Stimulation of G Protein–Coupled Bile Acid Receptor Enhances Vascular Endothelial Barrier Function via Activation of Protein Kinase A and Rac1 Ⓢ

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ABSTRACT

Bile acids are end products of cholesterol metabolism, and they constantly exist at high concentrations in the 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 the 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 the 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.

Introduction

Bile acids are end products of cholesterol metabolism. After being 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 the liver through the 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. (1999) first identified a nuclear receptor called farnesoid X receptor as a distinct bile acid receptor that is highly expressed in the liver and intestine. The following studies reported that farsenoid X receptor–mediated signaling regulates the transcription of genes involved in bile acid, lipid, and glucose homeostasis (Lefebvre et al., 2009). Thereafter, Maruyama et al. (2002) and Kawamata et al. (2003) discovered that a G protein–coupled receptor, GPBAR, is another bile acid receptor that is ubiquitously expressed in many types of tissues, such as the gallbladder, intestine, placenta, and spleen.

Some in vitro studies showed that GPBAR agonism suppresses proinflammatory 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; Wang et al., 2011). Thus, GPBAR-mediated signaling is currently highlighted as a potential therapeutic target for inflammatory diseases in enterohepatic tract.

Whereas the enterohepatic tract contains large amounts of bile acids, systemic blood also contains a 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 a great increase in serum bile acids, up to 100 μM (Mashige et al., 1981; Everson, 1987). Therefore, in health or in hepatic diseases, the innermost layer of vasculature, the 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).

ABBREVIATIONS: BAEC, bovine aortic endothelial cell; Epac, exchange protein directly activated by cAMP; FITC, fluorescein isothiocyanate; GPBAR, G protein–coupled bile acid receptor; NSC23766, 6-*N*-[2-[5-(diethylamino)pentan-2-ylamino]-6-methylpyrimidin-4-yl]-2-methylquinoline-4,6-diamine trihydrochloride; 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.

Vascular endothelial cells form a semiselective 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, which 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 the 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 the tumor microenvironment by suppressing vascular permeability (Murata et al., 2008). Thus, discovery of a novel endothelial barrier modulator and clarification of 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/protein kinase A (PKA)/Rac1-dependent signal pathway.

Materials and Methods

Chemicals. The chemicals used were as follows: TLCA, croton oil, and 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO); penicillin-streptomycin, dextran fluorescein 70,000 mol. wt. anionic lysine fixable [fluorescein isothiocyanate (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 (Merck 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 Hanks' balanced salt solution, endothelial cells were obtained from the luminal surface of aortas by digestion with trypsin-EDTA solution and scratching with a 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 passages 3–8 after starvation with the serum-free medium for 16–24 hours. For gene knockdown of GPBAR, BAECs at 40–60% confluence were transfected with a set of four RNAi small interfering RNAs (siRNAs) targeting GPBAR (Invitrogen, 25 nM each), using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. Cells were used for experiments at least 90 hours 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/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 seconds. To normalize the data, the TER values were divided by the value before stimulation.
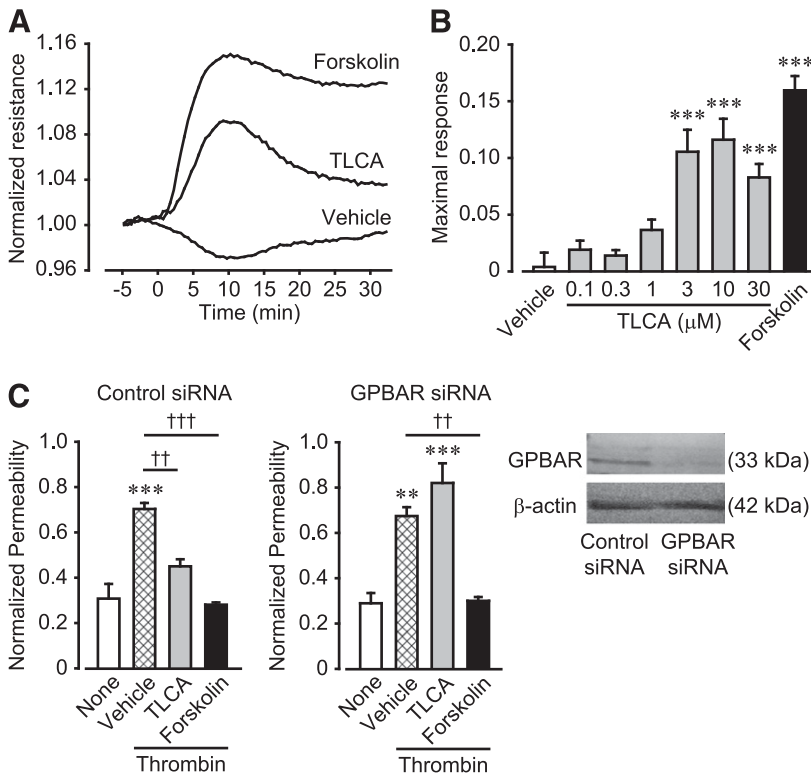

Fig. 1. GPBAR agonism enhances the endothelial barrier function. (A) Typical trace of TER changes in BAECs induced by TLCA (3 µM), as well as a positive control, forskolin (1 µM). (B) Maximal responses in TER in BAECs induced by various concentration of TLCA (0.1–30 µM), as well as forskolin (1 µM) (n = 4–11). (C) The relative amount of FITC-dextran that 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.

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 minutes after the thrombin treatment. Thirty minutes after the addition of FITC-dextran, 100 μl of media were collected from the lower chamber. The amount of FITC-dextran that had passed through the cell monolayer was determined by a fluorescence spectrophotometer (Wallac 1420 ARVOsx; Perkin-Elmer, Waltham, MA).

Immunostaining. BAECs on gelatin-coated sterilized glass coverslips were fixed with 4% paraformaldehyde for 5 minutes, followed by permeabilizing and blocking with 0.1% Triton X-100 and 3% bovine serum albumin for 30 minutes. The cells then were incubated with goat polyclonal anti-vascular endothelial (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 minutes. After stimulation, cells were lysed and the cAMP concentration in the lysate was measured by enzyme immunoassay according to the manufacturer's instruction (cAMP enzyme-linked immunosorbent assay kit; Enzo Life Sciences, Farmingdale, NY). The intracellular cAMP level 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 guidelines. 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 minutes, Evans blue (30 mg/kg) was injected intravenously. In another series of experiments, croton oil (2.5% in acetone) was applied on both sides of the ear. After 80 minutes, TLCA was intradermally injected as described herein, followed by intravenous injection of Evans blue 10 minutes later. Thirty minutes 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).

Statistical Analysis. The results are expressed as means ± S.E.M. Statistical evaluations of the data were 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.

Results

GPBAR Agonism Enhances the Endothelial Barrier Function. Transendothelial electrical resistance (TER) was measured as an index of endothelial barrier integrity. As shown in Fig. 1A, treatment with a bile acid that 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 10–15 minutes after stimulation, lasted at least 30 minutes, and the value gradually

returned to the basal level. The largest responses were obtained by 3–10 μM TLCA (Fig. 1B). 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 Fig. 1C, treatment with thrombin (1 U/ml, 40 minutes) significantly increased the FITC-labeled dextran leakage through a BAEC monolayer. This response was significantly inhibited by pretreatment with TLCA (3 μM, 5 minutes). 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 minutes) almost completely blocked the thrombin-induced dextran leakage in cells transfected with either control or GPBAR siRNA.

GPBAR Agonism Increases Intracellular cAMP Level. It has been reported that GPBAR agonism increases intracellular cAMP via activation of Gα_s (Maruyama et al., 2002; Kawamata et al., 2003; Keitel et al., 2007). As shown in Fig. 2A, treatment with TLCA (3–10 μM, 15 minutes) significantly increased intracellular cAMP amount in BAECs.
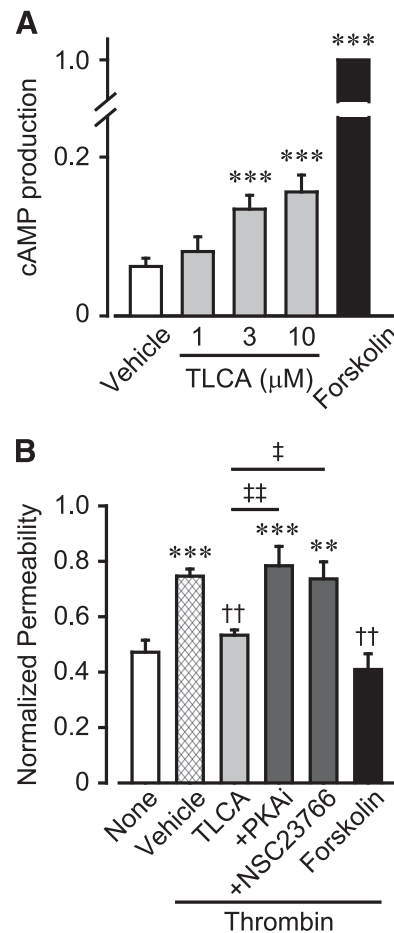

Fig. 2. GPBAR-mediated endothelial barrier enhancement is dependent on PKA and Rac1 activity. (A) The relative amount of intracellular cAMP in BAECs. The cells were pretreated with 3-isobutyl-1-methylxanthine (200 μM, 5 minutes) and then treated with each agent (n = 4–6). (B) The relative amount of FITC-dextran 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 + thrombin. ‡$P < 0.05$, ‡‡$P < 0.01$ compared between the indicated groups.

The dose-response relationship was similar to that of trans-endothelial resistance increase induced by TLCA (1–10 μM) (Fig. 1B). Forskolin (1 μM, 5 minutes) 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 Fig. 2B, the inhibitory effect of TLCA (3 μM, 5 minutes) 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 minutes). The effect of TLCA was also abolished by pretreatment with a Rac1 inhibitor, NSC23766, which interferes with Rac-specific guanine nucleotide exchange factors Tiam1 and Trio (Gao et al., 2004) (100 μM, 30 minutes). These results suggest the contributions 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 the adherens junction. In the adherens junction, VE-cadherin mediates adhesion of adjacent endothelial cells. Actin band recruited to the 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 (Fig. 3, first row), BAECs treated with TLCA (3 μM, 15 minutes) displayed increased formation of actin bundle in the cortical region (Fig. 3, second row). Pretreatment with a Rac1 inhibitor NSC23766 (100 μM, 30 minutes) inhibited the TLCA-induced actin cytoskeleton rearrangement (Fig. 3, third row). In BAECs treated with thrombin (1 U/ml, 20 minutes), 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 (Fig. 3, fourth row). These observations indicate that thrombin disrupted the adherens junction and endothelial barrier integrity. Pretreatment with TLCA (3 μM, 5 minutes)
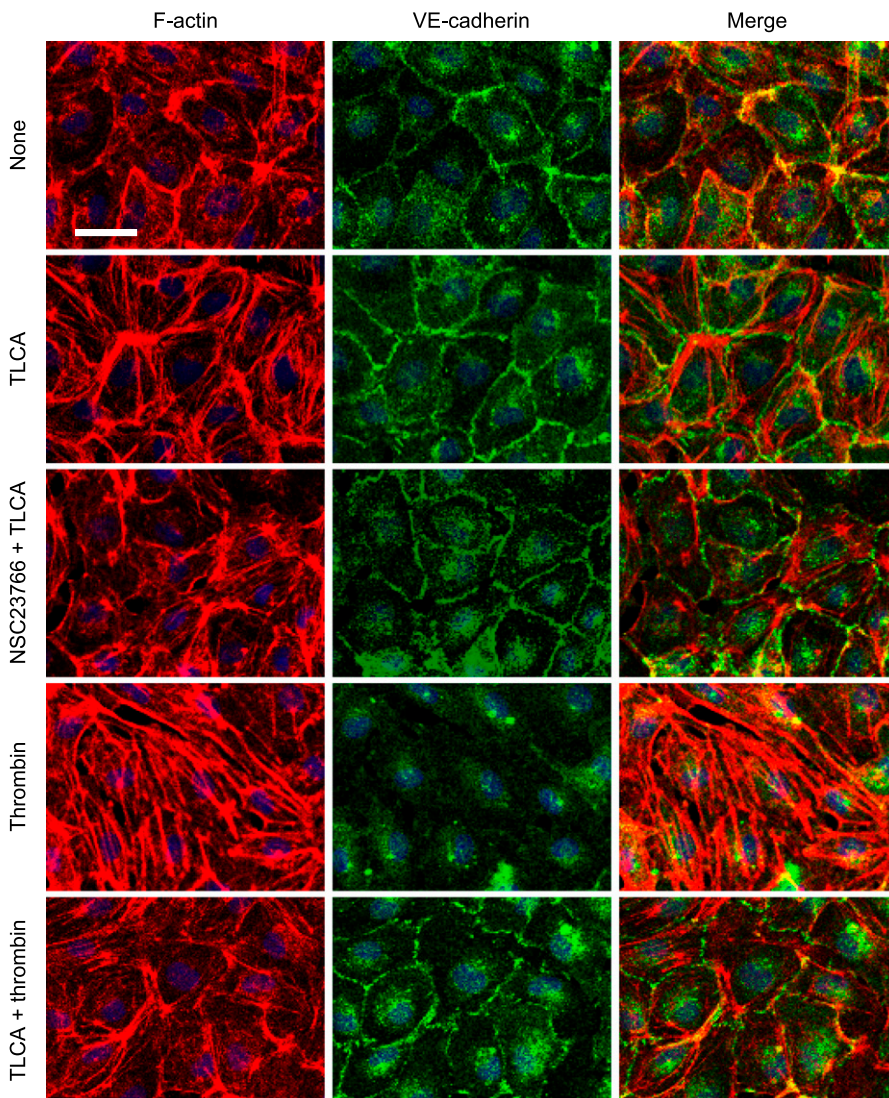

Fig. 3. GPBAR agonism induces actin cytoskeleton rearrangement. Typical pictures of filamentous actin stained with rhodamine phalloidin (left, red) and VE-cadherin immunostained with Alexa Fluor 488 (middle, green). The merged pictures are shown in right. Cell nuclei were also stained with 4′,6-diamidino-2-phenylindole (blue). $n = 6$. Scale bar: 25 μm.

inhibited the thrombin-induced actin stress fiber formation. This was accompanied with the recovery of VE-cadherin localization at the cell border (Fig. 3, fifth row).

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 hours), on a mouse ear increased Evans blue dye extravasation and the tissue thickness. Local administration of TLCA (100 ng, 1 hour 20 minutes after the oil application) significantly reduced the croton oil–induced dye extravasation and ear swelling (Fig. 4, A and B). VEGF directly stimulates endothelial cell and causes hyperpermeability without inflammation (Esser et al., 1998). Local administration of VEGF (30 ng, 35 minutes) increased dye extravasation and ear thickness. Concurrent administration of TLCA (100 ng) significantly reduced the VEGF-induced dye extravasation and ear swelling (Fig. 4, A and B).

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 (see Supplementary Fig. 1).

The anti-inflammatory roles of GPBAR in the liver are well documented. GPBAR deficiency exacerbated liver injury induced by lipopolysaccharide administration, accompanied by 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-mediated signaling can alleviate tissue inflammation by suppressing vascular permeability (Fig. 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 mM) of bile acid, deoxycholic acid or taurochenodeoxycholic acid, increased cerebrovascular permeability by disrupting plasma membrane and intercellular junction of endothelial cells in the 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 might not be due to GPBAR-mediated signaling but possibly are due to the physicochemical property of bile acids as detergents. At high concentrations, that is, in the range of 100 μM–1 mM, 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
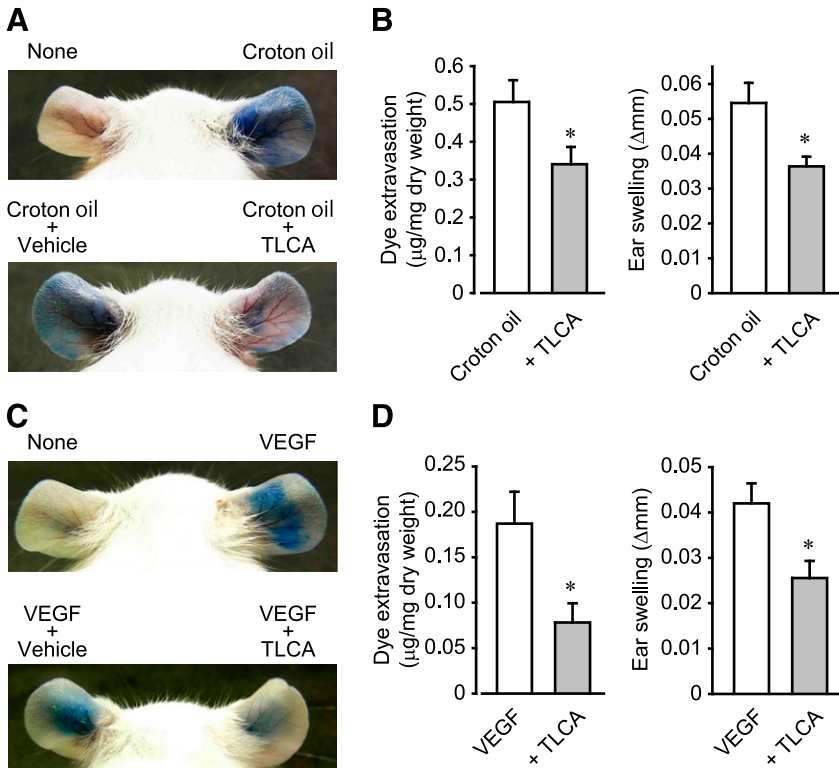

Fig. 4. GPBAR agonism inhibits vascular permeability in vivo. (A) Typical pictures and (B) quantitative representation of the croton oil–induced dye extravasation and the inhibitory effect of TLCA on it (n = 11). (C) Typical pictures and (D) quantitative representation of the VEGF-induced dye extravasation and the inhibitory effect of TLCA on it (n = 9–10). *$P < 0.05$ compared with croton oil or VEGF.

(Thomas et al., 2009), and an increase in energy expenditure in brown adipocytes and skeletal muscle cells (Watanabe et al., 2006), In this study, GPBAR agonism increased intracellular cAMP content in vascular endothelial cells (Fig. 2A). We also demonstrated that GPBAR-mediated endothelial barrier enhancement is dependent on a cAMP effector PKA activation (Fig. 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 the 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). Consistent with this, GPBAR-mediated endothelial barrier enhancement was accompanied with cortical actin polymerization, which was abolished by Rac1 inhibition (Fig. 3). Taken together, these results led us to conclude that GPBAR agonism induces cAMP/PKA/Rac1-dependent actin cytoskeleton rearrangement and enhances endothelial barrier function.

It is noteworthy that the cAMP content in forskolin-treated BAECs was much ($\sim5.7\times$) higher than that in TLCA-treated cells (Fig. 2A). Likewise, the endothelial barrier enhancement induced by forskolin was stronger than that induced by TLCA (Fig. 1), but this difference was not so remarkable as 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 the phosphoinositide 3-kinase/Akt pathway (Kida et al., 2013), and this signal is reported to enhance endothelial barrier function (Singleton et al., 2005). More detailed studies are needed to understand more clearly the involvement of phosphoinositide 3-kinase/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.

Authorship Contributions

Participated in research design: Kida, Murata.
Conducted experiments: Kida, Omori.
Contributed new reagents or analytic tools: Hori, Ozaki.
Performed data analysis: Kida, Omori, Murata.
Wrote or contributed to the writing of the manuscript: Kida, Murata.

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