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Research Article **Immunology**

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Anaphylactic shock depends on PI3K and eNOS-derived NO

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Introduction

Anaphylaxis is an acute, severe, and potentially fatal systemic allergic reaction that usually attacks the patient in the absence of a history of allergy. Causes of anaphylaxis include bee stings, medications, food, latex exposure, and exercise (1). Anaphylaxis is not as rare as generally believed and may affect as much as 1.2% to 15% of the US population (2). In addition, it is generally recognized that the prevalence of anaphylaxis is increasing significantly. During an anaphylactic reaction, serious cardiovascular or pulmonary dysfunction often leads to acute death, with severe hypotension as the cardinal clinical feature. Intravenous fluids and parenteral adrenaline administration are considered the only effective treatment; unfortunately, severe cardiovascular collapse during anaphylaxis is often resistant to this treatment (1, 3).

Platelet-activating factor (PAF) is a biologically active phospholipid. Although PAF was originally named after its ability to induce platelet aggregation, it stimulates a broad range of cell types and tissues by binding to a unique G protein–coupled 7 transmembrane receptor (PAF-R), activating a number of signaling pathways, including those that elevate intracellular calcium content and activate phospholipase A2 (PLA2) and PI3K (4). As PAF antagonists or PAF-R deficiency can prevent fatal anaphylaxis in animal models, PAF is thought to be a critical factor in the development of anaphylactic shock (5–9). In addition, PAF is known to contribute to hypotension and cardiac dysfunctions during hemorrhagic, traumatic, or septic shock (4). Nevertheless, the downstream molecular pathways beyond PAF-R triggering that direct its shock-inducing effect are still enigmatic. Both ROS and NO have been implicated in PAF shock, but whether their involvement is detrimental or beneficial is still a matter of debate (10–12). However, most studies were performed in animals under general anesthesia, which influences NO-mediated effects and blood pressure considerably (13–15). Also, conflicting results in anaphylactic shock models have been reported with regard to the possible involvement of NO (16–19).

NO may be endogenously produced by the inducible iNOS or by the constitutively expressed isoforms eNOS and neuronal NOS (nNOS). While the latter are believed to produce low amounts of NO to execute physiological and/or antiinflammatory functions, the inflammation-associated expression of iNOS and the subsequent overproduction of NO are assumed to be responsible for the cardiovascular failure, morbidity, and mortality associated with (septic) shock (20, 21). In addition, it is generally accepted that NO-mediated activation of soluble guanylate cyclase (sGC) is the key mechanism in the regulation of vascular tone during (septic) shock (22, 23).

In this study, we aimed to unequivocally elucidate the involvement and importance of NO, sGC, ROS, and PLA2 signaling in shock induced by intravenous PAF or anaphylaxis in conscious, nonanesthetized mice. We found that PAF-induced shock completely depended on PI3K signaling and on NO produced by the constitutive enzyme eNOS and not on NO produced by the inducible "inflammatory" iNOS. Downstream from NO, PAF shock appeared to be independent of sGC. Also, in 2 different models of active systemic anaphylaxis, absolute protection was obtained by inhibition of PI3K, Akt, or NOS or by eNOS deficiency. Although it is generally accepted that excessive iNOS-derived NO underlies the cardiovascular collapse associated with shock,

Nonstandard abbreviations used: EB, Evans blue; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MAP, mean arterial blood pressure; MB, methylene blue; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; PAF, platelet-activating factor; PLA2, phospholipase A2; sGC, soluble guanylate cyclase; WM, wortmannin.

Conflict of interest: The authors have declared that no conflict of interest exists.

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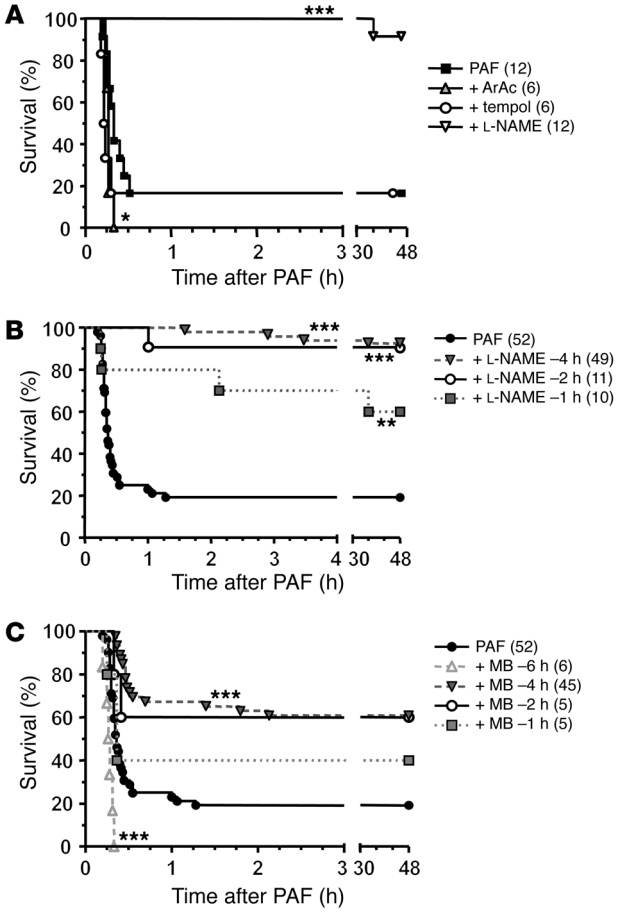

Figure 1
eNOS-derived NO is critical for PAF shock. C57BL/6 mice received various pretreatments and were challenged i.v. with 55 μg PAF; survival was monitored. (**A**) Pretreatment with vehicle, aristolochic acid (ArAc), tempol, or L-NAME (–4 hours). The number of mice is indicated between brackets. ***$P < 0.0001$; *$P = 0.0201$ compared with PAF. (**B**) Pretreatment with L-NAME 4, 2, or 1 hour before treatment with PAF. **$P = 0.0165$. (**C**) Pretreatment with MB 6, 4, 2, or 1 hour before PAF.

our data provide compelling new evidence for a shock-inducing function of eNOS-derived NO.

Results

NO is critical for PAF-induced shock. Intravenous PAF injection in conscious mice elicits rapid shock and results in death within 20–30 minutes (Figure 1A). When PAF engages its specific receptor, several intracellular signaling events are triggered, including PLA2 activation and superoxide production (4). Nevertheless, inhibition of these events by the PLA2 inhibitor aristolochic acid or the cell-permeable superoxide dismutase mimetic tempol did not provide any protection against PAF challenge (Figure 1A). In comparison, tempol or aristolochic acid provided complete protection against hyperacute shock induced by TNF in combination with the caspase inhibitor zVAD-fmk (24). As PAF may cause severe hypotension and iNOS induction (25), the vasorelaxant NO is generally assumed to be critically involved in PAF shock. Nonetheless, NOS inhibition cannot always preclude PAF-induced hypotension and sometimes even aggravates fatality (10–12). Since all of the aforementioned studies were performed in animals under general anesthesia, which is known to influence NO-mediated effects and blood pressure (13–15), we studied the effect of NOS inhibition on PAF hypotension and shock in conscious mice. We found that N^{ω}-nitro-L-arginine methyl ester (L-NAME) pretreatment completely protected against PAF lethality, implicating NO in a pivotal and detrimental function (Figure 1, A and B). Even when the PAF dose exceeded the LD_{100}, L-NAME still provided absolute protection (not shown).

NO is thought to relax vascular smooth muscle cells by activation of sGC and subsequent cyclic GMP–dependent modification of several intracellular processes, including the phosphorylation of proteins of the contractile apparatus and of pumps or channels involved in modulating intracellular calcium and potassium levels (26). Moreover, recent studies reported that methylene blue (MB), an inhibitor of sGC activation, can reverse clinical anaphylactic shock induced by injected contrast media and prolong survival of rabbits in experimental anaphylaxis (27, 28). Nevertheless, MB (15 mg/kg) did not prevent PAF shock to the same degree as L-NAME treatment, implying that sGC-independent NO activities are also involved (Figure 1, B and C). In comparison, MB provided 100% protection in a mouse model of TNF-induced shock as well as in a model of hyperacute shock induced by TNF in combination with the caspase inhibitor zVAD-fmk, as reported previously (24, 29). Also, lower doses of MB (5 and 10 mg/kg) still provided partial protection (data not shown). However, pretreatment with various doses (5, 10, 15, or 20 mg/kg) of another, more specific sGC inhibitor, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (23, 30), at different time points (4, 2, or 0.5 hours before challenge) did not protect (data not shown). In addition, $sGC\alpha1^{-/-}$ mice (31) deficient for 1 of the 2 isoforms of the α subunit of the heterodimeric sGC enzyme were also not protected against PAF-induced shock (not shown). Together, these results suggest that, downstream from NO, sGC-independent events are most important.

To examine the hypotensive response to PAF, mean arterial blood pressure (MAP) was measured in unrestrained conscious mice using a catheter placed in the femoral artery. As documented before (8), intravenously administered PAF has an extremely fast and drastic hypotensive effect (Figure 2, A and C). However, when mice were pretreated with L-NAME, PAF caused only a transient and mild drop in blood pressure, followed by a very fast recovery in all but 1 of the mice (Figure 2B). All mice treated with PAF died within 13 minutes (Figure 2A) while only 1 of 5 animals pretreated with L-NAME died, after 44 minutes (Figure 2B). The MAP of the 4 surviving L-NAME–treated mice 24 hours after PAF challenge was 132 ± 18 mmHg. We therefore conclude that NO plays a dominant role in acute PAF-induced hypotension. Nevertheless, other vasoactive mediators, such as histamine, serotonin, or leukotrienes, may also contribute in an NO-independent manner, albeit to a much smaller extent.

NO that causes PAF shock is produced by eNOS, activated via PI3K. To identify the NOS isoform involved, we used various NOS-deficient animals. Injection of PAF in iNOS-deficient mice caused a mortality that was similar to and as rapid as that observed in WT control animals, but age-matched eNOS-deficient animals were significantly protected (Figure 3A), indicating an unexpected pivotal role for eNOS in PAF shock. Studies in vitro have shown that eNOS can be activated by direct serine phosphorylation, obviating the need for calcium and calmodulin binding and causing increased NO production (32, 33). This phosphorylation is mediated by the

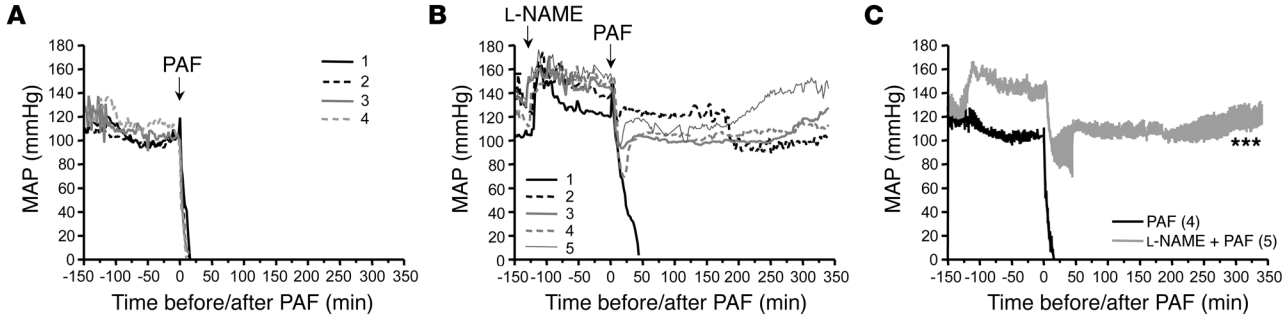

Figure 2
MAP measurements during PAF shock. C57BL/6 mice were challenged i.v. with 55 μg PAF at $t = 0$. (**A**) Individual MAP of PAF-challenged mice. (**B**) Individual MAP of mice treated with L-NAME (100 mg/kg, i.v., –2 hours) and PAF. (**C**) Data points show the mean MAP ± SD of the plots shown in **A** and **B**. Error bars are plotted as vertical lines above and below the curves. The number of mice is indicated between brackets. ***$P < 0.0001$.

PI3K/Akt pathway, which can be activated by receptor tyrosine kinases, G protein–coupled receptors, or mechanical forces such as shear stress. Pretreatment with wortmannin (WM), a specific inhibitor of PI3K, completely protected both WT and iNOS$^{-/-}$ mice (Figure 3A), demonstrating the critical importance of PI3K in PAF-induced shock. In addition, Western blot analysis of WT kidney homogenates revealed specific Ser1177 eNOS phosphorylation after PAF challenge, which can be efficiently prevented by WM pretreatment (Figure 3B).

Anaphylactic shock depends on PI3K and eNOS-derived NO. To evaluate the pathophysiological involvement of PI3K and eNOS-derived NO in shock in a clinically more relevant setting, we set up 2 different models of active systemic anaphylaxis, known to depend predominantly on PAF signaling (8, 9). Mice were first sensitized with BSA or OVA in the presence of adjuvants. A few weeks later, the animals were challenged intravenously with the same antigen. Soon after this challenge, mice developed severe hypothermia (demonstrated in Figure 4, B and D, for BSA-induced anaphylaxis) and rapidly succumbed to systemic shock reaction (Figure 4A). However, when mice were pretreated with WM or L-NAME, hypothermia was transient and less severe (Figure 4B). While 75% of the control mice died, all 8 animals pretreated with WM or L-NAME survived the anaphylactic challenge (Figure 4A). Similar results were obtained in the OVA-induced anaphylaxis model (data not shown). Western blot analysis of WT kidney homogenates confirmed specific eNOS phosphorylation after BSA challenge in sensitized mice, prevented by WM pretreatment (Figure 4E). Next, we compared the 2 models of anaphylaxis in WT and eNOS-deficient mice. Fatality in WT animals was 82% (9 of 11) for the BSA model and 92% (11 of 12) for the OVA model. In contrast, none of the eNOS-deficient mice died (Figure 4C; not shown for the OVA model). Moreover, for both anaphylaxis models, there was only a delayed, mild, and transient hypothermia in eNOS-deficient mice (Figure 4D). Neither BSA nor OVA had an effect on the temperature or behavior of unsensitized mice (Figure 4, B and D; not shown for OVA). These results reveal an unexpected but pivotal role for PI3K and eNOS-derived NO in anaphylactic shock.

During anaphylaxis, vasodilation and hypotension are often associated with excessive vascular permeability and fluid extravasation, resulting in a mixed distributive-hypovolemic shock. To assess vascular permeability, protein leakage was measured with the Evans blue (EB) technique. BSA or OVA in 1% EB was injected into the lateral tail vein, and subsequent EB extravasation into the ear tissue was analyzed (Figure 4F). In the BSA/BSA anaphylactic response, vascular permeability was significantly increased in WT mice but not in eNOS$^{-/-}$ animals (Figure 4F). Moreover, vascular leakage in BSA/BSA eNOS$^{-/-}$ mice was 2-fold lower than in WT mice ($P < 0.01$ by ANOVA). In OVA/OVA-induced shock, vascular permeability increased only in WT animals, and extravasation was again significantly lower in eNOS$^{-/-}$ OVA/OVA mice than in WT OVA/OVA animals ($P < 0.001$ by ANOVA) (not shown). In addition, pretreatment with WM or L-NAME clearly prevented EB extravasation in WT mice ($P < 0.001$, data not shown). Together, these data suggest the involvement of PI3K/eNOS in vascular leakage associated with anaphylaxis.

Once activated, PI3Ks generate phosphoinositide 3,4,5-trisphosphate (PIP_3), leading to the recruitment and activation of Akt (protein kinase B), which then activates a range of downstream targets,

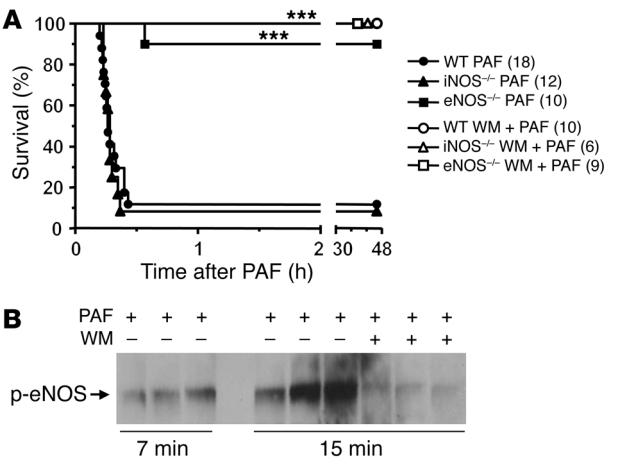

Figure 3
NO that causes PAF shock is produced by eNOS, activated via PI3K. (**A**) C57BL/6 (WT), iNOS$^{-/-}$, or eNOS$^{-/-}$ mice were injected i.v. with 55 μg PAF after pretreatment with vehicle or WM. Survival is presented as a combined Kaplan-Meier plot of 2 independent, representative experiments. The total number of mice is indicated between brackets. ***$P < 0.0001$ compared with PAF in WT mice. (**B**) Western blot for phosphorylated eNOS (p-eNOS) in kidney homogenates of individual mice 7 or 15 minutes after PAF injection. Where indicated, mice were pretreated with WM.

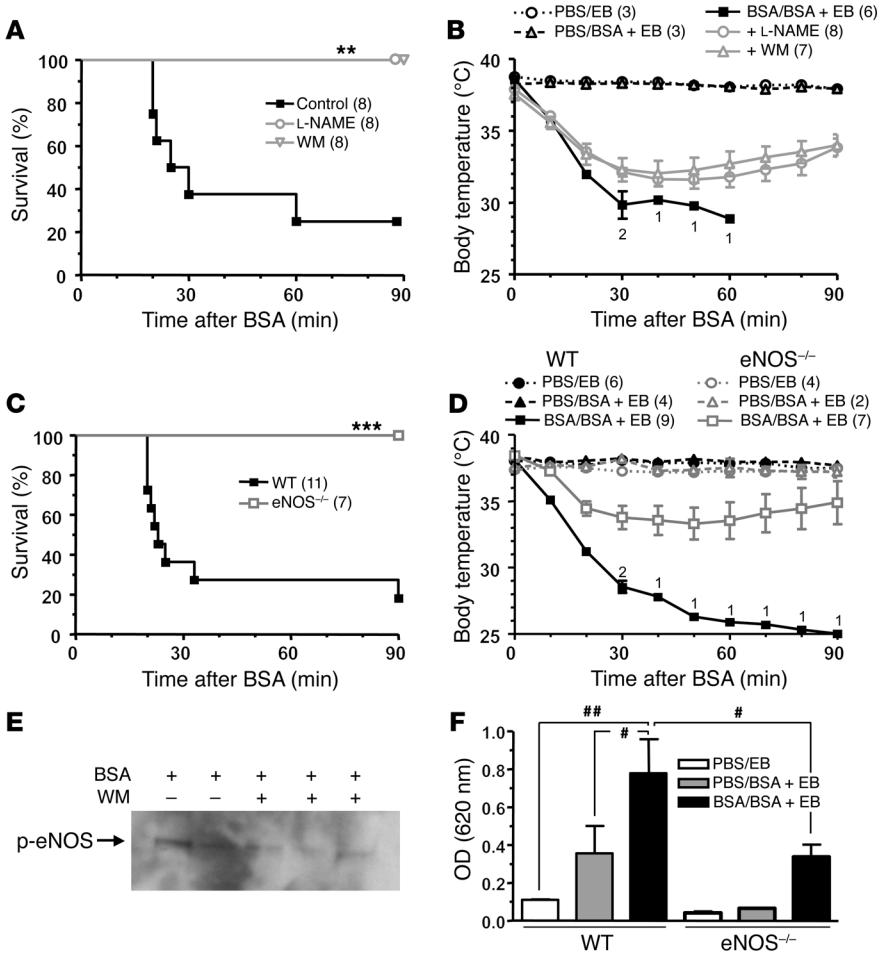

Figure 4

BSA anaphylactic shock in WT and eNOS$^{-/-}$ mice. (**A**) Survival plot of C57BL/6 mice sensitized with BSA, treated with vehicle (controls), L-NAME, or WM, and finally challenged with BSA in EB. The number of mice in each experimental group is shown in parentheses. $^{**}P = 0.0022$. (**B**) Temperature plot (mean ± SD) of mice sensitized by BSA or PBS and challenged 15 days later with BSA in EB (BSA + EB) or EB alone. The total number of mice is shown in parentheses; numbers beneath time points indicate the number of mice still alive at the indicated times. (**C**) Survival plot of WT and eNOS$^{-/-}$ mice sensitized with BSA and challenged with BSA in EB. Total number of mice is shown in parentheses. $^{***}P = 0.0007$. (**D**) Temperature plot (mean ± SD) of mice sensitized by BSA or PBS and challenged with BSA in EB or EB alone. The total number of mice is shown in parentheses, numbers above the time points indicate the number of mice still alive at the indicated times. (**E**) Western blot for p-eNOS in kidney homogenates of individual, BSA-sensitized mice 12 minutes after BSA challenge. Where indicated, mice had been pretreated with WM. (**F**) Vascular leakage assayed by EB extravasation into ear tissues. Inner ear rims were removed 90 minutes after i.v. challenge with BSA in EB or EB only and subsequently extracted in formamide. Plots show mean OD_{620} ± SD. $^{\#}P < 0.001$; $^{\#\#}P < 0.01$.

including eNOS. Akt exists as 3 isoforms (Akt1, Akt2, and Akt3) that share extensive structural similarity and that may have unique as well as common functions within the cell. To corroborate the importance of the PI3K/Akt pathway in anaphylaxis, we treated OVA-sensitized mice with perifosine, a lipid-based inhibitor of Akt for which phase I and II clinical trials are being conducted (34, 35). As depicted in Figure 5, A and B, all mice pretreated with perifosine suffered much less from hypothermia and survived the otherwise 100% lethal OVA challenge. For all Akt isoforms, knockout mice have been created (36–38). As Akt1 is the predominant Akt isoform in endothelial cells (39), we also studied anaphylactic shock in Akt1-deficient mice. However, Akt1$^{-/-}$ animals were not protected, except for a minor (but significant) delay in mortality, as compared with WT mice (Figure 5C).

To examine the effect of NOS deficiency on anaphylaxis-induced hypotension, MAP was measured in unrestrained conscious mice 3 days after surgical catheter insertion into the right femoral artery. In animals previously sensitized by BSA, intravenous BSA challenge had an immediate precipitous hypotensive effect, resulting in mortality, regardless of the challenging dose of BSA used (2, 1, or 0.1 mg) (Figure 6A). When mice were challenged with 2 mg BSA, pretreatment with L-NAME only prolonged survival: L-NAME mice died 40–81 minutes after challenge while all control animals were dead within 20 minutes. However, when 0.1 mg BSA was used as a challenge, the BSA challenge in L-NAME pretreated mice caused only a transient drop in blood pressure, followed by a very fast recovery in all but 1 mouse (Figure 6B). All mice treated with 0.1 mg BSA died within 15 minutes (Figure 6, A and C) while only 1 of 4 animals pretreated with L-NAME died, after 72 minutes (Figure 6B).

Discussion

NO has been shown to play a primary and harmful role in (septic) shock, causing progressive refractory hypotension and ultimately leading to multiple organ dysfunction and death. Constitutive NOS enzymes (eNOS and nNOS) are believed to produce low amounts of NO essential for various physiological and/or antiinflammatory events while higher amounts of NO are produced by inducible iNOS during inflammation (20, 21). It is generally accepted that during inflammation and shock, cardiovascular collapse is initiated and mediated by excessive production of NO due to the transcriptional induction of the inducible "inflammatory" isoform iNOS, a process that requires hours rather than minutes. Downstream, sGC is regarded as the principal vasorelaxing mediator of NO in the cardiovascular system (22, 23, 26).

As PAF can participate in the induction of iNOS (25), induced NO is assumed to mediate PAF-induced hypotension and shock. While some investigators have reported that NOS inhibitors can prevent PAF-induced hypotension (40, 41), others reported that it cannot (11, 12). However, all of these studies were performed in animals under general anesthesia, known to influence NO-mediated effects and blood pressure changes considerably (13–15). In addition, NOS inhibitors have also been reported to exacerbate PAF shock, including enhanced pulmonary hypertension (11), aggravated alveolar permeability (12), and even an increase in

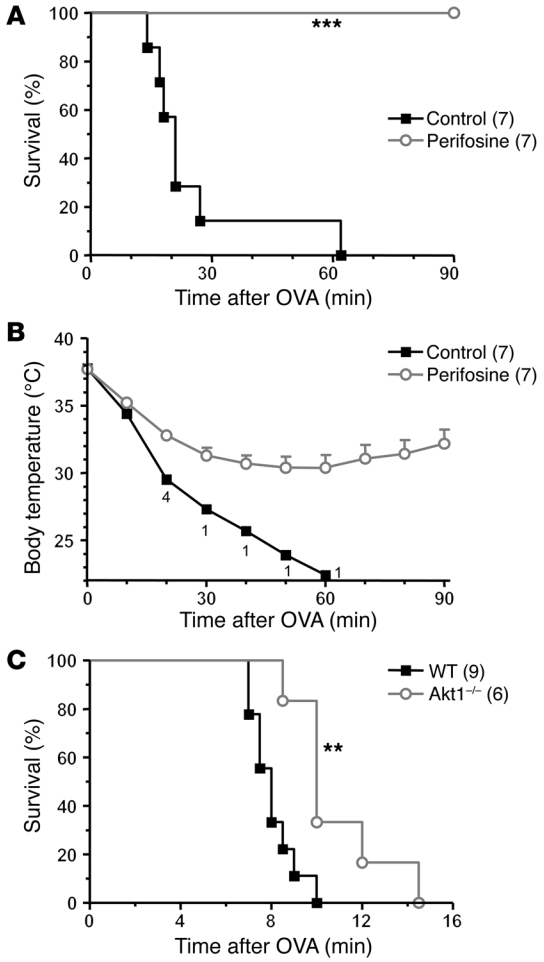

Figure 5
OVA anaphylactic shock in WT and Akt1$^{-/-}$ mice. (**A**) Survival plot of C57BL/6 mice sensitized with OVA, pretreated with vehicle (controls) or perifosine, and challenged 19 days later with OVA in 1% EB. The total number of mice in each experimental group is shown in parentheses. ***P = 0.0002. (**B**) Temperature plot (mean ± SD) of mice sensitized by OVA and challenged 19 days later with OVA in 1% EB. The total number of mice is shown in parentheses, and numbers below the time points indicate the number of mice still alive at the indicated times. (**C**) Survival plot of WT and Akt1$^{-/-}$ mice, sensitized with OVA and challenged 20 days later with OVA in 1% EB. The total number of mice is shown in brackets in the legend. **P = 0.0043.

mortality of experimental animals (10, 12). In vitro, PAF stimulates leukocyte superoxide production, chemotaxis, and adhesion (42). Although it has been suggested that some biological activities of PAF are mediated by ROS, antioxidants such as butylated hydroxytoluene, vitamin E, or N-acetylcysteine failed to inhibit PAF-induced mortality, disseminated intravascular coagulation, or pulmonary edema (43, 44). Furthermore, the exact role of NO in various anaphylactic shock models also remains unclear, with reports ranging from L-NAME–induced reduction of mortality (16) to reduced survival (17, 19).

In the present study, we set out to unequivocally elucidate the involvement and importance of NO, sGC, ROS, and PLA2 signaling in shock induced by intravenous PAF in conscious, nonanesthetized mice. To validate the results we obtained in the PAF-induced shock model, we also used 2 different models of active murine anaphylactic shock.

We found that PAF-induced mortality was not influenced by antioxidant treatment or PLA2 inhibition. Intravenous PAF injection in conscious mice elicited a very acute and drastic drop in blood pressure, resulting in death within only 20–30 minutes. As iNOS activation depends on de novo synthesis of both RNA and protein, several hours are required for its full activation. Surprisingly, however, NOS inhibition by L-NAME prevented PAF-induced hypotension and provided complete protection, even against PAF doses exceeding the LD_{100}. Moreover, whereas PAF injection in iNOS-deficient mice caused equally rapid mortality, eNOS-deficient animals were protected, indicating an unexpected pivotal role for eNOS-derived NO in PAF shock. Inhibitors of sGC protected either partially (MB) or not at all (ODQ), and sGC$\alpha1^{-/-}$ and WT mice suffered equally from PAF-induced shock. Catalytically active sGC heterodimers are composed of an α_1 or α_2 subunit combined with a β_1 subunit. Of interest, direct pharmacological sGC activation did not cause vasorelaxation or hypotension in sGC$\alpha1^{-/-}$ mice, indicating that sGC-dependent cardiovascular events predominantly rely on sGCα1 (31). Our results therefore imply that, downstream from NO, sGC-independent events are most important during PAF shock.

Traditionally, eNOS has been considered a constitutively expressed enzyme regulated by calcium and calmodulin. However,

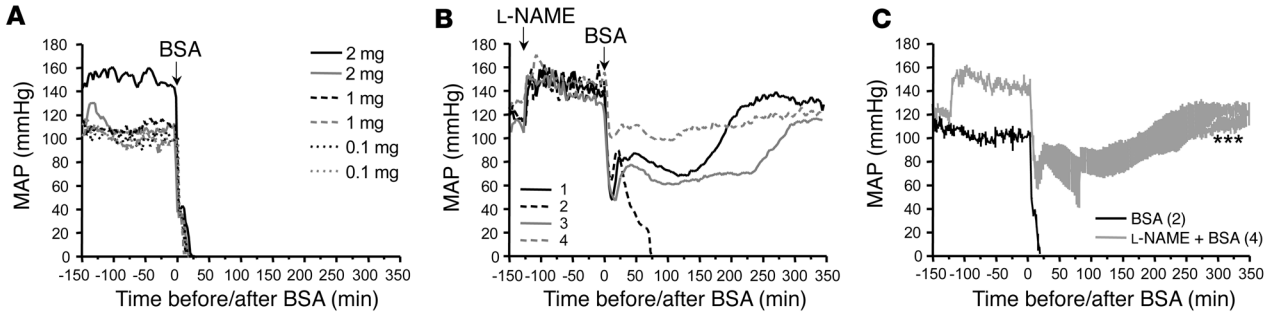

Figure 6
MAP measurements during anaphylactic shock. BSA-sensitized C57BL/6 mice were challenged i.v. with BSA at t = 0. (**A**) Individual MAP of mice challenged with 2, 1, or 0.1 mg BSA. (**B**) Individual MAP of mice pretreated with L-NAME (200 mg/kg, i.v.; –2 hours) and 0.1 mg BSA (t = 0). (**C**) Data points show the mean MAP ± SD of the 0.1 mg BSA plots shown in **A** and **B**. Error bars are plotted as vertical lines above and below the curves. Number of mice is indicated in parentheses. ***P < 0.0001.

phosphorylation of a C terminal serine residue through the PI3K/Akt pathway activates the eNOS enzyme at resting calcium levels and/or increases its activity (32, 33, 45). Interestingly, PI3K/Akt-dependent eNOS phosphorylation and ensuing NO production occur much faster than iNOS-dependent NO production, generally reaching a maximum within only 10 to 15 minutes of stimulation (32, 46, 47). In our study, pretreatment with WM, a specific PI3K inhibitor, prevented PAF-induced eNOS Ser1177 phosphorylation and efficiently protected both WT and iNOS-deficient mice from PAF shock. Hence, we conclude that there is a critical role for PI3K in the rapid activation of eNOS during PAF-induced shock.

To assess the pathophysiological role of PI3K/Akt and eNOS-derived NO in a clinically more relevant shock setting, we tested the effects of WM, perifosine, L-NAME, and eNOS or Akt1 deficiency in 2 different active and acute systemic anaphylaxis models. The experiments revealed a critical role for PI3K/Akt and eNOS-derived NO in anaphylactic shock. Following pretreatment with WM, perifosine, or L-NAME or in conditions of eNOS deficiency, anaphylaxis-induced hypothermia was mild and transient, vascular permeability was significantly lower, and no deaths occurred at all, indicating that PI3K/Akt-activated eNOS-derived NO is the most important vasodilator in anaphylactic shock.

Understanding the requirement of Akt in mammals is complicated by the existence of 3 highly conserved isoforms, Akt1, Akt2, and Akt3. Akt1-deficient mice show impaired overall growth, Akt2 knockout mice are insulin intolerant, demonstrating a diabetes-like syndrome, and mice lacking Akt3, which has more limited distribution, display a selective reduction in brain size (36–38, 48). Despite the protection obtained by Akt inhibition, $Akt1^{-/-}$ mice were not protected against anaphylactic shock, except for a minor delay in mortality. However, the viability of adult mice lacking each individual Akt isoform suggests that there is functional redundancy among these proteins. Interestingly, mice lacking both Akt1 and Akt2 die shortly after birth (49). In addition, endothelial cells of Akt1-deficient mice still show robust Akt levels and increased eNOS phosphorylation following VEGF treatment, which clearly provides evidence of the presence of compensatory mechanisms (39). This redundancy may explain why the Akt inhibitor perifosine is much more efficient than Akt1 deficiency in preventing anaphylactic shock.

Historically, the constitutively expressed eNOS enzyme was never seriously implicated as a detrimental mediator of inflammation. On the contrary, eNOS-derived NO was generally considered to exercise a rather antiinflammatory role (20). Nevertheless, the early activation of eNOS was already linked to an early, transient vascular hyporeactivity during both endotoxic and hemorrhagic shock more than a decade ago (50, 51). Although eNOS was originally believed to be constitutively expressed and modulated exclusively via changes in intracellular calcium concentration, it has now become clear that eNOS can be induced several-fold by multiple stimuli (such as shear stress, estrogen, and VEGF) and posttranslationally activated through protein phosphorylation or protein-protein interactions (45, 52). Moreover, eNOS deficiency or eNOS inactivation efficiently prevents the acute local inflammatory response induced by subplantar carrageenan injection (53, 54). Very recently, an important function for eNOS in facilitating iNOS expression and hypotension in endotoxemia was found, demonstrating a regulatory role for eNOS-derived NO in the gene expression of inflammatory mediators such as iNOS (55, 56). Our present study reveals what we believe is a new and surprising concept that eNOS-derived NO is the principal vasodilator in PAF-induced and anaphylactic shock, thereby strongly supporting the emerging belief in a possible detrimental role for eNOS in regulating inflammation and/or vascular function during shock.

In addition, MAP measurements in anaphylactic shock demonstrated that the abrupt and precipitous drop in blood pressure, which happens immediately after the challenge and lasts only a couple of minutes, is clearly NOS/NO-independent. This may be attributed to the rapid release of preformed vasoactive mediators, such as histamine and/or serotonin. However, following this initial dramatic blood pressure drop (of ± 80 mmHg), L-NAME–pretreated mice quickly recovered and eventually survived while control animals suffered a continued steep drop in blood pressure, leading to abrupt mortality. These data clearly demonstrate that eNOS-dependent vasorelaxation is key to the sustained hypotension causing shock and mortality during anaphylaxis.

Up until lately, very little was known about the role of PI3K in the cardiovascular system. Recently, the PAF/PI3K/eNOS pathway was reported to exert a potent negative inotropic effect on the heart and to be an important cause of myocardial dysfunction following ischemia/reperfusion (57, 58). Our data provide important additional information on a similar detrimental effect of the PAF/PI3K/eNOS pathway with respect to shock. In addition, we clearly demonstrate that anaphylactic shock in conscious mice is crucially orchestrated by PI3K/Akt and eNOS-derived NO. Although cyclic GMP/protein kinase G–dependent phosphorylation of the contractile machinery and calcium-regulating proteins is generally assumed to be the major mechanism by which NO causes pathological vascular smooth muscle relaxation, our results suggest that sGC-independent actions of NO may contribute significantly to its shock-inducing effect in vivo. As a general conclusion, we propose that selective targeting of eNOS and/or PI3K or Akt may provide new tools for the treatment of anaphylactic shock.

Methods

Mice. Female C57BL/6 mice were purchased from Elevage Janvier and $Akt1^{-/-}$ mice from Jackson Laboratory, where we also obtained iNOS-deficient (59) and eNOS-deficient (60) mice on a C57BL/6 background to breed in our facilities. The $sGC\alpha1^{-/-}$ mice were generated as reported (31), and littermates were used as controls. Mice were housed in temperature-controlled, air-conditioned facilities with 14-hour light/10-hour dark cycles and food and water ad libitum. All data were collected using mice 8–12 weeks of age. All experiments were approved by and performed according to the guidelines of the animal ethics committees of Ghent University, Belgium, and Maastricht University, The Netherlands.

Cytokines, reagents, injections, body temperature, and MAP measurements. PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; Sigma-Aldrich) was diluted in 200 μl endotoxin-free PBS supplemented with 0.25% BSA and injected i.v. Mortality was scored up to 7 days after challenge. The NOS inhibitor L-NAME was purchased from Novabiochem and injected i.v. at a dose of 100 mg/kg. Tempol (Sigma-Aldrich) was injected i.p. at 6 mg/mouse 1 hour before PAF. Aristolochic acid (Sigma-Aldrich) was administered s.c. in 50 μl DMSO 1.5 hours before PAF at 120 μg/mouse. MB in glucose solution suitable for i.v. injection (MB; Sterop) was used at a dose of 15 mg/kg unless otherwise stated. All of these treatments were based on the protective effects observed in our previous studies (24, 29). ODQ (Tocris Bioscience) was used i.p. in 50 μl DMSO at 20, 15, 10, or 5 mg/kg. WM (Sigma-Aldrich, 30 μg/mouse in 1.5% DMSO) was given i.p. 1.3 hours before the PAF challenge. Perifosine (octadecylphosphopiperidine; Keryx Biopharmaceuticals) was injected s.c. (1 mg/100 μl PBS/mouse) for

4 consecutive days, the last treatment being 2 hours before challenge. Rectal body temperature was recorded with an electronic thermometer (model 2001; Comark). Blood pressure and heart rate were measured continuously in conscious, permanently catheterized C57BL/6 mice, as described (24, 29). Briefly, a heat-stretched piece of polyethylene tubing was inserted into the right femoral artery. Similarly, a catheter was placed in the jugular vein for drug injections. After surgery, animals received Ringer solution (Baxter BV) and analgesic and were kept at 30°C to improve recovery. Experiments were performed 3 days after surgery, at which time the catheter was connected to a low-volume pressure transducer to record MAP and HR continuously throughout the study. The setup was allowed to stabilize for at least 40 minutes before the experiment was started.

Active systemic anaphylaxis models. Two different sensitization models were used: (a) Mice were given a single i.p. injection of 1 mg BSA (Sigma-Aldrich) mixed with 300 ng pertussis toxin (Sigma-Aldrich). Anaphylaxis was elicited 15 days later by i.v. injection of 2 mg of BSA. In the hemodynamic studies, the dose of BSA had to be lowered from 2 to 0.1 mg because the surgical procedures led to a sensitization to the BSA challenge. (b) Mice were sensitized by i.p. injection of 100 μg OVA (Sigma-Aldrich), aluminum hydroxide (Sigma-Aldrich, 1 mg) and pertussis toxin (300 ng). Mice were challenged 19–20 days later by i.v. injection of 150 μg OVA. When appropriate, mice were pretreated with L-NAME (i.v., –2.0 hours), WM (i.p., –1.3 hours), or perifosine (s.c.). Sensitizing i.p. injections were given in 0.5 ml of endotoxin-free PBS. To permit analysis of vascular permeability, challenges were injected into the lateral tail vein in 0.2 ml of 1% EB in endotoxin-free PBS, and ear biopsies were taken 90 minutes later. Control mice were challenged with EB only. As long as vascular permeability is unaffected, EB remains confined to the intravascular space. To quantify vascular leakage, ear biopsies were incubated in 0.3 ml formamide at 60°C for 4 days to extract EB from the tissue; absorption was measured at 620 nm.

Western blot analysis. Mice were pretreated with WM or vehicle and 1.3 hours later challenged with a lethal dose of PAF, BSA, or OVA. They were euthanized by cervical dislocation at the indicated time. Organs were dissected, snap-frozen in liquid nitrogen, and homogenized in a lysis buffer suitable for detecting phosphorylated proteins containing 50 mM HEPES, 5 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 0.15 μM aprotinin, 2.1 μM leupeptin, 1 mM Pefabloc, 25 mM β-glycerolphosphate, 0.2 mM sodium vanadate, and 10 mM sodium fluoride. After centrifugation of cell debris, protein concentrations were determined (Bio-Rad), and 20 mg were used for immunoprecipitation with mouse anti-eNOS (BD Biosciences — Pharmingen). Purified eNOS was resolved in a 4–12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose (Schleicher & Schuell BioScience). Filters were blocked for 1 hour at room temperature in TBS containing 0.1% Tween 20 (TBS/T) supplemented with 5% milk powder. The filters were incubated overnight at 4°C with a mAb against phosphorylated eNOS (Ser1177) (Cell Signaling Technology) in TBS/T with 5% BSA, washed 5 times with TBS/T, and incubated for 1 hour at room temperature with a goat anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling Technology). Afterwards, 5 washes with TBS/T preceded development with the Chemiluminescent Reagent Plus (ECL) detection reagent (PerkinElmer).

Statistics. Statistics were performed using GraphPad Prism version 4.0 (GraphPad Software; log-rank test for comparison of survival curves, χ^2 test to compare total mortality percentages, unpaired 2-tailed Student's *t* test to compare mean MAP data, and 1-way ANOVA with Bonferroni's multiple comparison test for EB extravasation data).

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