

## Research Article

# Internalization and Transportation of Endothelial Cell Surface $K_{Ca}2.3$ and $K_{Ca}3.1$ in Normal Pregnancy and Preeclampsia

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Altered redox state modulates the expression levels of endothelial  $K_{Ca}2.3$  and  $K_{Ca}3.1$  ( $K_{Ca}s$ ) in normal pregnancy (NP) and preeclampsia (PE), thereby regulating vascular contractility. The mechanisms underlying  $K_{Ca}s$  endocytosis and transportation remain unknown. We investigated the regulation of  $K_{Ca}s$  expression in plasma membrane (PM) during NP and PE. Cultured human uterine artery endothelial cells were incubated in serum from normal nonpregnant women and women with NP or PE, or in oxidized LDL-, or lysophosphatidylcholine- (LPC-) containing a medium for 24 hours. NP serum elevated PM levels of  $K_{Ca}s$  and reduced caveolin-1 and clathrin levels. PE serum, oxidized LDL, or LPC reduced PM levels of  $K_{Ca}s$  and elevated caveolin-1, clathrin, Rab5c, and early endosome antigen-1 (EEA1) levels. Reduced  $K_{Ca}s$  levels by PE serum or LPC were reversed by inhibition of caveolin-1, clathrin, or EEA1. Catalase and glutathione peroxidase 1 (GPX1) knockdown elevated PM-localized  $K_{Ca}s$  levels and reduced caveolin-1 and clathrin levels. Elevated  $K_{Ca}2.3$  levels upon catalase and GPX1 knockdown were reversed by PEG-catalase treatment. An  $H_2O_2$  donor reduced clathrin and Rab5c. In contrast, elevated clathrin, caveolin-1, or colocalization of caveolin-1 with  $K_{Ca}3.1$  by PE serum or LPC was reversed by NADPH oxidase inhibitors or antioxidants. A superoxide donor xanthine+xanthine oxidase elevated caveolin-1 or Rab5c levels. We concluded that  $K_{Ca}s$  are endocytosed in a caveola- or a clathrin-dependent manner and transported in a Rab5c- and EEA1-dependent manner during pregnancy. The endocytosis and transportation processes may slow down via  $H_2O_2$ -mediated pathways in NP and may be accelerated via superoxide-mediated pathways in PE.

## 1. Introduction

$K_{Ca}2.3$  and  $K_{Ca}3.1$  play an important role in endothelial control of vascular contractility. Activation of these  $K^+$  channels induces  $K^+$  efflux and endothelial hyperpolarization, which hyperpolarizes vascular smooth muscle cells (VSMCs) by activating inward-rectifier  $K^+$  channels and spreading to VSMCs through gap junctions, respectively [1–3]. In addition, endothelial hyperpolarization enhances  $Ca^{2+}$  entry through  $Ca^{2+}$ -permeable channels such as transient receptor potential channels by increasing its electrical driving force and elevates intracellular  $Ca^{2+}$  levels [4], which stimulates nitric oxide (NO) production in endothelial cells (ECs) [5]. NO and

VSM hyperpolarization relax blood vessels, thereby controlling vascular contractility. The contribution of NO and VSM hyperpolarization to the control of vascular contractility might vary between conduit arteries and resistant arteries. The contribution of NO was most prominent in the aorta, whereas that of VSM hyperpolarization was most prominent in the distal mesenteric arteries, suggesting that VSM hyperpolarization plays a more important role in the control of vasorelaxation in resistant arteries than in conduit arteries [6–9]. Since resistant arteries are the main regulators of systemic vascular resistance, endothelial  $K_{Ca}2.3$  and  $K_{Ca}3.1$  might play an important role in the regulation of blood pressure.

TABLE 1: Blood pressure levels of subject groups.

Group	Normal nonpregnancy ( $n = 8$ )	Normal pregnancy ( $n = 15$ )	Preeclamptic pregnancy ( $n = 12$ )
Systolic blood pressure (mmHg)	115.0 $\pm$ 1.0	117.7 $\pm$ 1.7	153.0 $\pm$ 3.2
Diastolic blood pressure (mmHg)	74.0 $\pm$ 1.7	77.4 $\pm$ 2.1	97.7 $\pm$ 2.9

Values shown are mean  $\pm$  SEM and exclusively composed of plasma donors.

Since endothelial hyperpolarization is caused by  $K_{Ca2.3}$  and  $K_{Ca3.1}$  activation, the magnitude of endothelial hyperpolarization may be affected by the expression levels of these  $K^+$  channels in plasma membrane (PM). Thus,  $K_{Ca2.3}$  and  $K_{Ca3.1}$  upregulation might increase endothelial hyperpolarization, thereby potentiating L-NAME-resistant,  $K_{Ca2.3}$  and  $K_{Ca3.1}$  activation-induced endothelium-dependent relaxation, as shown in blood vessels during aging process and normal pregnancy (NP) [10, 11]. On the contrary, downregulation of  $K_{Ca2.3}$  and  $K_{Ca3.1}$  activity and expression causes endothelial dysfunction, thereby developing vascular diseases, such as preeclampsia (PE) and Fabry disease [10, 12–14].

$K_{Ca2.3}$  and  $K_{Ca3.1}$  levels are affected by various stimuli, such as sphingolipids and redox state.  $K_{Ca3.1}$  upregulation was caused by an altered sphingolipid profile via a  $H_2O_2$ /Fyn-mediated pathway during the aging process, whereas globotriaosylceramide downregulated  $K_{Ca3.1}$  in Fabry disease [14].  $K_{Ca2.3}$  and  $K_{Ca3.1}$  levels were regulated by the altered redox state in pregnancy [10].  $K_{Ca2.3}$  and  $K_{Ca3.1}$  were upregulated by soluble serum factors, such as vascular endothelial growth factor (VEGF) in NP.  $H_2O_2$  plays an important role in  $K_{Ca2.3}$  and  $K_{Ca3.1}$  upregulation during aging [11] and NP [10]. On the other hand,  $K_{Ca2.3}$  and  $K_{Ca3.1}$  were downregulated by serum factors, such as progesterone and oxidized low-density lipoprotein (LDL) through superoxide generation in PE [10].

Our previous study explains how membrane levels of  $K_{Ca3.1}$  are reduced in Fabry disease. Clathrin-dependent internalization, Rab5c, early endosome antigen-1- (EEA1-) dependent transportation, and lysosomal degradation were involved in globotriaosylceramide-induced  $K_{Ca3.1}$  downregulation in Fabry disease [12]. However, little is known about how membrane levels of  $K_{Ca2.3}$  and  $K_{Ca3.1}$  are regulated in NP and PE. Thus, we investigated the regulation of  $K_{Ca2.3}$  and  $K_{Ca3.1}$  expression in PM during NP and PE and found that endothelial  $K_{Ca2.3}$  and  $K_{Ca3.1}$  are internalized from PM via caveola- or clathrin-dependent pathways. Rab5c and early EEA1 were involved in transportation of the internalized  $K^+$  channel proteins. Moreover,  $K_{Ca2.3}$  and  $K_{Ca3.1}$  internalization and transportation processes were delayed in NP and facilitated in PE.

## 2. Materials and Methods

Studies involving human subjects were approved by the local ethics committee, the Institutional Review Board of the Ewha Womans University Mokdong Hospital, and Korea University Guro Hospital and were conducted in accordance with the Declaration of Helsinki. All patients provided their written informed consent prior to the inclusion in this study. Experiments with mice were approved by the local ethics committee and the Institutional Review Board of the Ewha

Womans University Mokdong Hospital and were conducted in accordance with the Declaration of Helsinki, the Animal Care Guidelines of the Ewha Womans University, Medical School, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**2.1. Human Subjects.** The study population consisted of Asian women who were not pregnant or had either a NP or PE (Table 1). Pregnancies were considered normal when patients did not have medical and obstetric complications of pregnancy and delivered a newborn at a gestational age of 37–42 weeks. Preeclampsia was defined by a systolic blood pressure over 140 mmHg and a diastolic blood pressure over 90 mmHg after 20 weeks of gestation in a previously normotensive woman, and the onset of proteinuria exceeding 300 mg of protein during 24 hours of urine collection. Nonpregnant women were healthy premenopausal volunteers taking no medications. Preeclamptic patients and normal pregnant women were matched for age ( $\pm 3$  years) and gestational age ( $\pm 2$  weeks), and nonpregnant healthy female volunteers were matched for age ( $\pm 3$  years). Blood samples were obtained from subjects during the third trimester of pregnancy. The study population was monitored at the Department of Obstetrics and Gynecology from the first trimester until their pregnancy was completed without complications. Exclusion criteria included the following: altered renal function, diabetes or chronic diseases, twin pregnancies, recurrent miscarriages, fetal growth retardation, and *abruptio* placenta. Smokers and women with a history of essential hypertension were also excluded from this study. Gestational age was defined as the interval between the first day of the mother's last menstrual period and the date of delivery.

**2.2. Animals and Tissue Collection.** We studied young C57BL/6 wild-type mice (about 20-week-old;  $n = 24$ ), and catalase/glutathione peroxidase 1 (GPX1) double knockout (catalase<sup>-/-</sup>/GPX1<sup>-/-</sup>) mice (about 20-week-old;  $n = 18$ ), generously donated by Dr. Ye-Shih Ho (Wayne State Medical School, Detroit, MI) [15]. Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and sacrificed by cervical dislocation.

**2.3. Cell Culture and Serum Treatment.** Human uterine microvascular ECs (HUtMECs), which were purchased from PromoCell GmbH (Heidelberg, Germany), were maintained in EC Growth Medium MV2 (PromoCell GmbH). For serum treatment, HUtMECs were plated in 6-well plates for 24 hours. The concentration of fetal bovine serum in a culture medium was gradually decreased from 10% to 5, 2, and 0% over 30 minutes, and HUtMECs were incubated in a serum-free medium for 30 minutes. After that, a culture

medium was substituted with serum from normal nonpregnancy (NNP) women or women with NP or PE, and the cells were incubated for 24 hours.

Mouse aortic endothelial cells (MAECs) were isolated from the mouse aortas as described [16]. Briefly, periadventitial fats and connective tissues around the aorta were carefully cleaned in  $\text{Ca}^{2+}$ -free phosphate-buffered saline under a dissecting microscope. Matrigel (BD Biosciences, San Jose, CA) was plated and polymerized at  $37^{\circ}\text{C}$  for 30 minutes. After that, aorta pieces were placed with the intima side down on the Matrigel. To demonstrate the endothelial nature of the cell, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Biomedical Technologies Inc., Stoughton, MA) uptake assay was employed. MAECs were used within 2 passages and not above 3 passages.

**2.4. Immunoblotting and Immunoprecipitation.** For immunoblotting, cell lysates were used to examine the protein level. After proper processing of each type of sample, total protein was measured using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). The same amount of total protein was analyzed using SDS-PAGE on 7.5–12% gels and transferred to nitrocellulose membrane (Invitrogen, Eugene, OR). Membranes were blocked for 1 hour in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 and incubated overnight at  $4^{\circ}\text{C}$  with primary antibodies (Abs) diluted in blocking buffer. Membranes were then washed three times with Tris-buffered saline with 0.1% Tween-20 and incubated for 1 hour with horseradish peroxidase-conjugated secondary Abs diluted in blocking buffer. The immunoblots were visualized by chemiluminescence reagents bought from GE Healthcare (Piscataway, NJ). Data processing was performed using a luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan) and IMAGE GAUSE software.

For immunoprecipitation, cells were washed twice with phosphate buffer saline and lysed in lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at  $4^{\circ}\text{C}$  for 1 hour. The lysate samples were prepared by centrifugation at  $12000 \times g$  for 30 minutes to eliminate the cell debris. Total protein concentration was estimated as described above. The lysate samples were precleared with a nonspecific IgG Ab. Total  $30 \mu\text{L}$  of 50% protein G-coupled dynabead slurry (Invitrogen) was added to an Ab ( $1\text{--}5 \mu\text{g}$ ) diluted in  $200 \mu\text{L}$  phosphate buffer saline with Tween 20, incubated for 15 minutes at room temperature (about  $20^{\circ}\text{C}$ ) with rotating, and washed. Precleared lysate samples were incubated with the dynabeads-Ab by rotating at  $4^{\circ}\text{C}$  overnight or at room temperature for 30 minutes. Following that, the dynabeads-antigen-Ab complexes were washed three times with the lysis buffer, and the antigen was eluted in 2x SDS-PAGE sample buffer by heating at  $70^{\circ}\text{C}$  for 10 minutes. Immunoprecipitates were separated using 7.5–12% SDS-PAGE and analyzed by immunoblotting.

**2.5. Biotinylation of Cell Surface Protein.** Cells were washed twice with phosphate buffer saline and labeled with 1 mM sulfo-succinimidyl-2-(biotinamido)ethyl-1,

3-dithiopropionate (EZ-Link-sulfo-NHS-SS-biotin; Pierce Biotechnology) in labeling buffer ( $150 \text{ mM NaCl}$ ,  $20 \text{ mM HEPES}$ ,  $3 \text{ mM CaCl}_2$ , and  $1 \text{ mM MgCl}_2$ ) for 30 minutes to 1 hour at room temperature. After the cells were washed, any nonreacted biotinylation reagent was quenched with  $100 \text{ mM glycine}$ , the cells were lysed in NP40 lysis buffer, and the proteins were incubated with  $30 \mu\text{L}$  of dynabead M-280 streptavidin (Invitrogen) for 3 hours at  $4^{\circ}\text{C}$  with rotation and then washed three times with lysis buffer. The proteins were eluted from streptavidin bead in 2x SDS-PAGE sample buffer by heating at  $65^{\circ}\text{C}$  for 5 minutes. Supernatant was subjected to 7.5–12% SDS-PAGE and analyzed by immunoblotting.

**2.6. siRNA Transfection.** Negative control siRNAs (SN-1012) and siRNAs against EEA1 (SDH-1001) were purchased from Bioneer (Daejeon, Korea). Negative control siRNAs (sc-36869) and siRNAs against NADPH oxidase 4 (NOX4; sc-41586) were purchased from Santa Cruz Biotechnology. ECs were transiently transfected with the siRNAs using an siRNA transfection reagent (Santa Cruz Biotechnology) according to the procedure suggested by the manufacturer. Cell lysates were prepared 24 hours after transfection, and immunoblotting was performed using anti-EEA1 Abs.

**2.7. Electrophysiology.** The patch-clamp technique was used in whole-cell configurations at  $20\text{--}22^{\circ}\text{C}$ . Whole-cell currents were measured using ruptured patches and monitored in voltage-clamp modes with an EPC-9 (HEKA Elektronik, Lambrecht, Germany). The holding potential was  $0 \text{ mV}$ , and currents were monitored by the repetitive application of voltage ramps from  $-100$  to  $+100 \text{ mV}$  with a 10-second interval (sampling interval  $0.5$  milliseconds,  $650$  millisecond duration). The standard external solution contained (in mM)  $150 \text{ NaCl}$ ,  $6 \text{ KCl}$ ,  $1.5 \text{ CaCl}_2$ ,  $1 \text{ MgCl}_2$ ,  $10 \text{ HEPES}$ , and  $10 \text{ glucose}$ , pH adjusted to  $7.4$  with  $\text{NaOH}$ . The pipette solution for whole-cell recording contained (in mM)  $40 \text{ KCl}$ ,  $100 \text{ K-aspartate}$ ,  $2 \text{ MgCl}_2$ ,  $0.1 \text{ EGTA}$ ,  $4 \text{ Na}_2\text{ATP}$ , and  $10 \text{ HEPES}$ , pH adjusted to  $7.2$  with  $\text{KOH}$ . To buffer free  $\text{Ca}^{2+}$ , the appropriate amount of  $\text{Ca}^{2+}$  (calculated using CaBuf software; G. Droogmans, Leuven, Belgium) was added in the presence of  $5 \text{ mM EGTA}$ .

$\text{K}_{\text{Ca}3.1}$  currents were activated by loading  $1 \mu\text{M Ca}^{2+}$  via a patch pipette in whole-cell clamped MAECs.  $\text{K}_{\text{Ca}3.1}$  current was normalized to cell capacitance, and the selective  $\text{K}_{\text{Ca}3.1}$  blocker TRAM-34-sensitive current was measured as the  $\text{K}_{\text{Ca}3.1}$  current.

**2.8. Reagents.** Reagents were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in sterilized distilled water unless indicated otherwise. The cells were treated with xanthine+xanthine oxidase (X/XO), tert-butylhydroperoxide (TBHP), or oxidized LDL (Intracel Inc., Frederick, MD) or the major component of oxidized LDL, lysophosphatidylcholine (LPC; dissolved in chloroform:methanol, 2:1) for 24 hours. Abs specific to lectin-like oxidized LDL receptor 1 (LOX1; ab81709, Abcam, Cambridge, MA), chlorpromazine (CPZ; C8138), or methyl- $\beta$ -cyclodextrin ( $\text{M}\beta\text{CD}$ ; C4555) were pretreated for 1 hour. Abs against  $\text{K}_{\text{Ca}2.3}$  (sc-28621),

K<sub>Ca</sub>3.1 (sc-32949), GAPDH (sc-25778), and  $\beta$ -actin (sc-130656) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

The final concentration of DMSO, chloroform, or methanol in media was less than 0.1%, and these solvents did not have any effect on the experiments tested in this study (data not shown).

**2.9. Statistics.** Data represent mean  $\pm$  SEM. To prove the statistical significance between groups, one-way ANOVA with Bonferroni's post hoc or 2-tailed Student's *t*-test was used. A *P* value of 0.05 or lower was considered statistically significant. Calculations were performed with SPSS 14.0 for Windows (SPSS, Chicago, IL).

### 3. Results

**3.1. Endothelial Membrane Levels of K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 Are Altered during Pregnancy.** We compared the effects of soluble serum factors from NNP and NP women on PM-localized K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 levels by incubating HUtMECs in serum from NNP and NP women for 24 hours. K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 proteins were biotinylated at the cell surface and labeled with horseradish peroxidase-conjugated streptavidin. The levels of PM-localized K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 were significantly higher in ECs treated with NP serum than in ECs treated with NNP serum (Figure 1(a)). We then compared the effects of serum from NP and PE women on PM levels of K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 in HUtMECs. The levels of PM-localized K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 proteins were significantly lower in ECs treated with PE serum than in ECs treated with NP serum (Figure 2(b)). In addition, we compared the effect of serum from NNP, NP, and PE women on PM levels of K<sub>Ca</sub>2.3 (Figure 1(c)) or K<sub>Ca</sub>3.1 (Figure 1(d)) in HUtMECs. The levels of PM-localized K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 were not changed in ECs treated with NNP serum, compared to those treated with normal culture medium (CM), and were elevated in ECs treated with NP serum, compared to those treated with NNP serum or CM. Increases in the K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 levels were significantly reduced in ECs treated with PE serum than in ECs treated with NP serum. Since oxidized LDL is among the causative factors to induce endothelial dysfunction in PE, we examined the effects of oxidized LDL on PM levels of K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 by incubating HUtMECs in oxidized LDL containing a culture medium for 24 hours. We found that the levels of PM-localized K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 were significantly reduced upon incubation (Figure 1(e)). Reduced K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 levels by oxidized LDL treatment were reversed by blocking the oxidized LDL receptors using an anti-LOX1 Ab (Figure 1(e)). These results suggest that the expression and localization of K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 in PM are elevated in NP compared to that in NNP, and the elevation was attenuated in PE.

**3.2. Caveolae and Clathrin Are Involved in the Internalization of K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1.** The levels of PM-localized proteins, such as ion channels, can be modulated by caveola-dependent internalization. Caveolins, the essential structural elements of caveolae, are suggested to be scaffolding proteins

that facilitate the compartmentalization of various signaling molecules or proteins within caveolae. We thus investigated whether caveola-dependent internalization of K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 occurs during pregnancy by examining Cav-1 levels. Cav-1 levels were markedly lower in the ECs treated with NP serum than in ECs treated with NNP serum, and VEGF receptor (VEGFR) inhibition using an anti-VEGFR1 Ab or anti-VEGFR2 Ab enhanced Cav-1 levels in ECs treated with NP serum (Figure 2(a)), indicating that NP serum decreases Cav-1 levels via VEGFR activation. In contrast, Cav-1 levels were markedly higher in ECs treated with PE serum than in ECs treated with NP serum (Figure 2(b)). In addition, LPC, the major component of oxidized LDL, enhanced Cav-1 levels in a concentration-dependent manner (Figure 2(b)). To confirm the presence of Cav-1 in the inner leaflet of the PM, the cell surface was biotinylated with a membrane impermeable agent (NHS-SS-biotin). Biotinylated PM proteins from whole cell lysates were isolated on a streptavidin column and, following elution and SDS-PAGE, were blotted for Cav-1 with anti-Cav-1 Ab. PM localization of Cav-1 was significantly elevated upon treatment with oxidized LDL (Figure 2(c)). We then examined whether caveolae are involved in the regulation of the levels of K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1. Colocalization of K<sub>Ca</sub>3.1 with Cav-1 was examined using coimmunoprecipitation. Colocalization of K<sub>Ca</sub>3.1 with Cav-1 was markedly higher in ECs treated with PE serum than in ECs treated with NP serum (Figure 2(d)). Cav-1 inhibition using the Cav-1 inhibitor M $\beta$ CD elevated K<sub>Ca</sub>2.3 (Figure 2(e)) or K<sub>Ca</sub>3.1 (Figure 2(f)) levels in ECs treated with PE serum or LPC. These results suggested that caveola-dependent internalization is involved in regulating the PM localization of K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 during pregnancy. Caveola-dependent internalization process might be delayed in NP, whereas it might be facilitated in PE.

Previously, we reported that K<sub>Ca</sub>3.1 proteins on the PM are internalized via a clathrin-dependent process in Fabry disease [12]. We, therefore, examined whether clathrin-dependent internalization is involved in regulating PM localization of K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 during pregnancy. Clathrin levels were not altered in ECs treated with NNP or NP serum (Figure 3(a)). In contrast, clathrin levels were markedly higher in the ECs treated with PE serum than in ECs treated with the NP serum (Figure 3(b)). Then, clathrin protein was biotinylated at the cell surface. PM clathrin levels were significantly higher in ECs treated with PE serum than in ECs treated with NP serum (Figure 3(c)). Colocalization of K<sub>Ca</sub>3.1 with clathrin was markedly enhanced in ECs treated with PE serum than in ECs treated with NP serum, and LPC markedly increased colocalization of K<sub>Ca</sub>3.1 with clathrin (Figure 3(d)). Furthermore, the clathrin inhibitor chlorpromazine enhanced K<sub>Ca</sub>2.3 (Figure 3(e)) and K<sub>Ca</sub>3.1 levels (Figure 3(f)) in ECs treated with PE serum or LPC. These results suggest that K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 are internalized via a clathrin-dependent process. Clathrin-dependent internalization process might not be affected in NP, whereas it might be facilitated in PE.

**3.3. A Rab5c- and EEA1-Dependent Process Mediates K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 Degradation.** The small GTPases Rab5 and EEA1



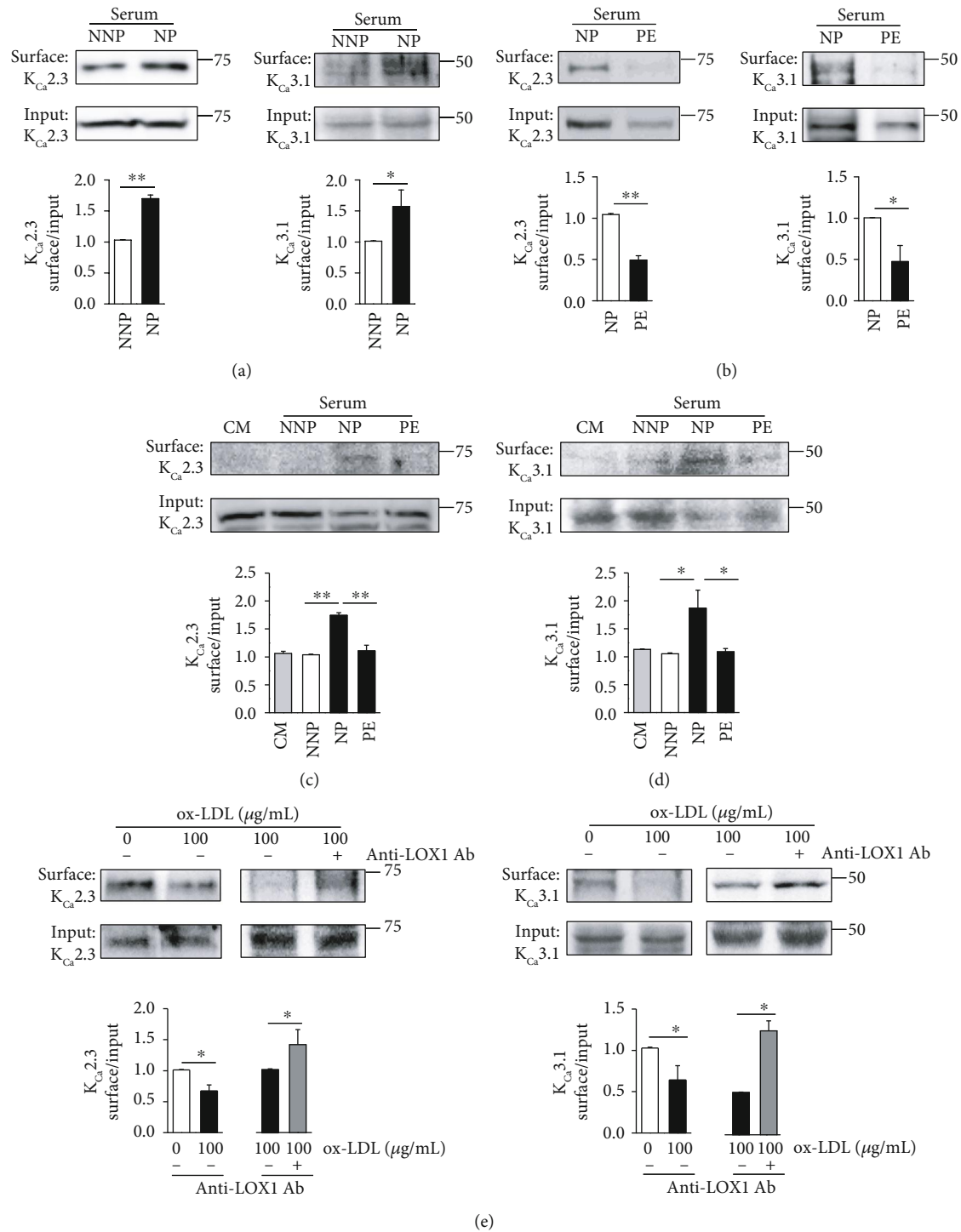
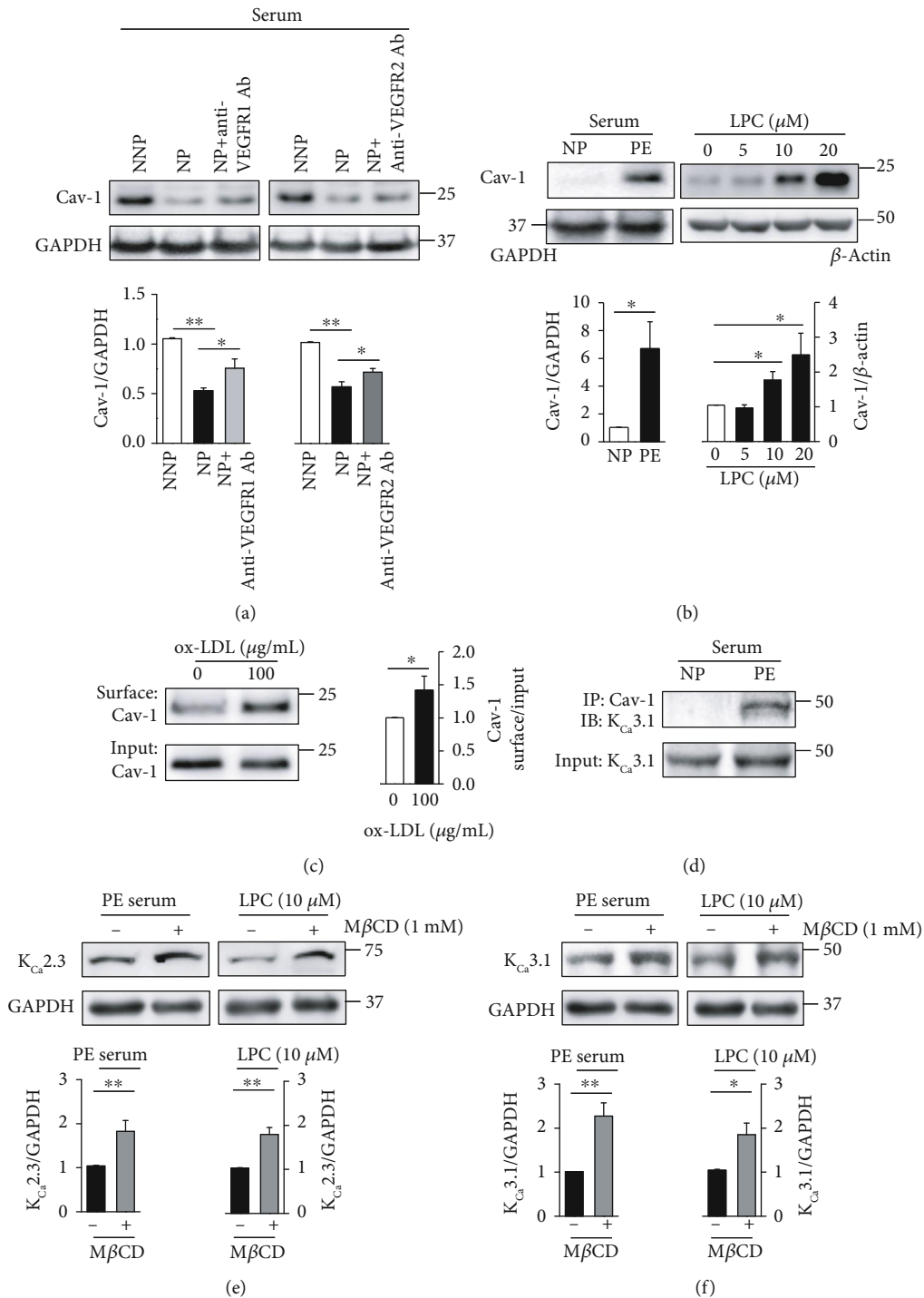


FIGURE 1: Membrane levels of  $K_{Ca}2.3$  and  $K_{Ca}3.1$  are altered in NP and PE. (a) PM levels of  $K_{Ca}2.3$  and  $K_{Ca}3.1$  in ECs treated with NNP or NP serum. (b) PM levels of  $K_{Ca}2.3$  and  $K_{Ca}3.1$  in ECs treated with NP or PE serum. (c, d) PM levels of  $K_{Ca}2.3$  (c) and  $K_{Ca}3.1$  (d) in ECs treated with CM, NNP serum, NP serum, or PE serum. (e) PM levels of  $K_{Ca}2.3$  or  $K_{Ca}3.1$  in ECs treated with oxidized LDL. Reduced  $K_{Ca}2.3$  or  $K_{Ca}3.1$  levels were reversed by blocking oxidized LDL receptor using an anti-LOX1 Ab. Blots shown are representative of the three to five experiments performed with three to five different cultures. Bar graphs represent the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

play a rate-limiting role in membrane docking or fusion in the early endocytic pathway [17, 18], and our previous study suggested that  $K_{Ca}3.1$  is transported into early endosomes via a Rab5c- and EEA1-dependent process in Fabry disease [12]. We thus examined whether a Rab5c- and EEA1-dependent

process mediates  $K_{Ca}2.3$  and  $K_{Ca}3.1$  transportation during pregnancy. Compared to ECs treated with NNP serum, levels of EEA1 (Figure 4(a)) or Rab5c (Figure 4(b)) were slightly decreased in the ECs treated with NP serum. However, significant difference was not found in ECs treated with NNP



**FIGURE 2:** Cav-1 is necessary for  $K_{Ca}2.3$  and  $K_{Ca}3.1$  internalization from the PM. (a) Cav-1 levels were reduced in ECs treated with NP serum, which were reversed by VEGFR inhibition using anti-VEGFR1 and anti-VEGFR2 Abs. (b) Cav-1 levels in ECs treated with NP serum or PE serum, and in ECs treated with LPC. LPC enhanced Cav-1 levels in ECs in a concentration-dependent manner. (c) PM localization of Cav-1 was increased by oxidized LDL. (d) Coimmunoprecipitation showing the interaction between Cav-1 and  $K_{Ca}3.1$  in ECs treated with NP or PE serum. Input is lysate without primary Ab. (e, f) Treatment with the Cav-1 inhibitor  $M\beta CD$  (1 mM) recovered  $K_{Ca}2.3$  (e) or  $K_{Ca}3.1$  (f) levels in ECs treated with PE serum or LPC. Blots shown are representative of three to six experiments performed with three to six different cultures. Bar graphs represent the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

or NP serum. Then, NP or PE serum was diluted with the culture medium. Compared to the diluted NP serum, the diluted PE serum (10%, 25%, and 100% serum) reduced  $K_{Ca}3.1$  levels

and increased Rab5c and EEA1 levels in a concentration-dependent manner (Figure 4(c)), indicating that  $K_{Ca}3.1$  levels are inversely related to Rab5c and EEA1 levels. In addition,

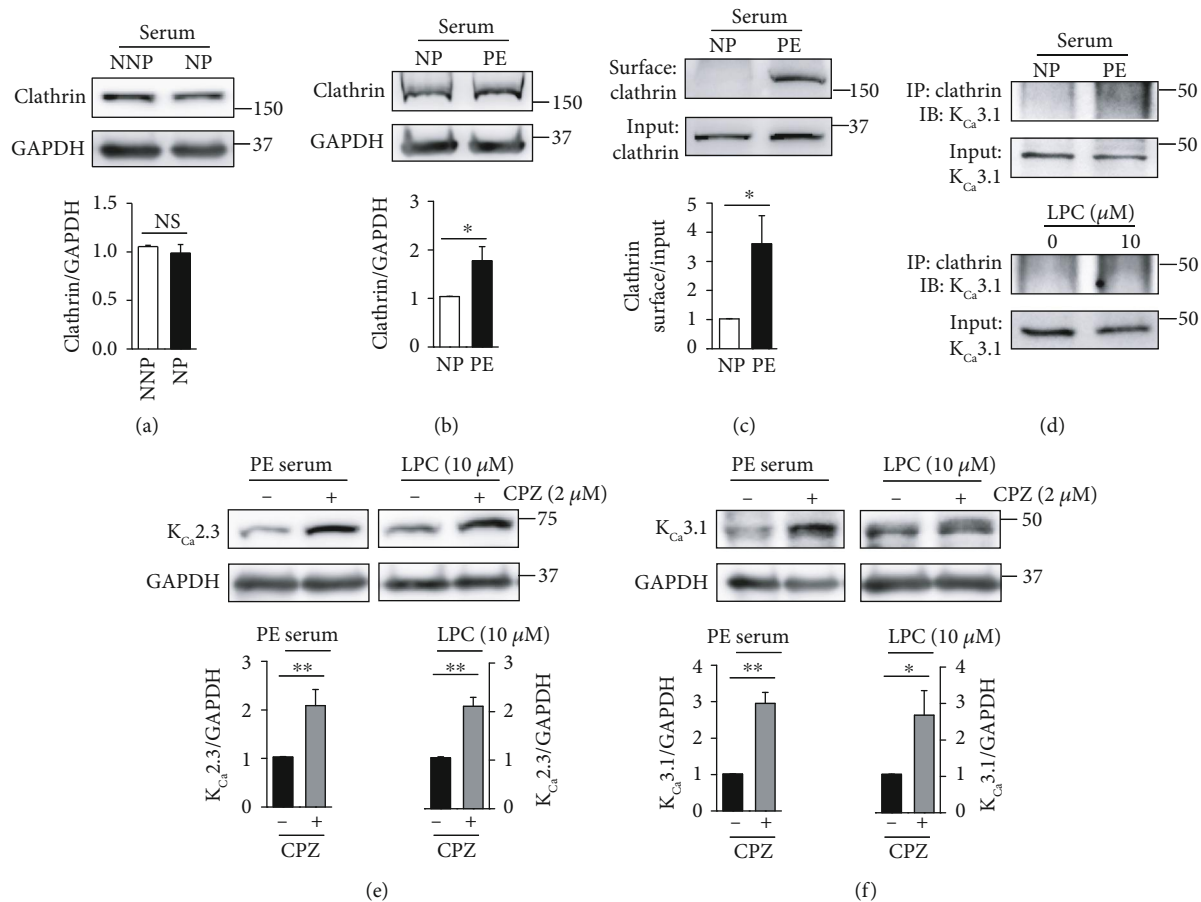


FIGURE 3: Role of clathrin in the  $K^+$  channel degradation. (a) Clathrin levels in ECs treated with NNP or NP serum. Alteration in clathrin levels was not found between ECs treated with NNP or NP serum. (b) Clathrin levels in ECs treated with NP or PE serum. (c) Membrane clathrin levels in ECs treated with NP or PE serum. (d) Colocalization of clathrin and  $K_{Ca}3.1$  was enhanced by PE serum or LPC. (e, f) Treatment with the clathrin inhibitor CPZ ( $2 \mu M$ ) recovered  $K_{Ca}2.3$  (e) or  $K_{Ca}3.1$  (f) levels in the ECs treated with PE serum or LPC. Blots shown are representative of three to six experiments performed with three to six different cultures. Bar graphs represent the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

LPC increased Rab5c and EEA1 levels in a concentration-dependent manner (Figure 4(d)). We examined whether Rab5c is involved in the regulation of the levels of  $K_{Ca}3.1$  using coimmunoprecipitation. LPC enhanced colocalization of Rab5c with  $K_{Ca}3.1$  (Figure 4(e)). We then examined the effect of EEA1 inhibition on LPC-induced downregulation of  $K_{Ca}2.3$  or  $K_{Ca}3.1$  by using siRNA against EEA1.  $K_{Ca}2.3$  (Figure 4(f)) and  $K_{Ca}3.1$  (Figure 4(g)) levels, which were reduced by PE serum or LPC, recovered upon EEA1 inhibition. These results suggest that Rab5c and EEA1 are involved in  $K_{Ca}2.3$  or  $K_{Ca}3.1$  downregulation in PE.

**3.4. A Redox State Regulates Membrane Levels of  $K_{Ca}2.3$  and  $K_{Ca}3.1$  during Pregnancy.** Previously, we showed that catalase and GPX downregulation increased  $H_2O_2$  levels, thereby upregulating  $K_{Ca}2.3$  and  $K_{Ca}3.1$  in NP [10]. In addition,  $K_{Ca}3.1$  levels were elevated in mouse aortic endothelial cells (MAECs) from catalase/GPX1 double knockout (catalase<sup>-/-</sup>/GPX1<sup>-/-</sup>) mice [11]. Thus, we examined whether catalase and GPX1 knockdown affects PM levels of  $K_{Ca}2.3$  or  $K_{Ca}3.1$  using wild-type and catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> mice.

PM-localized  $K_{Ca}2.3$  or  $K_{Ca}3.1$  levels were markedly increased in catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> MAECs, compared to wild-type MAECs (Figure 5(a)). We then compared Cav-1 or clathrin levels in wild-type and catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> MAECs. In catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> MAECs, levels of Cav-1 (Figure 5(b)) or clathrin (Figure 5(c)) were markedly reduced, and  $K_{Ca}2.3$  levels were elevated (Figure 5(c)). Thus, inverse relation between  $K_{Ca}2.3$  and clathrin levels was found. The increase in  $K_{Ca}2.3$  levels seen in these cells was reversed by treatment with polyethylene glycol- (PEG-) catalase (Figure 5(d)). Furthermore, a  $H_2O_2$  donor TBHP reduced clathrin (Figure 5(e)) and Rab5c (Figure 5(f)) levels in a concentration-dependent manner. These results suggest that catalase and GPX1 downregulation slows down the internalization and trafficking of  $K_{Ca}2.3$  and  $K_{Ca}3.1$  from PM via  $H_2O_2$ -mediated pathways.

We then compared  $K_{Ca}3.1$  currents in MAECs isolated from wild-type and catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> mice. Figure 5(g) shows  $K_{Ca}3.1$  currents activated by loading cells with  $1 \mu M$   $Ca^{2+}$  in the patch pipette (A) and the current-intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) relationship at +50 mV (B).

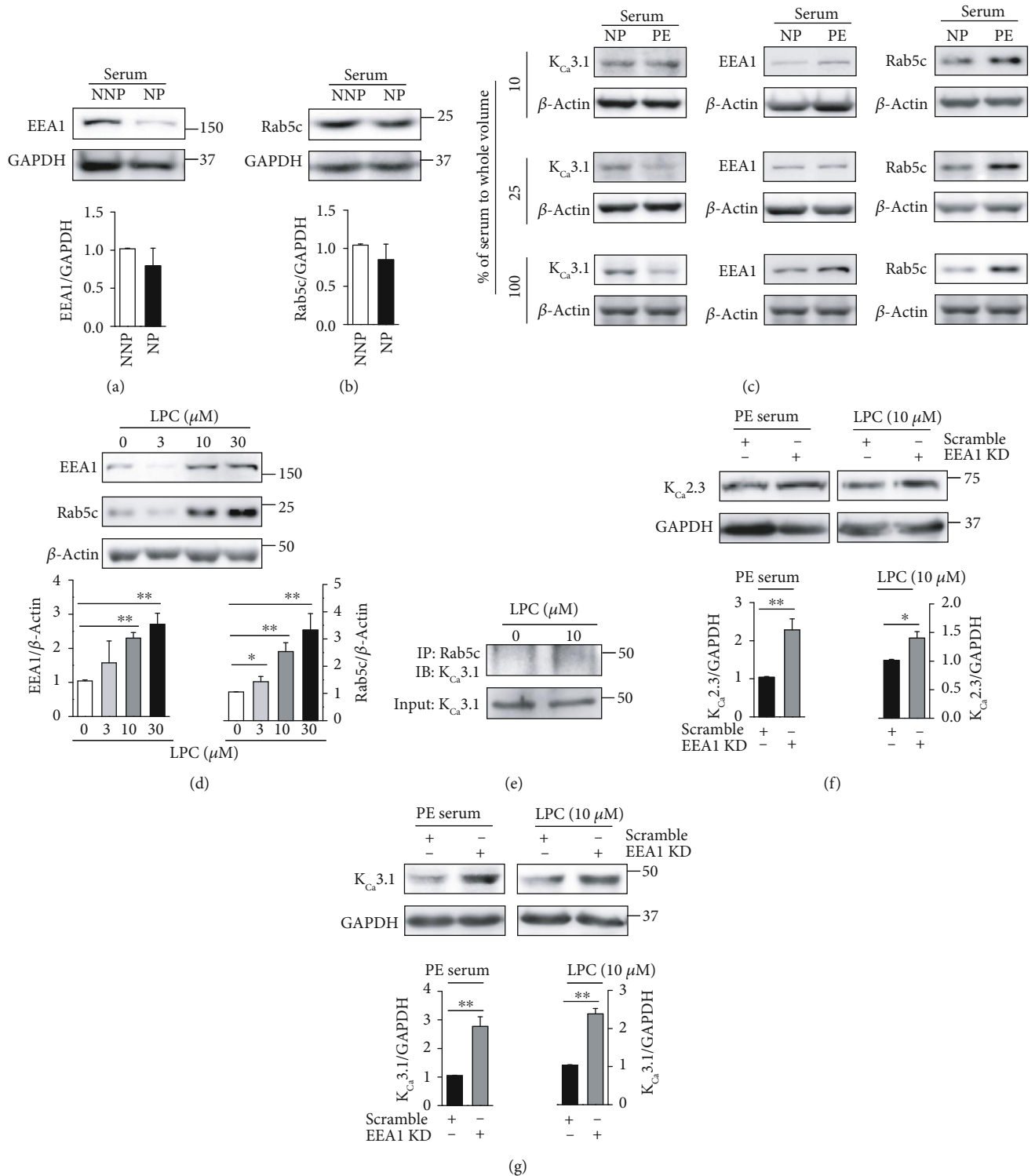


FIGURE 4: Rab5c- and EEA1-dependent transportation of  $K_{Ca}2.3$  and  $K_{Ca}3.1$  proteins. (a, b) Levels of EEA1 (a) or Rab5c (b) in ECs treated with NNP or NP serum. (c) PE serum reduced  $K_{Ca}3.1$  levels and enhanced Rab5c and EEA1 levels. (d) LPC increased EEA1 and Rab5c levels in a concentration-dependent manner. (e) Coimmunoprecipitation showing the interaction between Rab5c and  $K_{Ca}3.1$  in ECs treated with LPC. Input is lysate without primary Ab. (f, g) Treatment with siRNA against EEA1 recovered  $K_{Ca}2.3$  (f) or  $K_{Ca}3.1$  (g) levels in ECs treated with PE serum or LPC. Blots shown are representative of three to four experiments performed with three to four different cultures. Bar graphs represent the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .



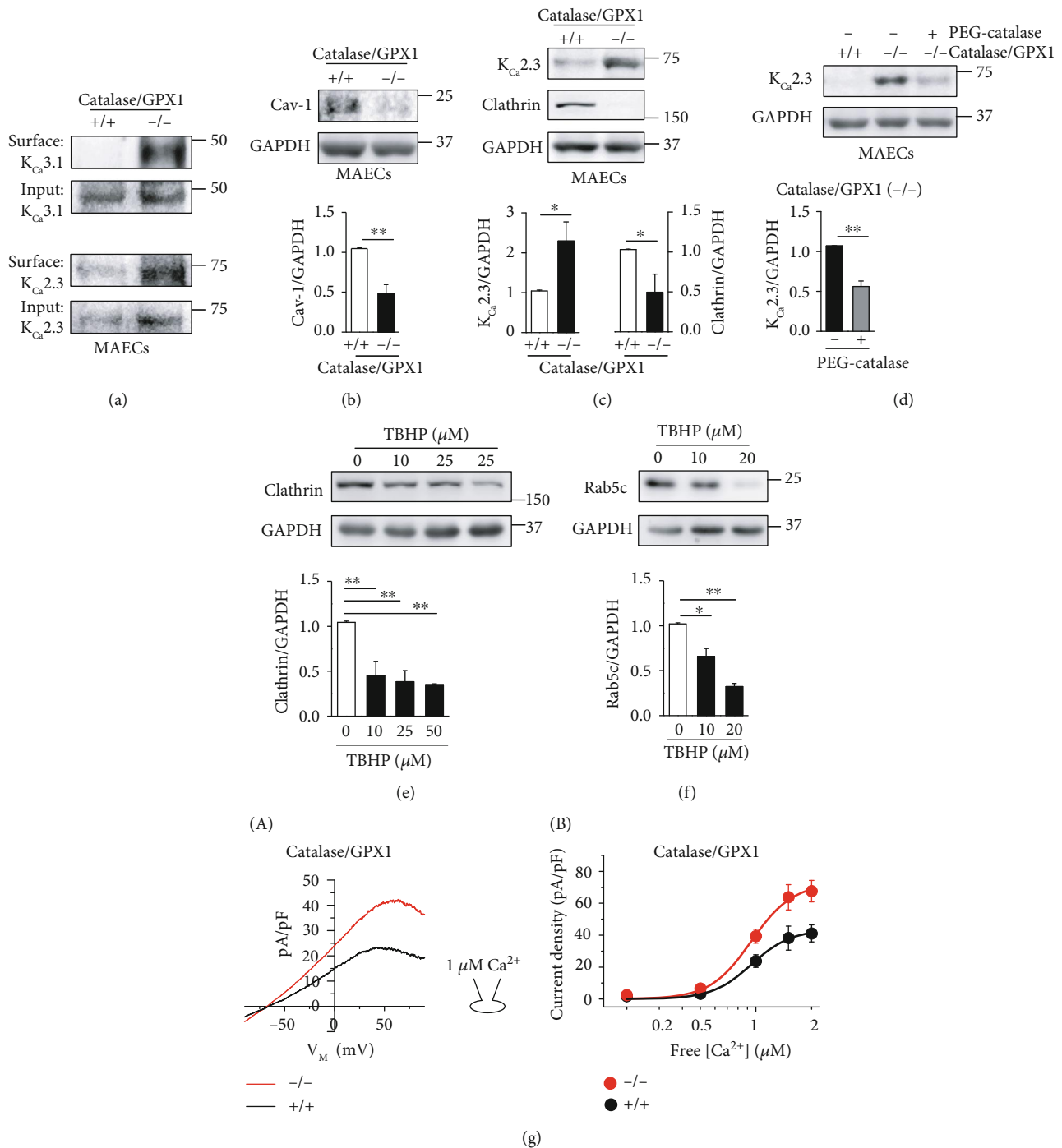


FIGURE 5: Catalase and GPX1 knockdown slows down K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 endocytosis. (a) PM levels of K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 in MAECs from wild-type and catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> mice. (b) Cav-1 levels in MAECs from wild-type and catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> mice. (c) K<sub>Ca</sub>2.3 and clathrin levels in MAECs from wild-type and catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> mice. (d) Elevated K<sub>Ca</sub>2.3 levels by catalase/GPX1 knockdown were reversed by a membrane permeable catalase, PEG-catalase. (e, f) A H<sub>2</sub>O<sub>2</sub> donor TBHP reduced clathrin (e) or Rab5c (f) levels in a concentration-dependent manner. (g) K<sub>Ca</sub>3.1 currents activated by loading cells with 1 μM Ca<sup>2+</sup> (A) and the current-[Ca<sup>2+</sup>]<sub>i</sub> relationship at +50 mV (B). The current densities at +50 mV are plotted against [Ca<sup>2+</sup>]<sub>i</sub>. Blots shown are representative of three to four experiments performed with three to four different cultures. Bar graphs represent the mean ± SEM. \*P < 0.05, \*\*P < 0.01.

The current densities were found to be dependent on [Ca<sup>2+</sup>]<sub>i</sub>. At 0.2, 0.5, 1, 1.5, and 2 μM [Ca<sup>2+</sup>]<sub>i</sub>, the current densities were 1.76 ± 0.21, 3.20 ± 1.06, 23.81 ± 3.96, 38.15 ± 7.46, and 41.10 ± 5.38 pA/pF in wild-type MAECs (n = 8), respectively,

and 2.39 ± 1.37, 6.42 ± 1.93, 39.33 ± 4.40, 63.72 ± 7.89, and 67.52 ± 6.73 pA/pF in catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> MAECs (n = 8), respectively, suggesting that K<sub>Ca</sub>3.1 currents were significantly enhanced in catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> MAECs, compared

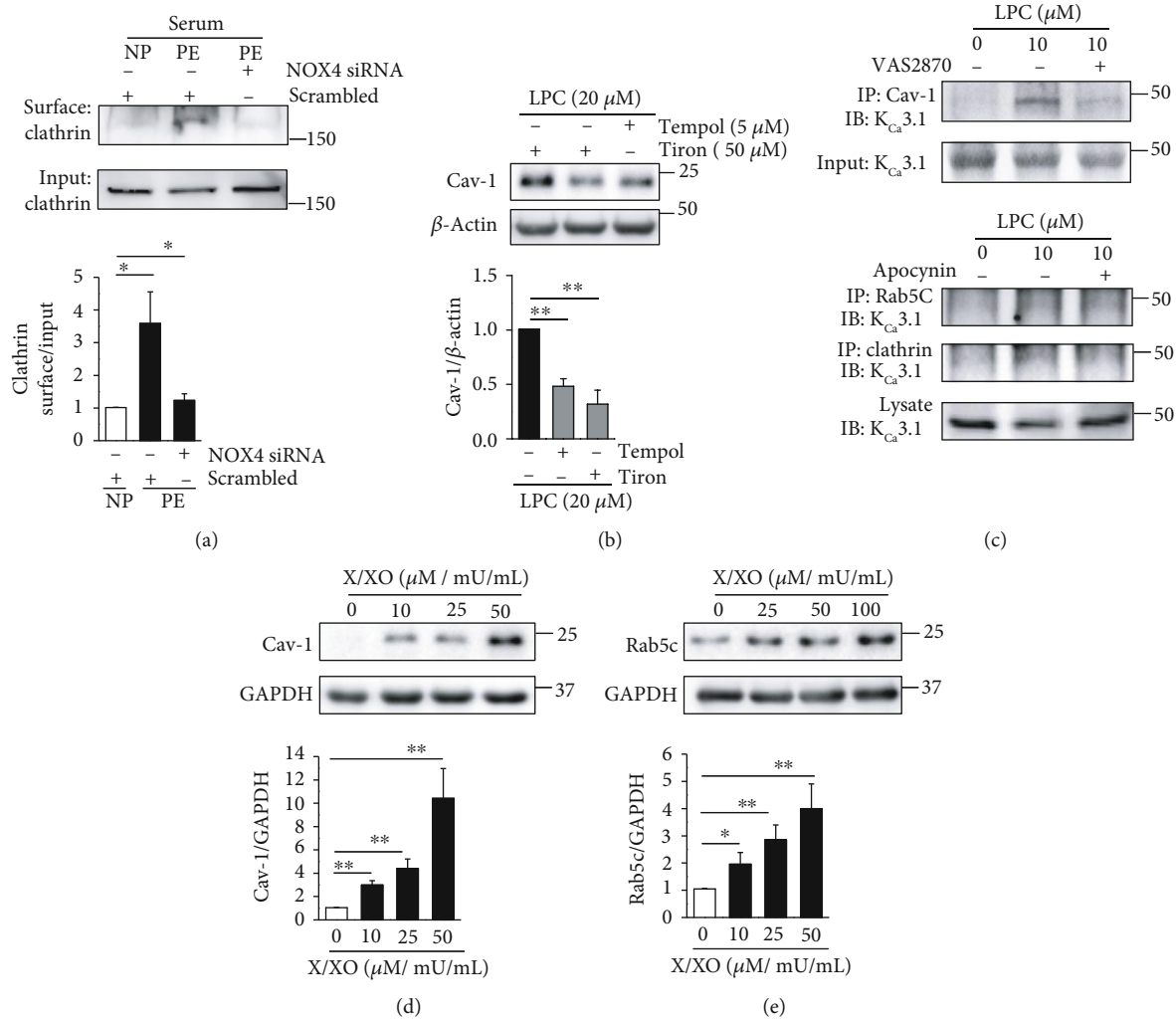


FIGURE 6: Superoxide facilitates K<sub>Ca</sub>2.3 or K<sub>Ca</sub>3.1 endocytosis. (a) Elevated PM clathrin levels by PE serum were reduced by NOX4 inhibition using NOX4 siRNA. (b) Elevated Cav-1 levels by LPC were reduced by the antioxidants, tempol or tiron. (c) Elevated colocalization of K<sub>Ca</sub>3.1 with Cav-1, clathrin, or Rab5c by LPC was reversed by the treatment with a pan-NOX inhibitor, VAS2870, or apocynin. (d, e) A superoxide donor, X/XO, elevated Cav-1 (d) or Rab5c (e) levels in a concentration-dependent manner. Blots shown are representative of three to four experiments performed with three to four different cultures. Bar graphs represent the mean  $\pm$  SEM. \**P* < 0.05, \*\**P* < 0.01.

to wild-type MAECs. However, no significant difference was found in EC<sub>50</sub> between wild-type and catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> MAECs ( $948 \pm 37$  nM [Ca<sup>2+</sup>]<sub>i</sub> and  $934 \pm 42$  nM [Ca<sup>2+</sup>]<sub>p</sub>, respectively). This implies K<sub>Ca</sub>3.1 upregulation enhances K<sub>Ca</sub>3.1 currents in catalase<sup>-/-</sup>/GPX1<sup>-/-</sup> MAECs.

On the other hand, we had previously reported that NADPH oxidase 2 (NOX2) and NOX4 upregulation and SOD downregulation enhance superoxide levels, thereby downregulating K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 in PE [10, 19]. We thus examined whether superoxide facilitates the internalization and transportation processes of K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 in PE. Compared to cells treated with NP serum, PM-localized clathrin levels were markedly elevated in ECs treated with PE serum, and the elevation was reversed by the treatment with NOX4 siRNA (Figure 6(a)). LPC elevated Cav-1 levels, and the elevation was reversed by the treatment with the antioxidants, tempol or tiron (Figure 6(b)). LPC increased colocalization of K<sub>Ca</sub>3.1 with Cav-1, clathrin, or Rab5c,

which was inhibited by a pan-NOX inhibitor VAS2870 or apocynin (Figure 6(c)). Furthermore, a superoxide donor X/XO increased Cav-1 (Figure 6(d)) or Rab5c (Figure 6(e)) levels in ECs in a concentration-dependent manner. These results suggest that the internalization and transportation processes of K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 facilitated by superoxide contribute to the downregulation of K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 in PE.

#### 4. Discussion

In this study, we observed for the first time that PM-localized K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 are internalized via caveola- or clathrin-dependent processes and transported via a Rab5c- and EEA1-dependent process in ECs (Figure 7). Compared to NNP, the internalization and transport processes are delayed in NP, thus elevating PM-localized K<sub>Ca</sub>2.3 and K<sub>Ca</sub>3.1 levels. However, compared to NP, the internalization and transport

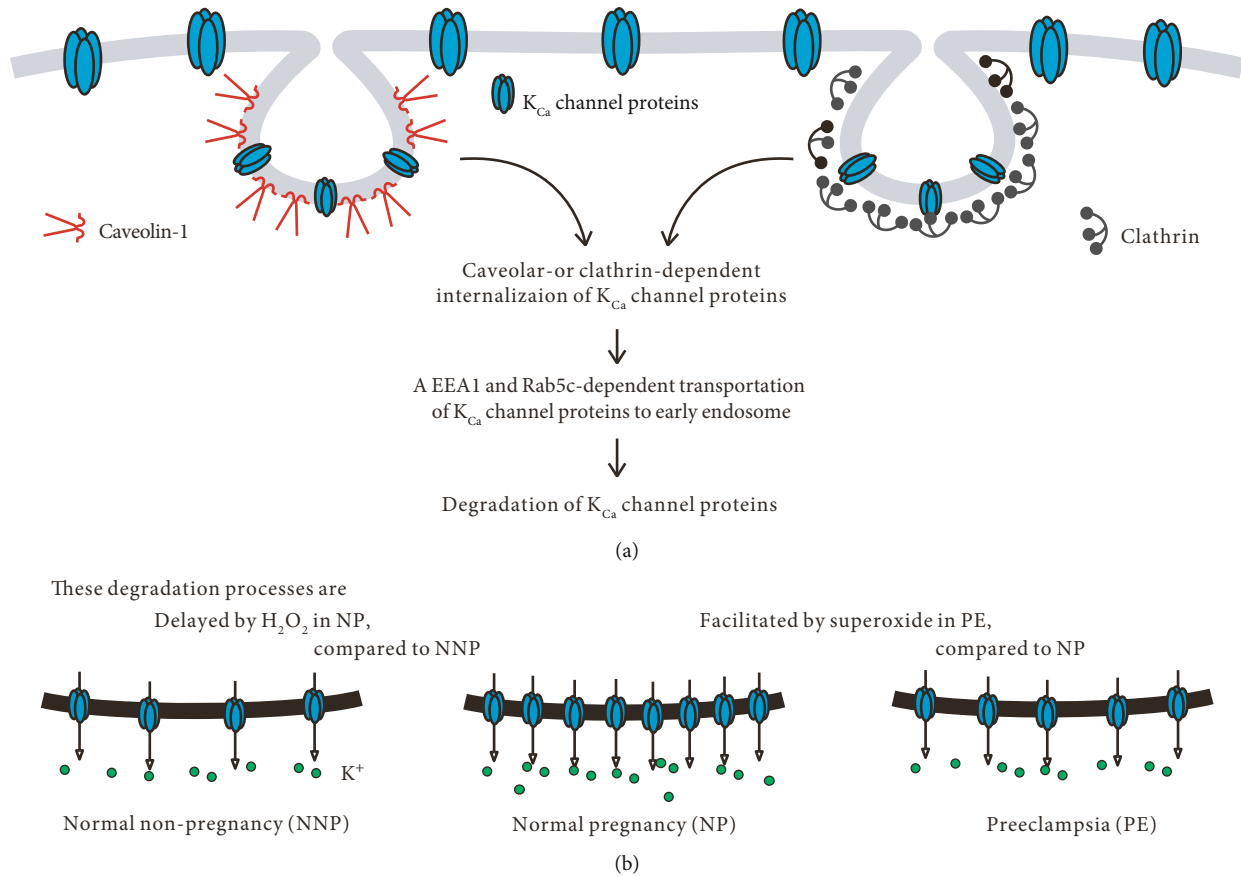


FIGURE 7: A schematic model for altered redox state-induced modulation of PM-localized  $K_{Ca}2.3$  and  $K_{Ca}3.1$  in NP and PE. (a) PM-localized  $K_{Ca}2.3$  and  $K_{Ca}3.1$  are internalized via caveola- or clathrin-dependent processes and transported via a Rab5c- and EEA1-dependent process to early endosome. (b) Compared to NNP, the internalization and transport processes are delayed by  $H_2O_2$  in NP, thus elevating PM-localized  $K_{Ca}2.3$  and  $K_{Ca}3.1$  levels. However, compared to NP, the internalization and transport processes are facilitated in PE, thus reducing PM-localized  $K_{Ca}2.3$  and  $K_{Ca}3.1$  levels.

processes are facilitated in PE, thus reducing PM-localized  $K_{Ca}2.3$  and  $K_{Ca}3.1$  levels. Soluble factors in PE serum, such as oxidized LDL, might induce internalization of  $K^+$  channel proteins from the PM via clathrin- or caveola-dependent processes and thereby attenuate the pregnancy-associated  $K_{Ca}2.3$  and  $K_{Ca}3.1$  upregulation. Since endothelial  $K_{Ca}2.3$  and  $K_{Ca}3.1$  play important roles in the control of vascular contractility, altered levels of these  $K^+$  channels might explain the hemodynamic changes seen during the progress of pregnancy, both NP and PE.

$K_{Ca}2.3$  was internalized from PM via a caveola- or clathrin-dependent process. The involvement of caveolae in the internalization of  $K_{Ca}2.3$  is consistent with the finding that  $K_{Ca}2.3$  is observed in caveolae, Cav-1-rich membrane fractions [20], and that the endocytosis of  $K_{Ca}2.3$  from the cell membrane is dependent upon both Cav-1 and dynamin II [21]. In addition, we showed that clathrin is involved in  $K_{Ca}2.3$  internalization. Internalized  $K_{Ca}2.3$  might be transported via a EEA1- and Rab5-dependent process, since LPC-induced reduced  $K_{Ca}2.3$  levels were reversed by siRNA against EEA1 (Figure 4(f)). Similarly, Gao et al. demonstrated the involvement of Rab5-containing endosome in the endocytosis of  $K_{Ca}2.3$  from PM [21]. These mecha-

nisms involved in  $K_{Ca}2.3$  internalization and transportation were similar to those for  $K_{Ca}3.1$  internalization and transportation.

Previously, we demonstrated that PM  $K_{Ca}3.1$  proteins are internalized via a clathrin-dependent process and transported in a Rab5c- and EEA1-dependent process in Fabry disease [12]. In addition, we demonstrated that caveolae are involved in  $K_{Ca}3.1$  internalization from cell membrane, although endothelial  $K_{Ca}3.1$  is suggested to be present in noncaveolar membrane fractions [20, 22]. However,  $K_{Ca}3.1$ , which is not associated with Cav-1 under baseline conditions, was found to colocalize with Cav-1 during shear stress conditions [23]. In addition, PE serum induced colocalization of  $K_{Ca}3.1$  with Cav-1 (Figure 2(d)). Thus, stimulation by serum components or shear stress might lead to colocalization of  $K_{Ca}3.1$  and Cav-1. In addition, clathrin-dependent internalization followed by Rab5c- and EEA1-dependent transportation was found to regulate membrane  $K_{Ca}3.1$  levels during pregnancy.

Altered redox state might be involved in the regulation of internalization and transportation of  $K_{Ca}2.3$  and  $K_{Ca}3.1$  in NP and PE. We previously showed that  $H_2O_2$  levels are elevated via downregulation of catalase and GPX1 during NP

[10]. Catalase and GPX1 knockdown elevated  $K_{Ca2.3}$  and  $K_{Ca3.1}$  levels in PM (Figure 5(a)) and reduced Cav-1 and clathrin levels (Figures 5(b) and 5(c)), suggesting that  $H_2O_2$  elevates PM levels of  $K_{Ca2.3}$  and  $K_{Ca3.1}$  by slowing down the internalization process of the  $K^+$  channel proteins from PM. In addition, reduction in Rab5c levels by the  $H_2O_2$  donor TBHP suggests that  $H_2O_2$  slows down the transportation process of the internalized  $K_{Ca2.3}$  and  $K_{Ca3.1}$ . Thus, reduced degradation of  $K_{Ca2.3}$  and  $K_{Ca3.1}$  might contribute to the upregulation of these  $K^+$  channel proteins during NP. In contrast, superoxide might facilitate the internalization and transportation processes of  $K_{Ca2.3}$  and  $K_{Ca3.1}$  from cell membrane. NOX2 and NOX4 upregulation and SOD downregulation enhanced superoxide levels, thereby downregulating  $K_{Ca2.3}$  and  $K_{Ca3.1}$  in PE [10].

Reduction in Cav-1 levels by NP serum was reversed by anti-VEGFR Abs, suggesting that soluble serum factors, such as VEGF, might inhibit caveola-dependent internalization of  $K_{Ca2.3}$  and  $K_{Ca3.1}$  through VEGFR activation during NP. Since serum levels of the soluble VEGF inhibitor, sFlt-1 are increased in PE, VEGFR activation might be suppressed during PE. Inhibition of VEGFR activation might contribute to the increase in Cav-1 levels by PE serum. In addition, cell membrane localization of Cav-1 and clathrin was enhanced by the treatment with oxidized LDL or LPC, and LPC increased colocalization of clathrin with  $K_{Ca3.1}$ . Thus, VEGFR inhibition or oxidized LDL might facilitate internalization of  $K_{Ca2.3}$  and  $K_{Ca3.1}$ , thereby reducing membrane levels of these  $K^+$  channels during PE.

Oxidative stress is increased during pregnancy (in both NP and PE) [24–26]. ROS reduces NO bioavailability by interacting directly with NO [27–29], suggesting that NO-induced vasorelaxation might be impaired during pregnancy. Since  $K_{Ca2.3}$  and  $K_{Ca3.1}$  activation induces NO-independent vasorelaxation by evoking hyperpolarization of VSMCs,  $K_{Ca2.3}$  and  $K_{Ca3.1}$  upregulation may compensate for decreased NO signaling, as shown in vascular aging [11].  $K_{Ca2.3}$  and  $K_{Ca3.1}$  upregulation in NP suggests that a similar compensation occurs in NP [10]. In contrast, ROS and superoxide production has been suggested to be more in PE than in NP, and superoxide decreased levels of endothelial NO synthases [30]. These results suggest that the impairment of NO-induced vasorelaxation might be greater in PE than in NP. In addition to the impaired NO-induced vasorelaxation, reduced  $K_{Ca2.3}$  and  $K_{Ca3.1}$  upregulation suggests that NO-independent, endothelium-dependent relaxation is also impaired in PE [10].

The present findings suggest that PM-localized  $K_{Ca2.3}$  and  $K_{Ca3.1}$  levels are regulated by similar internalization and transportation processes, and the regulation of the processes is affected by an altered redox state during NP and PE. We identified Cav-1, clathrin, Rab5c, and EEA1 as critical components in the regulation of PM-localized  $K_{Ca2.3}$  and  $K_{Ca3.1}$  levels during pregnancy. Inhibiting Cav-1, clathrin, or EEA1 using siRNAs against these components led to the recovery of PM-localized  $K_{Ca2.3}$  and  $K_{Ca3.1}$  levels, suggesting that siRNAs against these components can be used to treat endothelial dysfunction. To the best of our knowledge, this is the first study to reveal the mechanisms underlying

the regulation of membrane  $K_{Ca2.3}$  and  $K_{Ca3.1}$  levels during pregnancy.

## Abbreviations

Ab:	Antibody
Cav-1:	Caveolin-1
CM:	Culture medium
CPZ:	Chlorpromazine
EC:	Endothelial cell
EEA1:	Early endosome antigen-1
HUtMEC:	Human uterine microvascular EC
LDL:	Low-density lipoprotein
LOX:	Lectin-like oxidized LDL receptor
LPC:	Lysophosphatidylcholine
MAECs:	Mouse aortic endothelial cells
M $\beta$ CD:	Methyl- $\beta$ -cyclodextrin
NNP:	Normal nonpregnancy
NO:	Nitric oxide
NOX:	NADPH oxidase
NP:	Normal pregnancy
PE:	Preeclampsia
PM:	Plasma membrane
sFlt-1:	Soluble fms-like tyrosine kinase-1
VEGF:	Vascular endothelial growth factor
VEGFR:	VEGF receptor.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

No conflicts of interest, financial, or otherwise are declared by the authors.

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