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Calcitonin Gene-Related Peptide Rescues Proximity Associations of Its Receptor Components, Calcitonin Receptor-Like Receptor and Receptor Activity-Modifying Protein 1, in Rat Uterine Artery Smooth Muscle Cells Exposed to Tumor Necrosis Factor Alpha[1]

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ABSTRACT

Calcitonin gene-related peptide (CALCB), adrenomedullin (ADM), and ADM2/intermedin play critical roles in vascular adaptation during pregnancy through calcitonin receptor-like receptor (CALCRL) and receptor activity-modifying proteins (RAMPs). This study was designed to assess the predominant RAMP that associates with CALCRL to form a functional receptor in the rat uterine artery smooth muscle (RUASM). We also determined if these receptor component associations are decreased by tumor necrosis factor (TNF) alpha and if CALCB, ADM, or ADM2 can rescue CALCRL/RAMP associations. Using proximity ligation assay in RUASM cells, this study shows that CALCRL predominantly associates with RAMP1 forming a CALCB receptor, and minimally with RAMP2 and RAMP3 that confer specificity for ADM and ADM2. However, knockdown of RAMP1 mRNA increases the interaction between CALCRL and RAMP3 without affecting the association of CALCRL and RAMP2. Furthermore, CALCB, ADM, and ADM2 have no effects on the associations of CALCRL with any of the RAMPs in RUASM cells. Interestingly, CALCB reverses the TNFalpha-induced decreases in CALCRL/RAMP1 associations. Furthermore, CALCB increases ERK1/2 phosphorylation in a time-dependent manner in RUASM, and the protective effect of CALCB on TNFalpha-induced inhibition of CALCRL/RAMP1 associations was significantly blocked in presence of ERK inhibitor (PD98059). In conclusion, this study demonstrates that CALCRL predominantly associates with RAMP1 forming a CALCB-specific receptor complex in RUASM cells, which is dissociated by TNFalpha. Rescue of TNFalpha-induced dissociation of CALCRL/RAMP1 complex by CALCB in RUASM cells suggests a potential use of CALCB in developing therapeutic strategies for pregnancy-related complications that are vulnerable to abnormal levels of TNFalpha, such as fetal growth restriction and preeclampsia.

CALCB, pregnancy, uterine artery

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INTRODUCTION

The maternal cardiovascular system undergoes structural and functional changes to accommodate the increased nutrients and oxygen requirements of the growing fetus. These changes are most profound in the uteroplacental vasculature, where a marked increase in uteroplacental blood flow is achieved by a large reduction in vascular resistance [1] and pronounced enlargement and structural reorganization of the uterine spiral arteries [2]. Uterine arteries play a major role in regulating the uteroplacental blood flow. Failure to make or to sustain these changes may result in serious complications of human pregnancy, such as intrauterine fetal growth restriction (FGR) and preeclampsia [3, 4]. Furthermore, FGR and preeclampsia are associated with higher concentration of the proinflammatory cytokine tumor necrosis factor (TNF) α [5, 6] and its soluble receptor 1 (TNF-R1) in maternal serum [7, 8], and elevated levels of TNFα are known to cause endothelial dysfunction in preeclamptic vasculature [7]. Using uterine artery Doppler velocimetry examination, a strong correlation was reported between high levels of TNFα and abnormal pulsatile index of uterine artery in patients who developed preeclampsia [9]. However, the mechanisms underlying the TNFα effect on the uterine artery in pregnancy are not fully understood.

We and others have shown that vasoactive peptides, such as calcitonin gene-related peptide (CALCB), adrenomedullin (ADM), and ADM2/intermedin, support fetoplacental growth via their effect on pregnancy induced vascular adaptations in rats [10]. Furthermore, blocking the endogenous function of any of these peptides results in fetoplacental growth restriction [10]. All three peptides are potent vasodilators belonging to a unique group of calcitonin/CALCB family peptide hormones important for homeostasis in diverse tissues [11–15]. Elevated levels of CALCB [16–18], ADM [16, 17, 19], as well as ADM2 [7, 20] are associated with rat and human pregnancy. We reported earlier that CALCB, ADM, and ADM2 are shown to exhibit increased sensitivity to mesenteric, as well as uterine artery, relaxation in rat pregnancy in an endothelium-dependent and/or -independent manner, where the relaxant effects of CALCB are mediated through vascular smooth muscle cells and ADM effects are predominantly endothelium dependent [10, 15]. However, the effects of ADM2 are minimal on the uterine artery relaxation (Yallampalli and Chauhan, unpublished results).

All three peptides exert their effects through a single G-protein coupled receptor (GPCR) calcitonin receptor-like receptor (CALCRL), the ligand binding of which is dictated by the presence of one of the three receptor activity-modifying proteins (RAMPs) 1, 2, or 3. CALCRL in combination with RAMP1 gives rise to a CALCB receptor, whereas CALCRL in combination with RAMP2 or RAMP3 forms ADM receptors

[19, 20]. However, ADM2 is shown to be a nonselective agonist for RAMPs, but exhibits greater potency with CALCRL/RAMP1 and CALCRL/RAMP3 [20].

We showed previously that CALCRL and the three RAMPs are present in the uterine artery, and that pregnancy results in increased expression of CALCRL, RAMP1, and RAMP3, but not RAMP2 mRNA in the uterine artery, suggesting a potential involvement of these three receptor components in mediating the effects of CALCB and ADM in the uterine artery [15, 21, 22]. Although both CALCB and ADM relax the rat uterine artery in vitro, only CALCB stimulates cAMP production by rat uterine artery smooth muscle (RUASM) cells in vitro and relaxes endothelium-denuded uterine artery ex vivo, whereas both ADM and ADM2 fail to stimulate cAMP generation [15] as well as endothelium-independent uterine artery relaxation. This further confirms a functional role for CALCB in RUASM and smooth muscle-independent effects of ADM in the uterine artery, and suggests that these peptides may act on the rat uterine artery via distinct CALCRL/RAMP interaction in different vascular compartments. However, it is unknown which of the RAMPs is associated with CALCRL to exert the effect of CALCB in the uterine artery smooth muscle cells, and if TNFα has an adverse effect on the formation of CALCB-specific receptor complex in the uterine artery smooth muscle cells.

We hypothesize that TNFα disrupts the associations of receptor components of CALCB in vascular smooth muscle cells, and therefore could interfere with the actions of CALCB. The present study was designed to assess the basal association of CALCRL with various RAMPs in the uterine artery smooth muscle cells, to determine the effects of TNFα in the presence or absence of CALCB, ADM, and ADM2 on these associations by using state-of-the-art proximity ligation assay (PLA), and to identify the involved downstream signaling mechanisms.

MATERIALS AND METHODS

Animal Welfare and Ethics Statements

All procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and were approved by the Animal Care and Use Committee and by the Animal Research Ethics Committee of Baylor College of Medicine. All studies involving animals are reported in accordance with the ''Animal Research: Reporting of In Vivo Experiments'' guidelines for reporting experiments involving animals [23]. Female rats on Day 12 of pregnancy were obtained from Harlan Sprague Dawley. Rats were maintained in the colony room with a fixed photoperiod of 12L:12D and with access to water and rodent chow ad libitum, and killed when needed.

Isolation of RUASM Cells

All procedures were carried out under aseptic conditions. The uterine artery with all its major branches was excised from rats on Day 20 of pregnancy and placed in a Petri dish containing ice-cold Hanks Balanced Salt Solution (HBSS; Ca^{2+} and Mg^{2+} free; Gibco by Life Technologies) with 0.2 mM Ca^{2+}. The RUASM cells were harvested from enzymatically dissociated rat uterine arteries, per the method described previously [14]. Briefly, the cleaned uterine artery arcades (from at least two animals) were transferred into a 50-ml plastic tissue-culture flask containing 4.0 ml of enzymatic dissociation mixture: HBSS (Ca^{2+} and Mg^{2+} free), with 0.2 mM Ca^{2+}, 15 mM Hepes buffer (pH 7.2–7.3), 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 16.5-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; Gibco by Life Technologies) with all supplements. The cell pellets were resuspended 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 with HBSS in 48- to 72-h intervals, and typical confluent monolayers formed within 7 days. Each preparation usually yielded 5–6 × 10^5 cells with 80%–90% viability, as assessed by trypan blue exclusion. RUASM cells were maintained at 37°C in a humidified incubator in an atmosphere of 95% air and 5% carbon dioxide and studied at subconfluence. RUASM cells were grown on 16-well Lab Tek chamber slides (Electron Microscopy Sciences) in DMEM containing 100 μg penicillin-streptomycin/ml, and supplemented with 10% fetal bovine serum (Gemini Bioproducts) at 37°C in a humidified 5% CO_2 atmosphere. After overnight starvation in DMEM containing 0.5% bovine serum albumin (Fisher Scientific), the cells were treated with CALCB, AM, and ADM2, as designated.

RAMP Knockdowns in the Uterine Artery Smooth Muscle Cells

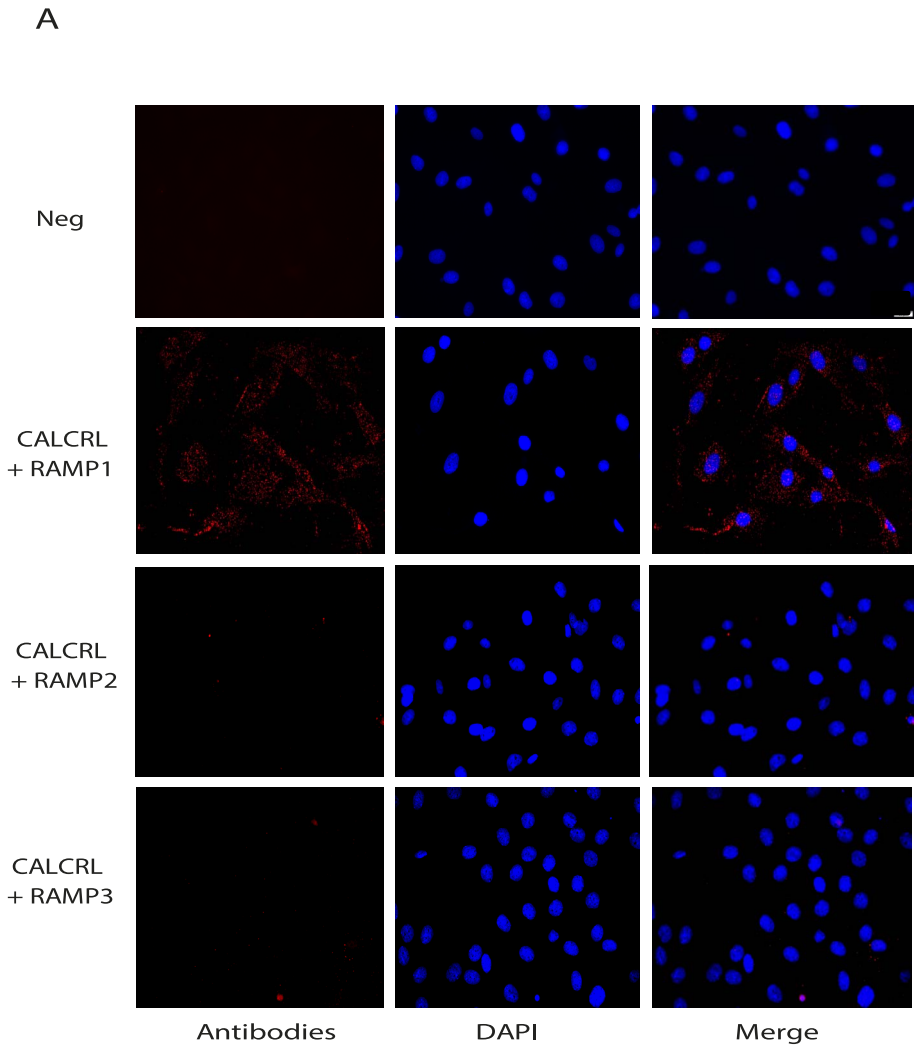
Vascular smooth muscle cells (passages 5–6) from the uterine artery were transfected with a 19-nt short hairpin RNA (shRNA) sequence specific for rat RAMP1 (Origene) by nucleofection, using a nucleofection kit specific for primary smooth muscle cell, per manufacturer's instructions (Lonza). Scramble shRNA was used as the control. Briefly, after nucleofection, cells were transferred to complete growth medium (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) for 24 h followed by addition of puromycin (3 μg/ml; Sigma) for selection of cells transfected with shRNA. Stable cell lines expressing either RAMP1-specific shRNA or scramble shRNA were established in about 10 days under the selection pressure of puromycin.

Proximity Ligation Assay

PLA was performed using a Duolink II Fluorescence kit (Olink Biosciences) according to the manufacturer's instructions. PLA technology extends the capabilities of traditional immunoassays to include direct detection of protein-protein interactions with high specificity and sensitivity, and thus readily detected and localized the target proteins with single-molecule resolution and objectively quantified their interaction in unmodified cells. PLA probe species-specific secondary antibodies, each with a unique short DNA strand attached to it, bind to the primary antibodies (two antibodies raised in different species against the target antigens of interest), and the DNA strands can interact through a subsequent addition of two other circle-forming DNA oligonucleotides. Interaction between the two proteins results in amplification of circularized DNA molecules, which can then be detected as bright fluorescent dots, providing a unique capability to study both stable and transient interactions at endogenous expression levels of the target protein(s). In the present study, RUASM cells (8000 cells/well) grown on 16-well Lab-Tek chamber slides were treated with CALCB, ADM, or ADM2 (100 nM, 4 h; American Peptide Co., Inc.) in the presence or absence of TNFα (10 ng/ml, 4 h; Sigma-Aldrich). Experiments were also performed to determine the time-dependent influence of CALCB on protein interactions in the presence or absence of TNFα (10 ng/ml, 4 h), in combination with the inhibitors of Akt (LY294002, 10 μM; Sigma-Aldrich) or Erk (PD98059, 10 μM; Sigma-Aldrich). The cells were washed with PBS and fixed using 4% paraformaldehyde at room temperature for 5 min. Fixed cells were incubated with primary antibodies to CALCRL (goat polyclonal antibody; Santa Cruz Biotechnology) in combination with RAMP1, -2, or -3 diluted 1:250 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 1 h and 30 min at 37°C. Slides were mounted with cover slips using Duolink II mounting medium and images were observed using fluorescence microscope (U-TV1 X; Olympus) under a 20× objective. Images were processed, and red spots were counted using CellSence software (Olympus Scientific) in five randomly selected images, with a total of 200 cells per replicate. Appropriate negative controls were included with no primary antibody or –ve IgG isotype of primary antibody.

Western Blotting

Western blotting was performed as described previously [10]. Briefly, equal amounts (17 μg) of protein were separated by PAGE and then electrotransferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% fat-free milk in TBS-T and incubated with primary antibodies for total and phosphorylated Akt (Cell Signaling), total and phosphorylated extracellular signal-related kinase (ERK) 1 and ERK2 (Cell Signaling), and βactin (Abcam)



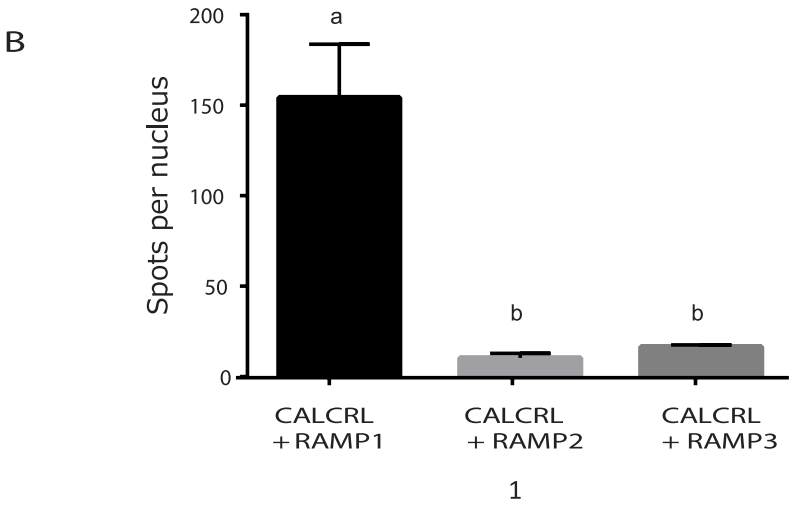


FIG. 1. Basal CALCRL/RAMP associations on RUASM cells. RUASM cells were sparsely grown on 16-well Lab-Tek chamber slides and subjected to PLA. **A**) Representative images of PLA for associations of CALCRL with RAMP1, CALCRL with RAMP2, and CALCRL with RAMP3 on RUASM cells incubated with or without primary antibodies against CALCRL, RAMP1, RAMP2, and RAMP3 or without primary antibodies (Neg). **B**) Comparison of the red spots per nucleus (blue, 4 ,6-diamidino-2-phenylindole [DAPI]) on RUASM cells are shown in bar graph as mean ± SEM of five random fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Different letters at the top of the bars indicate significant differences between groups ($P < 0.01$).

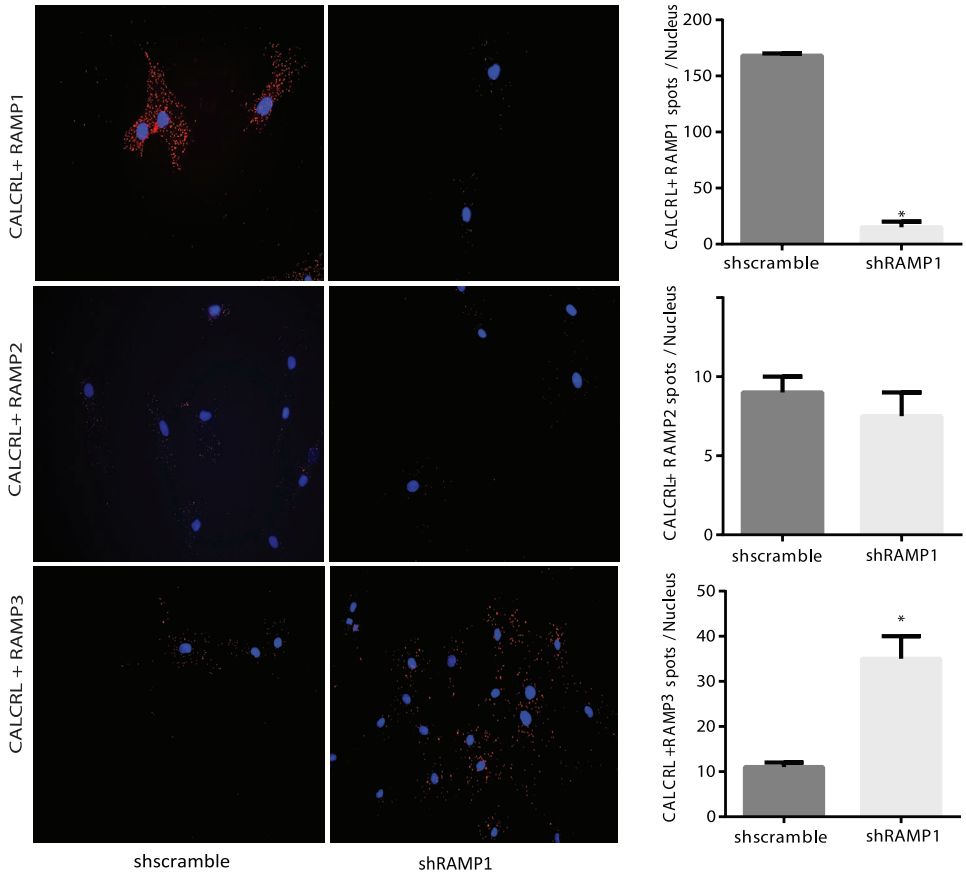


FIG. 2. CALCRL/RAMP associations on the RUASM cells with knockdown of RAMP1. Representative images of PLA for associations of CALCRL with RAMP1, CALCRL with RAMP2, and CALCRL with RAMP3 in cells transfected with scramble RAMP1 RNA or shRAMP1 RNA. Red spots per nucleus on cells are presented as bar graphs for mean ± SEM of five random fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Aterisks at the top of the bars indicate significant differences between groups ($P < 0.01$).

overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated antirabbit or antimouse secondary antibody (Southern Biotech). Target proteins were visualized with Pierce enhanced chemiluminescence detection (Thermo Scientific). The signals were quantified by using Odyssey Imaging System (LI-COR Biosciences).

Statistical Analysis

Data sets were analyzed by SigmaPlot 9.0 (Systat Software, Inc.) and Prism (GraphPad Software Inc.) employing appropriate statistical tools. The means of the various groups were analyzed by unpaired *t*-test or one-way ANOVA and subjected to the Newman-Keuls multiple comparison test or Bonferroni post hoc test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Cell Surface Associations of CALCRL with RAMPs on RUASM Cells

As shown in Figure 1A, PLA analysis demonstrated that RAMP1, -2, and -3 are each associated with CALCRL in close proximity, on the surface of the resting RUASM cells. The protein-protein interaction between CALCRL with RAMP1, as expressed by the number of red spots per cell (number of red spots/nucleus stained blue), was 154.3 ± 29.3 (Fig. 1B). The association of CALCRL with RAMP2 was 10.4 ± 2.5 red sports per cell, and of CALCRL with RAMP3 was 16.5 ± 1.0 red sports per cell. Thus, the interaction of CALCRL with RAMP1 was greater in the resting RUASM cells when compared to the associations of CALCRL with either RAMP2 or RAMP3 ($P < 0.01$), suggesting that the receptors for CALCB, but not for ADM and ADM2, are the predominant CALCB-family peptide receptors in these cells. However, knockdown of RAMP1 by shRNA specific for rat RAMP1 results in an increased association of CALCRL with RAMP3 compared to the scramble with no effect on the association of CALCRL with RAMP2 (Fig. 2).

The Effect of CALCB, ADM, and ADM2 on the Basal Associations of CALCRL with RAMPs

Treatment of RUASM cells with CALCB, ADM, and ADM2 at 100 nM for 2 min did not significantly alter the number of red fluorescent spots pertaining to the association of CALCRL with either RAMP1 (Fig. 3), or with RAMP2 and RAMP3 (data not shown).

The Effect of TNFα on the Association of CALCRL with RAMP1 on RUASM Cells

As shown in Figure 4, treatment with TNFα significantly inhibited the associations of CALCRL with RAMP1 (156.8 ± 25.3 red spots per cell in control vs. 17.8 ± 5.7 red spots per cell in TNFα-treated group; $P < 0.001$) on the RUASM cells.

The Effects of CALCB, ADM, and ADM2 on TNFα-Induced Reduction in CALCRL and RAMP1 Associations in RUASM

As shown in Figure 4, CALCB reverses the inhibitory effect of TNFα on the associations of CALCRL with RAMP1 (17.8

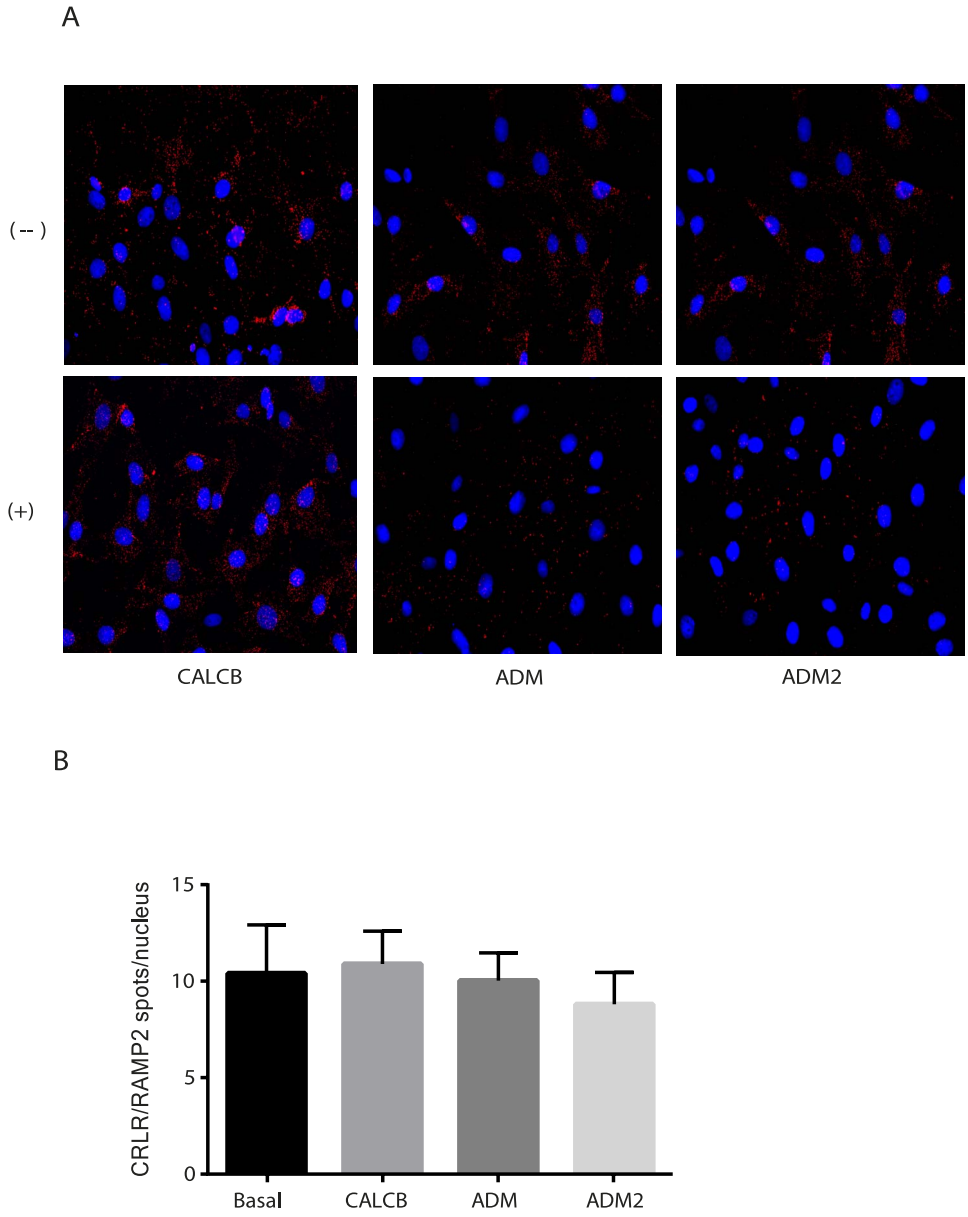


FIG. 3. The effect of CALCB, ADM, and ADM2 on basal CALCRL with RAMP1 associations on RUASM cells. **A**) Representative images of PLA for associations of CALCRL with RAMP1 on RUASM cells with or without CALCB, ADM, and ADM2 treatments (100 nM for 4 h). **B**) Comparison of the red spots per nucleus for CALCRL with RAMP1 on RUASM cells. Data shown are the means ± SEM of five randomly selected fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. No significant difference was observed between groups ($P > 0.05$).

± 5.7 red spots/nucleus in the TNFα-treated group vs. 156.8 ± 25.3 red spots/nucleus in TNFα + CALCB group, $P < 0.001$). However ADM (Fig. 5, 41.1 ± 6.8 red spots/nucleus in TNFα + ADM treated) and ADM2 (Fig. 6, 61.6 ± 6.1 red spots/nucleus in TNFα + ADM2 treated) did not display a profound effect on the CALCRL and RAMP1 associations in cells exposed to TNFα ($P > 0.05$).

The Time-Dependent Effect of CALCB on the Association of CALCRL with RAMP1 on RUASM Cells Exposed to TNFα

As shown in Figure 7, cells exposed to TNFα at 10 ng/ml for 4 h displayed a significant inhibition of the protein-protein interaction between CALCRL and RAMP1 when compared to the controls (Fig. 7, 113.6 ± 20.7 red spots/nucleus in control vs. 17.8 ± 5.7 red spots/nucleus in TNFα treated group; $P <$ 0.001). Treatments with CALCB (100 nM) for 1 h prior to the termination of 4-h incubation increased the TNFα-inhibited associations between CALCRL and RAMP1, whereas CALCB treatment for 2, 10, or 30 min failed to show any improvements. In the case of cotreatment of TNFα with CALCB for 4 h, the CALCB and RAMP1 associations were completely recovered at 4 h after CALCB treatment. Furthermore, as shown in Figure 8, when the RUASM cells were treated with TNFα (10 ng/ml) alone, the associations of CALCRL with RAMP1 were significantly reduced at 1 min and remained lower throughout 24 h of treatment. However, when cells were treated simultaneously with both TNFα (10 ng/ml) and CALCB (100 nM) for 1, 5, 10, and 30 min, and 1, 2, 4, and 24 h, the improvements in associations were observed as early as 5 min after treatment (11.1 ± 2.4 red spots/nucleus in TNFα only vs. 46.5 ± 7.4 red spots in TNFα + CALCB

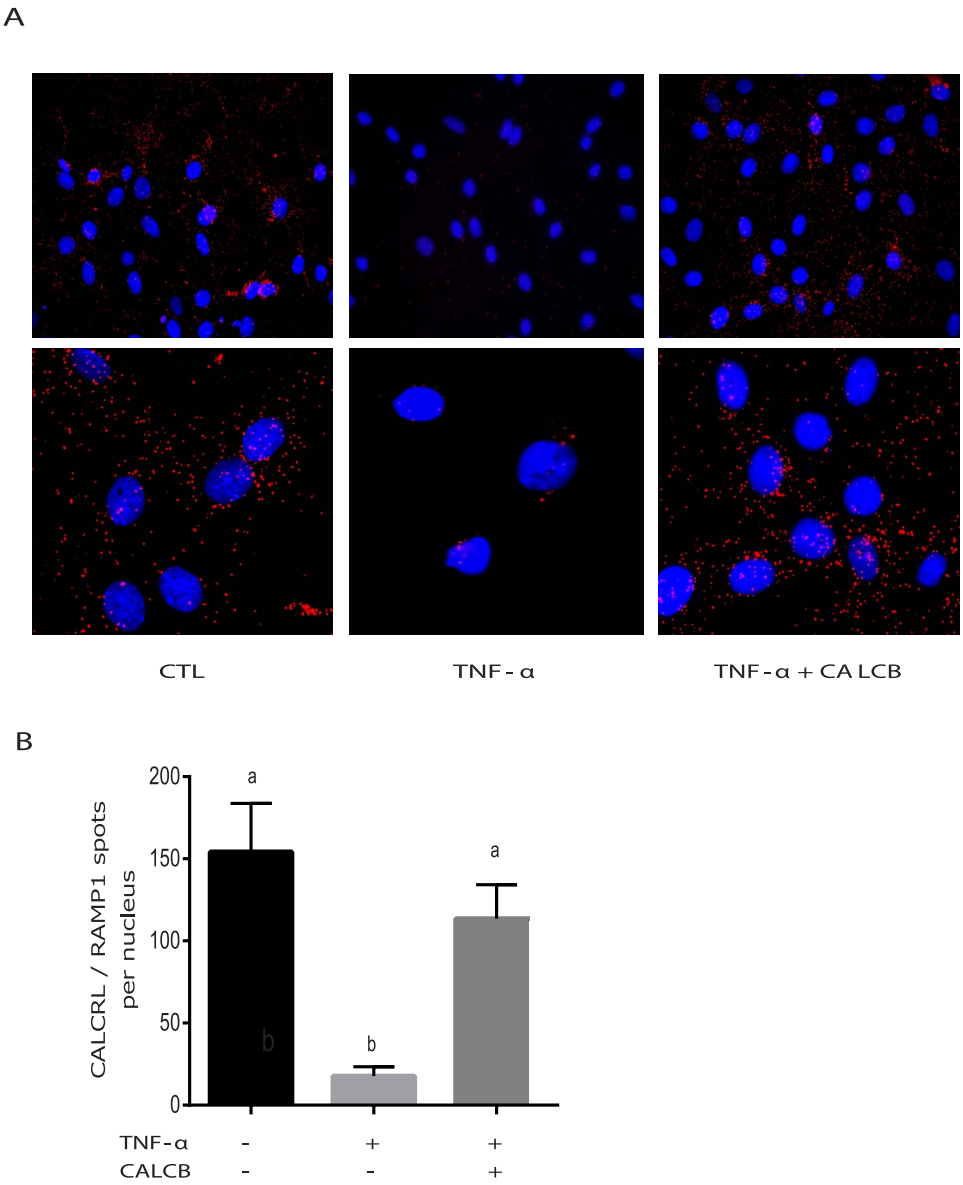


FIG. 4. The effect of CALCB on the associations of CALCRL with RAMP1 on RUASM cells exposed to TNFα. **A**) Representative images of PLA for associations of CALCRL with RAMP1 on RUASM cells with or without TNFα (10 ng/ml, 4 h) and CALCB treatments (100 nM for 4 h). Original magnification: ×100 for images in the top panel, ×400 for images in the bottom panel. **B**) Comparison of the red spots per nucleus on RUASM cells. Data shown are the means ± SEM of five randomly selected fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Different letters at the top of the bars indicate significant differences between groups ($P < 0.01$).

treatment; $P < 0.01$), and these associations were completely recovered at 1 h to the level of control cells and maintained thereafter up to 24 h.

The Underlying Mechanism of CALCB Actions on RUASM Cells

TNFα-induced reactions in CALCRL and RAMP1 associations were completely reversed by CALCB, and this reversal effect of CALCB was blocked by both Akt-signaling inhibitor, LY294002 (10 μM), and ERK-signaling inhibitor, PD98059 (10 μM) (Fig. 9). However, treatments with LY294002 alone, but not PD98059, significantly inhibited the basal CALCRL and RAMP1 associations (basal, 116.7 ± 14.6 red spots/nucleus; LY294002, 7.1 ± 1.1 red spots/nucleus; and PD98059, 81.29 ± 4.3 red spots/nucleus), suggesting that basal CALCRL and RAMP1 associations require an intact P13k/Akt signaling. Because PD98059 had no effects on the basal associations of CALCRL and RAMP1, but blocked the protective effect of CALCB on TNFα-induced inhibition of CALCRL/RAMP1 associations, we suggest that ERK, but not Akt signaling, is involved in the CALCB actions in rescuing TNFα-inhibited CALCRL and RAMP1 associations. In addition, CALCB (100 nM) induced phosphorylation of ERK1/2, but not Akt, in a time-dependent manner (Fig. 10).

DISCUSSION

The current study demonstrates that CALCRL predominantly associates with RAMP1 in RUASM cells forming CALCB receptors. The basal interaction of CALCRL with RAMP2 or RAMP3 is minimal, resulting in lower numbers of ADM- or ADM2-specific receptors. Treatment with CALCB, ADM, and ADM2 did not affect the basal associations of

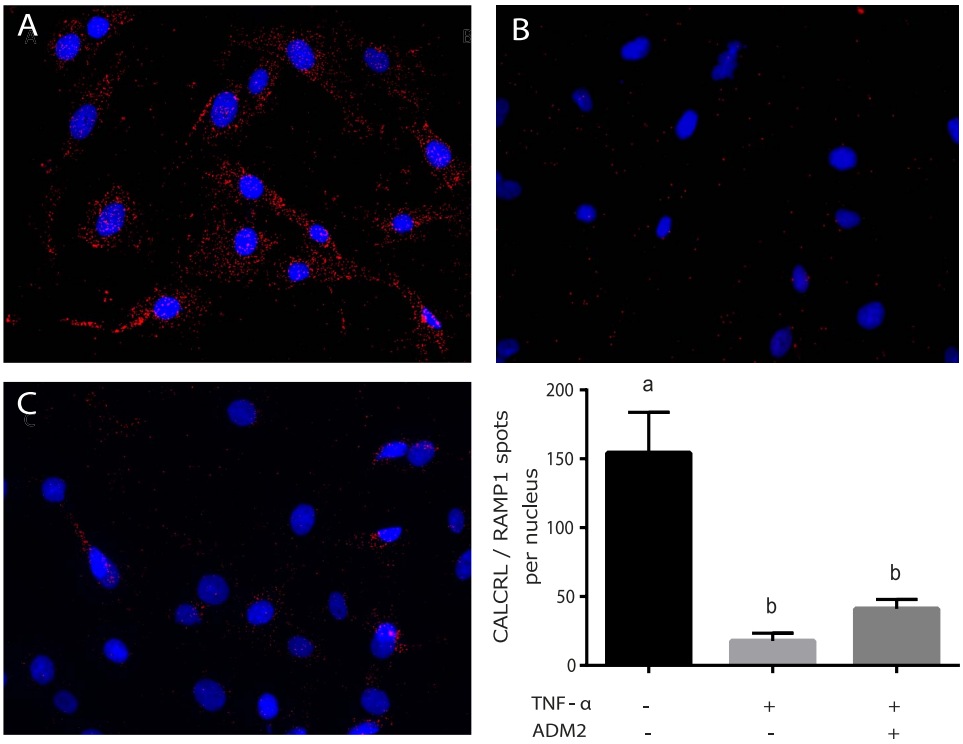


FIG. 5. The influence of ADM on CALCRL and RAMP1 associations on RUASM cells exposed to TNFα. Representative images of PLA for association of CALCRL with RAMP1 on RUASM cells in controls (**A**), those exposed to TNFα (10 ng/ml) (**B**), or those exposed to TNFα + ADM (100 nM) (**C**) for 4 h. Bar graph shows the comparison of the red spots per nucleus on RUASM cells. Data are the means ± SEM of five randomly selected fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Different letters at the top of the bars indicate significant differences between groups ($P < 0.01$).

CALCRL with any of the RAMPs in these cells. TNFα dramatically inhibited the associations of CALCRL with RAMP1, but no changes were observed in interaction of CALCRL with RAMP2 or RAMP3. Treatment with CALCB rescued TNFα-induced inhibition of CALCRL and RAMP1 interaction, whereas ADM and ADM2 were ineffective. Furthermore, ERK signaling appears to be involved in the CALCB effects in the rescue of CALCRL/RAMP association in the RUASM cells. Therefore, this study suggests that, among the group of CALCB family of peptides, CALCB plays a major role in enhancing uterine artery relaxation during pregnancy via its receptor complex, CALCRL/RAMP1, in RUASM cells. Furthermore, the rescue of TNFα-induced disruption of CALCB receptor complex by CALCB suggests a potential use for CALCB in developing therapeutic strategies for placental and fetal growth-related complications involving abnormal levels of TNFα, such as FGR and preeclampsia.

The CALCRL/RAMP1 Receptor Complex Is the Primary Functional CALCB Family Peptide Receptor in RUASM Cells

The current study shows that, in RUASM cells, CALCRL was primarily associated with RAMP1, which forms CALCB receptors, and very little with RAMP2 and RAMP3 (Fig. 1). This suggests that the receptors for CALCB are the predominant functional receptors in the uterine artery in pregnant rats. This supports our previous study showing that the majority of CALCB effects in the uterine artery are mediated through the direct effects of CALCB on vascular smooth muscle cells and that the lack of ADM and ADM2 effects on rat uterine artery are due, at least in part, to the minimal presence of associations between CALCRL and RAMP2 or -3. CALCB, ADM, and ADM2 are potent vasodilators that share the signaling mechanisms in smooth muscle and vascular endothelium [10]. The vascular relaxation effects of these peptides are greater during pregnancy compared to the nonpregnant state [21, 22]. Due to the structural similarities, CALCB, ADM, and ADM2 bind and activate the same GPCR CALCRL to elicit similar yet distinct physiological responses in a variety of cell types [10, 21, 24]. Among the three peptides, CALCB effects are primarily observed in smooth muscle cells that are antagonized by CALCB antagonist $CALCB_{8\text{-}37}$, whereas ADM is shown to exert its effects on both endothelium and smooth muscle cells, while the majority of ADM2 effects on the vasculature appear to be endothelium dependent [11, 14, 25]. Our previous study revealed that the relaxation effects of CALCB were significantly inhibited when uterine arteries were incubated with the antibody raised against Nt-CALCRL, and were totally abolished in the presence of antibodies for both Nt-CALCRL and Nt-RAMP1 [1]. These earlier studies further confirm our suggestion that CALCB effects in the uterine artery are mediated through the associations of CALCRL and RAMP1, and the interactions between CALCRL and RAMP1 mediate CALCB-induced uterine artery relaxation. In addition, our previous studies on rat uerine artery have also shown that both CALCB and ADM relax rat uterine artery in a dose-dependent manner, and the increased responsiveness during pregnancy is positively related with the mRNA and protein expressions of CALCRL, RAMP1, and RAMP3 [15, 22]. However, CALCB-induced uterine artery relaxation was not reduced by L-NAME, a nitric oxide inhibitor, suggesting that the endothelium and the release of nitric oxide in response to CALCB are not major contributors. In contrast, ADM-induced uterine artery relaxation was inhibited by denudation of endothelium and by L-NAME, suggesting that the endothelium and the release of

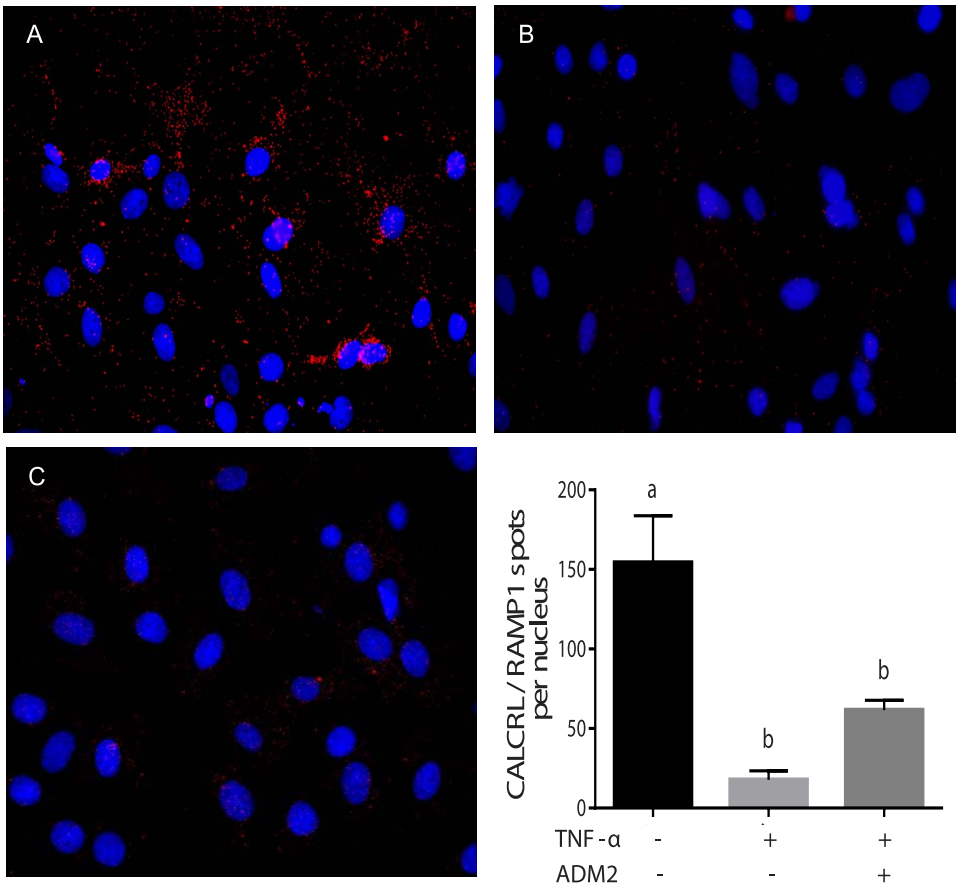


FIG. 6. The effect of ADM2 on CALCRL and RAMP1 associations on RUASM cells exposed to TNFα. Representative images of PLA for associations of CALCRL with RAMP1 on RUASM cells in control (**A**), those exposed to TNFα (10 ng/ml) (**B**), or those exposed to TNFα + ADM (100 nM) (**C**) for 4 h. Bar graph shows the comparison of the red spots per nucleus on RUASM cells. Data are the means ± SEM of five randomly selected fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Different letters at the top of the bars indicate significant differences between groups ($P < 0.01$).

nitric oxide in response to ADM are the major contributors in this setting. In a recent study, we also showed that all three peptides, CALCB, ADM, and ADM2, increase cAMP production in rat mesenteric artery smooth muscle cells, but with different potency [24, 26]. Although, all three peptides are functionally active in rat mesenteric artery, the uterine artery responds primarily to CALCB, where CALCB stimulates cAMP in RUASM cells in vitro and relaxes uterine artery ex vivo, whereas ADM and ADM2 fail to stimulate cAMP generation and endothelium-independent uterine artery relaxation [22]. However, the downstream molecular mechanisms involved in the action of these peptides in the uterine artery are not clear.

Since RAMPs determine the specificity of peptide binding and signaling through CALCRL, the type of RAMP present in close proximity to CALCRL determines the peptide specificity for the receptor complex. Interestingly, as shown in Figure 2, in the absence of RAMP1, the protein-protein interaction between CALCRL and RAMP3 is enhanced, whereas the basal association between CALCRL and RAMP2 is unaffected. Enhanced CARLCRL/RAMP3 complex formation in the absence of RAMP1 further supports our earlier study [25], and suggests that there exists a competitive association of RAMPs with CALCRL, where an absence of one isotype allows for increased association with the other.

TNFα Inhibits the Receptor Complex of CALCRL with RAMP1

Clinical studies have shown that circulating TNFα is elevated in pregnant women with preeclampsia and FGR [5, 6]. In addition, placental insufficiency is associated with higher serum levels of TNFα than those in the control group [27], suggesting that elevation in TNFα could be a specific phenomenon of certain subsets of FGR. Further evidence showed that treatment of pregnant baboons with low-dose TNFα causes vascular dysfunction, proteinuria, and increases in sFLT-1 [28], suggesting that increased concentrations of TNFα in the maternal and umbilical serum play a significant role in pathogenesis of preeclampsia. In addition, our previous studies have demonstrated that inadequate CALCB function in uteroplacental vasculature may have been involved in the pathogenesis of preeclampsia [6], but the influence of TNFα on the interactions of the receptor components for CALCB remain unknown. In this study, using a dose of TNFα that was previously utilized for vascular smooth muscle cells [29], we demonstrate significant inhibition of the association of CALCRL with RAMP1 on the RUASM cells (Fig. 4), with no effect on the association of CALCRL with either RAMP2 or RAMP3. Although the reported range for TNFα in the circulation of PE women was 150–650 pg/ml [30, 31], given that TNFα is of decidual and/or decidual macrophage origin [32] and placental origin [33, 34], local uteroplacental vascular levels may be higher than systemic circulating levels, and the

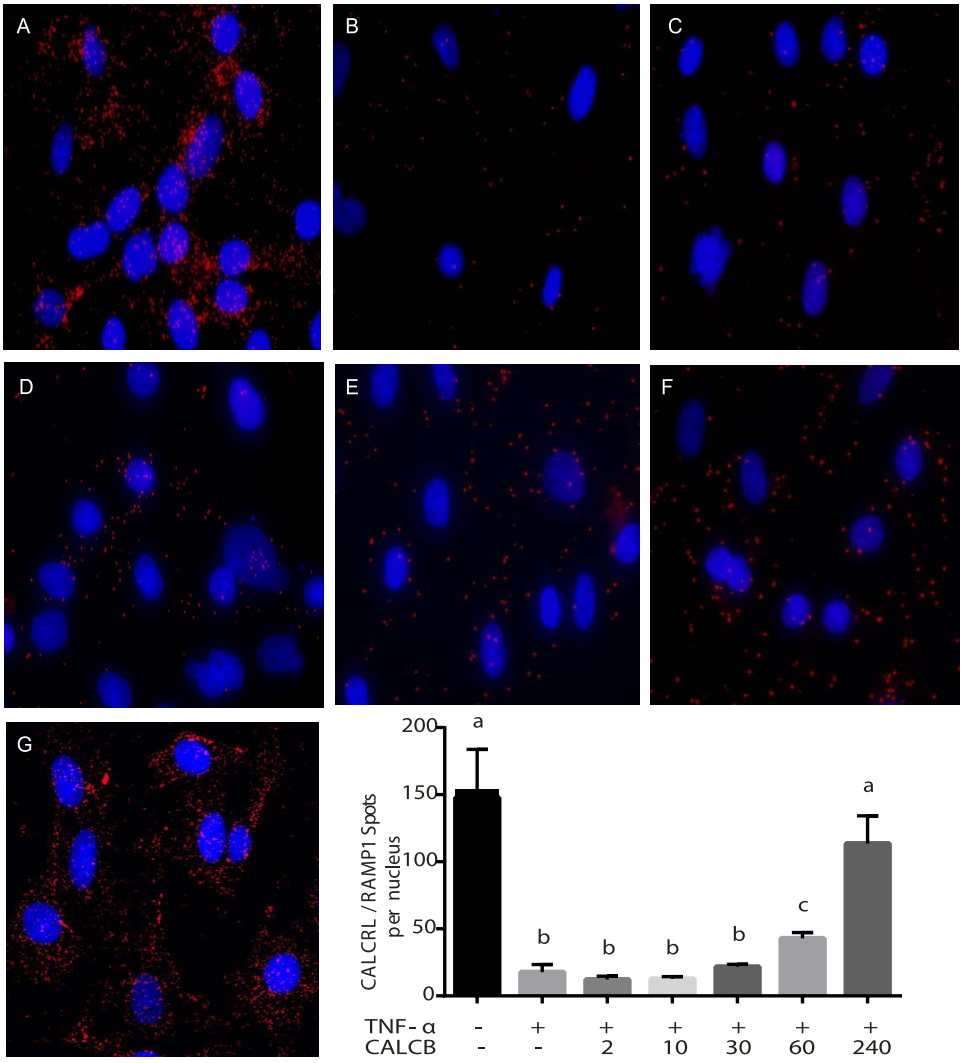


FIG. 7. Time-dependent rescue effects of CALCB on the associations of CALCRL with RAMP1 on RUASM cells exposed to TNFα for 4 h. The representative images of PLA for associations of CALCRL with RAMP1 on RUASM cells in controls (**A**), those exposed to TNFα (10 ng/ml) for 4 h (**B**), or those exposed to TNFα with addition of CALCB (100 nM) during the last 2 min (**C**), 10 min (**D**), 30 min (**E**), 1 h (**F**), or 4 h (**G**) prior to the termination of incubations at 4 h, respectively. Bar graph shows the comparison of the red spots per nucleus on RUASM cells, presented as mean ± SEM of five random fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Different letters at the top of the bars indicate significant differences between groups ($P < 0.01$).

dose we have used is appropriate for assessing the pharmacological effects in vitro. These results imply that TNFα could reduce the response of the uterine artery to CALCB through interrupting the associations of CALCRL with RAMP1, and thus may contribute to the insufficient uteroplacental blood flow seen in FGR and preeclampsia.

CALCB Rescues TNFα-Inhibited CALCRL and RAMP1 Associations

Our previous study in humans showed that both CALCRL and RAMP1 were expressed in fetoplacental vessels from normal pregnancies [35]. Furthermore, CALCRL produced a dose-dependent relaxation of serotonin-induced contraction of umbilical and chorionic arteries from normal pregnancies, but the response to CALCB was significantly attenuated in the vessels from preeclampsia. This suggests that CALCB-dependent vascular relaxation appears to be compromised in preeclamptic pregnancies, and inadequate CALCB-mediated vasodilator response may play a role in the pathophysiology of this pregnancy complication. However, it is unknown if supplementation of CALCB could be beneficial to the vasculature in the maternal-fetal unit that is compromised in these complications. Figure 7 shows that the cells exposed to TNFα at 10 ng/ml for 4 h significantly inhibited the association of CALCRL and RAMP1 compared to controls. Although CALCB supplementation (100 nM) for 2, 10, and 30 min was ineffective when the cells were pretreated with TNFα for 4 h, the associations were significantly increased at 1 h after the treatment with CALCB, and completely recovered 4 h after treatment, suggesting that CALCB is able to reverse the TNFα effects. Moreover, when the RUASM cells were treated simultaneously with both TNFα (10 ng/ml) and CALCB (100 nM) for 1, 5, 10, and 30 min, and 1, 2, 4, and 24 h, the improvements in associations were observed as early as 5 min after treatment, and the associations were completely restored to normal at 1 h, and remained so thereafter. These results suggest that CALCB is able to prevent TNFα-inhibited CALCRL and RAMP1 associations when given together with TNFα. In contrast, treatment with either ADM or ADM2 is

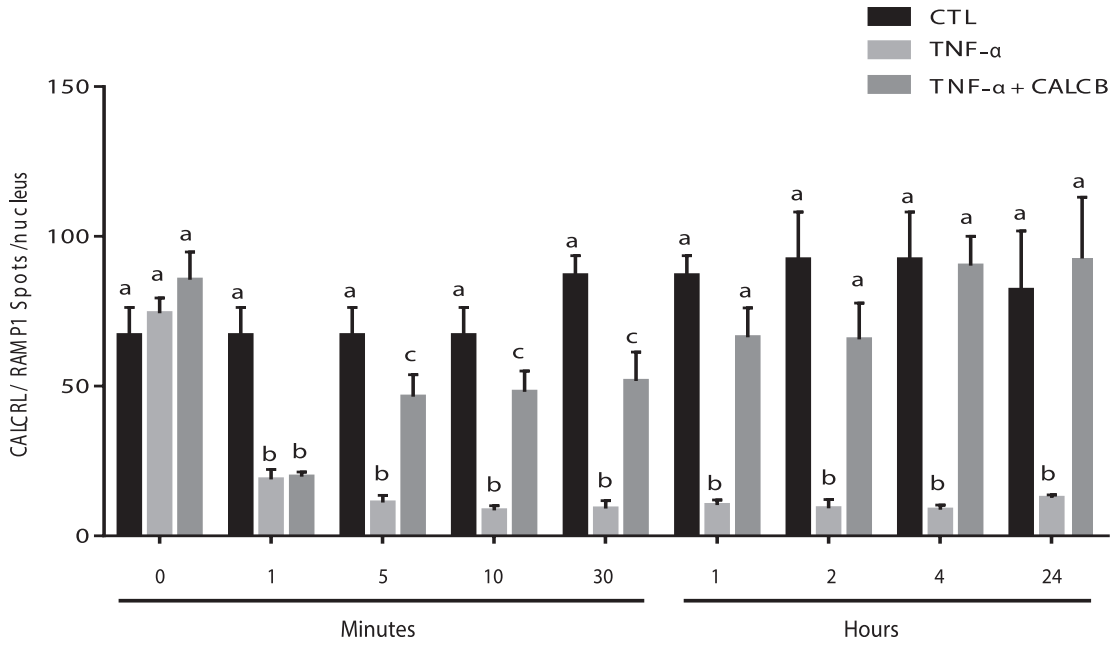


FIG. 8. Time-dependent effect of coincubation of CALCB with TNFα or TNFα alone for 24 h on the associations of CALCRL with RAMP1 on RUASM cells. RUASM cells treated with TNFα or simultaneously with both TNFα (10 ng/ml) and CALCB (100 nM) for 1, 5, 10, and 30 min, and 1, 2, 4, and 24 h, respectively. Association of CALCRL with RAMP1 on cells measured as red spots per nucleus in different groups are presented as mean ± SEM of five randomly selected fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Different letters at the top of bars indicate significant differences between groups ($P < 0.01$).

ineffective, perhaps due to the minimal association of CRLR with RAMP2 or RAMP3 in RUASM cells.

Underlying Mechanisms of CALCB Actions on RUASM Cells

The findings that CALCB acts as a potent dilator, and that pregnancy increases both the sensitivity to CALCB and the endogenous levels of CALCB, support the view that CALCB has a physiologic role in dilating the uterine vasculature, especially during pregnancy. To better understand the molecular mechanisms of CALCB actions on the uterine artery, a line of studies in humans and other animal species have been performed. In the rat uterine artery, the CALCB-induced relaxation was significantly attenuated by inhibitors of guanylate cyclase (ODQ), adenylate cyclase (SQ 22536), potassium channel blockers KATP (glybenclamide), and calcium-activated potassium (KCa) (tetraethylammonium) [22], suggesting the involvement of cGMP, cAMP, KATP, and KCa channels in the actions of CALCB on uterine artery relaxation. In the human uterine artery, CALCB produced a concentration-dependent relaxation of norepinephrine-induced contractions [36], and the relaxant effect of CALCB was inhibited by its receptor antagonist, $CALCB_{8-37}$, but was not affected by propranolol, indomethacin, methylene blue, or by the removal of the endothelium, indicating that the vasodilatory effect of CALCB in the human uterine artery is mediated by its receptors, and does not involve beta-adrenoceptors, vasodilator prostanoids, increased levels of guanosine 3′,5′-cyclic monophosphate, or endothelium-derived relaxing factor. However, because of the shared receptor system and existing overlap in the biological activities, and the specific receptor heterodimer that mediates the effects of CALCB in RUASM cells, the mechanisms of CALCB actions on rescuing TNFα-inhibited CALCRL and RAMP1 association are not known. It has been reported that the potential consequences of interaction of the RAMPs with the CALCRL include transport of the receptor, CALCRL, to the cell surface, modification of the receptor glycosylation, and direct and indirect modification of the ligand-binding site through association with the receptor at the cell surface [37]. In the present study, we showed that the inhibition of Akt signaling with LY294002 and the inhibition of ERK pathway with PD98059 significantly disrupts CALCB actions on rescuing TNFα-induced reduction in the associations of CALCRL with RAMP1 (Fig. 9). Furthermore, the basal CALCRL and RAMP1 associations were inhibited by Akt inhibitor LY294002 alone, but not by ERK inhibitor PD98059 alone, implying that Akt pathway mediates the basal interaction of CALCRL with RAMP1, but not TNFα-induced inhibition of CALRL and RAMP1 interactions. Although PD98059 is known to prevent stimulation of only new ERK activity, without affecting the basal ERK activity, absence of increases in ERK phosphorylation by CALCB in the presence or absence of TNFα suggests the involvement of ERK pathway (Figs. 9 and 10) in CALCB-mediated rescue. The majority of TNFα effects are mediated through two distinct receptors of the TNF receptor family—TNF-R1 and TNF-R2—and the pleotropic biological effects of TNFα can be attributed to its ability to simultaneously activate multiple signaling pathways in the cell [6]. Further studies are necessary to address the activation of precise signaling pathway targeted by TNFα to disrupt the cell surface association of CRLR and RAMP complexes. The current study demonstrates a major role for CALCB in the control of uterine artery relaxation via CALCRL and RAMP1, and suggests a potentially protective role for CALCB in rescuing impaired associations of CALCRL and RAMP1 in RUASM cells in pathologies involving TNFα. However, the precise mechanism underlying the actions of CALCB on the internalization, recycling, and resensitization of the CALCRL and RAMP1 warrant further investigation.

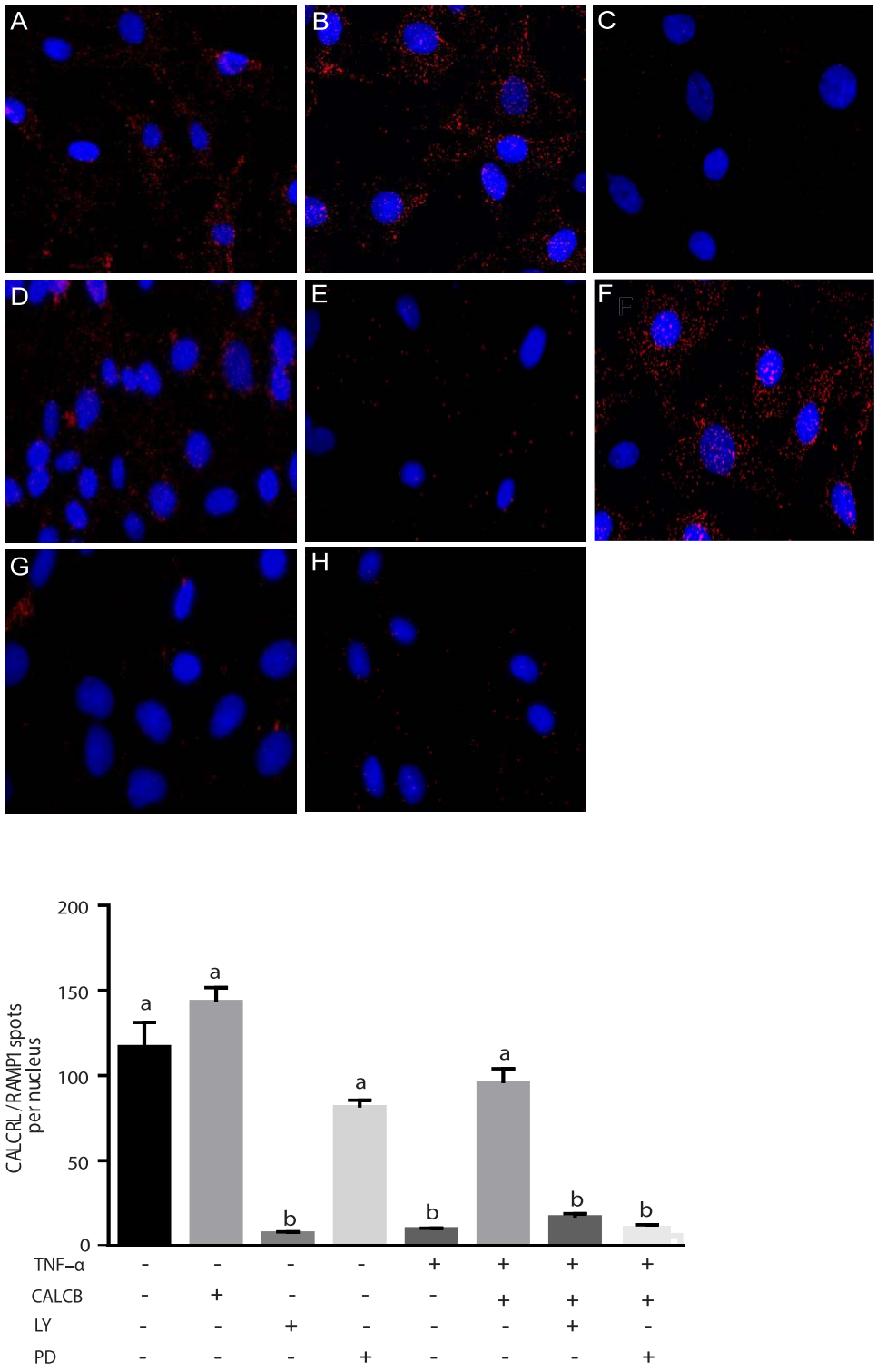


FIG. 9. Effect of LY294002 and PD98059 on the associations of CALCRL with RAMP1 on RUASM cells. Representative images of PLA for associations of CALCRL with RAMP1 on RUASM cells in control (**A**), in the presence of CALCB (100 nM, **B**), LY294002 (10 μM, **C**), PD98059 (10 μM, **D**), TNFα (10ng/ml, **E**), TNFα (10 nM) + CALCB (100 nM, **F**), TNFα + CALCB + LY294002 (**G**), or TNFα + CALCB + PD98059 (**H**) for 24 h. Bar graph shows comparison of the red spots per nucleus on RUASM cells between groups. Data are the means ± SEM of five random fields from three replicates. Statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison tests. Different letters at the top of the bars indicate significant differences between groups ($P < 0.01$).

In summary, the present study showed evidence of physical presence of complexes of all three RAMPs with CALCRL on RUASM cell surface in a competitive manner, with CALCRL + RAMP1 as the predominant complex. TNFα inhibits CALCRL association with RAMP1, indicating that CALCB-related mechanisms may be involved in TNFα-induced uterine artery dysfunction. Moreover, CALCB appears to rescue TNFα-induced dissociation of these receptor complexes, and

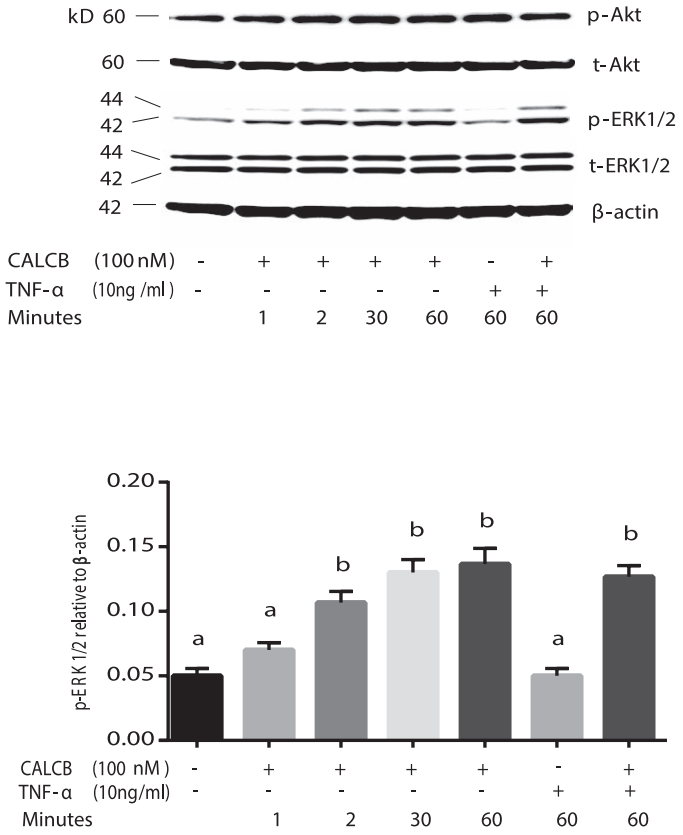


FIG. 10. Effect of CALCB on Akt and ERK1/2 pathway in RUASM cells. Representative Western blots for phosphor-Akt/t-Akt, phospho-ERK1/2/t-ERK1/2, and βactin in RUASM cells incubated with CALCB (100 nM) in the presence or absence of TNFα (10 ng/ml) for 24 h. The bar graph represents the mean ± SEM of the ratio of density of the phospho-ERK1/2 protein band to that of βactin (n = 3). *$P < 0.05$.

perhaps restore normal uterine artery relaxation and improve the TNFα-associated pathologies during pregnancy.

Perspective and Significance

A large body of evidence from both in vivo and in vitro studies suggests that GPCR CALCRL mediates important physiological or pathological functions of CALCB, ADM, and ADM2 in pregnant and nonpregnant vascular beds. Targeted blockade or activation of the peptide-specific CALCRL-RAMP-mediated signaling may provide novel approaches to treating vascular abnormalities involving these peptides. Due to the inherent complexity of the shared CALCRL-RAMP system for these peptides, the signaling mechanisms involved in their vascular functions are far from completely understood. Based on the data provided in this study, we anticipate an exciting future for the discovery of new drugs targeting CALCRL-RAMP complexes utilizing PLA technology for monitoring the functional CALCRL/RAMP dimerization and receptor/peptide interaction in the reproductive and vascular actions of CALCB family peptides.

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