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Anaphylactic shock depends on PI3K and eNOS-derived NO

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Anaphylactic shock is a sudden, life-threatening allergic reaction associated with severe hypotension. Platelet-activating factor (PAF) is implicated in the cardiovascular dysfunctions occurring in various shock syndromes, including anaphylaxis. Excessive production of the vasodilator NO causes inflammatory hypotension and shock, and it is generally accepted that transcriptionally regulated inducible iNOS is responsible for this. Nevertheless, the contribution of NO to PAF-induced shock or anaphylactic shock is still ambiguous. We studied PAF and anaphylactic shock in conscious mice. Surprisingly, hyperacute PAF shock depended entirely on NO, produced not by inducible iNOS, but by constitutive eNOS, rapidly activated via the PI3K pathway. Soluble guanlyate cyclase (sGC) is generally regarded as the principal vasorelaxing mediator of NO. Nevertheless, although methylene blue blue partially prevented PAF shock, neither 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) nor sGCα1 deficiency did. Also, in 2 different models of active systemic anaphylaxis, inhibition of NOS, PI3K, or Akt or eNOS deficiency provided complete protection. In contrast to the unsubstantiated paradigm that only excessive iNOS-derived NO underlies cardiovascular collapse in shock, our data strongly support the unexpected concept that eNOS-derived NO is the principal vasodilator in anaphylactic shock and define eNOS and/or PI3K or Akt as new potential targets for treating anaphylaxis.

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 PI3K or Akt 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 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 PI3K or Akt deficiency can prevent fatal anaphylaxis in animal models, PAF is thought to be a critical factor in the development of anaphylactic shock (5–9).

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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 Nω-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 LD100, l-NAME still provided absolute protection (not shown).

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.

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]quinolin-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, sGCα1−/− 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
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/OVA 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, which causes PAF shock is produced by eNOS, activated via PI3K. 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.

![Figure 3](http://www.jci.org)
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 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

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 OD492 ± SD, *P < 0.001; #P < 0.01.
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 \(\alpha1\) or \(\alpha2\) subunit combined with a \(\beta1\) 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 sGC\(\alpha1\) (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,
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, gener-
ally reaching a maximum within only 10 to 15 minutes of stimula-
tion (32, 46, 47). In our study, pretreatment with WM, a specific 
PI3K inhibitor, prevented PAF-induced eNOS Ser1177 phosphory-
lation 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 defi-
ciency 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 com-
plicated 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, demonstrat-
ing 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 inhibi-
tion, 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. Interest-
ingly, 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 
ever seriously implicated as a detrimental mediator of inflam-
mation. On the contrary, eNOS-derived NO was generally consid-
ered to exercise a rather antiinflammatory role (20). Nevertheless, 
the early activation of eNOS was already linked to an early, tran-
sient vascular hyporeactivity during both endotoxemic and hem-
orrhagic 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 inflam-
atory response induced by subplantar carrageenan injection (53, 
54). Very recently, an important function for eNOS in facilitat-
ing 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 con-
cept 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 regulat-
ing inflammation and/or vascular function during shock.

In addition, MAP measurements in anaphylactic shock demon-
strated that the abrupt and precipitous drop in blood pressure, 
which happens immediately after the challenge and lasts only a 
couple of minutes, is clearly NO/NO-independent. This may be 
attributed to the rapid release of preformed vasoactive mediators, 
such as histamine and/or serotonin. However, following this ini-
tial dramatic blood pressure drop (of ± 80 mmHg), l-NAME–pre-
treated mice quickly recovered and eventually survived while con-

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 hypoten-
sion 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 path-
way was reported to exert a potent negative inotropic effect on the 
heart and to be an important cause of myocardial dysfunction fol-
owing 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 con-
tactile machinery and calcium-regulating proteins is generally 
assumed to be the major mechanism by which NO causes patho-

gological 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 sGCα1−/− mice were generated as reported (31), and litter-
mates 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 guide-
lines 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 
infected 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 admin-
istered 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


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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 μg pertussis toxin (Sigma-Aldrich). Anaphylaxis was elicited 15 days later by i.v. injection of 2 μg 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 i-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 EB assays were taken 90 minutes later. Control mice were challenged with EB only. As long as vascular permeability is unaffected, BE remains confined to the intravascular space. To quantify vascular leakage, ear biopsies were incubated in 0.1 ml formalin 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 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 μg were used for immunoprecipitation with mouse anti-eNOS (BD Biosciences – Pharmin-gen). 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, χ² 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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