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Anaphylactic shock depends on endothelial G_q/G_{11}

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Anaphylactic shock is a severe allergic reaction involving multiple organs including the bronchial and cardiovascular system. Most anaphylactic mediators, like platelet-activating factor (PAF), histamine, and others, act through G protein–coupled receptors, which are linked to the heterotrimeric G proteins G_q/G_{11}, G_{12}/G_{13}, and G_i. The role of downstream signaling pathways activated by anaphylactic mediators in defined organs during anaphylactic reactions is largely unknown. Using genetic mouse models that allow for the conditional abrogation of G_q/G_{11}- and G_{12}/G_{13}-mediated signaling pathways by inducible Cre/loxP-mediated mutagenesis in endothelial cells (ECs), we show that G_q/G_{11}-mediated signaling in ECs is required for the opening of the endothelial barrier and the stimulation of nitric oxide formation by various inflammatory mediators as well as by local anaphylaxis. The systemic effects of anaphylactic mediators like histamine and PAF, but not of bacterial lipopolysaccharide (LPS), are blunted in mice with endothelial $G\alpha_q/G\alpha_{11}$ deficiency. Mice with endothelium-specific $G\alpha_q/G\alpha_{11}$ deficiency, but not with $G\alpha_{12}/G\alpha_{13}$ deficiency, are protected against the fatal consequences of passive and active systemic anaphylaxis. This identifies endothelial G_q/G_{11}-mediated signaling as a critical mediator of fatal systemic anaphylaxis and, hence, as a potential new target to prevent or treat anaphylactic reactions.

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Abbreviations used: BAC, bacterial artificial chromosome; cGMP, cyclic guanosine monophosphate; EC, endothelial cell; eNOS, endothelial NOS; GPCR, G protein–coupled receptor; HSA, human serum albumin; L-NAME, Nω-nitro-L-arginine methylester; LPA, lysophosphatidic acid; MLC, myosin light chain; NO, nitric oxide; NOS, NO synthase; PAF, platelet-activating factor.

Anaphylaxis is a serious allergic reaction with a rapid onset and potentially fatal outcome. It can be induced by insect venoms, food, drugs, latex, and other allergens and may affect as much as 1–15% of the population with an increasing prevalence (1–4). Anaphylaxis is characterized by severe hypotension, vascular leakage, cardiac arrhythmia, hypothermia, and bronchial constriction as well as gastrointestinal and skin symptoms. In particular, cardiovascular and pulmonary dysfunction often lead to death.

Most anaphylactic reactions are caused by IgE-mediated hypersensitivity reactions resulting from cross-linking of allergen-specific IgE molecules bound to the IgE receptor on tissue mast cells and basophils. However, an alternative mechanism involving IgG and macrophages has also been described (5). The allergen-induced activation of mast cells results in the formation and release of multiple mediators that are responsible for the acute and potentially life-threatening symptoms of anaphylactic reactions (6). These mediators include preformed substances like histamine or the proteases tryptase and chymase, which are released upon mast cell activation, and lipid mediators like platelet-activating factor (PAF), cysteinyl leukotrienes, or prostaglandin D_2, which are newly synthesized (7). In the context of anaphylactic reactions, these mediators have been shown to act on multiple organs. PAF, histamine, and tryptase activate endothelial cells (ECs) to induce vasodilatation and increased capillary leakage (8–12). PAF, especially, can activate leukocytes (13, 14), and most anaphylactic mediators induce the contraction

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of bronchial smooth muscles (9, 15, 16). Other organs and cells, such as the heart (9, 10, 17), nervous system (9, 18), platelets (10, 19), or vascular smooth muscle cells (9, 16), are also directly affected by anaphylactic mediators.

Most of the anaphylactic mediators exert their effects through G protein–coupled receptors (GPCRs), which are linked to heterotrimeric G proteins of the G_i, G_q/G_{11}, and G_{12}/G_{13} families (8, 12, 20–24). The G proteins G_q/G_{11} couple receptors to β isoforms of phospholipase C resulting in inositol-1,4,5-trisphosphate–mediated mobilization of intracellular Ca^{2+} and diacylglycerol-dependent activation of protein kinase C, whereas G_{12}/G_{13} couple receptors to the activation of the Rho/Rho kinase–mediated signaling pathway. G_i-type G proteins couple receptors in an inhibitory fashion to adenylyl cyclase and, in addition, serve as the major source of G protein βγ complexes which can regulate a variety of channels and enzymes (25–28).

Many mediators of the effector phase of anaphylactic reactions have been described, and their cellular effects in the heart and the vascular, bronchial, and immune systems have been analyzed. However, the downstream signaling pathways mediating the effects in defined organs during anaphylaxis remain largely unclear. In this study, we analyzed the role of defined endothelial G protein–mediated signaling pathways in anaphylaxis. By conditional mutagenesis of genes encoding particular G protein α subunits, we show that the endothelium-specific ablation of the G_q/G_{11}-mediated signaling pathway, but not the G_{12}/G_{13}-mediated signaling pathway, blocks nitric oxide (NO) formation and loss of the endothelial barrier function in response to various vasoactive stimuli. Lack of endothelial G_q/G_{11} also protects mice from the deleterious consequences of PAF injection as well as of active and passive systemic anaphylaxis. Our data identify endothelial G_q/G_{11}-mediated signaling as an essential mediator of systemic anaphylaxis.

RESULTS

Endothelial effects of inflammatory mediators acting via GPCRs are mediated primarily by G_q/G_{11}

To analyze the role of G_q/G_{11}- and G_{12}/G_{13}-mediated signaling in endothelial responses to vasoactive mediators, we generated ECs lacking the α subunits of G_q/G_{11} or G_{12}/G_{13}. We have previously generated floxed alleles of the genes encoding $G\alpha_q$ (*Gnaq*) and $G\alpha_{13}$ (*Gna13*) which allow the conditional inactivation of these genes in $G\alpha_{11}$- or $G\alpha_{12}$-deficient backgrounds (29, 30). To induce $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ double deficiency, we prepared pulmonary microvascular ECs from WT, $Gnaq^{flox/flox}$;$Gna11^{-/-}$, and $Gna12^{-/-}$;$Gna13^{flox/flox}$ mice and infected them with an adenovirus transducing the recombinase Cre. As shown in Fig. 1 A, expression of Cre recombinase in $Gnaq^{flox/flox}$;$Gna11^{-/-}$ or $Gna12^{-/-}$;$Gna13^{flox/flox}$ ECs resulted in $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ deficiency, respectively.

We then analyzed the role of G_q/G_{11}- and G_{12}/G_{13}-mediated signaling in the regulation of endothelial NO formation by known endothelial stimuli acting via GPCRs. To determine NO-dependent activation of guanylyl cyclase, we performed a transfer bioassay in which (cyclic guanosine monophosphate) cGMP levels were determined in RFL6 fibroblasts incubated with WT, $G\alpha_q/G\alpha_{11}$-deficient, or $G\alpha_{12}/G\alpha_{13}$-deficient lung ECs treated without or with thrombin, PAF, or ionomycin (Fig. 1 B). Although thrombin and PAF induced a significant increase in cGMP levels in cocultures containing WT and $G\alpha_{12}/G\alpha_{13}$-deficient ECs, the effects in cocultures containing $G\alpha_q/G\alpha_{11}$-deficient ECs were strongly reduced. None of the stimuli induced guanylyl cyclase activation when added to RFL6 fibroblasts or ECs alone (unpublished data). The effect of ionomycin was not affected by $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ deficiency in ECs. This indicates that G_q/G_{11}, but not G_{12}/G_{13}, are critically involved in thrombin- and PAF-induced NO-dependent stimulation of guanylyl cyclase activity.

Because the phosphorylation state of the myosin light chain (MLC) is a critical determinant of endothelial contractility, we analyzed the effect of thrombin on MLC phosphorylation in WT, $G\alpha_q/G\alpha_{11}$-deficient, and $G\alpha_{12}/G\alpha_{13}$-deficient ECs. As shown in Fig. 1 (C and E), thrombin induced a rapid increase in MLC phosphorylation that was maximal after ~3 min, whereas thrombin had no effect on MLC phosphorylation in ECs lacking $G\alpha_q/G\alpha_{11}$. The defect of thrombin-induced MLC phosphorylation in $G\alpha_q/G\alpha_{11}$-deficient cells could be rescued by adenovirus-mediated expression of $G\alpha_q$ (Fig. 1 D). Lack of $G\alpha_{12}/G\alpha_{13}$ did not completely block thrombin-induced MLC phosphorylation but led to a reduced and more transient response to thrombin. Interestingly, the abrogation of thrombin-induced MLC phosphorylation in cells lacking $G\alpha_q/G\alpha_{11}$ was not accompanied by any defect in thrombin-induced RhoA activation, whereas thrombin-induced RhoA activation was abrogated in ECs lacking $G\alpha_{12}/G\alpha_{13}$ (Fig. 1 F).

Generation of mice with EC-specific $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ deficiency

For in vivo experiments, we restricted $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ double deficiency to ECs by using a bacterial artificial chromosome (BAC) transgenic mouse line that expresses a fusion protein of the Cre recombinase with the modified estrogen receptor binding domain (*Cre*ER^{T2}) (31) under the control of the *tie2* promoter (see Materials and methods). The inducible endothelium-specific Cre transgenic mouse line (*tie2-Cre*ER^{T2}) did not show any Cre activity in the absence of tamoxifen when crossed with the *Gt(ROSA)26Sor* Cre reporter mouse line (Fig. 2 A). However, after treatment of animals with tamoxifen, ECs showed Cre-mediated recombination, indicating that Cre had been activated with high efficacy. Cre-mediated recombination was exclusively observed in ECs of various organs (Fig. 2 A). The lack of $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ in ECs of tamoxifen-treated *tie2-Cre*ER^{T2};$Gnaq^{flox/flox}$;$Gna11^{-/-}$ (EC-$G\alpha_q/G\alpha_{11}$-KO) and *tie2-Cre*ER^{T2};$Gna12^{-/-}$;$Gna13^{flox/flox}$ (EC-$G\alpha_{12}/G\alpha_{13}$-KO) mice was verified by Western blotting of pulmonary EC lysates from the respective mouse lines (Fig. 2 B). Western blot analysis of platelets, leukocytes, and vascular smooth muscle cells showed no difference between WT and EC-$G\alpha_q/G\alpha_{11}$-KO mice with

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regard to $G\alpha_q/G\alpha_{11}$ expression (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20082150/DC1).

Blockade of endothelial G_q/G_{11}-mediated signaling, but not G_{12}/G_{13}-mediated signaling, inhibits local extravasation in response to various stimuli

We then analyzed the effect of various vasoactive substances on the vascular permeability in EC-$G\alpha_q/G\alpha_{11}$-KO and EC-$G\alpha_{12}/G\alpha_{13}$-KO mice. In the absence of any intradermal injection, the vascular leakage of Evans blue given i.v. was negligible (unpublished data). Intradermal injection of lysophosphatidic acid (LPA), the protease-activated receptor 1 (PAR-1)–activating peptide SFLLRN-NH_2, histamine, PAF, and leukotriene C_4 each induced a dose-dependent increase in the leakage of Evans blue dye (Fig. 3, A and B; Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20082150/DC1). In addition, intradermal injection of control buffer resulted in a small extravasation of Evans blue that was significantly smaller than the one seen in response to the vasoactive stimuli, suggesting that the manipulation resulted in the local release or production of some active mediators. Both basal vascular permeability and stimulus-induced increases in vascular permeability were severely reduced in mice with endothelial-specific $G\alpha_q/G\alpha_{11}$ deficiency but not

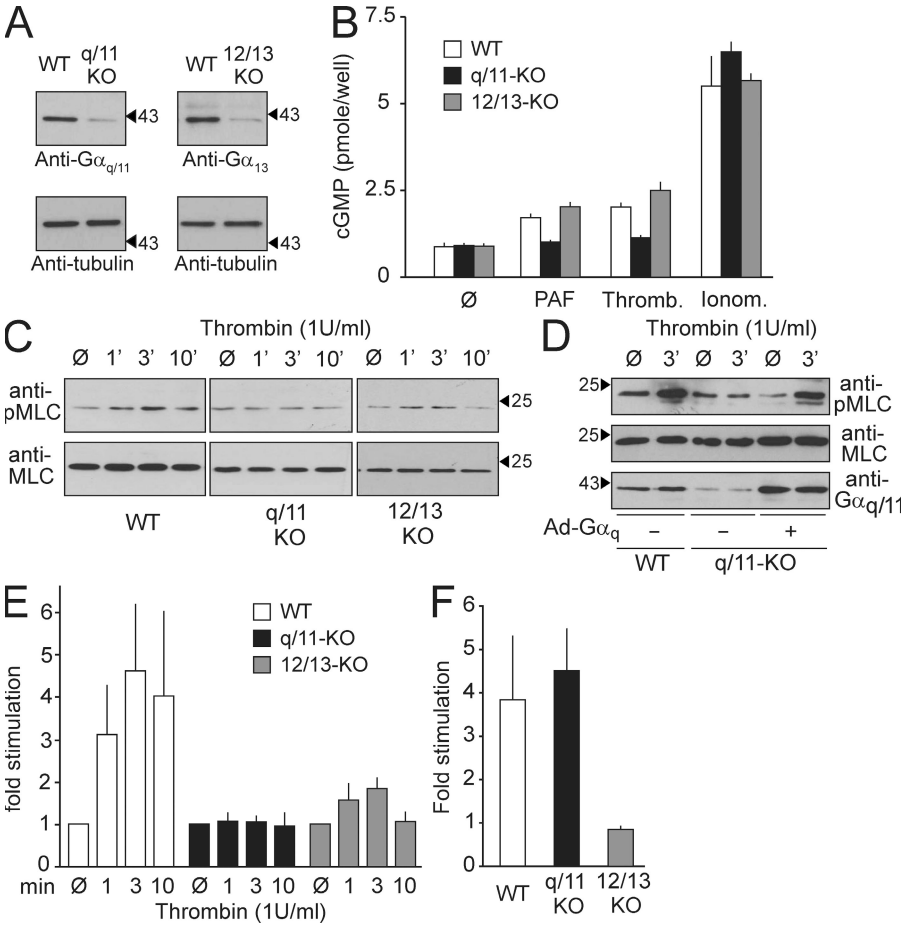

Figure 1. The role of G_q/G_{11} and G_{12}/G_{13} in the regulation of NO production and MLC phosphorylation in pulmonary ECs. (A) Lysates of pulmonary ECs prepared from WT, *Gnaq*$^{flox/flox}$;*Gna11*$^{-/-}$ (q/11-KO), or *Gna12*$^{-/-}$;*Gna13*$^{flox/flox}$ (12/13-KO) mice were infected with Cre-transducing adenovirus and were analyzed by Western blotting with antibodies directed against $G\alpha_q/G\alpha_{11}$, $G\alpha_{13}$, or α-tubulin. Arrowheads indicate the position of the 43-kD marker protein. The presented data are representative of at least five experiments performed with samples from different animals. (B) WT $G\alpha_q/G\alpha_{11}$-deficient (q/11-KO) and $G\alpha_{12}/G\alpha_{13}$-deficient (12/13-KO) ECs were incubated without and with 1 U/ml thrombin (thromb.), 100 nM PAF, or 100 nM ionomycin (ionom.), and NO bioavailability was assessed in a transfer bioassay by determining cGMP production in detector RFL6 fibroblasts by radioimmunoassay. Shown are the results of three separate experiments (mean values ± SEM). (C–E) WT, $G\alpha_q/G\alpha_{11}$- (q/11-KO), and $G\alpha_{12}/G\alpha_{13}$-deficient (12/13-KO) ECs were incubated in the absence or presence of 1 U/ml thrombin for 1, 3, or 10 min, and the amount of phosphorylated MLC (pMLC) was determined using a phosphorylation site-specific antibody (see Materials and methods). Where indicated (Ad-$G\alpha_q$ +), cells had been transfected with $G\alpha_q$ using an adenoviral transfection system. Shown are representative Western blots of cell lysates using the indicated antibodies (C and D) and the results of the densitometric evaluation of three independently performed experiments (E). Shown are mean values ± SEM. Arrowheads indicate the position of the 25- or 43-kD (D, bottom) marker proteins. (F) Effect of 1 U/ml thrombin on RhoA activity in WT, $G\alpha_q/G\alpha_{11}$-deficient (q/11-KO), and $G\alpha_{12}/G\alpha_{13}$-deficient lung ECs (12/13-KO). Data are from three independently performed experiments (mean values ± SD).

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in mice lacking $G\alpha_{12}/G\alpha_{13}$ in ECs. The small remaining response to the PAR1-activating peptide observed in EC-$G\alpha_q/G\alpha_{11}$-KO mice was not further reduced in mice lacking both $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ in ECs (Fig. 3 B). To test the regulation of the endothelial barrier in a more complex model of local anaphylaxis, we sensitized mice by intradermal injection of anti-DNP IgE antibodies and subsequently injected DNP–human serum albumin (HSA) systemically. In addition, in this IgE-mediated model of local anaphylaxis opening of the endothelial barrier was not significantly affected in EC-$G\alpha_{12}/G\alpha_{13}$-KO mice, whereas mice with endothelium-specific $G\alpha_q/G\alpha_{11}$ deficiency showed strongly reduced vascular permeability (Fig. 3 C). Thus, local regulation of vascular permeability requires G_q/G_{11}-mediated signaling in ECs but not G_{12}/G_{13}.

Systemic effects of histamine and PAF but not of LPS are blocked in EC- $G\alpha_q/G\alpha_{11}$-KO mice

i.v. injection of histamine induced a rapid and transient drop in the systolic blood pressure to levels of ~50 mmHg in WT mice (Fig. 4 A). Normal values were restored ~90 min after the application of histamine. In EC-$G\alpha_q/G\alpha_{11}$-KO mice, the same dose decreased blood pressure for only ~20 min with maximal hypotensive values of ~90 mmHg, whereas mice with endothelium-specific $G\alpha_{12}/G\alpha_{13}$ deficiency responded comparable to WT mice (Fig. 4 A). The strongly reduced hypotensive response of EC-$G\alpha_q/G\alpha_{11}$-KO mice to histamine was not caused by a general defect in the regulation of the vascular tone, as is indicated by the indistinguishable response of WT, EC-$G\alpha_q/G\alpha_{11}$-KO, and EC-$G\alpha_{12}/G\alpha_{13}$-KO mice to the NO-donor sodium nitroprusside as well as to the

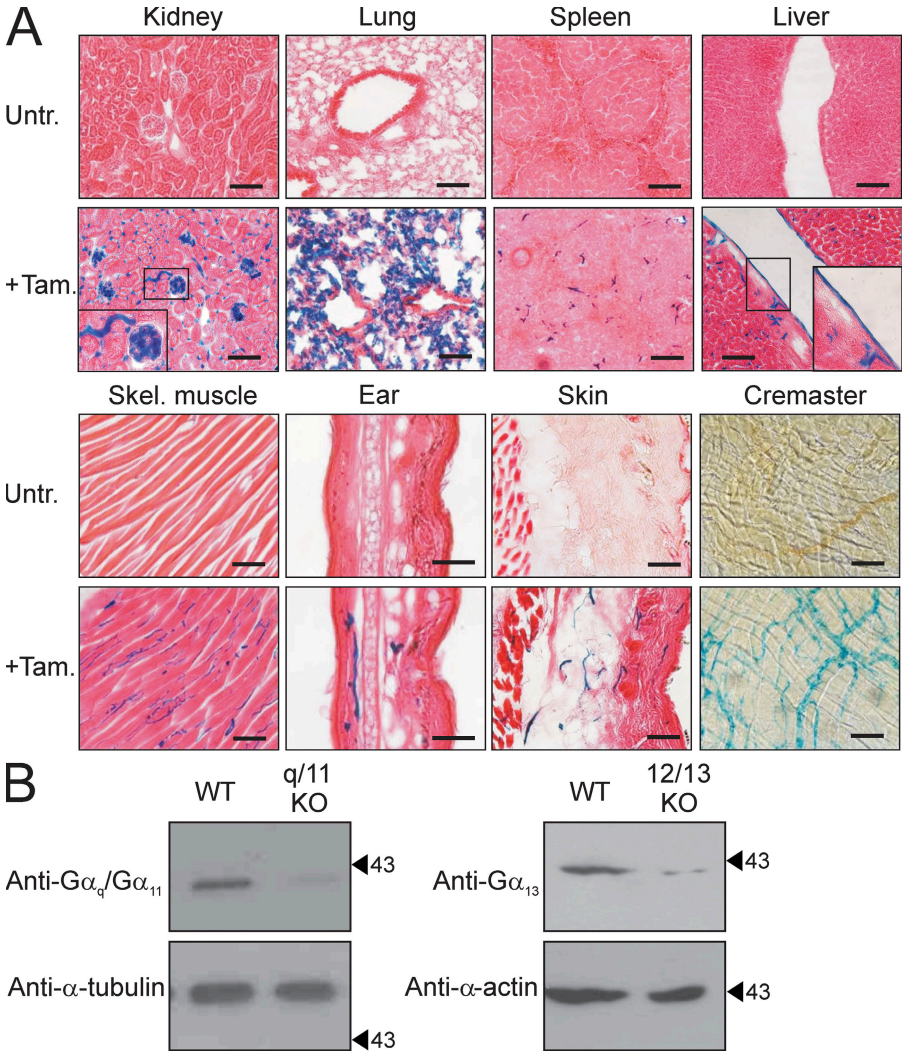

Figure 2. Generation of mice with EC-specific $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ deficiency. (A) Gt(ROSA26)SorCre reporter mice carrying the *tie2-Cre*ER^{T2} transgene were treated with vehicle alone (untr.) or with tamoxifen (+Tam.) and then killed. The indicated organs were sectioned and stained for β-galactosidase activity. Bars, 50 µm. Inserts represent 2× magnifications of the indicated areas. (B) Lysates from lung ECs prepared from tamoxifen-treated WT, EC-$G\alpha_q/G\alpha_{11}$-KO (q/11-KO), or EC-$G\alpha_{12}/G\alpha_{13}$-KO (12/13-KO) mice were analyzed by Western blotting with antibodies directed against $G\alpha_q/G\alpha_{11}$, $G\alpha_{13}$, α-tubulin, or β-actin. Arrows indicate the position of the 43-kD marker protein. Shown are representative data from three independently performed experiments.

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NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; Fig. 4, B and C).

We then tested the effect of endothelium-specific $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ deficiency on the systemic response to PAF, which is thought to be a critical mediator of anaphylactic shock (32–34). i.v. injection of PAF induced severe hypothermia (Fig. 4 D) and resulted in the death of WT and EC-$G\alpha_{12}/G\alpha_{13}$-KO mice within 20 min (Fig. 4 E). However, mice with endothelial $G\alpha_q/G\alpha_{11}$ deficiency were protected from PAF-induced shock, and all of the animals assessed survived the injection of PAF with only a transient drop in body temperature (Fig. 4, D and E). Mice lacking only $G\alpha_{11}$ demonstrated an intermediate phenotype with more severe hypothermia than EC-$G\alpha_q/G\alpha_{11}$-KO mice and a survival rate of only 25% (two of eight tested animals; unpublished data). Interestingly, the intraperitoneal injection of the endotoxin LPS induced a severe hypotension and eventual lethality in WT and EC-$G\alpha_{12}/G\alpha_{13}$-KO as well as in EC-$G\alpha_q/G\alpha_{11}$-KO mice (Fig. 4 and not depicted). Thus, endothelial $G\alpha_q/G\alpha_{11}$ deficiency does not protect from endotoxic shock.

Anaphylactic shock depends on endothelial G_q/G_{11}

To further evaluate the role of endothelial G protein–mediated signaling pathways under pathophysiologically more relevant conditions, we set up models for passive and active systemic anaphylaxis. To test the role of endothelial G_q/G_{11} and G_{12}/G_{13} in passive systemic IgE-dependent anaphylaxis, we injected WT and EC-$G\alpha_q/G\alpha_{11}$-KO and EC-$G\alpha_{12}/G\alpha_{13}$-KO mice i.v. with anti-DNP IgE and challenged them 24 h later with DNP-HSA. As shown in Fig. 5 A, WT and EC-$G\alpha_{12}/G\alpha_{13}$-KO mice responded with a rapid drop in systolic blood pressure down to values of ~35 mmHg. After a few minutes, the blood pressure started to slowly rise but remained hypotensive for more than 90 min. Both lines also showed a strong increase in their hematocrit when determined 10 min after application of the allergen as an indicator of severe extravasation

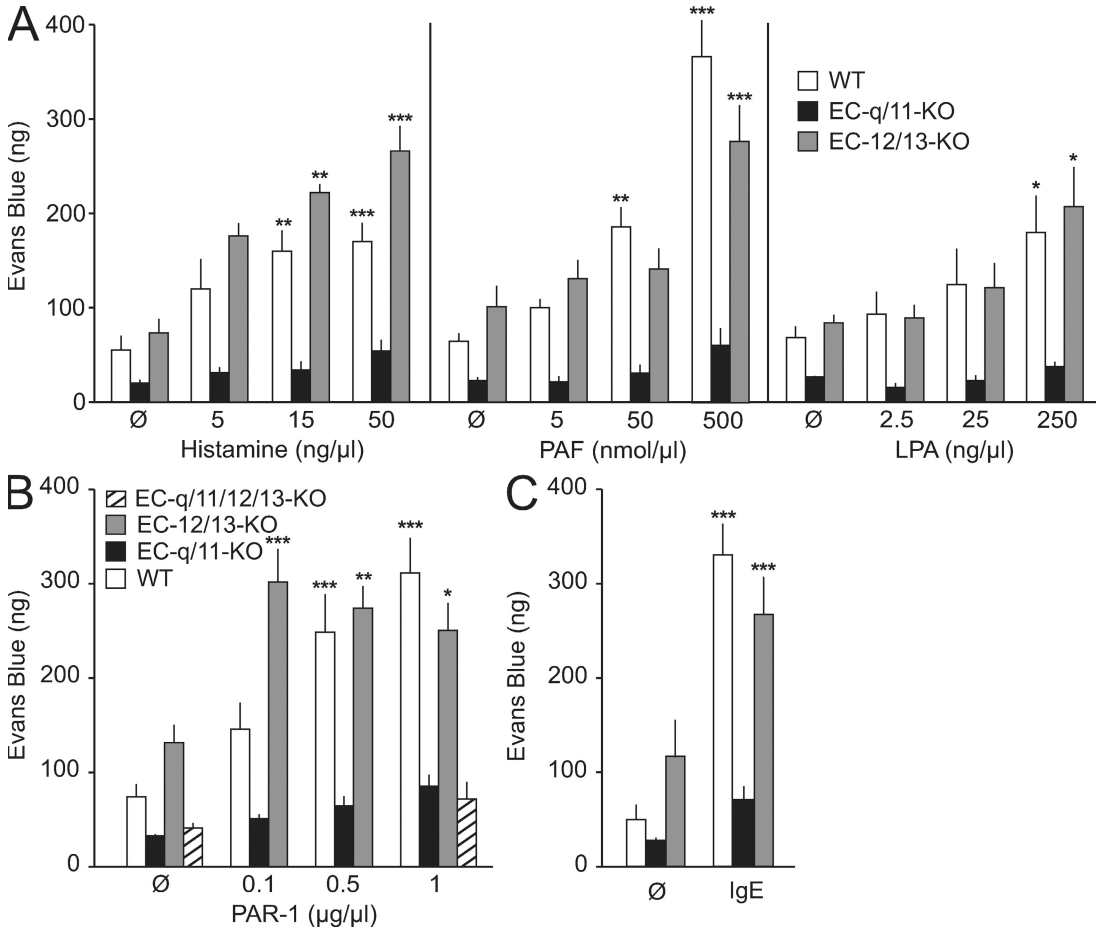

Figure 3. Basal and stimulated endothelial permeability in EC-specific $G\alpha_q/G\alpha_{11}$- and $G\alpha_{12}/G\alpha_{13}$-deficient mice. (A and B) Evans blue extravasation was determined in five to eight mice per genotype after intracutaneous injection of 20 µl of the indicated doses of PAF, histamine, LPA (A), or the PAR-1 peptide SFLLRN-NH_2 (B). Shown are the amounts of Evans blue determined in skin explants as described in the Materials and methods. (C) At least five mice per genotype were sensitized by intracutaneous injection of anti-DNP IgE antibodies. 24 h later, DNP-HSA was injected i.v., and Evans blue extravasation was determined as described in the Materials and methods. Values are means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with basal).

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of plasma (Fig. 5 B). Under the same conditions, mice with endothelial lack of $G\alpha_q/G\alpha_{11}$ showed only a small and very transient reduction in blood pressure, and the hematocrit of EC-$G\alpha_q/G\alpha_{11}$-KO mice remained unchanged after allergen administration (Fig. 5, A and B).

We then actively sensitized mice with BSA together with adjuvant. 2 wk later, mice were challenged with an i.v. injection of the same allergen. Within minutes after this challenge, all mice developed severe hypothermia (Fig. 5 C), and WT and EC-$G\alpha_{12}/G\alpha_{13}$-KO mice died within 20 min (Fig. 5 D). However, mice with endothelium-specific $G\alpha_q/G\alpha_{11}$ deficiency recovered from hypothermia after ~1 h, and all of the tested animals (n = 5) survived the anaphylactic challenge (Fig. 5, C and D). Mice lacking only $G\alpha_{11}$ exhibited an intermediate phenotype in the active systemic anaphylaxis model showing a survival rate of 20% (2 of 10 animals; unpublished data).

DISCUSSION

The pathological processes induced by mediators of anaphylaxis involve diverse organs such as the bronchial and immune systems, blood vessels, or the heart and require complex cell–cell and mediator–mediator interactions which involve various signaling pathways (5, 35, 36). In this study, we addressed the role of defined endothelial G protein–mediated signaling pathways in the pathomechanism of systemic anaphylaxis.

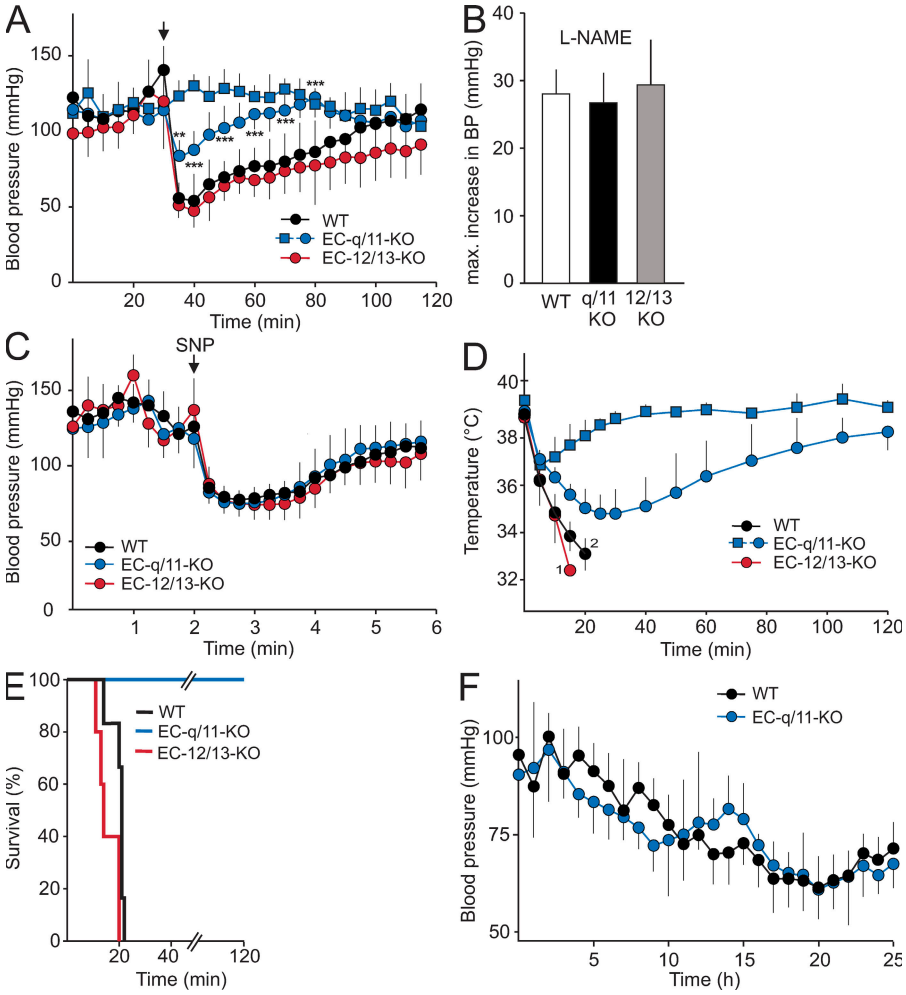

Figure 4. Role of endothelial G_q/G_{11} and G_{12}/G_{13} in the systemic effects of histamine, PAF, and LPS. (A) Arterial blood pressure was monitored telemetrically in mice before and after i.v. injection of carrier solution (squares) or 10 mg/kg histamine (circles). Shown are mean values of five to seven animals per genotype ±SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (compared with WT). The arrow indicates the time point of injection. (B) Arterial blood pressure was monitored telemetrically in anesthesized mice ($n \geq 5$ per genotype) before and after i.v. injection of 50 mg/kg L-NAME. Shown is the maximal blood pressure change, in millimeters of mercury, after injection of the NOS inhibitor. Values are the means ± S.D. (C) Arterial blood pressure was monitored telemetrically in mice before and after i.v. injection of 1 mg/kg sodium nitroprusside. Shown are mean values of 5–8 animals per genotype ± S.D. (D and E) Five to six mice per genotype were injected i.v. with 1.9 µg/g PAF, and body temperature (D) and survival (E) were monitored over 120 min. Numbers below the time points of the temperature plot indicate the number of animals still alive at the indicated times (mean values ± SD). (F) Three WT and EC-$G\alpha_q/G\alpha_{11}$-KO mice were injected i.p. with 80 µg/g LPS, and the blood pressure was monitored telemetrically for the indicated time period. Shown are the mean values ± SD.

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We report here that the endothelium-specific ablation of G_q/G_{11} prevents the loss of the endothelial barrier function induced by various inflammatory mediators as well as by local anaphylaxis. The systemic effects of anaphylactic mediators like histamine and PAF as well as of IgE-mediated passive anaphylaxis were blunted in EC-$G\alpha_q/G\alpha_{11}$-KO mice, and mice with endothelium-specific $G\alpha_q/G\alpha_{11}$ deficiency, but not with $G\alpha_{12}/G\alpha_{13}$ deficiency, were protected against the fatal consequences of active systemic anaphylaxis. Thus, the blockade of endothelial G_q/G_{11} signaling is sufficient to protect against fatal anaphylactic shock, indicating that endothelial G_q/G_{11}-mediated signaling is critically involved in local and systemic anaphylactic reactions. In contrast, endothelial G_q/G_{11} does not appear to play a role in septic shock as the degree of hypotension and the lethality after systemic administration of LPS was indistinguishable between WT and EC-$G\alpha_q/G\alpha_{11}$-KO mice.

The analysis of the role of G_q/G_{11}- and G_{12}/G_{13}-mediated signaling pathways in the adult endothelium under in vivo conditions has been hampered by the fact that mice lacking the α subunits of these G proteins are embryonic lethal (37–39). By crossing a newly generated inducible and endothelium-specific Cre transgenic mouse line with conditional and null alleles of the genes encoding $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$, we were able to study the role of G_q/G_{11} and G_{12}/G_{13} in the endothelium of adult animals in which lack of $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ did not lead to any obvious defects. There was also no acute or delayed change in the systemic blood pressure after induction of endothelial $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ deficiency (unpublished data). At the same time, the short and transient drop in blood pressure induced by i.v. injection of histamine was strongly reduced in EC-$G\alpha_q/G\alpha_{11}$-KO mice, indicating that pharmacological responses were affected. Thus, although endothelial G_q/G_{11} and G_{12}/G_{13} are obviously not critically involved in the regulation of vascular functions under basal physiological conditions, G_q/G_{11}-mediated signaling plays a crucial role in the regulation of endothelial functions under inflammatory and anaphylactic conditions. Studies in mice lacking $G\alpha_{13}$ have indicated a critical role of endothelial G_{13} in embryonic angiogenesis (38, 40). Female EC-$G\alpha_{12}/G\alpha_{13}$-KO mice are fertile, and we have not observed any defects in wound healing suggesting that endothelial G_{12}/G_{13} are not required for adult angiogenesis in the female reproductive system or during wound healing. However, the potential role of G_{13} in tumor angiogenesis remains to be evaluated.

The stimulation of endothelial permeability by inflammatory and anaphylactic mediators like thrombin, bradykinin, histamine, PAF, etc. requires the retraction of ECs as a result

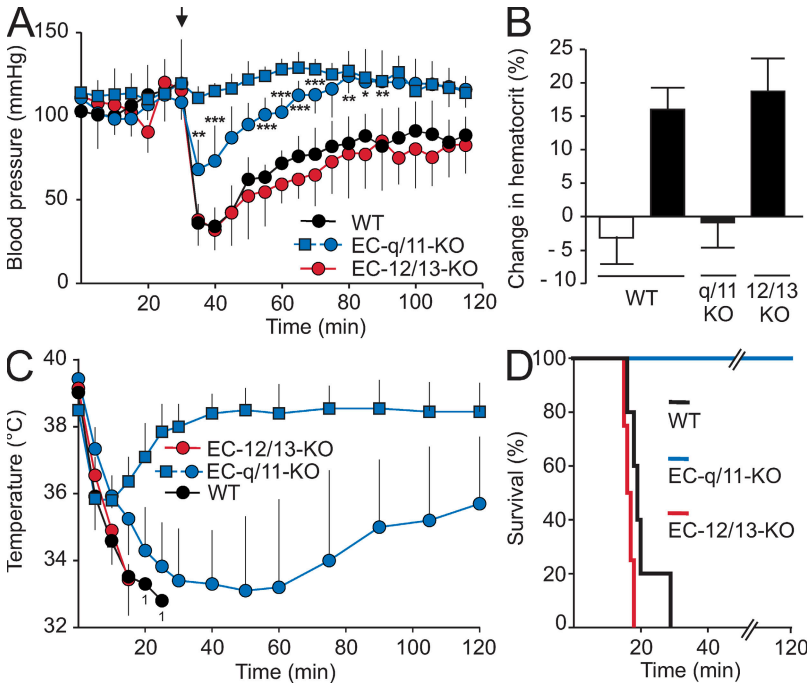

Figure 5. Passive and active anaphylaxis in endothelium-specific $G\alpha_q/G\alpha_{11}$- and $G\alpha_{12}/G\alpha_{13}$-deficient mice. (A and B) Mice were either sensitized with anti-DNP IgE antibodies (A, circles; B, black bars) or received buffer (A, squares; B, white bars). 24 h later, animals were challenged by i.v. injection of DNP-HSA as described in Materials and methods. Shown is the arterial blood pressure monitored telemetrically before and after administration of DNP-HSA (A) as well as the change in hematocrit 10 min after administration of DNP-HSA (B). The data represent mean values of five to six animals per group ±SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with WT). The arrow in A indicates the time point of DNP-HSA injection. (C and D) Body temperature (C) and survival (D) of mice sensitized with BSA and challenged 14 d later with BSA (circles) or buffer (squares). Experiments were performed with a total of five WT, four EC-$G\alpha_{12}/G\alpha_{13}$-KO, five EC-$G\alpha_q/G\alpha_{11}$-KO (immunized), and three EC-$G\alpha_q/G\alpha_{11}$-KO (nonimmunized) mice. Numbers below the time points of the temperature plot indicate the number of mice still alive at the indicated times. Shown are mean values ± SD.

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of increased actomyosin-mediated contraction as well as the disruption of cell–cell contacts (41, 42). Endothelial contraction is regulated by the phosphorylation state of the MLC which in its phosphorylated form allows myosin to interact with actin and to generate contractile forces (43, 44). Analogous to the situation in smooth muscle cells (45–47), the dual regulation of MLC phosphorylation in ECs via the Ca^{2+}-dependent MLC kinase activation and the Rho/Rho kinase–mediated myosin phosphatase inhibition is believed to be initiated by the dual coupling of receptors to G_q/G_{11} and G_{12}/G_{13}, respectively (44). Our in vitro studies using $G\alpha_q/G\alpha_{11}$- and $G\alpha_{12}/G\alpha_{13}$-deficient pulmonary ECs indicate that thrombin-induced MLC phosphorylation is abrogated in the absence of G_q/G_{11}, a defect which can be rescued by transfection of cells with $G\alpha_q$, whereas RhoA activation by thrombin was not affected in $G\alpha_q/G\alpha_{11}$-deficient ECs. In cells lacking G_{12}/G_{13}, MLC phosphorylation in response to thrombin was only reduced and RhoA activation was blocked. This indicates that the G_{12}/G_{13}-RhoA–mediated signaling pathway plays only a minor role in thrombin-induced MLC phosphorylation in primary pulmonary ECs. This is consistent with our in vivo data, which show that endothelial $G\alpha_{12}/G\alpha_{13}$ deficiency has no effect on vascular leakage induced by thrombin, PAF, histamine, or anaphylactic reactions, whereas $G\alpha_q/G\alpha_{11}$ deficiency blocked these effects. A predominant role of G_q/G_{11} compared with G_{12}/G_{13} was recently also demonstrated for thrombin-induced increase in endothelial permeability analyzed in vitro (48). Thus, G_q/G_{11}-mediated signaling, rather than G_{12}/G_{13}, is critically involved in the regulation of endothelial barrier function by inflammatory mediators acting via GPCRs.

The role of NO in systemic anaphylaxis has been controversial (49, 50). Recently, it was shown that the systemic inhibition of NOSs prevented mortality in various models of anaphylaxis in mice (51). This effect could also be seen in mice lacking the endothelial NOS (eNOS) but not the inducible NOS (iNOS). Although eNOS is expressed in ECs, it can also be found in various other tissues, and it has been suggested that it is the NO production in non-ECs which is involved in anaphylaxis (52). Our data indicate that the stimulation of NO formation in isolated ECs depends on G_q/G_{11} but not on G_{12}/G_{13}. In addition, endothelium-specific lack of G_q/G_{11} results in a strong reduction in histamine-induced hypotension and various anaphylactic reactions very similar to the effects seen in mice lacking eNOS (51, 53). Thus, our data are consistent with a primary role of endothelial NOS in systemic anaphylaxis.

Using conditional mutagenesis, we have generated mice with inducible endothelium-specific $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ deficiency. When challenged with anaphylactic mediators or subjected to systemic anaphylaxis, EC-$G\alpha_q/G\alpha_{11}$-KO mice were protected, whereas mice with endothelium-specific $G\alpha_{12}/G\alpha_{13}$ deficiency responded like WT animals. Endothelial $G\alpha_q/G\alpha_{11}$ deficiency blocked MLC phosphorylation and NO formation as well as increases in vascular permeability induced by various inflammatory and anaphylactic mediators. This study identifies endothelial G_q/G_{11}-mediated signaling as a critical process in the pathophysiology of systemic anaphylaxis. Because lack of G_q/G_{11}-mediated signaling does not affect basal physiological regulation of endothelial function, it may be an interesting target to treat systemic anaphylaxis.

MATERIALS AND METHODS

Chemicals and antibodies. For Western blotting, the following antibodies were used: anti-$G\alpha_{q/11}$ and anti-$G\alpha_{13}$ (Santa Cruz Biotechnology, Inc.), anti–α-tubulin and anti-MLC (Sigma-Aldrich), and anti-pMLC (Cell Signaling Technology). Histamine, thrombin, PAF, LPA, PAR-1 peptide (SFLLRN-NH_2), Evans blue, anti–DNP-IgE, DNP-HSA, and BSA were obtained from Sigma-Aldrich. Ionomycin was obtained from Invitrogen.

Genetic mouse models. All procedures of animal care and use in this study were approved by the local animal ethics committee (Regierungspräsidium Karlsruhe, Germany). The generation of floxed alleles of the genes encoding $G\alpha_q$ (*Gnaq*) and $G\alpha_{13}$ (*Gna13*) and of null alleles of the genes encoding $G\alpha_{11}$ (*Gna11*) and $G\alpha_{12}$ (*Gna12*) have been described previously (29, 30, 37, 39).

To generate an inducible EC-specific Cre transgenic mouse line, a cassette consisting of the *Cre*ER^{T2} followed by a polyadenylation signal from bovine growth hormone and a module containing the β-lactamase gene flanked by frt sites was introduced into the coding ATG of the mouse *tie2* gene carried by a BAC using ET recombination as previously described (54–56). Correct recombinants were verified by Southern blotting. After FLPe-mediated recombination, the recombined BAC was injected into male pronuclei derived from fertilized FvB/N oozytes. Transgenic offspring were analyzed for BAC insertion by genomic PCR amplification. To verify inducibility and activity of the Cre fusion protein, *tie2-Cre*ER^{T2} mice were mated with animals of the Cre reporter transgenic line Gt(ROSA)26Sortm1sor (ROSA26-LacZ). Cotransgenic progeny from these matings were treated i.p. with 5 × 1 mg/d tamoxifen or vehicle alone and were killed 14 d after induction. For histological analysis of β-galactosidase activity, staining was performed on 10–12-µm cryosections followed by eosin counterstaining.

Isolation of mouse primary pulmonary ECs. Mouse lung ECs were isolated as described previously (57). Lungs were minced and digested in 50 U/ml dispase for 1 h at 37°C with shaking (350 rpm). After filtration, the cells were washed in PBS containing 0.5% BSA. Cells were incubated with anti-CD144 antibody-coated (BD) magnetic beads (Invitrogen) for 1 h at room temperature, washed, and isolated with a magnet (Invitrogen). Cells were grown in DMEM/F12 (Invitrogen) supplemented with 10% FBS, penicillin/streptomycin, and EC growth supplement with heparin (PromoCell) on fibronectin-coated wells. To induce Cre-mediated recombination or to express $G\alpha_q$, the cells were infected with 5×10^7 PFU of Adeno-Cre-GFP virus (Vector Laboratories) or Adeno-$G\alpha_q$ virus (58, 59) 72 h before the experiments.

RhoA activation assay. RhoA activation in primary ECs was detected by a luminescence-based G-LISA RhoA activation assay kit (tebu-bio) according to the manufacturer's instructions. Briefly, mouse primary lung ECs were grown on 12-well plates and stimulated with 1 U/ml thrombin for 1 min, washed with 1.5 ml of ice-cold PBS, and lysed in 150 µl of lysis buffer on ice. Protein concentrations were measured and equalized with lysis buffer if necessary.

Detection of MLC phosphorylation. For detection of MLC phosphorylation, mouse primary ECs were cultured on 24-well plates. The cells were stimulated with 1 U/ml thrombin for the indicated time periods and lysed in 2× Laemmli buffer, incubated for 10 min at 100°C, and then loaded on 12% SDS PAGE gels. MLC phosphorylation was detected by Western blotting using an anti-pMLC antibody (1:1,000).

Determination of NO production. NO formation was determined as previously described (60). Lung ECs from WT, $Gnaq^{flox/flox}$;$Gna11^{-/-}$, or

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Gna12$^{-/-}$;*Gna13*$^{flox/flox}$ mice were cultured and treated with Cre transducing adenovirus as described. Cells were then suspended by treatment with accutase (PAA Laboratories) and washed in Hepes-buffered Tyrode solution containing 0.1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and 100 U/ml of superoxide dismutase. Approximately 5×10^4 cells were added to RFL6 fibroblasts cultured in 24-well plates and incubated (37°C) for 5 min in the absence or presence of the indicated stimuli. Thereafter, the incubation was stopped by the addition of trichloroacetic acid (6%), and the concentration of cyclic GMP was determined by a radioimmunoassay (GE Healthcare).

Vascular permeability assay. We determined agonist-induced vascular permeability changes using Evans blue dye. Mice were anaesthetized with 50 mg/kg pentobarbital sodium and injected i.v. with 0.04 μg/g Evans Blue in saline. After 1 min, different doses of agonists (histamine, PAF, PAR-1 peptide, and LPA) in 20 μl of PBS were injected into the shaved back skin. PBS was injected as control. Mice were killed after 10 min, and ~1 cm^2 of skin containing the site of injection was removed. The skin punches were incubated in 500 μl of formamide at 55°C for 48 h, and the Evans blue content was determined by absorption at 595 nm.

Passive cutaneous anaphylaxis. 30 ng of anti-DNP IgE in 20 μl of sterile 0.9% NaCl was injected into the dorsal skin of the right ear. The left ear of mice received an equal volume of saline and served as control. After 24 h, we challenged the passively immunized mice by an i.v. injection of 0.5 mg of DNP-HSA together with 0.08 μg/g Evans blue in saline. Mice were killed 30 min after the challenge by cervical dislocation, and ear biopsies were collected. Evans blue was extracted in 400 μl of formamide at 55°C for 24 h and quantified by measuring light absorption at 595 nm.

Telemetric blood pressure and body temperature measurements. We used a radiotelemetry system (PA-C10; Data Sciences International) to monitor blood pressure in conscious unrestrained mice, as described previously (46). Pressure sensing catheters were implanted into the left carotid artery, and the transducer unit was inserted into a subcutaneous pouch along the right flank. After a recovery period of at least 1 wk, arterial pressure recordings were collected, stored, and analyzed with Dataquest A.R.T. software (version 4.0; Data Sciences International). We collected data for basal blood pressure measurements with a 10-s scheduled sampling every 5 min and used the 24-h mean values for analysis. For analyzing the acute effects of agonists, we collected data continuously in 5-s intervals for different time periods as indicated in the figures. The body temperature was measured with a temperature control module (TKM-0902; Föhr Medical Instruments GmbH).

Passive systemic anaphylaxis. To induce passive systemic anaphylaxis, we injected mice i.v. with 20 μg of anti-DNP IgE. After 24 h, we challenged these passively immunized mice by an i.v. injection of 1 mg DNP-HSA. Control mice were injected with saline and challenged as described for immunized mice. For determining hematocrit, blood samples were collected before and 10 min after the challenge. Blood pressure measurements were done using the telemetric system.

Active systemic anaphylaxis. For inducing active systemic anaphylaxis, we first immunized mice with i.p. injection of 1 mg BSA and 300 ng pertussis toxin as adjuvant in pyrogen-free 0.9% NaCl. After 14 d, mice were challenged with i.v. injection of 2 mg BSA. We monitored the body temperature and survival of the mice for 120 min after the challenge.

Online supplemental material. Fig. S1 shows that the expression of $G\alpha_q$/$G\alpha_{11}$ did not differ between various non-ECs prepared from WT and EC-$G\alpha_q$/$G\alpha_{11}$-KO mice. Fig. S2 shows the effect of leukotriene C_4 on the extravasation of Evan's blue in WT and the absence of this effect in EC-$G\alpha_q$/$G\alpha_{11}$-KO. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082150/DC1.

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