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Stimulation of G Protein-coupled Bile Acid Receptor Enhances Vascular Endothelial Barrier Function via Activation of PKA and Rac1

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Running title: GPBAR enhances endothelial barrier function

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Abbreviations:

BAEC, bovine aortic endothelial cell; Epac, exchange protein directly activated by cAMP; FITC, fluorescein isothiocyanate; G protein-coupled bile acid receptor, GPBAR; HUVEC,

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human umbilical vein endothelial cells; HPAEC, human pulmonary arterial endothelial cells; HDMVEC, human dermal microvascular endothelial cells; PKA, protein kinase A; siRNA, small interfering RNA; TER, transendothelial electrical resistance; TLCA, taurolithocholic acid; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor.

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Abstract

Bile acids are end products of cholesterol metabolism, which constantly exist at high concentration in blood. Since vascular endothelial cells express G protein-coupled bile acid receptor (GPBAR), bile acids potentially modulate endothelial function. Here, we investigated whether and how GPBAR agonism affects endothelial barrier function. In bovine aortic endothelial cells (BAECs), treatment with a GPBAR agonist, taurolithocholic acid (TLCA), increased the transendothelial electrical resistance. In addition, TLCA suppressed the thrombin-induced dextran-infiltration through endothelial monolayer. Knockdown of GPBAR abolished the inhibitory effect of TLCA on hyperpermeability. These results indicate that stimulation of GPBAR enhances endothelial barrier function. TLCA increased intracellular cAMP production in BAECs. Inhibition of protein kinase A (PKA) or Rac1 significantly attenuated the TLCA-induced endothelial barrier protection. TLCA induced cortical actin polymerization which was attenuated by a Rac1 inhibitor. In vivo, local administration of TLCA into mouse ear significantly inhibited vascular leakage and edema formation induced by croton oil or vascular endothelial growth factor. These results indicate that stimulation of GPBAR enhances endothelial barrier function by cAMP/PKA/Rac1-dependent cytoskeletal rearrangement.

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Introduction

Bile acids are end-products of cholesterol metabolism. After produced in the liver, bile acids are secreted to the intestine through the bile duct. It is well-known that bile acids emulsify dietary lipids and facilitate their absorption from the intestine. Most bile acids are reabsorbed from intestine, going back to liver through portal vein. Recent studies have shown that bile acids act as signaling molecules as well as lipid emulsifiers (Hylemon et al., 2009; Lefebvre et al., 2009). Makishima et al. first identified a nuclear receptor called farnesoid x receptor (FXR) as a distinct bile acid receptor, which is highly expressed in liver and intestine (Makishima et al., 1999). The following studies reported that FXR-mediated signaling regulates the transcription of genes involved in bile acid, lipid, and glucose homeostasis (Lefebvre et al., 2009). Thereafter, Maruyama et al. and Kawamata et al. discovered that a G protein-coupled receptor GPBAR is another bile acid receptor which is ubiquitously expressed in many types of tissues such as gall bladder, intestine, placenta, and spleen (Maruyama et al., 2002; Kawamata et al., 2003). Some in vitro studies showed that GPBAR agonism suppresses pro-inflammatory cytokine production and phagocytotic function of macrophages (Kawamata et al., 2003; Keitel et al., 2008). In vivo studies demonstrated the significance of anti-inflammatory roles of GPBAR in lipopolysaccharide-induced liver injury and 2,4,6-trinitrobenzenesulfonic acid or dextran sodium sulfate-induced colitis by suppressing local inflammation (Cipriani et al., 2011;

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Wang et al., 2011). Thus, GPBAR-mediated signaling is currently highlighted as a potential therapeutic target for inflammatory diseases in enterohepatic tract.

Whereas enterohepatic tract contains large amount of bile acids, systemic blood also contains high concentration of bile acids. The serum bile acid level fluctuates from ~2 μM between meals to ~15 μM after a meal (Kida et al., 2009). Patients with hepatic diseases such as cirrhosis display great increase in serum bile acids up to 100 μM order (Mashige et al., 1981; Everson, 1987). Therefore, in health and/or in hepatic diseases, the innermost layer of vasculature, endothelial cells are constantly exposed to a certain amount of bile acids. It can be assumed that bile acids act on endothelial cells and modulate their inflammatory responses by stimulating GPBAR. Previously we demonstrated that GPBAR agonism suppresses an inflammatory response, monocyte adhesion to endothelial cells, by stimulating endothelial nitric oxide production (Kida et al., 2013).

Vascular endothelial cells form a semi-selective barrier between blood and interstitium to regulate vascular permeability (Komarova and Malik, 2010). The integrity of this barrier is indispensable for maintaining tissue homeostasis, and its disruption is a crucial process of inflammatory responses. In acute inflammation, various proinflammatory substances are produced in injured tissues. They stimulate endothelial cells and disrupt the barrier. This

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leads to extravasation of leukocytes and tissue edema (Vestweber et al., 2009). Sustained vascular hyperpermeability leads to persistent leukocyte infiltration and retention of growth factors and cytokines. These reactions are involved in progression of chronic inflammatory diseases such as rheumatoid arthritis (Binstadt et al., 2006; Goel et al., 2011). Some endogenous lipid metabolites are known to tighten the endothelial barrier and alleviate inflammation. A lipid phosphate produced in sphingosine metabolism, sphingosine-1-phosphate, as well as phospholipid oxidation products called oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine are known to reduce vascular hyperpermeability and inflammation in acute lung injury (Nonas et al., 2006; Tauseef et al., 2008). A cyclooxygenase metabolite prostaglandin D_2 modulates inflammatory responses in tumor microenvironment by suppressing vascular permeability (Murata et al., 2008). Thus, discovery of a novel endothelial barrier modulator and clarifying the mechanism of its action can provide a therapeutic option for inflammatory diseases.

In the present study, we investigated whether and how bile acid/GPBAR signal affects endothelial barrier formation and found that GPBAR agonism enhances endothelial barrier function via cAMP/PKA/Rac1-dependent signal pathway.

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Materials and Methods

Chemicals

The chemicals used were as follows: TLCA, croton oil, and 4',6-diamidino-2-phenylindole (Sigma, St. Louis, MO); penicillin-streptomycin, dextran fluorescein 70,000 MW anionic lysine fixable (FITC-dextran), and rhodamine phalloidin (Invitrogen, Carlsbed, CA); forskolin, 3-isobutyl-1-methylxanthine and recombinant human vascular endothelial growth factor (VEGF)-A_{165} (Wako Pure Chemical, Osaka, Japan); PKA Inhibitor 14-22 Amide, Cell-Permeable, Myristoylated (Merk KGaA, Darmstadt, Germany); and NSC23766 (Tocris Bioscience, Bristol, UK).

Cell culture procedure

BAECs were freshly isolated from bovine aortas purchased at a local slaughterhouse. After several washings with Hank's balanced salt solution, endothelial cells were obtained from the luminal surface of aortas by digestion with trypsin-EDTA solution and scratching with scalpel blade. Isolated cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Confluent BAECs were used for experiments at passage 3-8 after starvation with the serum-free medium for 16-24 h. For gene knockdown of GPBAR, BAECs at 40-60% confluence were transfected with a set of 4 RNAi^{TM} siRNAs targeting GPBAR (invitrogen,

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25 nM each), using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instruction. Cells were used for experiments at least 90 h after the transfection, when GPBAR protein expression was reduced to ~60%, and cells showed similar viability to that of non-transfected cells.

Transendothelial electrical resistance measurement

Transendothelial electrical resistance (TER) was measured using xCELLigence real time cell analyzer DP system (Roche, Basel, Switzerland) according to the manufacturer's protocol. BAECs (10,000 cells per well) were seeded on golden electrodes and incubated until the TER value reached steady state level. Then the cells were stimulated with each agent, and the TER was measured every 30 s. To normalize the data, the TER values were divided by the value before stimulation.

Transwell endothelial permeability assay

BAECs were grown on the transwell inserts with 1 μm pores (BD Biosciences, San Jose, CA) to form a confluent monolayer. Test agents including thrombin were added to the upper chamber. FITC-dextran (20 μg/ml) was added to the upper chamber 10 min after the thrombin treatment. Thirty min after the addition of FITC-dextran, 100 μl media were collected from the lower chamber. The amount of FITC-dextran which had passed through

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the cell monolayer was determined by a fluorescence spectrophotometer (Wallac 1420 ARVOsx, PerkinElmer, Waltham, MA).

Immunostaining

BAECs on gelatin-coated sterilized glass coverslips were fixed with 4% paraformaldehyde for 5 min, followed by permeabilizing and blocking with 0.1% Triton X-100 and 3% bovine serum albumin for 30 min. Then the cells were incubated with goat polyclonal anti-VE-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100 dilution) and labeled with Alexa Fluor 488-conjugated donkey anti-goat IgG antibody (Invitrogen, 1:300 dilution) and rhodamine phalloidin (1:33 dilution). Cells were finally incubated with 4',6-diamidino-2-phenylindole (1 μg/mL) to stain nuclei. The images were captured using a confocal microscope (Eclipse Ti with C1 system, Nikon, Tokyo, Japan) equipped with an argon laser.

Intracellular cAMP content measurement

BAECs were pretreated with a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (200 μM), for 5 min. After stimulation, cells were lysed and the cAMP concentration in the lysate was measured by enzymeimmunoassay, according to the manufacturer's instruction (cAMP ELISA kit, Enzo Life Sciences, Farmingdale, NY). The intracellular cAMP level

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was normalized to the total protein content, and shown as a ratio with the value of forskolin-treated cells set at 1.

Modified Miles assay

Animal experiments were approved by the institutional animal care and use committees of the University of Tokyo, and conform to the guideline. The experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Male FVB mice were anesthetized with inhalatory isoflurane. The adequacy of anesthesia was monitored by the absence of eyelid reflex. Saline (10 μl) containing VEGF (30 ng) with or without TLCA (100 ng) was intradermally injected to the dorsal ear skin. After 5 min, Evans blue (30 mg/kg) was intravenously injected. In another series of experiments, croton oil (2.5% in acetone) was applied on both sides of the ear. After 80 min, TLCA was intradermally injected as described above, followed by intravenous injection of Evans blue at 10 min later. Thirty min after the Evans blue injection, the ear thickness was measured by a vernier caliper. Mice were then euthanized by cervical dislocation, and the ears were excised and dried in a constant-temperature oven. Evans blue extravasated in the ear was extracted in formamide, and the content was quantified spectrophotometrically (Wallac 1420 ARVOsx, PerkinElmer).

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Statistical analysis

The results are expressed as means ± S.E.M. Statistical evaluation of the data was performed by one-way analysis of variance followed by Dunnett's test or Bonferroni's test for comparison among more than three groups, and by unpaired Student's t test for comparison between two groups. A value of $p<0.05$ was taken as significant.

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Results

GPBAR agonism enhances the endothelial barrier function.

Transendothelial electrical resistance (TER) was measured as an index of endothelial barrier integrity. As shown in Figure 1A, treatment with a bile acid which has the highest affinity to GPBAR, TLCA (3 μM) (Maruyama et al., 2002; Kawamata et al., 2003), rapidly increased the TER of BAECs. This response reached its peak at 10-15 min after stimulation, lasted at least 30 min and the value gradually returned to the basal level. The largest responses were obtained by 3-10 μM TLCA (Figure 1B). In consistent with a previous report (Fukuhara et al., 2005), an adenylate cyclase activator, forskolin (1 μM), strongly increased the TER. We next investigated whether GPBAR agonism can counteract the endothelial hyperpermeability induced by a barrier-disrupting serine protease, thrombin. As shown in Figure 1C, treatment with thrombin (1 U/ml, 40 min) significantly increased the FITC-labeled dextran leakage through a BAEC monolayer. This response was significantly inhibited by pretreatment with TLCA (3 μM, 5 min). The siRNA knockdown of GPBAR abolished this suppression by TLCA. Western blot analysis confirmed the effective siRNA knockdown of GPBAR. Pretreatment with forskolin (1 μM, 5 min) almost completely blocked the thrombin-induced dextran leakage in cells transfected with either control or GPBAR siRNA.

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GPBAR agonism increases intracellular cAMP level

It is reported that GPBAR agonism increases intracellular cAMP via activation of Gαs (Maruyama et al., 2002; Kawamata et al., 2003; Keitel et al., 2007). As shown in Figure 2A, treatment with TLCA (3-10 μM, 15 min) significantly increased intracellular cAMP amount in BAECs. The dose-response relationship was similar to that of transendothelial resistance increase induced by TLCA (1-10 μM) (Figure 1B). Forskolin (1 μM, 5 min) also elevated intracellular cAMP level, and it was even larger, ~12 fold increase of vehicle.

GPBAR-mediated endothelial barrier enhancement is dependent on PKA and Rac1 activity.

Increased intracellular cAMP leads to activation of cAMP effectors, PKA (Lum et al., 1999; Fukuhara et al., 2005). which in turn activates a RhoGTPase family member Rac1. Activated Rac1 stabilizes endothelial adherens junction by rearranging actin cytoskeleton (Komarova and Malik, 2010; Spindler et al., 2010). As shown in Figure 2B, the inhibitory effect of TLCA (3 μM, 5 min) on thrombin-induced dextran leakage was abolished when the cells were pretreated with a competitive peptide substrate inhibitor of PKA (Cheng et al., 1986) (30 μM, 30 min). The effect of TLCA was also abolished by the pretreatment with a Rac1 inhibitor NSC23766, which interfere with Rac-specific guanine nucleotide exchange factors, Tiam1 and Trio (Gao et al., 2004) (100 μM, 30 min). These results suggest the contributions

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of PKA- and Rac1-signal pathways in GPBAR-mediated endothelial barrier enhancement.

GPBAR agonism induces actin cytoskeleton rearrangement in the cortical region

The endothelial barrier function is largely dependent on the integrity of an intercellular structure called adherens junction. In adherens junction, vascular endothelial (VE)-cadherin mediates adhesion of adjacent endothelial cells. Actin band recruited to cortical region also contributes to stabilizing the junction as its scaffold (Vandenbroucke et al., 2008). Therefore, the effects of GPBAR agonism on actin cytoskeleton and VE-cadherin distribution were assessed. Compared with the resting state (Figure 3, first row), BAECs treated with TLCA (3 μM, 15 min) displayed increased formation of actin bundle in the cortical region (Figure 3, second row). Pretreatment with a Rac1 inhibitor NSC23766 (100 μM, 30 min) inhibited the TLCA-induced actin cytoskeleton rearrangement (Figure 3, third row). In BAECs treated with thrombin (1 U/ml, 20 min), actin stress fiber clearly appeared running across the cells, suggesting that a centripetal contractile force is generated. In addition, localization of VE-cadherin at the cell border was lost (Figure 3, fourth row). These observations indicate that thrombin disrupted the adherens junction and endothelial barrier integrity. Pretreatment with TLCA (3 μM, 5 min) inhibited the thrombin-induced actin stress fiber formation. This was accompanied with the recovery of VE-cadherin localization at the cell border (Figure 3, fifth row).

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GPBAR agonism inhibits vascular permeability in vivo

We next verified the protective effect of GPBAR signal in endothelial barrier integrity in vivo. Application of a commonly-used inflammatory irritant, croton oil (2.5% in acetone, 2 h), on a mouse ear increased Evans blue dye extravasation and the tissue thickness. Local administration of TLCA (100 ng, 1 h and 20 min after the oil application) significantly reduced the croton oil-induced dye extravasation and ear swelling (Figure 4A and B). VEGF directly stimulates endothelial cell and causes hyperpermeability without inflammation (Esser et al., 1998). Local administration of VEGF (30 ng, 35 min) increased dye extravasation and ear thickness. Concurrent administration of TLCA (100 ng) significantly reduced the VEGF-induced dye extravasation and ear swelling (Figure 4A and B).

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Discussion

The current study highlights a novel role of hepatic metabolites of cholesterol, bile acids, to modulate vascular endothelial function. Here, we demonstrated that a bile acid receptor GPBAR-mediated signaling enhances endothelial barrier function. This effect is due to cAMP/PKA/Rac1-dependent actin cytoskeleton rearrangement (Supplementary Figure 1).

The anti-inflammatory roles of GPBAR in the liver are well documented. GPBAR-deficiency exacerbated liver injury induced by lipopolysaccharide administration, accompanied with increased expression of inflammatory mediators in the liver (Keitel et al., 2008; Wang et al., 2011). Thus, in the state of hepatic diseases, bile acids-GPBAR signaling may have protective effects in the liver. In addition, we demonstrated that GPBAR-medaiated signaling can alleviate tissue inflammation by suppressing vascular permeability (Figure 4). This result may strengthen the potential of GPBAR stimulation for therapeutic application to hepatic inflammation as well as systemic inflammation which is frequently associated with hepatic diseases (Boermeester et al., 1995).

Although the current findings allow us to assume that serum bile acids contribute to maintaining endothelial barrier integrity, some previous studies suggested the opposite effect of bile acids on endothelial barrier. Treatment with a relatively high concentration (1.5-2

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mM) of bile acids, deoxycholic acid or taurochenodeoxycholic acid, increased cerebrovascular permeability by disrupting plasma membrane and intercellular junction of endothelial cells in blood brain barrier (Greenwood et al., 1991). A well-established cirrhosis model, common bile duct-ligated rats displayed vascular hyperpermeability which was accompanied with high serum bile acids (Chang and Ohara, 1993; Ohara et al., 1993). Although the detailed mechanisms are unclarified, these barrier-disrupting effects of bile acids may not be due to GPBAR-mediated signaling but possibly due to the physicochemical property of bile acids as detergents. At high concentration as 100 μM-1 mM order, the detergent effect of bile acids may outweigh the GPBAR-mediated barrier protection. Further studies are required to understand the GPBAR-dependent or independent effects of serum bile acids on endothelial barrier function.

Previous studies reported that intracellular cAMP increase is important for many GPBAR-mediated cellular responses such as suppression of inflammatory cytokine production in macrophages (Kawamata et al., 2003; Pols et al., 2011), improvement of glucose control by intestinal endocrine cells (Thomas et al., 2009), and energy expenditure increase in brown adipocytes and skeletal muscle cells (Watanabe et al., 2006), In this study, GPBAR agonism increased intracellular cAMP content in vascular endothelial cells (Figure 2A). We also demonstrated that GPBAR-mediated endothelial barrier enhancement is

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dependent on a cAMP effector PKA activation (Figure 2B). In addition to PKA, exchange protein directly activated by cAMP (Epac) is known as a cAMP effector (Fukuhara et al., 2005). It remains to be clarified whether Epac is involved in the GPBAR-mediated endothelial barrier enhancement. Downstream of cAMP/PKA signal, a RhoGTPase family member Rac1 has emerged as a key effector to mediate dynamic interaction of adherens junction and actin cytoskeleton (Komarova and Malik, 2010; Spindler et al., 2010). In consistent with this, GPBAR-mediated endothelial barrier enhancement was accompanied with cortical actin polymerization, which was abolished by Rac1 inhibition (Figure 3). Taken all together, we conclude that GPBAR agonism induces cAMP/PKA/Rac1-dependent actin cytoskeleton rearrangement and enhances endothelial barrier function.

Of note, the cAMP content in forskolin-treated BAECs was much (~5.7 times) higher than that in TLCA-treated cells (Figure 2A). Likewise, the endothelial barrier enhancement induced by forskolin was stronger than that induced by TLCA (Figure 1), but this difference was not so remarkable than that in cAMP production. A possible explanation for the discrepancy is that cAMP/PKA is not the only signal pathway that regulates endothelial barrier function downstream of GPBAR. As we previously showed, GPBAR agonism in endothelial cells results in activation of PI3K/Akt pathway (Kida et al., 2013), and this signal is reported to enhance endothelial barrier function (Singleton et al., 2005). More

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detailed studies are needed to better understand the involvement of PI3K/Akt and other signals in the GPBAR-mediated endothelial barrier enhancement.

In conclusion, we found the novel function of a bile acid receptor GPBAR to enhance endothelial barrier integrity. The current report would provide useful information to understand the role of serum bile acid and GPBAR in vascular functions.

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Authorship Contributions

Participated in research design: Kida and Murata.

Conducted experiments: Kida and Omori.

Contributed new reagents or analytic tools: Hori and Ozaki

Performed data analysis: Kida, Omori, and Murata.

Wrote or contributed to the writing of the manuscript: Kida and Murata.

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Footnotes

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Figure Legends

Figure 1

GPBAR agonism enhances the endothelial barrier function.

(A) Typical trace of TER changes in BAECs induced by TLCA (3 μM), as well as a positive control, forskolin (1 μM). (B) Maximal responses in TER in BAECs induced by various concentration of TLCA (0.1-30 μM), as well as forskolin (1 μM) (n=4-11). (C) The relative amount of FITC-dextran which has passed through the control siRNA or GPBAR siRNA-transfected BAEC monolayer (n=4). The effective knockdown of GPBAR expression was confirmed by Western blot. ** p<0.01, *** p<0.001 compared with vehicle or none. †† p<0.01, ††† p<0.001 compared between the indicated groups.

Figure 2

GPBAR-mediated endothelial barrier enhancement is dependent on protein kinase A (PKA) and Rac1 activity.

(A) The relative amount of intracellular cAMP in BAECs. The cells were pretreated with 3-isobutyl-1-methylxanthine (200 μM, 5 min), and then treated with each agent (n=3). (B) The relative amount of fluorescein isothiocyanate (FITC)-dextran passed through the BAEC monolayer treated with each agent (n=4-8). PKAi, a protein kinase A inhibitory peptide. ** p<0.01, *** p<0.001 compared with vehicle or none. †† p<0.01 compared with vehicle +

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thrombin. ‡ $p<0.05$, ‡‡ $p<0.01$ compared between the indicated groups.

Figure 3

GPBAR agonism induces actin cytoskeleton rearrangement.

Typical pictures of filamentous actin stained with rhodamine phalloidin (left, red) and VE-cadherin immunostained with Alexa Fluor 488 (middle, green). The merged pictures are shown in right. Cell nuclei were also stained with 4',6-diamidino-2-phenylindole (blue). n=6. Scale bar: 25 μm.

Figure 4

GPBAR agonism inhibits vascular permeability *in vivo.*

(A) Typical pictures and (B) quantitative representation of the croton oil-induced dye extravasation and the inhibitory effect of TLCA on it (n=11). (C) Typical pictures and (D) quantitative representation of the vascular endothelial growth factor (VEGF)-induced dye extravasation and the inhibitory effect of TLCA on it (n=9-10). * $p<0.05$ compared with croton oil or VEGF.

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Figure 1

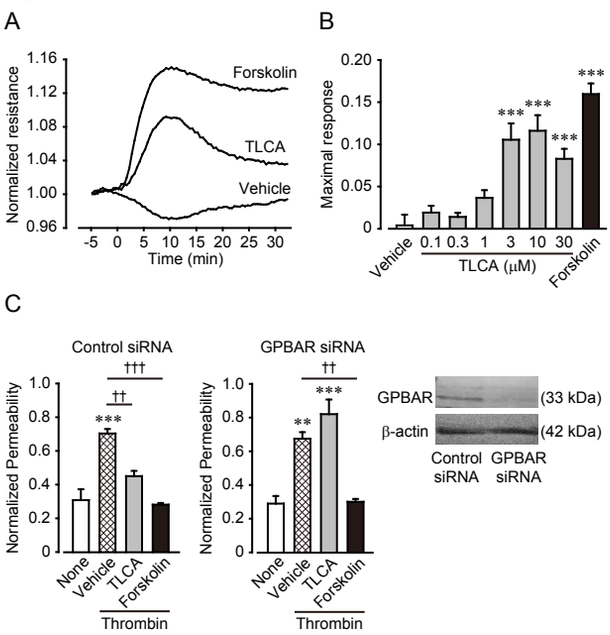

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Figure 2

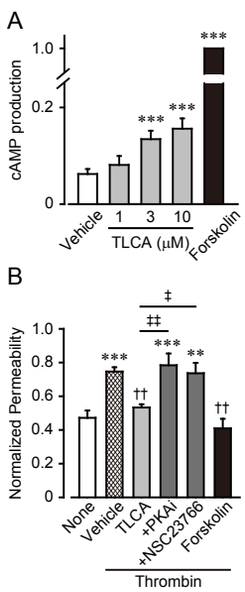

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Figure 3

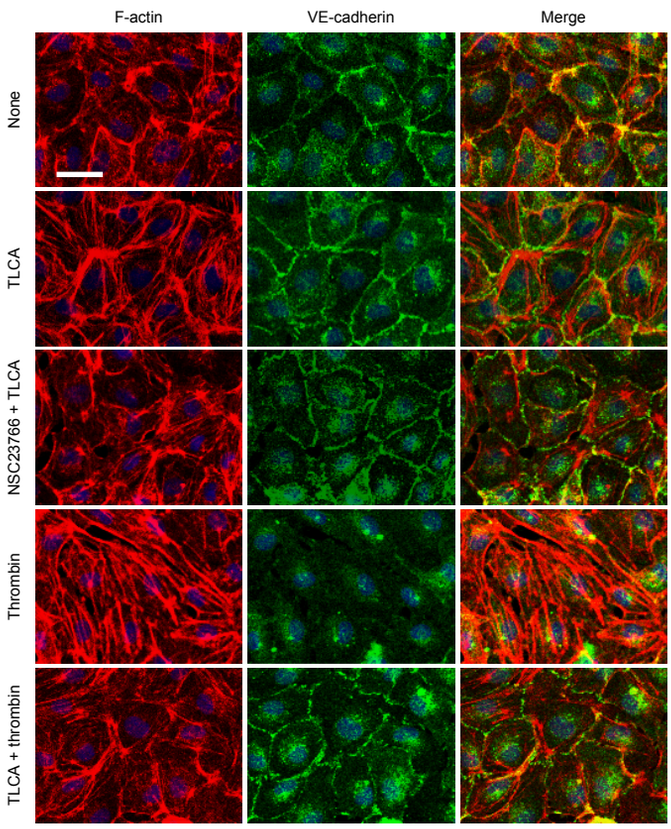

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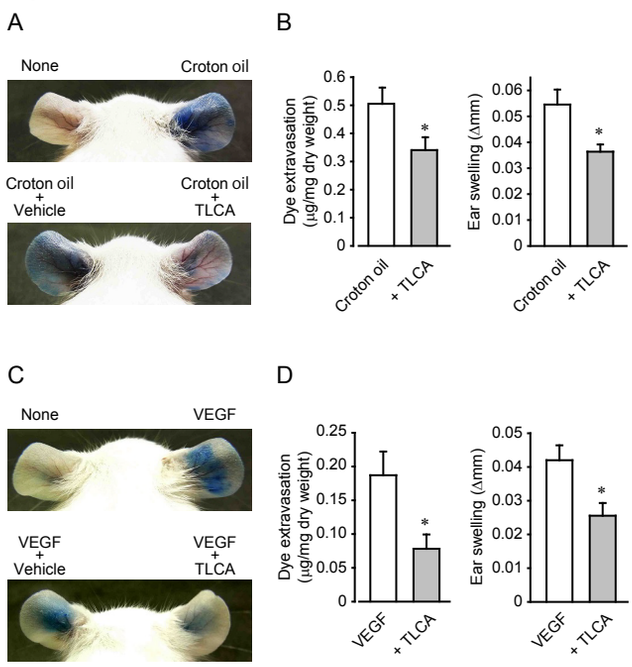
Figure 4



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