Anaphylactic shock depends on endothelial $G_q/G_{11}$

Hanna Korhonen,1 Beate Fisslthaler,2 Alexandra Moers,1 Angela Wirth,1 Daniel Habermehl,3 Thomas Wieland,4 Günther Schütz,3 Nina Wettschureck,1 Ingrid Fleming,2 and Stefan Offermanns1,5

1Institute of Pharmacology, University of Heidelberg, 69120 Heidelberg, Germany
2Institute for Cardiovascular Physiology, Goethe University Frankfurt, 60590 Frankfurt, Germany
3Division Molecular Biology of the Cell 1, German Cancer Research Center, 69120 Heidelberg, Germany
4Institute for Experimental and Clinical Pharmacology and Toxicology, Medical Faculty Mannheim, University of Heidelberg, 68169 Mannheim, Germany
5Department of Pharmacology, Max-Planck Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany

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 $G_q$, $G_{11}$, $G_{12}$, and $G_13$. 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 $G_q/G_{11}$ deficiency. Mice with endothelium–specific $G_{12}/G_{13}$ deficiency, but not with $G_q/G_{11}$ 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.
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<sub>q</sub>, G<sub>11</sub>, and G<sub>12</sub>/G<sub>13</sub> families (8, 12, 20–24). The G proteins G<sub>q</sub>/G<sub>11</sub> couple receptors to β isoforms of phospholipase C resulting in inositol-1,4,5-trisphosphate–mediated mobilization of intracellular Ca<sup>2+</sup> and diacylglycerol-dependent activation of protein kinase C, whereas G<sub>12</sub>/G<sub>13</sub> couple receptors to the activation of the Rho/Rho kinase–mediated signaling pathway. G<sub>q</sub>-type G proteins couple receptors in an inhibitory fashion to adenyl 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<sub>q</sub>/G<sub>11</sub>–mediated signaling pathway, but not the G<sub>12</sub>/G<sub>13</sub>–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<sub>q</sub>/G<sub>11</sub> also protects mice from the deleterious consequences of PAF injection as well as of active and passive systemic anaphylaxis. Our data identify endothelial G<sub>q</sub>/G<sub>11</sub>–mediated signaling as an essential mediator of systemic anaphylaxis.

RESULTS
Endothelial effects of inflammatory mediators acting via GPCRs are mediated primarily by G<sub>q</sub>/G<sub>11</sub>

To analyze the role of G<sub>q</sub>/G<sub>11</sub>– and G<sub>12</sub>/G<sub>13</sub>–mediated signaling in endothelial responses to vasoactive mediators, we generated ECs lacking the α subunits of G<sub>q</sub>/G<sub>11</sub> or G<sub>12</sub>/G<sub>13</sub>. We have previously generated floxed alleles of the genes encoding Gα<sub>q</sub> (Gnaq) and Gα<sub>13</sub> (Gna13) which allow the conditional inactivation of these genes in G<sub>q</sub>/G<sub>11</sub>– or G<sub>12</sub>/G<sub>13</sub>–deficient backgrounds (29, 30). To induce Gα<sub>q</sub>/Gα<sub>11</sub> or Gα<sub>12</sub>/Gα<sub>13</sub> double deficiency, we prepared pulmonary microvascular ECs from WT, Gna<sub>q</sub><sup>fl ox/</sup>fl ox;Gna11<sup>−/−</sup>, and Gna12<sup>−/−</sup>;Gna13<sup>−/−</sup> mice and infected them with an adenovirus transducing the recombinase Cre. As shown in Fig. 1 A, expression of Cre recombinase in Gna<sub>q</sub><sup>fl ox/</sup>fl ox;Gna11<sup>−/−</sup> or Gna12<sup>−/−</sup>;Gna13<sup>−/−</sup> mice resulted in Gα<sub>q</sub>/Gα<sub>11</sub> and Gα<sub>12</sub>/Gα<sub>13</sub> deficiency, respectively.

We then analyzed the role of G<sub>q</sub>/G<sub>11</sub>– and G<sub>12</sub>/G<sub>13</sub>–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, Gα<sub>q</sub>/Gα<sub>11</sub>–deficient, or Gα<sub>12</sub>/Gα<sub>13</sub>–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 Gα<sub>q</sub>/Gα<sub>11</sub>–deficient ECs, the effects in cocultures containing Gα<sub>q</sub>/Gα<sub>11</sub>–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 Gα<sub>q</sub>/Gα<sub>11</sub> or Gα<sub>12</sub>/Gα<sub>13</sub> deficiency in ECs. This indicates that Gα<sub>q</sub>/Gα<sub>11</sub>, but not Gα<sub>12</sub>/Gα<sub>13</sub>, 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, Gα<sub>q</sub>/Gα<sub>11</sub>–deficient, and Gα<sub>12</sub>/Gα<sub>13</sub>–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 Gα<sub>q</sub>/Gα<sub>11</sub>. The defect of thrombin–induced MLC phosphorylation in Gα<sub>q</sub>/Gα<sub>11</sub>–deficient cells could be rescued by adenovirus–mediated expression of Gα<sub>q</sub> (Fig. 1 D). Lack of Gα<sub>12</sub>/Gα<sub>13</sub> 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 Gα<sub>q</sub>/Gα<sub>11</sub> was not accompanied by any defect in thrombin–induced RhoA activation, whereas thrombin–induced RhoA activation was abrogated in ECs lacking Gα<sub>12</sub>/Gα<sub>13</sub> (Fig. 1 F).

Generation of mice with EC–specific Gα<sub>q</sub>/Gα<sub>11</sub> and Gα<sub>12</sub>/Gα<sub>13</sub> deficiency

For in vivo experiments, we restricted Gα<sub>q</sub>/Gα<sub>11</sub> and Gα<sub>12</sub>/Gα<sub>13</sub> 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 (CreER<sup>12</sup>) (31) under the control of the tie2 promoter (see Materials and methods). The inducible endothelium–specific Cre transgenic mouse line (tie2-CreER<sup>12</sup>) 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 Gα<sub>q</sub>/Gα<sub>11</sub> and Gα<sub>12</sub>/Gα<sub>13</sub> in ECs of tamoxifen–treated tie2-CreER<sup>12</sup>;Gna<sub>q</sub><sup>fl ox/</sup>fl ox;Gna11<sup>−/−</sup> (EC–Gα<sub>q</sub>/Gα<sub>11</sub>–KO) and tie2-CreER<sup>12</sup>;Gna12<sup>−/−</sup>;Gna13<sup>−/−</sup> (EC–Gα<sub>12</sub>/Gα<sub>13</sub>–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–Gα<sub>q</sub>/Gα<sub>11</sub>–KO mice with
regard to Goq/Gα11 expression (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20082150/DC1).

**Blockade of endothelial Goq/Gα11-mediated signaling, but not Go12/Gα13-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-Goq/Gα11-KO and EC-Go12/Gα13-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-NH2, histamine, PAF, and leukotriene C4 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 Goq/Gα11 deficiency but not

![Figure 1](image-url)
in mice lacking Gα12/Gα13 in ECs. The small remaining response to the PAR1-activating peptide observed in EC-Gαq/Gα11-KO mice was not further reduced in mice lacking both Gαq/Gα11 and Gα12/Gα13 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-Gα12/Gα13-KO mice, whereas mice with endothelium-specific Gαq/Gα11 deficiency showed strongly reduced vascular permeability (Fig. 3 C). Thus, local regulation of vascular permeability requires Gq/G11-mediated signaling in ECs but not G12/G13.

**Systemic effects of histamine and PAF but not of LPS are blocked in EC-Gαq/Gα11-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-Gαq/Gα11-KO mice, the same dose decreased blood pressure for only ~20 min with maximal hypotensive values of ~90 mmHg, whereas mice with endothelium-specific Gα12/Gα13 deficiency responded comparable to WT mice (Fig. 4 A). The strongly reduced hypotensive response of EC-Gαq/Gα11-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-Gαq/Gα11-KO, and EC-Gα12/Gα13-KO mice to the NO-donor sodium nitroprusside as well as to the

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**Figure 2. Generation of mice with EC-specific Gαq/Gα11 and Gα12/Gα13 deficiency.** (A) Gt[ROSA26]SorCre reporter mice carrying the tie2-CreER	extsuperscript{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-Gαq/Gα11-KO (q/11-KO), or EC-Gα12/Gα13-KO (12/13-KO) mice were analyzed by Western blotting with antibodies directed against Gαq/Gα11, Gα13, α-tubulin, or β-actin. Arrows indicate the position of the 43-kD marker protein. Shown are representative data from three independently performed experiments.
NO synthase (NOS) inhibitor Nω-nitro-l-arginine methyl ester (l-NNAME; Fig. 4, B and C).

We then tested the effect of endothelium-specific $\alpha_q/\alpha_{11}$ and $\alpha_q/\alpha_{11}/\alpha_{12}/\alpha_{13}$ 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-$\alpha_q/\alpha_{11}$/KO mice within 20 min (Fig. 4 E). However, mice with endothelial $\alpha_q/\alpha_{11}$ 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 $\alpha_{11}$ demonstrated an intermediate phenotype with more severe hypothermia than EC-$\alpha_q/\alpha_{11}$-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-$\alpha_{12}/\alpha_{13}$-KO as well as in EC-$\alpha_q/\alpha_{11}$-KO mice (Fig. 4 and not depicted). Thus, endothelial $\alpha_q/\alpha_{11}$ deficiency does not protect from endotoxic shock.

Anaphylactic shock depends on endothelial $\alpha_q/\alpha_{11}$

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 $\alpha_q/\alpha_{11}$ and $\alpha_{12}/\alpha_{13}$ in passive systemic IgE-dependent anaphylaxis, we injected WT and EC-$\alpha_q/\alpha_{11}$-KO and EC-$\alpha_{12}/\alpha_{13}$-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-$\alpha_{12}/\alpha_{13}$-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.
of plasma (Fig. 5 B). Under the same conditions, mice with endothelial lack of Gαq/11 showed only a small and very transient reduction in blood pressure, and the hematocrit of EC-Gαq/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-Gα12/13-KO mice died within 20 min (Fig. 5 D). However, mice with endothelium-specific Gαq/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 Gα11 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.

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**Figure 4. Role of endothelial Gαq/G11 and G12/G13 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 ≥ 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-Gαq/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.
we were able to study the role of \( \alpha_q/\alpha_{11} \) and \( \alpha_{12}/\alpha_{13} \) in the endothelium of adult animals in which lack of \( \alpha_q/\alpha_{11} \) or \( \alpha_{12}/\alpha_{13} \) did not lead to any obvious defects. There was also no acute or delayed change in the systemic blood pressure after induction of endothelial \( \alpha_q/\alpha_{11} \) or \( \alpha_{12}/\alpha_{13} \) 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-\( \alpha_q/\alpha_{11} \)-KO mice, indicating that pharmacological responses were affected. Thus, although endothelial \( \alpha_q/\alpha_{11} \) and \( \alpha_{12}/\alpha_{13} \) are obviously not critically involved in the regulation of vascular functions under basal physiological conditions, \( \alpha_q/\alpha_{11} \)-mediated signaling plays a crucial role in the regulation of endothelial functions under inflammatory and anaphylactic conditions. Studies in mice lacking \( \alpha_{13} \) have indicated a critical role of endothelial \( \alpha_{13} \) in embryonic angiogenesis (38, 40). Female EC-\( \alpha_{12}/\alpha_{13} \)-KO mice are fertile, and we have not observed any defects in wound healing suggesting that endothelial \( \alpha_{12}/\alpha_{13} \) are not required for adult angiogenesis in the female reproductive system or during wound healing. However, the potential role of \( \alpha_{13} \) 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

We report here that the endothelium-specific ablation of \( \alpha_q/\alpha_{11} \) 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-\( \alpha_q/\alpha_{11} \)-KO mice, and mice with endothelium-specific \( \alpha_q/\alpha_{11} \) deficiency, but not with \( \alpha_{12}/\alpha_{13} \) deficiency, were protected against the lethal consequences of active systemic anaphylaxis. Thus, the blockade of endothelial \( \alpha_q/\alpha_{11} \) signaling is sufficient to protect against fatal anaphylactic shock, indicating that endothelial \( \alpha_q/\alpha_{11} \)-mediated signaling is critically involved in local and systemic anaphylactic reactions. In contrast, endothelial \( \alpha_q/\alpha_{11} \) 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-\( \alpha_q/\alpha_{11} \)-KO mice.

The analysis of the role of \( \alpha_{12}/\alpha_{13} \) and \( \alpha_{12}/\alpha_{13} \)-mediated signaling pathways in the adult endothelium under in vivo conditions has been hampered by the fact that mice lacking the \( \alpha \) 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 \( \alpha_q/\alpha_{11} \) and \( \alpha_{12}/\alpha_{13} \), we were able to study the role of \( \alpha_q/\alpha_{11} \) and \( \alpha_{12}/\alpha_{13} \) in the endothelium of adult animals in which lack of \( \alpha_q/\alpha_{11} \) or \( \alpha_{12}/\alpha_{13} \) did not lead to any obvious defects. There was also no acute or delayed change in the systemic blood pressure after induction of endothelial \( \alpha_q/\alpha_{11} \) or \( \alpha_{12}/\alpha_{13} \) 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-\( \alpha_q/\alpha_{11} \)-KO mice, indicating that pharmacological responses were affected. Thus, although endothelial \( \alpha_q/\alpha_{11} \) and \( \alpha_{12}/\alpha_{13} \) are obviously not critically involved in the regulation of vascular functions under basal physiological conditions, \( \alpha_q/\alpha_{11} \)-mediated signaling plays a crucial role in the regulation of endothelial functions under inflammatory and anaphylactic conditions. Studies in mice lacking \( \alpha_{13} \) have indicated a critical role of endothelial \( \alpha_{13} \) in embryonic angiogenesis (38, 40). Female EC-\( \alpha_{12}/\alpha_{13} \)-KO mice are fertile, and we have not observed any defects in wound healing suggesting that endothelial \( \alpha_{12}/\alpha_{13} \) are not required for adult angiogenesis in the female reproductive system or during wound healing. However, the potential role of \( \alpha_{13} \) 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
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 Ca²⁺-dependent MLC kinase activation and the Rho/Rho kinase-mediated myosin phosphorylation inhibition is believed to be initiated by the dual coupling of receptors to G₉/₁₁ and G₁₂/₁₃, respectively (44). Our in vitro studies using Gα₉/Gα₁₁- and Gα₁₂/Gα₁₃-deficient pulmonary ECs indicate that thrombin-induced MLC phosphorylation is abrogated in the absence of G₉/₁₁, a defect which can be rescued by transfection of cells with Gα₉, whereas RhoA activation by thrombin was not affected in Gα₉/Gα₁₁-deficient ECs. In cells lacking G₁₂/G₁₃, MLC phosphorylation in response to thrombin was only reduced and RhoA activation was blocked. This indicates that the G₁₂/G₁₃-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 Gα₉/Gα₁₃ deficiency has no effect on vascular leakage induced by thrombin, PAF, histamine, or anaphylactic reactions, whereas Gα₉/Gα₁₁ deficiency blocked MLC phosphorylation and increased vascular permeability analyzed in vitro (48). Thus, G₉/₁₁-mediated signaling, rather than G₁₂/G₁₃, 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 NOs prevented mortality in various models of anaphylaxis in mice (51). This effect could also be seen in mice lacking the endothelial NO (eNOS) but not the inducible NO (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 G₉/₁₁ but not on G₁₂/G₁₃. In addition, endothelium-specific lack of G₉/₁₁ 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 NO in systemic anaphylaxis.

Using conditional mutagenesis, we have generated mice with inducible endothelium-specific Gα₉/Gα₁₁ or Gα₁₂/Gα₁₃ deficiency. When challenged with anaphylactic mediators or subjected to systemic anaphylaxis, EC-Gα₉/Gα₁₁-KO mice were protected, whereas mice with endothelium-specific Gα₁₂/Gα₁₃ deficiency responded like WT animals. Endothelial Gα₉/Gα₁₁ 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 G₉/₁₁-mediated signaling as a critical process in the pathophysiology of systemic anaphylaxis. Because lack of G₉/₁₁-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-Gα₉ (Santa Cruz Biotechnology, Inc.), anti-Gα₁₁ (Santa Cruz Biotechnology, Inc.), anti-α-tubulin and anti-MLC (Sigma-Aldrich), and anti-p-MLC (Cell Signaling Technology). Histamine, thrombin, PAF, LPA, PAR-1 peptide (SFLKNN-NH₂), Evans blue, anti-DNP-IgE, DNP-HSA, and BSA were obtained from Sigma-Aldrich. lonomycin 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 Gα₉ (Gnaq) and Gα₁₁ (Gna13) and of null alleles of the genes encoding Gα₁₂ (Gna12) and Gα₁₃ (Gna13) have been described previously (29, 30, 37, 39).

To generate an inducible EC-specific Cre transgenic mouse line, a cassette consisting of the CreER³² followed by a polyadenylation signal from bovine growth hormone and a module containing the β-lactamase gene flanked by flp 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 recombinated BAC was injected into male pronuclei derived from fertilized FvB/N oocytes. Transgenic offspring were analyzed for BAC insertion by genomic PCR amplification. To verify inducibility and activity of the Cre fusion protein, tie²-CreER³² 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 cosin 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-C144 antibody-coated (BID) 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 Gα₉, the cells were infected with 5 × 10⁶ PFU of Adeno-Cre-GFP virus (Vector Laboratories) or Adeno-Gα₉ virus (S8, 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 (ebu-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-p-MLC antibody (1:1,000).

Determination of NO production. NO formation was determined as previously described (60). Lung ECs from WT, Gnaqfl/;Gα₁₁ −/−, or
A6Gi1α/Gα11-KO mice were cultured and treated with Cre transducing adenovirus as described. Cells were then suspended by treatment with acctusase (PAA Laboratories) and washed in Heps-buffered Tyrode solution containing 0.1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and 100 U/ml of superoxide dismutase. Approximately 5 × 10⁶ 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 anesthetized 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 cm² 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 measured. The Evans blue was extracted in 400 μl of formamide at 55°C for 24 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 μg 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; Führ 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 Gα11/Gqα11 did not differ between various non-EC50 prepared from WT and EC-Gα11/Gqα11-KO mice. Fig. S2 shows the effect of leukotriene C4 on the extravasation of Evan’s blue in WT and the absence of this effect in EC-Gα11/Gqα11-KO. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082150/DC1.
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