM, and ADM2-mediated relaxation is retained in PE.

Summary sentence

sFLT-1 and Ang2 impair smooth muscle cell mediated functional responses of CALCB family peptides which can be inhibited by respective peptide treatment.

Graphical Abstract

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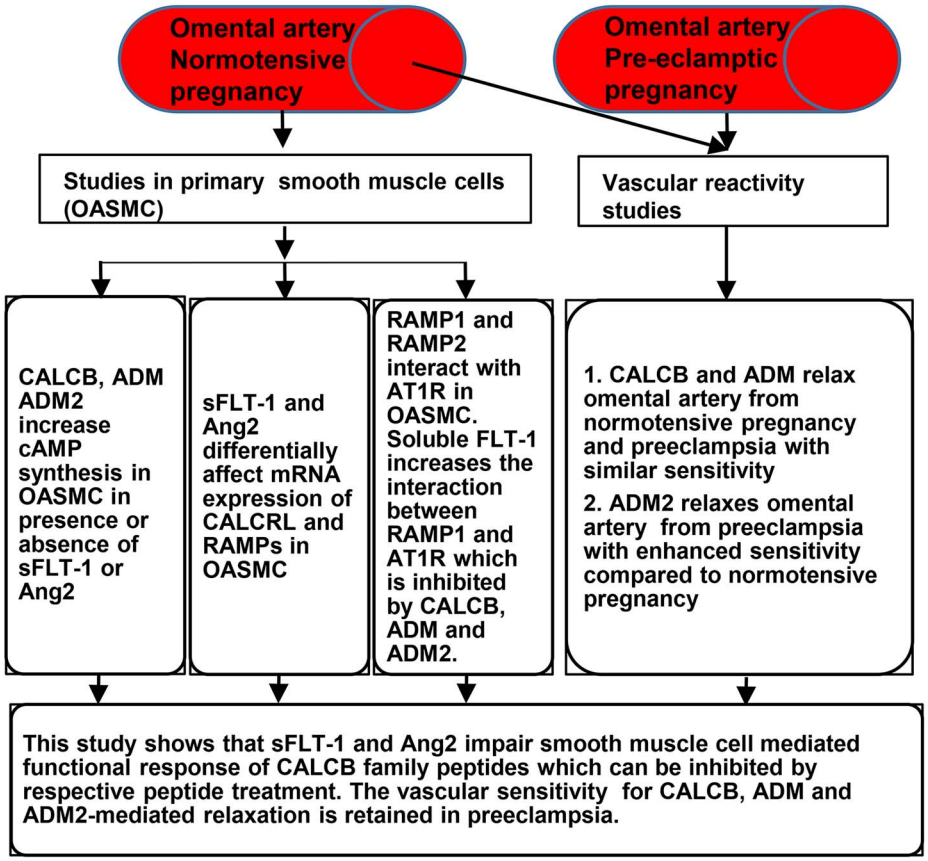

Key words: angiotensin 2, calcitonin gene-related peptides, cAMP, pregnancy, sFLT-1, vascular smooth muscle cells.

Introduction

The calcitonin gene-related peptide (CALCB) family of peptides, CALCB, adrenomedullin (ADM), and adrenomedullin2 (ADM2) are hypotensive peptides involved in several biological functions including cardiovascular adaptations during pregnancy and utero-placental functions to facilitate a healthy pregnancy in rodents [1–3]. We reported earlier that CALCB family peptides play a major role in maintaining decreased vascular contraction in rat pregnancy, and inhibition of endogenous actions of CALCB, ADM, and ADM2 by their respective receptor antagonists in pregnant rat resulted in impaired placental vasculature and fetal growth restriction (FGR) [1, 2, 4, 5]. Interestingly, these three peptides share a single 7TM G-protein coupled receptor (GPCR), calcitonin-receptor-like receptor (CALCRL) whose ligand binding is dictated by co-expression of three receptor activity-modifying proteins (RAMP1, 2, or 3) [2]. Hetero-dimer of CALCRL with RAMP1 or RAMP3 binds to CALCB, heterodimer of CALCRL with RAMP2 or RAMP3 binds to ADM, while heterodimer of CALCRL with any one of the three RAMPs can exhibit ADM2 effects.

Circulatory levels of CALCB, ADM, and ADM2 are elevated during human pregnancy. We reported earlier that although all three peptides relax omental artery (OA) in non-pregnant women, the sensitivity of OA for CALCB and ADM2, but not ADM is elevated in human pregnancy [6]. This suggests that these peptides differentially regulate omental vascular tone to facilitate vascular adaptation in human pregnancy. This is supported by our recent study showing that the sensitivity for all three peptides is impaired in OA from pregnant women with FGR [7], and serum levels of CALCB, ADM, and ADM2 are downregulated in spontaneous abortion and pre-eclampsia (PE) [3, 5, 8]. More importantly, recent reports show that ADM and ADM2 levels are lower during second trimester in pregnant women who are destined to develop PE [5, 9–11]. Thus, CALCB, ADM, and ADM2 appear to have an important role in the pathophysiology of pregnancy across species.

Pre-eclampsia is a hypertensive disorder of pregnancy associated with vascular dysfunction due to increased secretion of soluble fms-like tyrosine kinase-1 (sFLT-1), elevated Angiotensin2 (Ang2) sensitivity, and increased production of Ang2 type-1 receptor (AT1R) in vascular smooth muscle cells (VSMCs; [12]). Vascular sensitivity

for Ang2 is decreased in normal pregnancy that contributes to low systemic vascular resistance compared with the non-pregnant state [3, 5, 13]. However, vascular sensitivity to Ang2 is increased in PE in addition to concomitant decreases in potent vasodilators such as nitric oxide (NO), CALCB, ADM, and ADM2 [11, 13, 14, 17]. We reported earlier that CALCB decreased blood pressure (BP) in Nitro-L-arginine methyl ester (L-NAME) treated rat model of PE and its effects are NO independent [14]. Interestingly, CALCB is shown to reverse Ang2-induced hypertension in rat [15] and ADM and ADM2 inhibit Ang2-induced proliferation, collagen production and phenotype conversion of myocardial fibroblasts with effects of ADM2 being more potent than ADM [16]. In addition, ADM2 inhibits Ang2-induced hypertrophy and apoptosis in cardio myocytes [17]. Thus, all three peptides appear to render protection from the adverse effects of Ang2 in vasculature. However, their mechanism of action is not well studied in maternal vasculature during human pregnancy. It is not known if their functions are impaired by PE associated vasoconstrictors like sFLT-1 and Ang2 which could contribute immensely to vascular adaptation during pregnancy. Further, it is not known if the sensitivity of PE vasculature for the relaxation responses of CALCB, ADM, and ADM2 also decrease with the reported decreases in the serum level of these peptides in PE [11, 13, 14].

Effects of Ang2 on blood pressure involve central nervous system mechanisms and VSMC tone [18]. While vascular endothelial growth factor (VEGF)-A is important in vascular development and maintenance of endothelial integrity, VEGF-A and VEGF receptors (FLT-1) are also expressed in SMC that may have discrete autocrine and/or paracrine function in SMCs [19–22]. Therefore, the goal of this study was to assess CALCB, ADM, and ADM2 mediated cAMP synthesis as a surrogate for functional response of these peptides in OA smooth muscle cells (OASMC) isolated from normotensive pregnant women, and assess the effect of sFLT-1 and Ang2 on their function and expression of their receptor system in OASMC. In addition, as hetero-dimerization of RAMPs and AT1R with other receptor proteins is reported to cause modulation of their efficacy and potency for their putative ligands [23–28], we also examined if RAMPs interact with AT1R receptor in OASMC. Finally, we tested if the relaxation effects CALCB, ADM, and ADM2 as observed in OA from normotensive pregnancy [7] is retained in PE.

Materials and methods

Human samples

This study was approved by the Baylor College of Medicine Institutional Review Board and conducted according to the Declaration of Helsinki principles. Omental fat tissue was collected from pregnant patients undergoing cesarean delivery who had PE or gestational age-matched normal pregnancy after getting their informed consent. Patients were excluded from the study if they had diabetes, fetal anomalies, multifetal pregnancy, or any clinical evidence of maternal or fetal infection.

Isolation of OASMC

Omental arteries were cleaned off of fat and enzymatically digested as per the method described [30]. All procedures were carried out under aseptic conditions. The cleaned arteries were transferred into a 50 ml flask containing 4.0 ml of enzymatic dissociation mixture: Hanks balanced salt solution (HBSS, Ca^{2+}, and Mg^{2+} free) with 0.2 mM added Ca^{2+}, 15-mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7. 3), 0.125 mg/ml elastase, 0.375 mg/ml soybean trypsin inhibitor, 1 mg/ml collagenase, and 2 mg/ml bovine albumin. After incubation at 37°C for 90 min in a gyratory shaker water bath, the tissues were triturated 10 times into a 10-ml plastic syringe with a $16\frac{1}{2}$-gauge needle and passed through a 100-µm nylon mesh to separate the dispersed cells from undigested vessel wall fragments and debris. The filtered suspension was centrifuged in a siliconized conical plastic tube for 5 min at 200 × *g*, and the pellet was washed once with Dulbecco modified Eagle medium (DMEM) with all supplements. The cell pellet was suspended in 5 ml of DMEM (high glucose) supplemented with 25-mM HEPES buffer, 2-mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% (vol/vol) heat-inactivated calf serum, and the dispersed cell suspension was aliquoted into a 25-cm^2 flask. After 18–24 h, the cultures were washed once with HBSS. Cells were cultured in DMEM containing 10% serum with media changes at 48–72 h intervals and typical confluent monolayers formed within 7 days. Each preparation typically yielded 5–6 × 10^5 cells with 80–90% viability assessed by trypan blue exclusion. Omental artery SMCs were maintained at 37°C in a humidified incubator in an atmosphere of 95% air and 5% carbon dioxide and studied at sub-confluence. Before any treatment, cells were rendered quiescent by deprivation of serum and maintained in a serum-free medium for 6–12 h. Cyclic AMP generation and RT-PCRs show consistent responses through the sixth passage and beyond. Cells up to fourth passage were used in this study.

Isolation of total RNA and reverse transcription

Omental arteries SMCs were grown in DMEM Grand Island Biological Company (GIBCO) containing 10% fetal bovine serum (FBS). Cells (80% confluent) were starved overnight in serum free DMEM and treated with sFLT-1(10 ng/ml) or Ang2 (10^{-7} M), in presence or absence of CALCB, ADM, and ADM2 (10^{-8} M) for 24 h to assess the chronic effect of sFLT-1 or Ang2 treatments on the transcription of CALCRL and RAMPs. After 24 h total RNA was extracted followed by RNA cleanup (Qiagen, Valencia, CA) treatment to remove DNA contamination. For RT, 1 µg of total RNA was mixed with 3.0 nmol of random primer (Invitrogen, Carlsbad, CA), 200-µM dinucleotide triphosphate solution (Sigma, St. Louis, MO), and 10 units of AMV reverse transcriptase (Promega, Madison, WI) in the presence of 5 units of RNase inhibitor (Invitrogen, Carlsbad, CA). Complimentary DNA synthesis was performed using 1 cycle at 28°C for 15 min, 42°C for 30 min, 99°C for 5 min, and 4°C for 5 min.

Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time (qPCR) was performed by using TaqMan probes for CALCRL, RAMP1, RAMP2, RAMP3, and VEGF-A (Life Technologies, Grand Island, NY). Amplification of housekeeping GAPDH (forward primer: 5′-GGTCTCCTCTGACTTCAACA-3′, and reverse primer: 5′-AGCCAAATTCGTTGTCATAC-3′) served as an endogenous control to standardize the amount of sample RNA added to a reaction. The PCR conditions used were 2 min at 50°C and 10 min at 95°C for 1 cycle, then 15 s at 95°C and 1 min at 60°C for 40 cycles. All experiments were performed in triplicate. For negative controls RNA templates were replaced with nuclease-free water. For no-RT control, nuclease-free water was used in place of the reverse transcriptase. Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed in fold increase/decrease of the gene of interest.

Cyclic adenosine monophosphate (cAMP) assay

Human OA cells were cultured in 12-well plates for experiments to assess cAMP production. To assess the relative effect of peptides on cAMP synthesis, cells were starved for 6 h in serum-free DMEM. Following starvation, 100-µM phosphodiesterase inhibitor, isobutyl-1-methyl-xanthine (IBMX; Sigma) was added to all wells prior to the treatments with the three peptides (10^{-8} M) or Forskolin (100 µM, Sigma) for 5 min at 37°C under 95% O_2 and 5% CO_2. The cells were lysed in buffer provided in the cAMP kit as per the manufacturer instructions (Amersham Biosciences, Inc.). Effect of chronic treatment with sFLT-1 on peptides induced cAMP synthesis was assessed by treating OASMC with or without different doses of sFLT-1 (0.1–10 ng/ml) for 24 h. This was followed by addition of 100-µM phosphodiesterase inhibitor, isobutyl-1-methyl-xanthine (IBMX; Sigma) to all wells prior to treatments with the three peptides (10^{-8} M) or Forskolin (100 µM, Sigma) for 5 min at 37°C under 95% O_2 and 5% CO_2. To assess the acute effect of sFLT-1 and Ang2 treatments on the desensitization of CALCB, ADM, and ADM2 specific receptor system, stimulation of cAMP synthesis by the three peptides was measured after pretreating the cells with sFLT-1 or Ang2 in serum free medium for 30 min. Briefly, cells were starved in serum free medium for 6 h and treated with sFLT-1(10 ng/ml) or Ang2 (10^{-7} M) for 30 min, washed with the PBS twice and incubated in DMEM without serum again for 30 min at 37°C under 95% O_2 and 5% CO_2. Cells were then treated with CALCB (10^{-8} M), ADM (10^{-8} M), or ADM2 (10^{-8} M) for 5 min followed by cell lyses for the cAMP assay as per the manufacturer instructions. The intra-assay and inter-assay variation for cAMP assay was 8% and 11%, respectively. The dose of peptides used in these experiments was based on our earlier studies performed in rat SMCs [29].

Proximity ligation assay

Proximity ligation assay (PLA) was performed using a Duolink II Fluorescence kit (Olink Biosciences) according to the manufacturer instructions. This assay allows the traditional immunoassays to include direct detection of protein–protein interactions with high specificity and sensitivity. The technique involves two PLA probes for the two target proteins. Each probe is a unique short DNA strand attached to secondary antibody which is complimentary to the DNA strand of probe attached to secondary antibody for the other interacting protein. The probes attached to the secondary antibody binds to the primary antibodies raised in different host species. Close proximity of the two proteins brings the DNA strands on the probe in close proximity to each other, resulting in amplification of circularized DNA molecules detected as bright fluorescent signal. This technique provides a unique capability to study both stable and transient interactions at endogenous expression levels of the target proteins. Fixed cells were incubated with primary antibodies to AT1R (goat polyclonal antibody; Abcam) in combination with RAMP1 or RAMP2 antibody (rabbit polyclonal at 1:250 dilution; Alpha Diagnostics, USA) in blocking solution for 90 min at 37°C in a humid chamber, followed by incubation with rabbit plus and mouse minus PLA probes for 60 min at 37°C. Cells were then incubated with ligation mixture for 30 min at 37°C, followed by incubation with amplification mixture for 90 min at 37°C. Slides were mounted with cover slips using Duolink II mounting medium. The images were observed using fluorescence microscope (U-TV1 X; Olympus) under a 20X objective and the fluorescent red dots per nuclei were counted. Absence of probe in the reaction served as negative control.

Co-immunoprecipitation

Omental artery Cells (passages 2–4) were treated with or without sFLT-1 (10 ng/ml) in presence or absence of CALCB, ADM, or ADM2 at a dose of 10^{-8} M for 24 h in serum free DMEM. Cells were washed with PBS (10 ml × 2 times) after 24 h followed by centrifugation at 100 × *g* and cell lyses using the protocol provided by the manufacturer for non-denatured proteins (Abcam, USA). Immunoprecipitation was done using IgG, RAMP1, or RAMP2 antibodies (Alpha diagnostic, USA) and purified using anti-AT1R agarose slurry. The purified protein (20 ug) was used for western blot and probed with AT1R antibody (Abcam, USA) or with antibodies for respective input proteins RAMP1 and RAMP2.

Isolation of OA segments and wire myograph studies

Omental fat tissue collected from pregnant women was immediately placed in cold oxygenated physiological salt solution, consisting of 118.2-mM NaCl, 24.8-mM NaHCO3, 4.6-mM KCl, 1.2-mM KH2PO4, 1.2-mM MgSO4, 2.5-mM CaCl2, and 10.0-mM dextrose. A small segment of artery (200-µm normalized internal diameter) was cleaned off of fat, and cut into 2-mm rings followed by mounting on a Multi Wire Myograph System (Model 620M, Danish Myo Technology) for isometric tension recording, as previously described [7]. The arterial rings were precontracted with thromboxane agonist U46619 (10^{-6} M). Cumulative dose–response curves were constructed for CALCB, ADM, ADM2 (10^{-10} to 10^{-7} M), and Bradykinin (10^{-11} to 10^{-6} M). The relaxation responses were calculated as percent inhibition of the U46619 initially induced contraction and the maximum possible effect for the reagent (dose for half maximal response [EC_{50}] and maximal relaxation response [E_{max}]) was calculated accordingly as described in our earlier reports [6, 7]. The arteries with intact endothelium that responded well to Bradykinin mediated relaxation were used in this study.

Drugs

CALCB and ADM were purchased from Phoenix Pharmaceutical Inc., ADM2 from Alpha Diagnostics, and U46619 and bradykinin were purchased from Sigma Chemical.

Statistical analysis

All data are presented as mean ± SEM. Relaxation responses to CALCB family peptides are expressed as percent relaxation of the initial U46619-induced contraction. Vasodilator concentration response curves were fitted to a log-logistic sigmoid relation, and EC_{50} and Emax were calculated by using GraphPad Prism. Repeated measures analysis of variance (ANOVA) (treatment and time as factors) with a Bonferroni post hoc were used for comparisons of dose response curves between the groups. Expression levels of mRNA were compared between control and treatment groups using unpaired Student *t*-test. Statistical significance was defined as $P < 0.05$.

Results

Cyclic AMP synthesis and VEGF-A mRNA synthesis by CALCB peptides in OASMC, and the effect of sFLT-1 on peptides induced cAMP synthesis

Primary OASMCs at passage four were used to assess the effect of CALCB peptides on cAMP synthesis as a surrogate for mechanism involved in their relaxation responses. Figure 1A shows that all three peptides increase cAMP synthesis in OASMC ($P < 0.05$). Data show

that CALCB is the most potent in stimulating cAMP generation in OASMC compared with ADM and ADM2. Thus, the comparative order of efficacy for these peptides in stimulating cAMP synthesis in OASMC is CALCB > ADM = ADM2. Figure 1B demonstrates that all three peptides stimulate an increase in the synthesis of VEGF-A mRNA in presence or absence of sFLT-1in OASMC ($P < 0.05$). Figure 1C shows cAMP levels in OASMC treated with different doses of sFLT-1 for 24 h and the effect of peptides on cAMP synthesis in sFLT-1 treated cells. Figure 1C demonstrates that sFLT-1 at all doses results in significant decrease in the cAMP synthesis in OASMC ($P < 0.05$). Interestingly, in presence of CALCB, sFLT-1 mediated decrease in cAMP synthesis was completely abolished at all doses ($P < 0.05$). While ADM treatment rescued the effects of two lower doses of sFLT-1 treatment (0.1 ng–1 ng/ml; $P < 0.05$), ADM2 inhibited the effects of only the lowest dose of sFLT-1 (0.1 ng/ml, $P < 0.05$) in OASMC. Thus, the rank order of peptide induced inhibition of sFLT-1 effects and increasing the cAMP synthesis in OASMC is CALCB > ADM > ADM2.

Effect of sFLT-1 and Ang2 treatment on the expression of CALCB, ADM, and ADM2 receptor components in OASMC

Figure 2A shows the effect sFLT-1 treatment (24 h) on the mRNA expression of GPCR CALCRL and its three receptor activity-modifying proteins (RAMP1, RAMP2, and RAMP3) in OASMC. While there is no significant effect of sFLT-1 on the expression of CALCRL mRNA, sFLT-1 treatment at all doses resulted in decreased expression of both RAMP1 and RAMP2 mRNA expression in OASMC ($P < 0.05$). However, the expression of RAMP3 mRNA was not detectable in OASMC. Further, Figure 2B shows that treatment (24 h) with Ang2 decreased the expression of CALCRL and RAMP2 ($P < 0.05$) with no effect on the expression of RAMP1 mRNA in OASMC.

Effect of acute treatment with sFLT-1 and Ang2 on cAMP generation by CALCB, ADM, and ADM2 in OASMC

In Figure 3A, human OASMCs pretreated with sFLT-1 for 30 min were assessed for cAMP synthesis in response to stimulation by CALCB, ADM, and ADM2 for 5 min, as described in the methods. The cAMP synthesis induced by CALCB, ADM, or ADM2 are similar ($P < 0.05$) with or without sFLT-1 treatment and therefore does not appear to interfere with the functional receptor heterodimer formation/function. To assess if Ang2 pretreatment desensitizes the receptor system specific for CALCB, ADM, or ADM2 in OASMC, cells were incubated with Ang2 (10^{-7} M) for 30 min followed by washes and treatments with CALCB, ADM, or ADM2 for 5 min, as described in the methods. Figure 3B shows that the cells pretreated with Ang2 exhibit decreased stimulation of cAMP synthesis by CALCB and ADM2 ($P < 0.05$), while the response for ADM on cAMP synthesis remained unaltered.

Association of AT1R with RAMP1 and RAMP2

Figure 4A shows a Proximity ligation assay performed in unstimulated OASMC using two combinations of antibody: (i) AT1R antibody and RAMP1 antibody and (ii) AT1R antibody and RAMP2 antibody. The red fluorescent signal shown in both the combinations of antibody used suggest that AT1R associates with RAMP1 as well as RAMP2 in OASMC with significant higher interaction between RAMP1 and AT1R compared with that between RAMP2 and AT1R ($P < 0.05$; Magnification 20X). Using Co-immunoprecipitation methods, we further assessed the effect of sFLT-1 treatment on the interaction of AT1R with RAMP1 and RAMP2 proteins in OASMC and identify if the effects of sFLT-1 are altered in presence of CALCB, ADM, or ADM2. Figure 4B is a western blot of protein lysate of cells that were immuno-precipitated with RAMP1 or RAMP2 antibody. The precipitated protein was purified, electrophoresed and probed with AT1R antibody. RAMP3 was not tested because RAMP3 expression was not detected in OASMC. Interestingly, sFLT-1 treatment increases the interaction of RAMP1 with AT1R but does not have any effect on the interaction of RAMP2 and AT1R. Moreover, treatment with all three peptides inhibits the association of RAMP1 with AT1R in presence or absence of sFLT-1 with no effect on RAMP2 and AT1R interaction.

Vascular relaxation of OA from pre-eclamptic and normotensive pregnancies

Omental artery from PE and normal (normotensive) pregnancies was tested for its sensitivity for CALCB, ADM, ADM2, and Bradykinin induced relaxation in Figure 5. Segments of OA were precontracted with thromboxane agonist U46619 (1 μM) before treating with different doses of CALCB, ADM, or ADM2 as per the method used in earlier reports [6, 7]. Figure 5A shows that the maximal relaxation response of OA for CALCB in PE ($n = 5$) is similar to that in normotensive pregnancy ($n = 10$). The dose–response curves indicate an EC_{50} for CALCB as -7.49 ± 0.64 and E_{max} as 55.48 ± 1.90 in normotensive versus -8.10 ± 0.77 and 55.36 ± 1.87 respectively in PE women ($P < 0.05$). Figure 5B shows segments of OA precontracted with thromboxane agonist U46619 (10^{-6} M) and treated with different doses of ADM. As shown in the Figure 5B, the relaxation responses of ADM in OA from PE are similar to the responses of ADM in OA from normotensive pregnancy (28.71 ± 2.43 in normotensive versus 28.34 ± 2.44 in PE). EC_{50} could not be calculated for ADM as the maximal relaxation effect was <50% U466 contraction. Figure 5C shows a dose response curve for ADM2 treatment indicating an enhanced maximal relaxation response of OA for ADM2 in PE ($n = 7$) compared with the normotensive pregnancy ($n = 10$) [EC_{50} of -7.01 ± 0.60 and E_{max} as 27.40 ± 2.30 in normotensive versus an EC_{50} of -7.38 ± 0.92 ($P > 0.05$) and E_{max} of 39.42 ± 2.89 ($P < 0.05$) in PE pregnancy]. Therefore, the vascular sensitivity of OA in PE is maintained in the pathological condition with elevated effect for ADM2 in PE compared with the normotensive pregnancy. However, the maximal relaxation effect of CALCB are greater than ADM2 in OA from normotensive (E_{max} 40.01 ± 2.44 for CALCB versus 27.40 ± 2.30 for ADM2 in normotensive) as well as in OA from PE pregnancy (56.16 ± 2.58 for CALCB versus 39.42 ± 2.89 for ADM2 in PE). Further, Figure 5D shows increased relaxation responses of OA for Bradykinin (BK) in PE ($n = 7$) compared with the normotensive pregnancy ($n = 10$) with EC_{50} of -6.44 ± 0.88 and E_{max} of 54.12 ± 1.76 in normotensive versus EC_{50} of $-8.14 \pm 0.1$and E_{max} of 78.61 ± 2.99 in PE pregnancy ($P < 0.05$).

Discussion

Current study is first to demonstrate that CALCB family of peptides increase the synthesis of cAMP in the order of efficacy CALCB > ADM = ADM2 ($P < 0.05$) and expression of VEGF-A mRNA in OASMC from pregnant women. Soluble FLT-1 interferes with cAMP generation by CALCB, ADM, and ADM2 peptides,

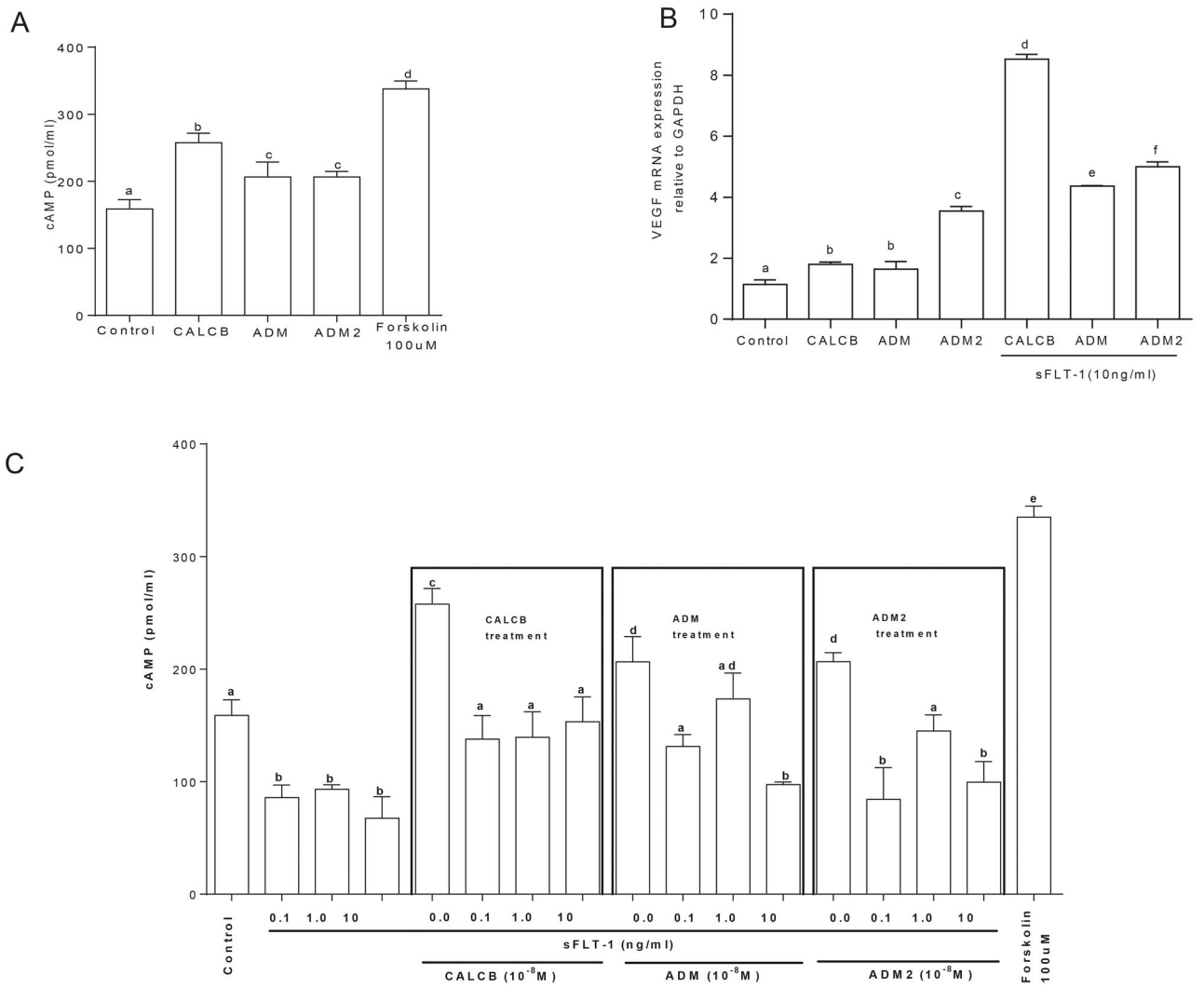

Figure 1. Stimulation of cAMP production and VEGF-A mRNA transcription by CALCB, ADM, and ADM2 in OASMC. (A) All three peptides (10^{-8} M) increase the synthesis of cAMP in the order of efficacy CALCB > ADM = ADM2 compared with the cells treated with IBMX alone (Control). Intracellular cAMP was quantified using Enzyme linked immunoassay (EIA) kit. Forskoline was used as a positive control. Data are presented as mean ± SEM for three replicates. Bars with different letters differ significantly among the groups ($P \leq 0.05$); (B) Effect of CALCB, ADM, and ADM2 treatment (10^{-8} M) on the expression of VGEF-A mRNA by qPCR. The bar graph represents mRNA levels of VEGF-A in OASMC treated with different concentrations of sFLT-1 in presence or absence of sFLT-1(10 ng/ml) for 24 h ($N = 3$). Bars with different letters differ significantly among the groups ($P \leq 0.05$), and (C) Effect of sFLT-1 treatment for 24 h on cAMP synthesis in presence or absence of CALCB, ADM, and ADM2 in OASMC. Intracellular cAMP was quantified using EIA. As shown, sFLT-1 treatment decreases the synthesis of cAMP in OASMC and all three peptides differentially inhibit the sFLT-1 induced decreases in cAMP levels in OASMC. Data are presented as mean ± SEM for three replicates and bars with different letters indicate significant difference between the groups ($P \leq 0.05$).

and treatment with these peptides differentially inhibits the adverse effects of sFLT-1 in OASMC. Further, this study shows that mRNA expression of receptor components, RAMP1 and RAMP2 is inhibited by sFLT-1 while that of CALCRL and RAMP2 is inhibited by Ang2 in these cells. In addition, pretreatment of OASMCs with sFLT-1 desensitizes the receptor system for ADM2 peptide only, while treatment with Ang2 results in desensitization of both CALCB and ADM2 effects in OASMC. Further, RAMPs interact with AT1R in OASMC and the level of interaction is greater between RAMP1 and AT1R. Interestingly, sFLT-1 treatment increases the interaction between RAMP1 and AT1R in OASMC but not between RAMP2 and AT1R, which is inhibited in presence of CALCB, ADM, or ADM2. Therefore, this study suggests that although SMC mediated functional responses of CALCB peptides could be the potential targets of sFLT-1 and Ang2-related hypertension, supplementation with these peptides provide a beneficial effect by increasing cAMP synthesis in presence or absence of these vasoconstrictors in addition to increasing VEGF expression in OASMC. This is further supported by the ability of these peptides in relaxing OA in PE pregnancy with an efficacy either similar to that in normotensive pregnancy as in the case of CALCB and ADM, or with enhanced sensitivity for ADM2.

CALCB, ADM, and ADM2 are hypotensive peptides that function through a single 7TM GPCR CALCRL and the ligand specificity of CALCRL is dictated by its interaction with RAMP1, 2, or 3. Activation of CALCRL/RAMPs heterodimer leads to the stimulation of intracellular cAMP synthesis [2]. Figure 1A shows that CALCB, ADM, and ADM2 stimulate an increase in cAMP synthesis in OASMC isolated from normotensive pregnant women with an

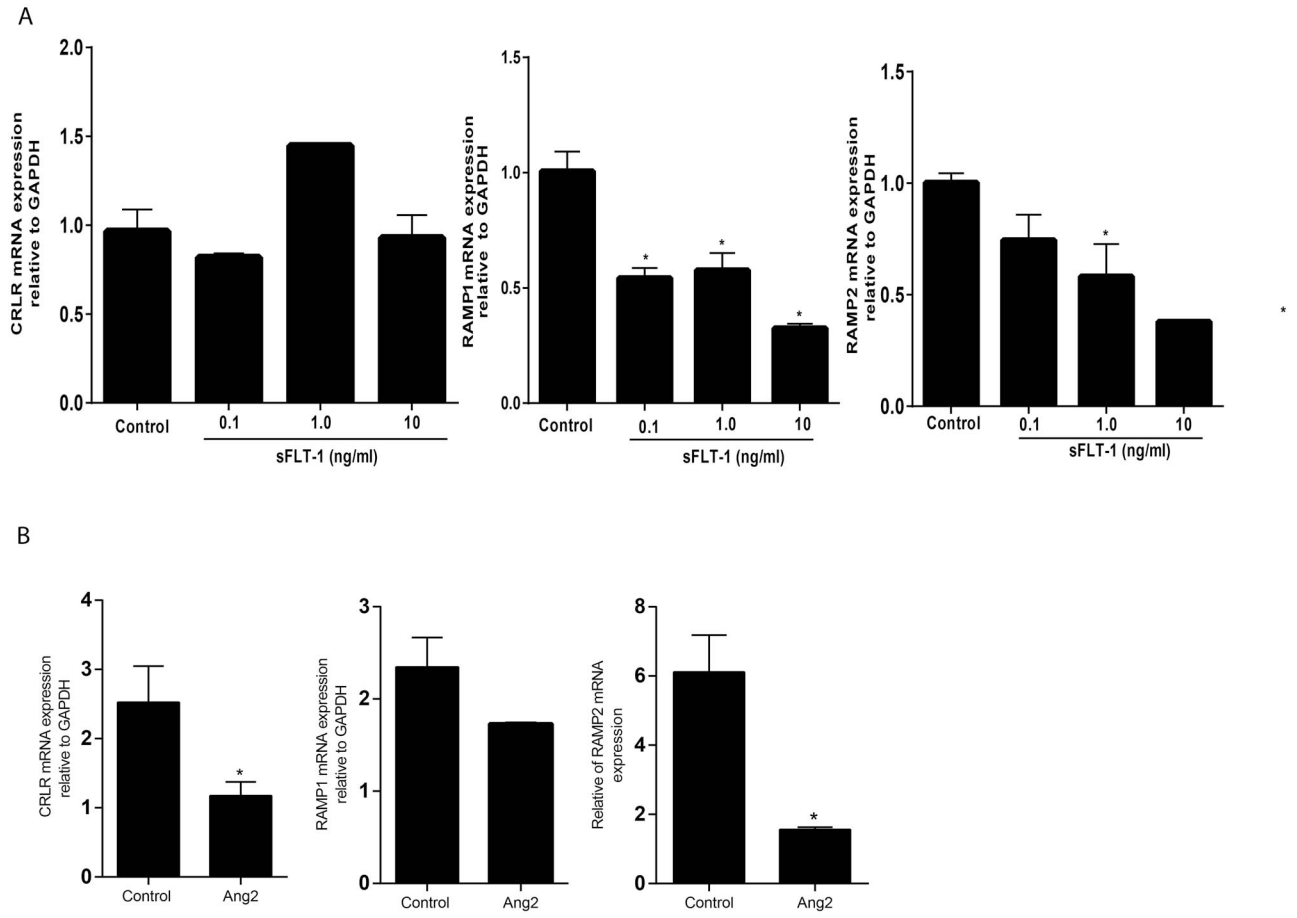

Figure 2. Effect of sFLT-1 and Ang2 treatment on the mRNA expression of CALCB, ADM, and ADM2 receptor system in OASMC. (A) Effect of sFLT-1 on the expression of CALCRL, RAMP1, and RAMP2 mRNA in OASMC. The bar graph represents mRNA levels of RAMP1 and RAMP2 in OASMC treated with different concentrations of sFLT-1 for 24 h ($N = 3$). As shown, sFLT-1 decreases the mRNA expression of both RAMP1 and RAMP2 in OASMC. The mRNA levels were normalized to the mRNA expression of GAPDH. Data are presented as mean ± SEM. Asterisk indicates $P < 0.05$ compared with the control. (B) Effect of Ang2 on the expression of CALCRL, RAMP1, and RAMP2 mRNA in OASMC. The bar graph represents mRNA levels of RAMP1 and RAMP2 in OASMC treated with Ang2 (10^{-7} M) for 24 h ($N = 3$). As shown, Ang2 decreases the mRNA expression of CALCRL and RAMP2 with no significant effect on the expression of RAMP1 in OASMC. The mRNA levels were normalized to the mRNA expression of GAPDH. Data are presented as mean ± SEM. Asterisk indicates $P < 0.05$ compared with the control.

efficacy of CALCB > ADM = ADM2 ($P < 0.05$). Smooth muscle cells express VEGF-A and its receptors suggesting its autocrine and/or paracrine role in SMC [19–22]. Basal expression of VEGF is high in SMC compared with the other vascular cells types and its expression is increased by cAMP synthesis in SMC [30]. This study shows that all three peptides increase cAMP synthesis (Figure 1A) and expression of VEGF (Figure 1B) in OASMC.

Soluble FLT-1 is believed to sequester VEGF, leading to systemic vascular dysfunction and hypertension in PE [13, 31]. Figure 1C shows that sFLT-1 treatment dose dependently decreased cAMP synthesis and sFLT-1 mediated decreases in cAMP synthesis is inhibited in presence of CALCB, ADM, or ADM2 with the effect of CALCB being most potent ($P < 0.05$). This correlates with the increase in VEGF mRNA expression induced by peptides in presence or absence of sFLT-1 as shown in Figure 1B. Interestingly, RAMP3 was not detectable in OASMC (not shown) suggesting CALCRL-RAMP1 or CALCRL-RAMP2 heterodimer to form the functional receptor system for these peptides in OASMC. Figure 2A shows that sFLT-1 treatment decreases both RAMP1 and RAMP2 mRNA expression suggesting that sFLT-1 induced decrease in RAMPs would impair the vascular SMC mediated responses of CALCB, ADM, and ADM2 in pathologies involving elevated sFLT-1 levels such as in PE. Strikingly, despite sFLT-1 mediated decrease in the synthesis of cAMP and mRNA levels of RAMPs in OASMC, all three peptides rescued the decreased cAMP levels in OASMC with the effect of CALCB > ADM > ADM2 (Figure 1C). Thus, in the event of decreased mRNA levels of RAMPs, an increase in cAMP levels in presence of sFLT-1 by the three peptides could be due to peptides induced increases in the cell surface association of CALCRL with the RAMPs resulting in increased ligand-receptor binding. This notion is supported by our earlier report showing peptide induced increase in the cell surface association of CALCRL with RAMPs [29], and by the data in Figure 3A showing that, pretreatment of OASMC with sFLT-1 partially blocks ADM2 induced cAMP production in OASMC and it has no effect on the cAMP synthesis induced by CALCB and ADM. In addition, this study suggests that sFLT-1 mediated decreases in cAMP levels involve desensitization of ADM2 specific CALCRL-RAMP complex in OASMC with no effect on the functional receptor system for CALCB or ADM (Figure 3A). This allows us to speculate that in the event of vascular dysfunction

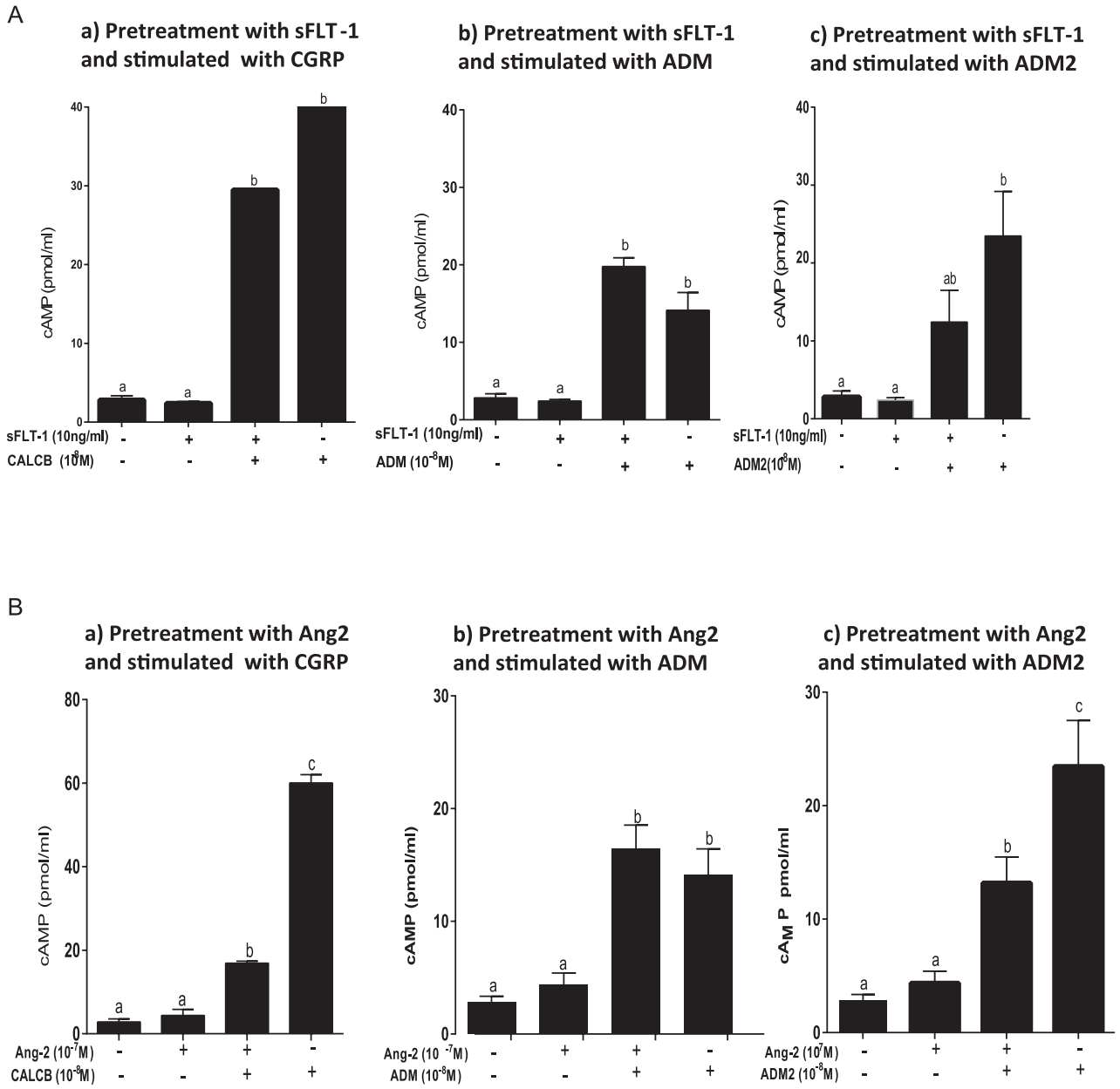

Figure 3. Acute effect of sFLT-1 and Ang2 pretreatment on CALCB, ADM, and ADM2 mediated cAMP synthesis in OASMC. (A) Effect of sFLT-1 on desensitization of CALCB, ADM, and ADM2 specific receptor for cAMP generation in OASMC. Cells were pretreated with or without sFLT-1 (10 ng/ml) for 30 min followed by treatment with or without peptide (10^{-8} M) for 5 min. Intracellular cAMP was quantified using EIA kit. As shown, all three peptides, CALCB (a), ADM (b), and ADM2(c) retain their effects on cAMP synthesis in OASMC pre-incubated with sFLT-1 compared with the cells without any pretreatment (Control). Data are presented as mean ± SEM for three replicates and bars with different letters differ significantly among the groups ($P \leq 0.05$). (B) Effect of Ang2 on desensitization of CALCB, ADM, and ADM2 specific receptor for cAMP generation in OASMC. Cells were pretreated with or without Ang2 (10^{-7} M) for 30 min followed by treatment with or without peptide (10^{-8} M) for 5 min. Intracellular cAMP was quantified using EIA kit. As shown, CALCB (a) and ADM2 (c) mediated cAMP synthesis is blocked, while ADM (b) effects are retained in OASMC pre-incubated with Ang2 compared with the cells without any pretreatment (Control). Data are presented as mean ± SEM for three replicates and bars with different letters differ significantly among the groups ($P \leq 0.05$).

caused by sFLT-1 mediated sequestration of VEGF, treatment with these peptides will replenish the VEGF by increasing its synthesis and render a protective effect.

Factors regulating sFLT-1 expression in PE are just beginning to emerge [12, 32]. It is known that agonistic autoantibodies to the Ang2 type-1 receptor (AT1AA) derived from the serum of women with PE produce PE-like syndrome in pregnant rats and increases AT1R levels and placental secretion of sFLT-1, suggesting a role for AT1R in pathogenic up-regulation of sFLT-1 in PE [32]. Ang2 mediates its vasoconstrictive effect in hypertensive disorders via AT1R which is usually present in monomeric form in normal pregnancy and in heteromeric form in PE, where it becomes insensitive to inactivation and highly sensitive to Ang2 binding [23]. It was recently reported that over expression of RAMP1 in mice rendered protection against Ang2-induced vascular dysfunction [33]. However, Figure 2B shows that Ang2 decreases the expression of CALCRL and RAMP2

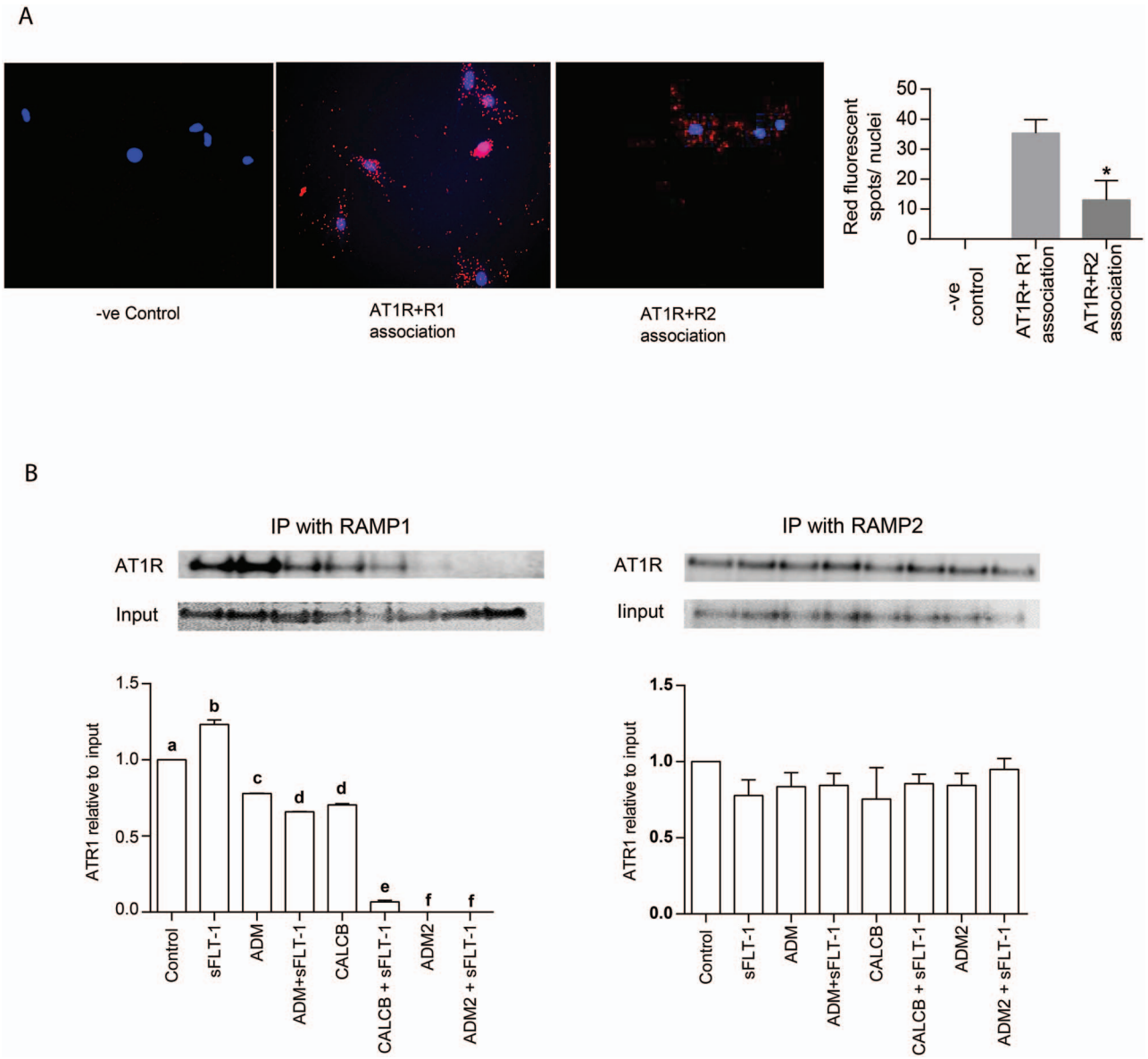

Figure 4. Association of AT1R with RAMPs in OASMC: (A) Proximity ligation assay showing representative images of the associations between AT1R with RAMP1 and RAMP2. The fluorescent red signal is indicative of protein–protein interaction between the two target proteins. The red spots were quantified and normalized with the blue nuclear staining. DAPI was used for nuclear staining and absence of probe served as the control (Magnification = 20X). The bar graph shows the number of red spots/nuclei ($n = 3$ replicate experiments); (B) Co-immunoprecipitation (Co-IP) of Ang2 receptor type 1 (AT1R) with RAMP1 and RAMP2 protein in OASMC treated with sFLT-1 in presence or absence of peptides. Cells were pretreated with or without sFLT-1 (10 ng/ml), peptides (10^{-8} M), or sFLT-1 (10 ng/ml) with the three peptides for 24 h. This was followed by cell lysis and immuno-precipitation (IP) with RAMP1 or RAMP2 antibody followed by elution using anti-AT1R agarose slurry. Western blot analysis was performed by probing with antibodies for AT1R or their respective input samples. Figure shows AT1R co-immuno-precipitates with both RAMP1 (a) and RAMP2 (b) protein in OASMC. Bar graph below shows the relative fold change in the band densities normalized with the band density of the respective input samples. Different letters indicate significant difference between the groups $P < 0.05$. Data are presented as mean ± SEM of three replicate experiments. IP (immunoprecipitation), AT1R (Ang2 type-1 receptor), R1 (RAMP1), and R2 (RAMP2)

mRNA but not RAMP1 in OASMC. This suggests that in addition to the adverse effects of sFLT-1 (Figure 2A), Ang2 also adversely affects the receptor system of CALCB family of peptides by targeting mRNA transcription of the shared GPCR, CALCRL, and its activity-modifying protein RAMP2 with no effect on the mRNA expression of RAMP1. Interestingly, Figure 3B shows that pretreatment of OASMC with Ang2 inhibits CALCB and ADM2 mediated cAMP synthesis, that function through CALCRL and RAMP1 receptor components, while it has no effect on the activity of ADM, which requires RAMP2 as a cofactor for its receptor function. This suggests that although Ang2 does not affect the transcription of RAMP1 in OASMC, it desensitizes the activity/receptor system of CALCB and ADM2 peptides.

RAMPs serve as a cofactor for several other GPCRs [34–36]. However, it is not known if RAMPs interact with AT1R, and if there is a crosstalk between sFLT-1, AT1R, and RAMPs in OASMC that may interfere with the function of CALCRL-RAMPs receptor system. Interestingly, both RAMP1 and 2 interact with AT1R

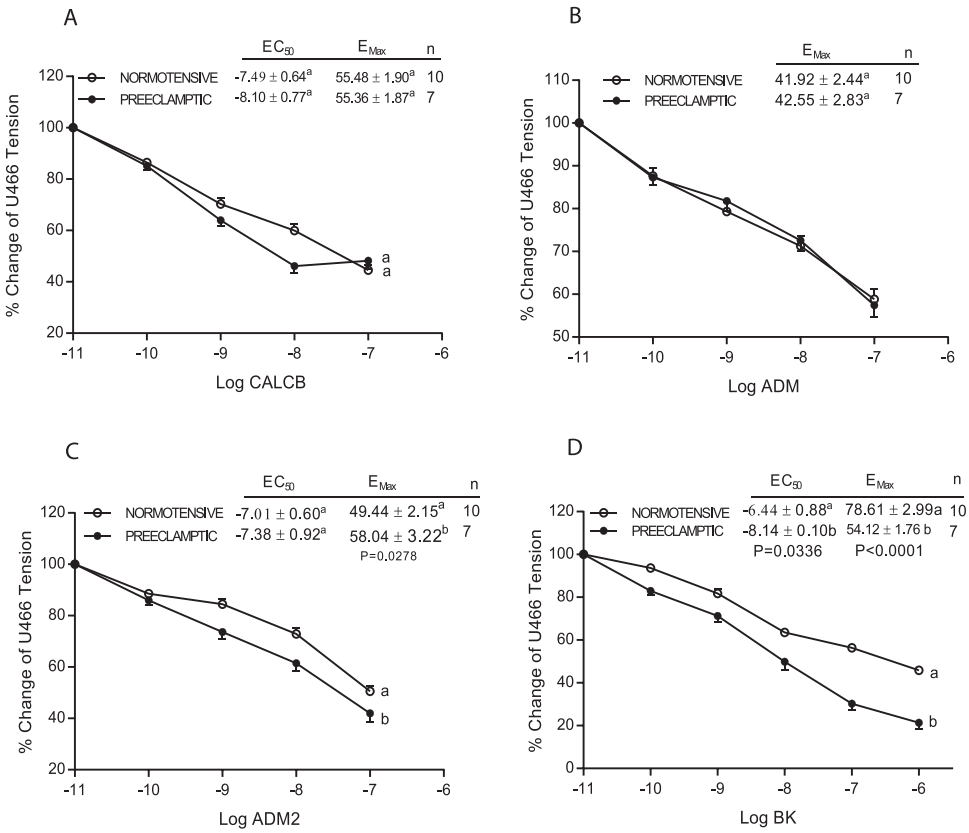

Figure 5. Relaxation responses of omental arteries (OA) to CALCB, ADM, ADM2, and Bradykinin. (A) Relaxation induced by CALCB in OA from pregnant women with normotensive ($n = 10$) and pre-eclamptic pregnancy (PE; $n = 5$). Dose response curves show no significant difference in the relaxation by CALCB in OA from PE ($n = 10$) compared with normotensive group ($n = 7$). (B) Relaxation induced by ADM in OA from pregnant women with normotensive ($n = 10$) and PE ($n = 7$). The dose–response curves indicate no significant difference in response to ADM between the two groups. (C) Relaxation induced by ADM2 in OA from pregnant women with normotensive ($n = 10$) and PE ($n = 7$). Figure shows enhanced relaxation to ADM2 in OA from PE ($n = 10$) compared with normotensive group ($n = 7$), and (D) Relaxation induced by Bradykinin (BK) in OA from pregnant women with normotensive ($n = 10$) and PE ($n = 7$). Figure shows enhanced relaxation to Bradykinin in OA from PE ($n = 10$) compared with that in the normotensive group ($n = 7$). Results are presented as mean ± SEM. Different letters indicate significant differences between groups ($P < 0.05$).

with a greater interaction occurring between RAMP1 and AT1R (Figure 4A; $P < 0.05$). However, sFLT-1 increases the interaction between RAMP1 and AT1R but not RAMP2 and AT1R, which is inhibited by all three peptides in presence or absence of sFLT-1 treatment (Figure 4B).

Assessing the consequence and mode of AT1R and RAMP1 interaction is beyond the scope of this study. However, it raises a possibility that AT1R may be structurally compatible to form a functional heterodimer with RAMPs and this association may define a mechanism of cell-specific modulation of efficacy and potency of AT1R for Ang2. It is likely that sFLT-1 induced inhibition of mRNA for RAMP1 and RAMP2 (Figure 2A) along with sFLT-1 induced increases in the interaction of RAMP1 with AT1R (Figure 4B), will render decreased access of RAMP1 to dimerize with CALCRL resulting in a compromised receptor system for CALCB family peptides. In pathological conditions such as PE pregnancy where the peptides levels are already downregulated, the elevated sFLT-1 levels and increased Ang2 sensitivity would not only limit the vascular relaxation induced by CALCB peptides, but also decrease peptides induced VEGF transcription (Figure 1B) and thus contribute to the decreased availability of VEGF. Therefore, PE associated decreases in CALCB family peptides along with impairment in CALCRL/RAMPs system resulting from PE associated increases in sFLT-1 levels, is likely to adversely impact the vascular function and blood pressure regulation. Interestingly, data in Figures 1C and 4B suggest that despite the adverse effects of sFLT-1 and Ang2 on the CALCRL/RAMPs system in OASMC, treatment with the peptides may reverse the sFLT-1 and AT1R mediated vasoconstriction by preventing decreases in cAMP synthesis and inhibiting AT1R and RAMP1 interactions. This notion is supported by vascular reactivity performed in segments of OA isolated from PE (Figure 5), showing the potential of these peptides in causing vascular relaxation with similar or elevated efficacy compared with the normotensive pregnancy. This is of clinical importance, as published reports not only show a strong association of PE pathology with decreases in the levels of these peptides in pregnant women at term, but also during second trimester of pregnancy who are destined to develop PE [5, 9–11, 37, 38]. Along with increased sensitivity to Ang2 [13], the putative mechanisms mediating the vascular dysfunction in PE include reduced NO-dependent dilation due to decreases in NO levels or unavailability of bioactive NO [39]. Interestingly, our earlier report shows CALCB-induced reversal of PE-like symptoms in rat model of PE generated by L-NAME mediated inhibition of endogenous NO synthesis. This further supports the protective effect of CALCB supplementation in a hypertensive pregnancy [14].

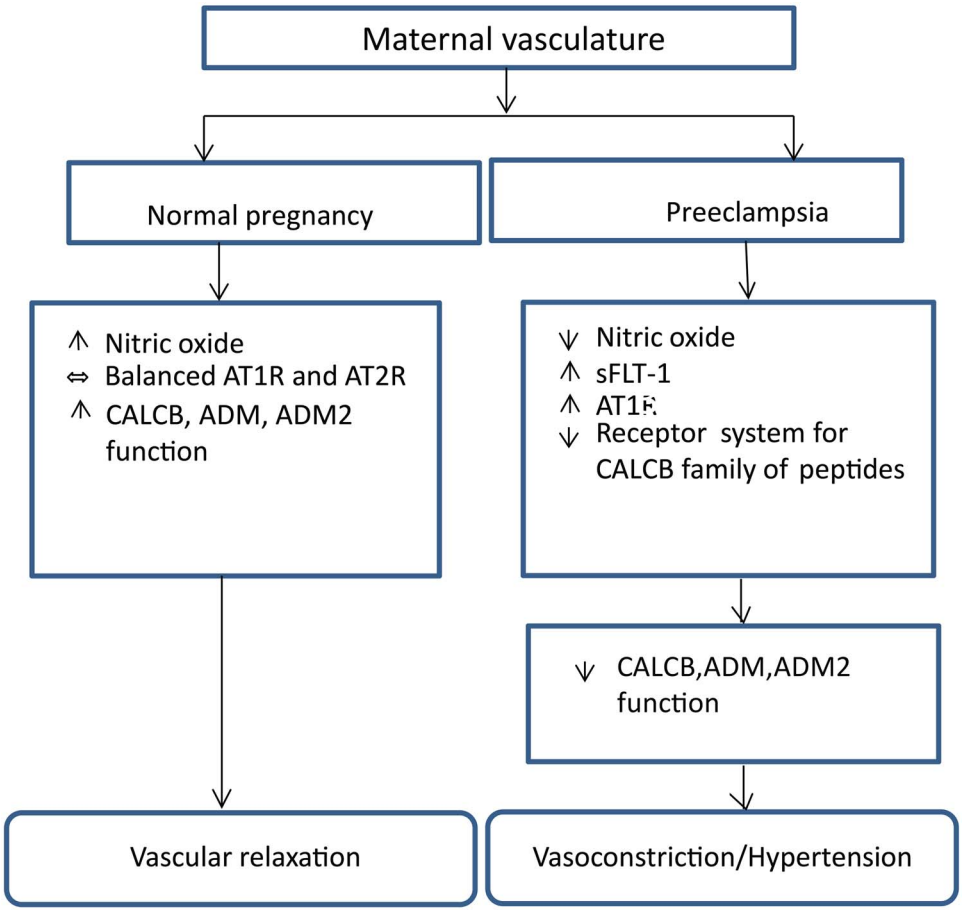

Figure 6. Flow chart showing potential involvement of CALCB family peptides, Ang2 and sFLT-1 system in maternal circulation during normal pregnancy and pregnancy complicated by PE. In normal (normotensive) pregnancy, maternal vasculature is relaxed due to increase in NO system, balanced AT1R and AT2R levels, and increased expression and function of CALCB family peptides. In PE, decreases in NO system and elevated sFLT-1 levels are accompanied with down regulation of ligand-receptor system of CALCB family of hypotensive peptides resulting in vasoconstriction. AT1R (Ang2 type-1 receptor), AT2R (Ang2 type-1 receptor), and sFLT-1.

Although, at a very preliminary stage, this study demonstrates an interaction of sFLT-1/Ang2 system with the receptor system for CALCB family of peptide in maternal vasculature that needs further characterization. Of clinical importance is the vascular data showing relaxation responses with either similar efficacy as in the case of CALCB and ADM or increased ADM2-mediated relaxation in OA from PE pregnancy compared with that in normal pregnancy (Figure 5), suggesting a potential of these peptides in reversing/normalizing PE associated vasoconstriction when their endogenous levels are down regulated. Therefore, this study suggests an involvement of these peptides in the pathophysiology of human pregnancy as proposed in Figure 6.

In summary, current study demonstrates vascular SMC mediated functional response of CALCB, ADM, and ADM2 through CALCRL-RAMPs receptor system. Soluble FLT-1 and Ang2 adversely affect the receptor system for these peptides in OASMC which is differentially protected by peptide supplementation. Further, vascular sensitivity of OA is retained for the relaxation effects of all three peptides in PE with enhanced responses for ADM2 compared with the normotensive pregnancy. Therefore, this study provides a basis to further explore the importance of these peptides in diseases involving elevated levels of sFLT-1 and warrants further studies to identify the mechanism of their action and their interaction with sFLT-1 and Ang2 system in maternal vasculature of human pregnancy.

Conflict of interest

None.

Author contributions

CY and MC designed the study and wrote the manuscript; MC, AB, MB, and AM performed the experiments; and KF and MB were involved in collection of human tissues.

Data availability

The data underlying this article are available in the article.

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