Anaphylactic shock depends on endothelial G\(_q/G_{11}\)

Hanna Korhonen, Beate Fisslthaler, Alexandra Moers, Angela Wirth, Daniel Habermehl, Thomas Wieland, Günther Schütz, Nina Wettschureck, Ingrid Fleming, and Stefan Offermanns

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\(_{12}/G_{13}\) deficiency. Mice with endothelium–specific G\(_{12}/G_{13}\) deficiency, but not with G\(_q\) 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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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 Gα, Gq/G11, and G12/G13 families (8, 12, 20–24). The G proteins Gq/G11 couple receptors to the β isoforms of phospholipase C resulting in inositol-1,4,5-trisphosphate–mediated mobilization of intracellular Ca2+ and diacylglycerol-dependent activation of protein kinase C, whereas G12/G13 couple receptors to the activation of the Rho/Rho kinase–mediated signaling pathway. Gq-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 Gq/G11–mediated signaling pathway, but not the G12/G13–mediated signaling pathway, blocks nitric oxide (NO) formation and loss of the endothelial barrier function in response to various vasoactive stimuli. Lack of endothelial Gq/G11 also protects mice from the deleterious consequences of PAF injection as well as of active and passive systemic anaphylaxis. Our data identify endothelial Gq/G11–mediated signaling as an essential mediator of systemic anaphylaxis.

RESULTS
Endothelial effects of inflammatory mediators acting via GPCRs are mediated primarily by Gq/G11
To analyze the role of Gq/G11– and G12/G13–mediated signaling in endothelial responses to vasoactive mediators, we generated ECs lacking the α subunits of Gq/G11 or G12/G13. We have previously generated floxed alleles of the genes encoding Gαq (Gnaq) and Gα13 (Gna13) which allow the conditional inactivation of these genes in Gq/G11– or G12/G13–deficient backgrounds (29, 30). To induce Gαq/G11 or Gα12/G13 double deficiency, we prepared pulmonary microvascular ECs from WT, Gnaqfl ox/fox;Gna11fl ox/fox, and Gna12−/−;Gna13fl ox/fox mice and infected them with an adeno virus transducing the recombinase Cre. As shown in Fig. 1A, expression of Cre recombinase in Gnaqfl ox/fox;Gna11−/− or Gna12−/−;Gna13fl ox/fox ECs resulted in Gαq/Gα11 and Gα12/Gα13 deficiency, respectively.

We then analyzed the role of Gq/G11– and G12/G13–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, Gαq/Gα11–deficient, or Gα12/Gα13–deficient lung ECs treated without or with thrombin, PAF, or ionomycin (Fig. 1B). Although thrombin and PAF induced a significant increase in cGMP levels in cocultures containing WT and Gαq/Gα11–deficient ECs, the effects in cocultures containing Gαq/Gα11–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 Gαq/Gα11 or Gα12/Gα13 deficiency in ECs. This indicates that Gq/G11, but not G12/G13, 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, Gαq/Gα11–deficient, and Gαq/Gα13–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 Gαq/Gα11. The defect of thrombin–induced MLC phosphorylation in Gαq/Gα11–deficient cells could be rescued by adeno virus–mediated expression of Gαq (Fig. 1D). Lack of Gα12/Gα13 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 Gαq/Gα13 was not accompanied by any defect in thrombin–induced RhoA activation, whereas thrombin–induced RhoA activation was abrogated in ECs lacking Gα12/Gα13 (Fig. 1F).

Generation of mice with EC-specific Gαq/Gα11 and Gα12/Gα13 deficiency
For in vivo experiments, we restricted Gαq/Gα11 and Gα12/Gα13 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.T2) (31) under the control of the tie2 promoter (see Materials and methods). The inducible endothelium-specific Cre transgenic mouse line (tie2-CreER.T2) did not show any Cre activity in the absence of tamoxifen when crossed with the Gi(Rosa26Sor 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 Gαq/Gα11 and Gα12/Gα13 in ECs of tamoxifen–treated tie2-CreER.T2;Gnaqfl ox/fox;Gna11−/− (EC-Gαq/Gα11–KO) and tie2-CreER.T2;Gna12−/−;Gna13fl ox/fox (EC-Gαq/Gα13–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-Gαq/Gα11–KO mice with
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\(\alpha_q/G\alpha_{11}\)-mediated signaling, but not G\(\alpha_{12}/G\alpha_{13}\)-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

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**Figure 1. The role of G\(\alpha_q/G\alpha_{11}\) and G\(\alpha_{12}/G\alpha_{13}\) in the regulation of NO production and MLC phosphorylation in pulmonary ECs.** (A) Lysates of pulmonary ECs prepared from WT, Gnaq\(^{-}\)/Gnaq\(^{-}\); Gna11\(^{-}\)/Gna11\(^{-}\) (q/11-KO), or Gna12\(^{-}\)/Gna12\(^{-}\); Gna13\(^{-}\)/Gna13\(^{-}\) (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\(\alpha_{12}\), or \(\alpha\)-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\(\alpha_q/G\alpha_{11}\)-deficient (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).
Systemic effects of histamine and PAF but not of LPS are blocked in EC-\(\text{G}_q/\text{G}_{11}\)-KO mice

i.v. injection of histamine induced a rapid and transient drop in the systolic blood pressure to levels of \(\sim 50\) mmHg in WT mice (Fig. 4 A). Normal values were restored \(\sim 90\) min after the application of histamine. In EC-\(\text{G}_q/\text{G}_{11}\)-KO mice, the same dose decreased blood pressure for only \(\sim 20\) min with maximal hypotensive values of \(\sim 90\) mmHg, whereas mice with endothelium-specific \(\text{G}_{12}/\text{G}_{13}\) deficiency responded comparable to WT mice (Fig. 4 A). The strongly reduced hypotensive response of EC-\(\text{G}_q/\text{G}_{11}\)-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-\(\text{G}_q/\text{G}_{11}\)-KO, and EC-\(\text{G}_{12}/\text{G}_{13}\)-KO mice to the NO-donor sodium nitroprusside as well as to the

Figure 2. Generation of mice with EC-specific \(\text{G}_q/\text{G}_{11}\) and \(\text{G}_{12}/\text{G}_{13}\) deficiency. (A) G\(\text{t}[\text{ROSA26}]/\text{Cre}\) reporter mice carrying the \(\text{tie}2\)-Cre\(\text{ER}\) transgene were treated with vehicle alone (untr.) or with tamoxifen (+Tam.) and then killed. The indicated organs were sectioned and stained for \(\beta\)-galactosidase activity. Bars, 50 \(\mu\)m. Inserts represent \(2\times\) magnifications of the indicated areas. (B) Lysates from lung ECs prepared from tamoxifen-treated WT, EC-\(\text{G}_q/\text{G}_{11}\)-KO (\(q/11\)-KO), or EC-\(\text{G}_{12}/\text{G}_{13}\)-KO (12/13-KO) mice were analyzed by Western blotting with antibodies directed against \(\text{G}_q/\text{G}_{11}\), \(\text{G}_{13}\), \(\beta\)-tubulin, or \(\beta\)-actin. Arrows indicate the position of the 43-kD marker protein. Shown are representative data from three independently performed experiments.
NO synthase (NOS) inhibitor N\textsuperscript{\textdegree}-nitro-L-arginine methyl ester (L-NAME; Fig. 4, B and C).

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

Anaphylactic shock depends on endothelial Go\textsubscript{q}/Go\textsubscript{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 Go\textsubscript{q}/Go\textsubscript{11} and Go\textsubscript{12}/Go\textsubscript{13} in passive systemic IgE-dependent anaphylaxis, we injected WT and EC-Go\textsubscript{q}/Go\textsubscript{11}-KO and EC-Go\textsubscript{12}/Go\textsubscript{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-Go\textsubscript{12}/Go\textsubscript{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 Go\textsubscript{q}/Go\textsubscript{11}– and Go\textsubscript{12}/Go\textsubscript{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\textsubscript{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).
of plasma (Fig. 5 B). Under the same conditions, mice with endothelial lack of $\alpha_q/\alpha_{11}$ showed only a small and very transient reduction in blood pressure, and the hematocrit of EC-$\alpha_q/\alpha_{11}$-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-$\alpha_{12}/\alpha_{13}$-KO mice died within 20 min (Fig. 5 D). However, mice with endothelium-specific $\alpha_q/\alpha_{11}$ 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 $\alpha_{11}$ 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.
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-$G_q/G_{11}$-KO mice, and mice with endothelium-specific $G_q/G_{11}$ deficiency, but not with $G_q/G_{12}/G_{13}$ 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-$G_q/G_{11}$-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 $G$ 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 $G_q/G_{11}$ and $G_{12}/G_{13}$, 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 $G_q/G_{11}$ or $G_{12}/G_{13}$ did not lead to any obvious defects. There was also no acute or delayed change in the systemic blood pressure after induction of endothelial $G_q/G_{11}$ or $G_{12}/G_{13}$ 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-$G_q/G_{11}$-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 $G_{13}$ have indicated a critical role of endothelial $G_{13}$ in embryonic angiogenesis (38, 40). Female EC-$G_{12}/G_{13}$-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 $G_q/G_{11}$- and $G_{12}/G_{13}$-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-$G_q/G_{11}$-KO, five EC-$G_q/G_{11}$-KO (immunized), and three EC-$G_q/G_{11}$-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.
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 Gq/G11 and G12/G13, respectively (44). Our in vitro studies using Goq/Gα11- and Gα12/Gα13-deficient pulmonary ECs indicate that thrombin-induced MLC phosphorylation is abrogated in the absence of Gq/G11, a defect which can be rescued by transfection of cells with Goq, whereas RhoA activation by thrombin was not affected in Gαq/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 Goq/Gα13 deficiency has no effect on vascular leakage induced by thrombin, PAF, histamine, or anaphylactic reactions, whereas Goq/Gαq deficiency blocked these effects. A predominant role of Goq/G11 compared with G12/G11 was recently also demonstrated for thrombin-induced increase in endothelial permeability analyzed in vitro (48). Thus, Goq/G11-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 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 Goq/G11 but not on G12/G13. In addition, endothelium-specific lack of Goq/G11 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 Goq/Gα11 or Gα12/Gα13 deficiency. When challenged with anaphylactic mediators or subjected to systemic anaphylaxis, EC-Goq/Gα11-KO mice were protected, whereas mice with endothelium-specific Gα12/Gα13 deficiency responded like WT animals. Endothelial Goq/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 Goq/G11-mediated signaling as a critical process in the pathophysiology of systemic anaphylaxis. Because lack of Goq/G11-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-Gαq (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 (SFLKNN-NH2), 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 Goq (Gnaq), and Gα11 (Gna11) and Goq/Gα11 and Gα12/Gα13 have been described previously (29, 30, 37, 39).

To generate an inducible EC-specific Cre transgenic mouse line, a cassette consisting of the CreER 32 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 pronuclear derived from fertilized FvB/N oocytes. Transgenic offspring were analyzed for Cre activity and expression using PCR amplification. To verify inducibility and activity of the Cre fusion protein, tie2-CreER 32 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-CD144 antibody-coated (Bi) magnetic beads (In Vitrogen) for 1 h at room temperature, washed, and isolated with a magnet (In Vitrogen). Cells were grown in DMEM/F12 (In Vitrogen) 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 Goq, the cells were infected with 5 × 105 PFU of Adeno-Cre-GFP virus (Vector Laboratories) or Adeno-Gαq virus (58, 59) 2 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 NO production. NO formation was determined as previously described (60). Lung ECs from WT, Gαq flox/tie2-Gαq11 Cre-lox /−, or
Gna12+/−;Gna13blue−/− 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 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; 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 Gαq/11,Gαq/11,KO did not differ between various non-EC53 prepared from WT and EC53,Gαq/11,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 EC53,Gαq/11,KO. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082150/DC1.

REFERENCES


SUPPLEMENTAL MATERIAL

Korhonen et al., http://www.jem.org/cgi/content/full/jem.20082150/DC1

Figure S1. Expression of Gαq/Gα11 in various nonendothelial cells prepared from WT and EC-Gαq/Gα11-KO (KO) mice. Lysates from platelets, peritoneal cells, leukocytes, and vascular smooth muscle cells prepared from tamoxifen-treated WT and EC-Gαq/Gα11-KO (KO) mice were analyzed by Western blotting with antibodies directed against Gαq/Gα11 and anti-α-tubulin.

Figure S2. Effect of leukotriene C4 (LTC4) on endothelial permeability in WT and EC-Gαq/Gα11-KO mice (q/11–KO). Evans blue extravasation was determined in mice after intracutaneous injection of 20 µl of buffer or 0.5 ng/µl LTC4. Shown are the amounts of Evans blue determined in skin explants. Values are means ± SEM. *, p < 0.05; n.s., nonsignificant (compared to basal).