

# Reactive oxygen species and small-conductance calcium-dependent potassium channels are key mediators of inflammation-induced hypotension and shock

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**Abstract** Septic shock is associated with life-threatening vasodilation and hypotension. To cause vasodilation, vascular endothelium may release nitric oxide (NO), prostacyclin (PGI<sub>2</sub>), and the elusive endothelium-derived hyperpolarizing factor (EDHF). Although NO is critical in controlling vascular tone, inhibiting NO in septic shock does not improve outcome, on the contrary, precipitating the search for alternative therapeutic targets. Using a hyperacute tumor necrosis factor (TNF)-induced shock model in mice, we found that shock can develop independently of the known vasodilators NO, cGMP, PGI<sub>2</sub>, or epoxyeicosatrienoic acids. However, the antioxidant tempol efficiently prevented hypotension, bradycardia, hypothermia, and mortality, indicating the decisive involvement of reactive oxygen species (ROS) in these phenomena. Also, in classical TNF or lipopolysaccharide-induced shock models, tempol protected significantly. Experiments with (cell-permeable) superoxide dismutase or catalase, N-acetylcysteine and apocynin suggest that the ROS-dependent shock depends on intracellular •OH radicals. Potassium channels activated by ATP (K<sub>ATP</sub>) or calcium

(K<sub>Ca</sub>) are important mediators of vascular relaxation. While NO and PGI<sub>2</sub>-induced vasodilation involves K<sub>ATP</sub> and large-conductance BK<sub>Ca</sub> channels, small-conductance SK<sub>Ca</sub> channels mediate vasodilation induced by EDHF. Interestingly, also SK<sub>Ca</sub> inhibition completely prevented the ROS-dependent shock. Our data thus indicate that intracellular •OH and SK<sub>Ca</sub> channels represent interesting new therapeutic targets for inflammatory shock. Moreover, they may also explain why antioxidants other than tempol fail to provide survival benefit during shock.

**Keywords** Shock · Hypotension · ROS · Nitric oxide · Potassium channels · Apamin

## Introduction

Septic shock has become the main cause of death in intensive care units [1]. In the first week, refractory hypotension is the leading pathogenic feature underlying mortality; later deaths are mainly due to multiple organ failure resulting from hypoxia, cytotoxicity, mitochondrial dysfunction, and prolonged hypotension. Hypotension is caused by profound vasodilation due to the endothelial release of the vasodilators nitric oxide (NO), prostacyclin (PGI<sub>2</sub>), and/or endothelium-derived hyperpolarizing factor (EDHF), as well as decreased production of and refractoriness to vasoconstrictors and vasopressors [2, 3]. EDHF seems to be particularly important in the microcirculation, where systemic vascular resistance is regulated. Despite the on-going dispute over its identity, it is generally accepted that small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (SK<sub>Ca</sub>) are critical for EDHF-dependent vasodilation [3–5]. NO can be produced by three different NO synthases (NOS); of which the inducible isoform iNOS (NOS2) is responsible

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for excessive and prolonged NO production following inflammation. Nevertheless, NO derived from the constitutive endothelial NOS isoform (eNOS and NOS3) may also mediate hypotension in both endotoxin-induced [6] and anaphylactic shock models [7, 8]. Besides modulating the contractile apparatus and intracellular calcium flux in vascular smooth muscle cells, NO also influences membrane potential and vascular tone via activating ATP-dependent ( $K_{ATP}$ ) and large-conductance  $Ca^{2+}$ -activated  $K^+$  channels ( $BK_{Ca}$ ) [8, 9]. In septic shock, NO plays a critical but controversial role. Although inhibitors of NOS can improve hemodynamics, they also increase toxicity [10], and a phase III clinical trial had to be prematurely terminated because of increased mortality [11]. While part of this paradox might be explained by protective properties of NO such as its antioxidative effects [12], there is also growing evidence that the pathogenesis of inflammation-associated shock involves oxidative stress next to nitrosative stress.

The sequential donation of electrons to oxygen generates reactive oxygen species (ROS), which may cause oxidative damage at high concentrations, but whose signaling capacities and regulatory functions are becoming more and more evident as well. Donation of one electron to oxygen results in the formation of superoxide radicals ( $O_2^{\bullet-}$ ). Donation of a second electron generates peroxide, which then undergoes protonation to hydrogen peroxide ( $H_2O_2$ ). Donation of a third electron, such as occurs in the Fenton reaction mediated by free  $Fe^{2+}$ , results in the production of hydroxyl radicals ( $\bullet OH$ ). On the whole,  $O_2^{\bullet-}$  is believed to contribute to cardiovascular pathologies such as hypertension and atherosclerosis because it reduces the bioavailability and the effects of NO [13, 14]. To the best of our knowledge, the contribution of various endogenous ROS ( $O_2^{\bullet-}$ ,  $H_2O_2$ ,  $\bullet OH$ ) to inflammation-induced hypotension has not been extensively studied yet. The evaluation of the involvement of ROS in inflammatory hypotension in vivo is a complicated matter, given the vast amounts of NO produced following inflammation-induced iNOS transcription and the highly interactive chemistry of reactive oxygen and nitrogen species [15].

This study was therefore conducted as a proof-of-principle, to evaluate whether ROS can cause inflammatory hypotension and shock in vivo. For this purpose, we used a rapid caspase-independent tumor necrosis factor (TNF)-induced murine shock model that starts to develop already before systemic NO production occurs, and that is accompanied by excessive oxidative stress [16]. Using this model, we found that ROS can indeed cause hypotension in inflammatory shock, and that  $Ca^{2+}$ -dependent small-conductance  $SK_{Ca}$  channels are the only  $K^+$  channels that play a prominent role in this ROS-dependent shock.

## Materials and methods

### Mice

Female C57BL/6J were from Janvier (France);  $gp91phox^{-/-}$ ,  $15\text{-lipoxygenase}^{-/-}$ ,  $iNOS^{-/-}$  [17], and  $eNOS^{-/-}$  [18] mice on a C57BL/6J background were from The Jackson Laboratory (Bar Harbor, ME, USA). To obtain double-deficient  $ixeNOS^{-/-}$  animals, we crossed  $iNOS^{-/-}$  with  $eNOS^{-/-}$  mice and verified the knockout by polymerase chain reaction. Mice were housed in temperature-controlled, air-conditioned facilities with 14–10-h light/dark cycles and food and water ad libitum, and used at 8–12 weeks. All experiments were approved by the animal ethics committees of Ghent University, Belgium, and Maastricht University, The Netherlands.

### Cytokines, reagents, and injections

Recombinant mouse TNF was produced in and purified from *Escherichia coli*; lipopolysaccharide (LPS) content was  $<0.02$  ng/mg (chromogenic *Limulus* amoebocyte lysate assay, Kabivitrium, Sweden). TNF or ultrapure *E. coli* LPS (Invivogen, serotype 0111:B4) were injected i.v. in LPS-free phosphate buffered saline (PBS). The TNF  $LD_{100}$  was determined before each experiment and ranged from 8 to 18  $\mu g$  depending on the TNF lot. zVAD-fmk (Bachem, Switzerland) was suspended in DMSO at 50 mg/ml, further diluted in PBS, and injected i.p. 15 min before (0.25 mg) and 1 h after TNF (0.1 mg). Mortality was scored up to 7 days.  $N^G$ -nitro-L-arginine methyl ester (L-NAME, Novabiochem, 100 mg/kg) was given i.v. either 2 h or 30 min before, or together with TNF. For the hemodynamic studies, L-NAME or tempol were injected 45 min before TNF. Indomethacin (Sigma, 125  $\mu g$ ) was injected i.p. 1 h before challenge. Tempol (6 mg), SOD (7,500 U), PEG-SOD (400 U), catalase (7,500 U), and PEG-catalase (7,500 U), all from Sigma, were dissolved in PBS and injected i.p. 40 min before challenge. ABT (Sigma, 2.5 mg), SKF-525A (Biomol, 1.5 mg), and fluconazole (Pfizer, 1.2 mg) were injected i.p. 2 h before TNF. For  $K^+$  channel inhibition, apamin (ICN, 0.5 mg/kg i.v.), charybdotoxin (Sigma, 33  $\mu g/kg$  i.v.), iberiotoxin (Sigma, 33  $\mu g/kg$  i.v.), TEA (Sigma, 50 mg/kg i.p.), and glibenclamide (ICN, 25 mg/kg i.p.) were injected as previously described [19]. All treatment schedules and doses were based on other rodent studies in which they had a positive modulatory effect.

### $NO_x^-$ and cGMP measurement

For  $NO_x^-$  determination, serum was diluted 1:1 with  $5.10^9$  CFU/ml *Pseudomonas oleovorans* suspension (reducing nitrate to nitrite) and incubated at 37°C for 3–4 h. After centrifugation, the supernatant was diluted 1:2 with Griess reagent, and proteins were precipitated with 10% TCA. The

absorbance of the supernatant was measured at 540 nm. Total  $\text{NO}_x^-$  was calculated from a nitrate standard curve. cGMP was determined by EIA (Amersham Pharmacia Biotech) after acetylation of the samples, according to the manufacturer's instructions. Plasma was collected by cardiac puncture using 7.5 mM EDTA and kept on ice until centrifugation. Dissected organs were immediately immersed in liquid nitrogen and stored at  $-20^\circ\text{C}$  before homogenization and purification. Kidney cGMP levels were plotted as picomoles cGMP per milligram of TCA-precipitable protein solubilized with 1 N NaOH.

#### Body temperature, mean arterial pressure, and HR measurements

Rectal body temperature was recorded with an electronic thermometer (model 2001; Comark Electronics, Littlehampton, UK). Blood pressure and heart rate were measured continuously in conscious mice, either in tethered permanently catheterized mice, as described [16], or via radiotelemetry. For the latter, PA-C10 telemetry probes (Data Sciences International) were surgically implanted. Mice were anesthetized with 2% isoflurane, and the cervical area skin was cleaned with povidone-iodine and alcohol. A 1-cm vertical incision was made in the left neck, and the left carotid artery was cannulated. The artery was punctured with a 26-gauge needle, the catheter tip of the transmitter was advanced to the aortic arch, and the catheter was sutured in place. A subcutaneous pocket was excavated over the right flank, the transmitter body was inserted into this, and the incision was closed with sutures. Ibuprofen was given in the drinking water from the day before surgery until 4 days after recovery. At day 10 after surgery, continuous, 24-h data collection began using the Dataquest ART Acquisition System (Data Sciences International, version 4.1).

#### Statistics

Statistics was performed using GraphPad Prism.  $\text{NO}_x^-$  or cGMP levels and body temperatures are presented as mean  $\pm$  SEM. They were compared with a one-way analysis of variance test, with a Bonferroni post-test for comparison of all pairs. Survival curves and total percent mortality were compared with a log-rank (Mantel–Cox) test and a Chi-square test, respectively.

## Results

### NO or PGI2 do not mediate zVAD+TNF shock

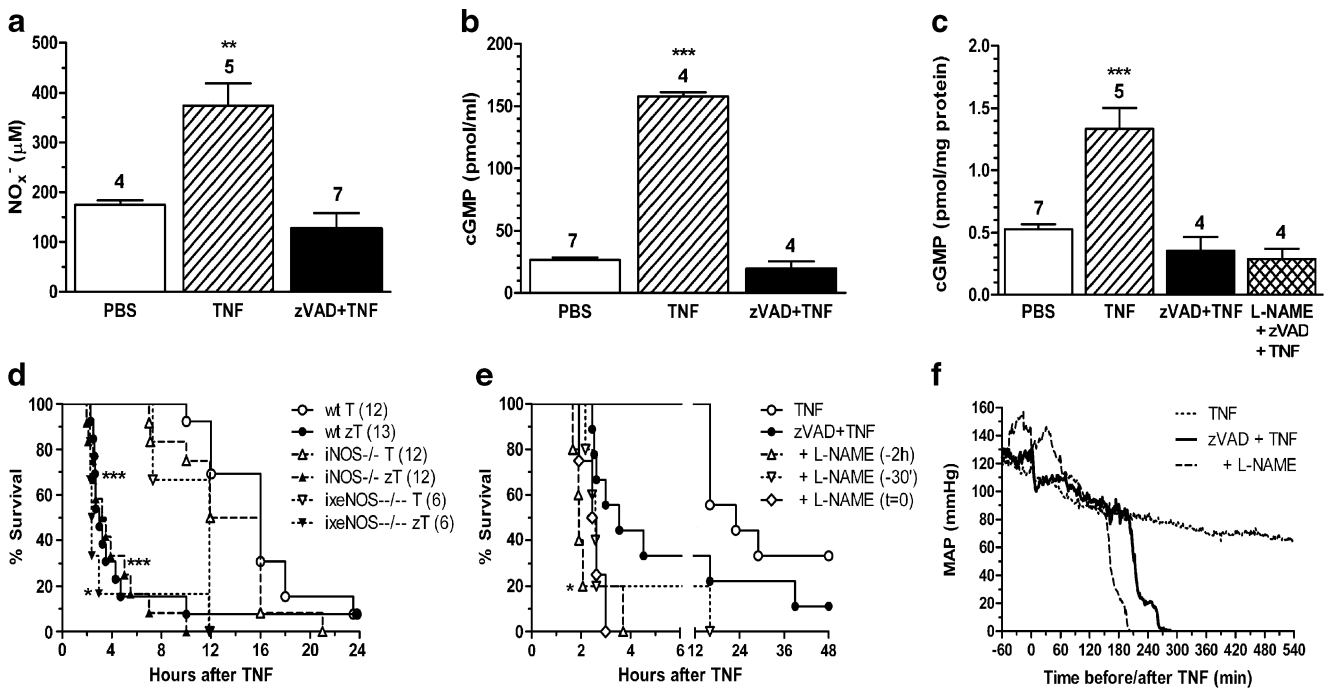
To evaluate the potential of ROS to cause hypotension, we used a hyperacute inflammatory shock model where

excessive oxidative stress is a key feature [16]. In this model, TNF is combined with the caspase inhibitor zVAD, leading to abrupt, hyperacute hypotension and death within only a few hours after challenge. It is generally believed that inflammation-induced cardiovascular collapse is predominantly orchestrated by iNOS-derived NO [10]. However, the iNOS enzyme needs to be transcriptionally induced, and its action thus requires several hours [9]. In agreement with this, we find detectable systemic  $\text{NO}_x^-$  at the earliest 2–3 h after TNF [20]. Since the abrupt zVAD+TNF hypotension starts even before that [16], the involvement of iNOS-derived NO seems improbable. Nevertheless, we have identified eNOS-derived NO as the principal vasodilator in anaphylactic shock, causing hypotension within less than half an hour [7], making also this a potential candidate to mediate hyperacute shock. To evaluate the possible involvement of NO and its downstream mediator cGMP in the zVAD+TNF shock model, we first determined endogenously induced  $\text{NO}_x^-$  and cGMP, which were not increased at all but rather reduced to background levels (Fig. 1a–c). In addition, deficiency for iNOS, or iNOS and eNOS, could not protect mice from the abrupt zVAD+TNF toxicity (Fig. 1d), and the NOS inhibitor L-NAME even aggravated toxicity (Fig. 1e). To evaluate the effect of NOS inhibition on hypotension, we measured blood pressure in permanently catheterized conscious mice. After L-NAME treatment, zVAD+TNF still induced an acute and steep drop in blood pressure, which generally developed even more rapidly than without L-NAME (Fig. 1f). Together, these results imply that the hyperacute zVAD+TNF shock develops NO-independently.

Next to NO, endothelial cells may also produce vaso-relaxing PGI<sub>2</sub>. However, inhibition of PGI<sub>2</sub> producing cyclooxygenases with indomethacin could not prevent zVAD+TNF shock [16] and even exacerbated it (not shown).

### EDHF candidates do not mediate zVAD+TNF shock

NO- and PGI<sub>2</sub>-independent vasodilation is especially significant in resistance vessels, which are particularly important for blood pressure regulation, and is mediated via a mechanism collectively labeled EDHF [3]. The (patho)physiological involvement of EDHF *in vivo* is hard to investigate, as there is still no consensus about its nature and exact mode of action.  $\text{H}_2\text{O}_2$  and epoxyeicosatrienoic acids (EETs) have been proposed as EDHF candidates [3]. To investigate the contribution of  $\text{H}_2\text{O}_2$ , we treated mice with catalase or cell-permeable PEG-catalase. However, they could not protect and rather aggravated the acute toxicity (Fig. 2a). In addition, also the CYP inhibitors 1-aminobenzotriazole (ABT), fluconazole, or SKF-525A could not provide significant protection, excluding a role



**Fig. 1** NO and cGMP in zVAD+TNF shock. **a**  $\text{NO}_x^-$  ( $\text{NO}_2^- + \text{NO}_3^-$ ) in serum collected 3 h after PBS or TNF (*n* above the bars). **b** cGMP in plasma collected 3 h after PBS or TNF (*n* above the bars). **c** cGMP in homogenates from kidneys, collected 3 h after PBS or TNF (*n* above the bars). \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , compared with PBS. **d** WT,  $\text{iNOS}^{-/-}$ , or double  $\text{iNOS}^{-/-}$  and  $\text{eNOS}^{-/-}$  mice ( $\text{ixeNOS}^{-/-}$ ) were injected with TNF (T, open symbols) or zVAD+TNF (zT, filled symbols; *n* in the legend). \*\*\* $P < 0.001$ , \* $P < 0.05$ , compared with TNF alone. **e** WT mice were injected with TNF

(open circles, *n*=9) or TNF+zVAD (closed circles, *n*=9), and the effect of L-NAME 2 h (triangles, *n*=5) or 30 min (triangles, *n*=5) before TNF or together with TNF (diamonds, *n*=8) was tested. \* $P < 0.05$ , compared with zVAD+TNF. **f** Mean arterial pressure in conscious free-moving catheterized mice injected through a catheter with TNF, with or without zVAD i.p. To analyze the role of NO, a group of zVAD+TNF mice was treated with L-NAME 45 min before TNF. *n*=5 for each group, plotted is the median response

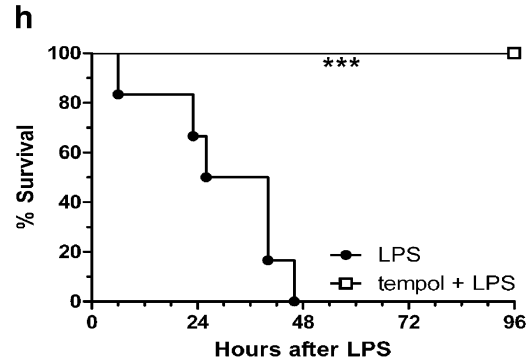
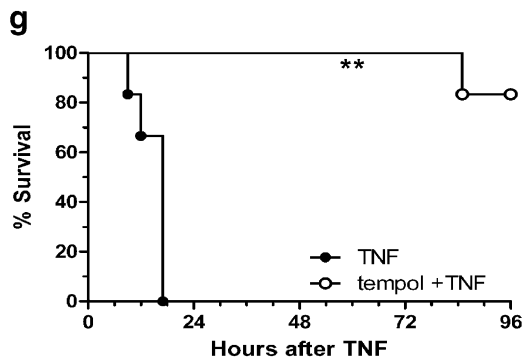
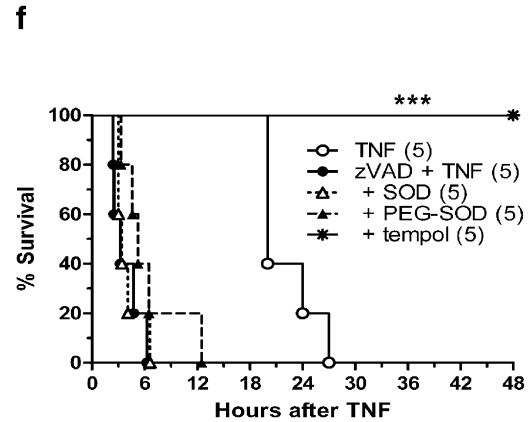
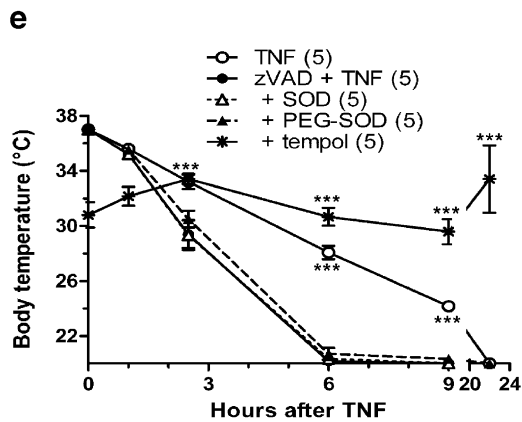
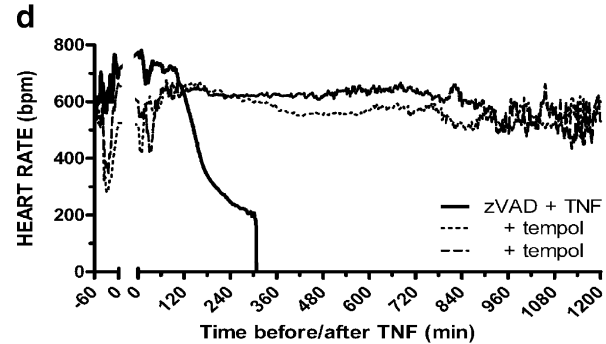
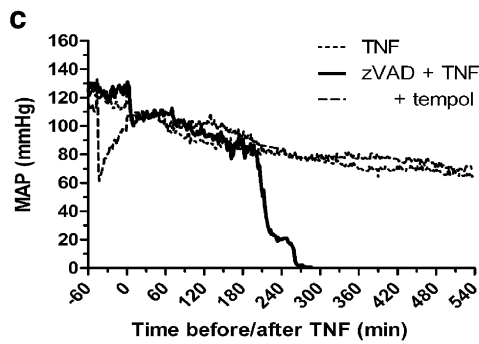
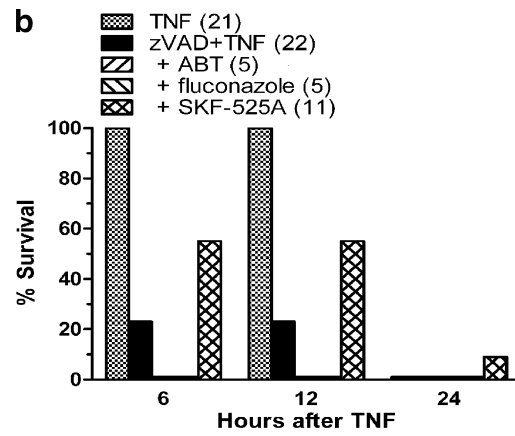
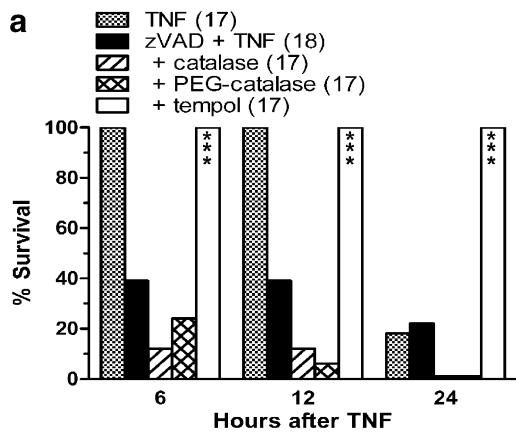
for EETs in zVAD+TNF shock (Fig. 2b). Also a 15-lipoxygenase derivative has been proposed as a vasorelaxing arachidonic acid metabolite [3], but 15-lipoxygenase $^{-/-}$  mice were as sensitive to zVAD+TNF shock as wild-type animals (not shown).

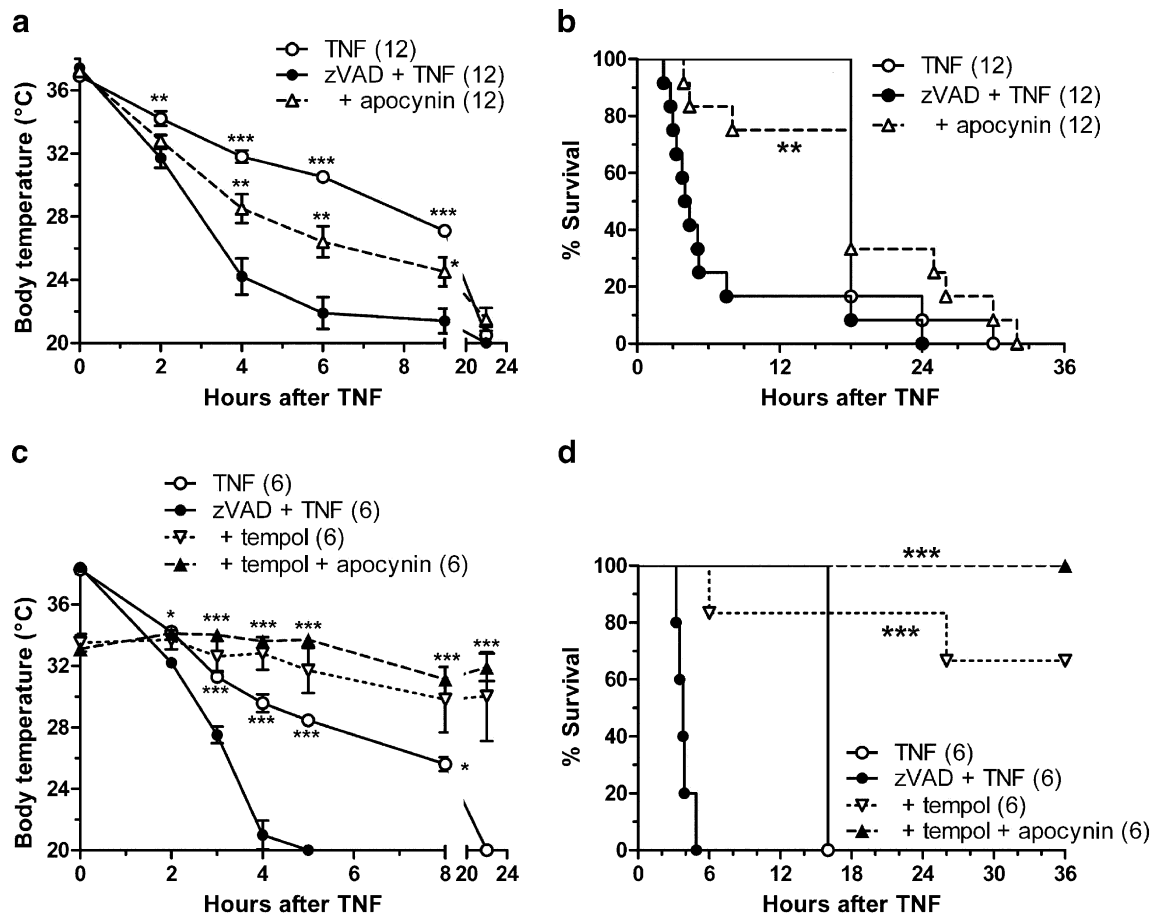
#### Antioxidant protection against shock induced by zVAD+TNF, TNF, or LPS

ROS are thought to contribute to morbidity in sepsis because of their direct cytotoxic and organ-damaging capacities. To investigate whether they also might be involved in causing the hypotension, we measured blood pressure in conscious radiotelemetred mice pretreated with tempol, a cell-permeable SOD mimetic, radical scavenger and inhibitor of the Fenton reaction [21, 22]. Surprisingly, while NOS inhibition did not prevent the abrupt zVAD+TNF hypotension at all (Fig. 1f), tempol very efficiently did (Fig. 2c). Moreover, while the blood pressure of mice treated with TNF or zVAD+TNF dropped substantially following TNF challenge, blood pressure did not decrease at all for at least 2 h if the mice had been pretreated with tempol (Fig. 2c). In addition, pretreatment with tempol

completely prevented the severe bradycardia that normally develops shortly after zVAD±TNF challenge (Fig. 2d). The effect of tempol on blood pressure and heart rate was mirrored by its effect on peripheral body temperature (Fig. 2e).

**Fig. 2** ROS and EETs in zVAD+TNF toxicity. **a** Effect of catalase, PEG-catalase, or tempol on mortality induced by zVAD±TNF. Plotted is the percent survival of all mice used in three independent experiments; the total number is indicated between brackets in the legend. Sensitization by zVAD corresponds to mortality within 6 h. Mice injected with TNF alone are also plotted for comparison. \*\*\* $P < 0.0001$  compared with zVAD+TNF (black bar). **b** Effect of the CYP inhibitors ABT, fluconazole, and SKF-525A. Plotted is the percent survival of all mice used in up to four independent experiments; the total number is indicated between brackets in the legend. Differences between zVAD+TNF and SKF-525A+zVAD+TNF are not significant ( $P > 0.07$ ). **c** Blood pressure and **d** heart rate were monitored in conscious radiotelemetred mice injected with zVAD±TNF. Two mice were treated with tempol 45 min before TNF. **e, f** Effect of tempol, SOD, or PEG-SOD on hypothermia (**e**) and mortality (**f**) induced by zVAD+TNF (*n*=5), \*\*\* $P < 0.001$  compared with zVAD+TNF. **g** Tempol protects against TNF shock. Mice were injected i.v. with a lethal dose of TNF alone (*n*=6), \*\* $P = 0.0012$ . **h** Tempol protects against LPS shock. Mice were injected i.v. with a lethal dose of LPS (*n*=6), \*\*\* $P = 0.0006$





**Fig. 3** Apocynin protects against acute zVAD+TNF shock. **a** Effect of apocynin on hypothermia induced by zVAD+TNF, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with zVAD±TNF. **b** Effect of apocynin

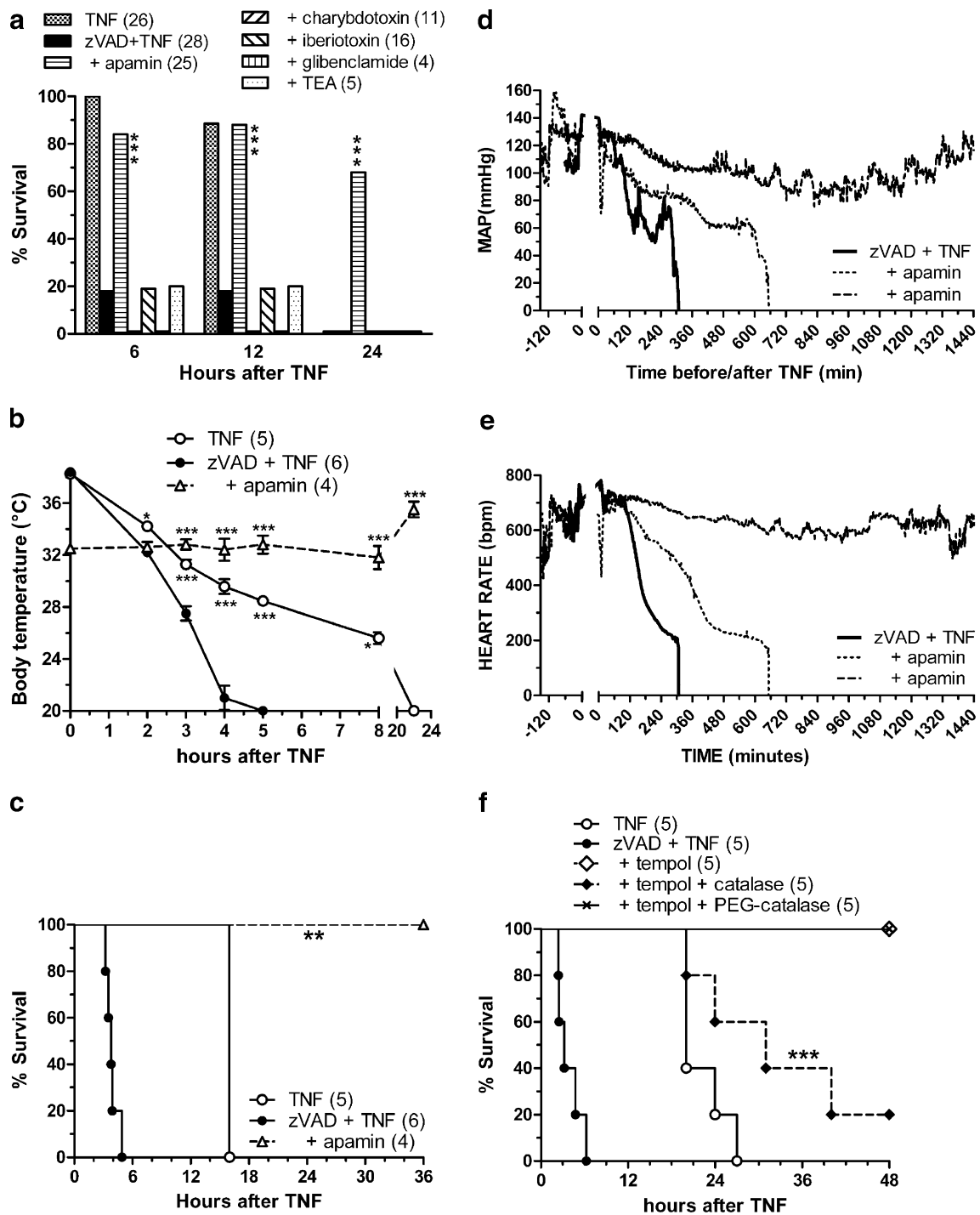
on zVAD±TNF mortality, \*\* $P = 0.0013$  compared with zVAD±TNF. **c, d** Effect of apocynin on protection by tempol. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with zVAD±TNF

To investigate the involvement of ROS in other models, we used tempol in shock induced by a high dose of TNF or LPS. Also in these models, tempol completely prevented mortality (Fig. 2g, h).

To better understand the exact mechanism of tempol's protective action, we compared its effect on ROS-dependent zVAD+TNF shock with SOD and cell-permeable PEG-SOD. Surprisingly, only tempol fully protected against acute hypothermia (Fig. 2e) and prevented both early and late mortality (Fig. 2a, f). As tempol is also an efficient scavenger of  $\bullet\text{OH}$  radicals and reduces the formation of  $\bullet\text{OH}$  [21, 22], these results suggest that not  $\text{O}_2^{\bullet-}$  but  $\bullet\text{OH}$  radicals are crucial. Apocynin, an NADPH oxidase (NOX) inhibitor and  $\bullet\text{OH}$  scavenger [23], also prevented hyperacute zVAD+TNF shock, but could not offer long-term protection (Fig. 3a, b). In combination with tempol, apocynin also conferred some additional protection against both hypothermia (Fig. 3c) and mortality (Fig. 3d).

$\text{SK}_{\text{Ca}}$  channels are essential for ROS-mediated zVAD+TNF shock

NO and PGI<sub>2</sub> activate large-conductance  $\text{BK}_{\text{Ca}}$  and  $\text{K}_{\text{ATP}}$  channels [2, 8, 9]. Vascular smooth muscle  $\text{BK}_{\text{Ca}}$  channels may also be activated by EETs or  $\text{H}_2\text{O}_2$ , which have been suggested as EDHFs in certain systems [3]. In contrast, EDHF-dependent hyperpolarization specifically depends on endothelial  $\text{SK}_{\text{Ca}}$  channels, and not on  $\text{BK}_{\text{Ca}}$  or  $\text{K}_{\text{ATP}}$  channels [3, 4]. To study  $\text{K}^+$  channels, we used apamin ( $\text{SK}_{\text{Ca}}$  inhibitor), iberiotoxin ( $\text{BK}_{\text{Ca}}$  inhibitor), charybdotoxin (inhibits  $\text{BK}_{\text{Ca}}$ ,  $\text{IK}_{\text{Ca}}$ , and certain voltage-gated  $\text{K}_{\text{v}}$ ), glibenclamide ( $\text{K}_{\text{ATP}}$  inhibitor), and TEA (inhibits  $\text{BK}_{\text{Ca}}$ ,  $\text{K}_{\text{ATP}}$ , and some  $\text{K}_{\text{v}}$ ). Only apamin completely prevented hyperacute (within 6 h) zVAD+TNF-induced hypothermia and mortality (Fig. 4a–c). Furthermore, apamin also significantly protected against long-term TNF-induced mortality (Fig. 4a, c). To evaluate the cardiovascular effects of  $\text{SK}_{\text{Ca}}$  inhibition, blood pressure and heart rate were



**Fig. 4** The role of  $K^+$  channels and  $H_2O_2$  in ROS-dependent zVAD+TNF shock. **a** Effect of different  $K^+$  channel inhibitors. Plotted is the percent survival of all mice used in up to five independent experiments; total numbers are indicated *between brackets* in the legend.  $***P < 0.0001$  compared with zVAD+TNF (black bar). **b, c** Effect of apamin on hypothermia (**b**) and mortality (**c**) induced by zVAD±TNF in a representative experiment (*n* in the legend),  $*P < 0.05$ ,  $**P =$

$0.0049$ ,  $***P < 0.001$  compared with zVAD±TNF. **d, e** Mean arterial pressure and HR were monitored in conscious radiotelemetred mice injected with zVAD±TNF. Three mice were treated with apamin 2 h before TNF, plotted are the non-survivor and one of the two survivors. **f** Effect of catalase and PEG-catalase on protection by tempol,  $***P < 0.001$  compared with tempol+zVAD+TNF (diamonds); data shown are from one individual representative experiment

measured in radiotelemetred mice. In two of the three animals pretreated with apamin, hypotension and bradycardia were efficiently prevented (one representative mouse is shown in Fig. 4d, e), resulting in survival. In one mouse, apamin could not prevent but delayed hypotension, bradycardia, and mortality (Fig. 4d, e, dotted line)

#### H<sub>2</sub>O<sub>2</sub> antagonizes TNF-induced shock

As shown in Fig. 2a, catalase could not improve but rather worsened zVAD+TNF shock. When catalase was combined with a protective tempol treatment, it even reversed the long-term protection provided by tempol (Fig. 4f). PEG-catalase, accumulating intracellularly, did not revert tempol protection (Fig. 4f), suggesting that extracellularly produced H<sub>2</sub>O<sub>2</sub> may counteract shock.

### Discussion

Our results indicate a key role for ROS and SK<sub>Ca</sub> channels in inflammatory hypotension and shock. To prove the involvement of ROS in inflammation-induced shock is not easy, as ROS and their production systems are efficiently antagonized by NO, which is massively produced as soon as iNOS is transcribed. Therefore, we used this specific TNF-induced caspase-independent hyperacute shock model as a proof-of-principle, because it develops even before NO metabolites can be detected in circulation [16]. In this shock model, we found that NOS inhibition or deficiency did not prevent hypotension and shock, while the antioxidants N-acetylcysteine (NAC), butylated hydroxyanisole (BHA), or tempol completely precluded the fast hyperacute mortality induced by zVAD±TNF [16]. In general, antioxidants are thought to protect against morbidity and mortality in sepsis because they prevent the cytotoxic and tissue-damaging effects of ROS. Our cardiovascular studies now reveal that tempol not just protects against oxidative toxicity and injury but also prevents the dramatic drop in mean arterial pressure and HR that normally develops abruptly after zVAD±TNF. The possible involvement of ROS in hypotension creates an intriguing paradox, since vascular ROS (particularly superoxide) have traditionally been associated with hypertension. A possible answer to this paradox could be that different ROS have different specific properties [24]. Therefore, we tried to pinpoint which ROS exactly could be the hypotensive culprit in the ROS-dependent zVAD+TNF shock. Tempol, which was clearly very proficient to prevent hypotension and mortality, is generally referred to as a “cell-permeable SOD mimetic.” However, since cell-permeable SOD could not protect, the ROS-dependent shock is most probably not directly triggered by O<sub>2</sub><sup>•-</sup>. Tempol is also an efficient radical

scavenger, as well as an inhibitor of Fenton-type reactions that occur between peroxide and transition metals and result in the formation of •OH radicals, and it has already been suggested that many of tempol's effects are probably due to its ability to scavenge •OH radicals [21, 22]. Also, the NOX inhibitor apocynin significantly delayed the hyperacute shock, in contrast to NOX2-deficiency [16]. This may indicate the involvement of NOX1 or NOX4, but the protective effect of apocynin may also be due to its •OH scavenging potential [23]. Interestingly, endothelial NOX was recently identified as a functionally relevant mediator of vasodilation in human coronary microcirculation [25]. We have previously shown that the hyperacute shock induced by zVAD+TNF may also be prevented by NAC [16], a scavenger of •OH radicals and precursor for cytoplasmic glutathione essential to prevent intracellular •OH production [26, 27]. Together, the protective effects of tempol, apocynin, and NAC, compared with the failure of (cell-permeable) SOD or catalase to protect, thus suggest the possibility that intracellular •OH radicals are the shock-causing ROS. The fact that NOS inhibition significantly exacerbated hypotension and toxicity may further corroborate this hypothesis, since NO can not only scavenge •OH radicals at near diffusion control but also prevent •OH formation from H<sub>2</sub>O<sub>2</sub> by scavenging superoxide radicals at diffusion control (thus inhibiting Fe<sup>3+</sup> reduction in ferritin and Fe-S clusters) and by reacting directly with free Fe<sup>2+</sup> to form dinitrosyliron complexes [15, 28]. The vasorelaxing and maybe even hypotensive potential of •OH radicals had already been suggested in the past [29, 30] but has not been given much attention since.

Surprisingly, catalase, but not PEG-catalase, aggravated zVAD+TNF toxicity and even reverted tempol's protection, demonstrating the detrimental effect of extracellular H<sub>2</sub>O<sub>2</sub> removal. Tempol is known to increase H<sub>2</sub>O<sub>2</sub> formation, vasoconstriction, and hypertension in vivo, effects that are abolished by catalase coinfusion [31]. Together, this suggests that extracellular H<sub>2</sub>O<sub>2</sub> may exert a protective role in shock, and that part of the protective capacity of tempol may reside in its ability to allow H<sub>2</sub>O<sub>2</sub> accumulation while efficiently interfering with intracellular •OH formation and accumulation at the same time. In this way, our results may explain why antioxidants other than tempol fail to provide survival benefit during shock [32], because they inhibit the accumulation of not only the detrimental •OH but also of the protective H<sub>2</sub>O<sub>2</sub>. Despite the fact that H<sub>2</sub>O<sub>2</sub> has been put forward as a potential EDHF [3, 33], due to the capability of catalase to diminish NO- and PGI<sub>2</sub>-independent vasorelaxation in isolated arteries, H<sub>2</sub>O<sub>2</sub> has also been demonstrated to antagonize vascular relaxation through various mechanisms [33–35] and was even identified as an important endogenous vasoconstrictor contributing to blood pressure regulation in vivo [36].



In addition to the effect of tempol on blood pressure, whose decline was prevented the first hours after TNF injection, there was also a remarkable effect on HR. Following injection with zVAD±TNF, mice respond with a transient compensatory tachycardia and a severe loss of HR variability, followed shortly afterwards by severe and abrupt bradycardia. Although the loss of HR variability was not affected by tempol, the initial tachycardia was not followed by bradycardia, indicating that ROS are involved in cardiac dysfunction during shock as well.

The opening of potassium ( $K^+$ ) channels is the main determinant of cell membrane potential, and thus the activation of vascular  $K^+$  channels is critical for the regulation of vascular tone. The vasodilators NO, PGI<sub>2</sub>, and EDHF may all activate various  $K^+$  channels in either endothelial or smooth muscle membranes, allowing  $K^+$  efflux out of the cell, causing hyperpolarization and vasodilation. More specifically, NO and PGI<sub>2</sub> are known to hyperpolarize vascular smooth muscle cells by activating  $Ca^{2+}$ -dependent large-conductance  $BK_{Ca}$  channels or ATP-sensitive  $K_{ATP}$  channels [2]. Although the exact molecular identity and mechanism of EDHF is still a matter of debate, it is generally agreed that EDHF-dependent hyperpolarization depends on endothelial  $SK_{Ca}$ , and not on  $BK_{Ca}$  or  $K_{ATP}$  channels [3, 4]. Using different inhibitors, we could clearly demonstrate the decisive contribution of  $SK_{Ca}$  channels to ROS-dependent shock, as apamin efficiently prevented hypotension, bradycardia, hypothermia, and mortality. Interestingly, both SK2 and SK3 channel conductances have been demonstrated to be increased by intracellular oxidative stress [37].

In conclusion, our data suggest that in an early phase of inflammation, important ROS-dependent cardiovascular effects are taking place. Interfering with these ROS-dependent effects efficiently antagonizes the progressive hypotension and bradycardia that normally ensues, resulting in fast recovery and survival. In general models of (septic) shock, the ROS-dependent effects may be easily overlooked, because of concomitant iNOS induction and massive NO production, which scavenges ROS and prevents further ROS production [15]. By acutely exacerbating oxidative stress (for example, by inhibiting caspases via the application of zVAD-fmk) and/or inhibiting NO production, the ROS-induced hypotension is amplified and results in lethal shock that is entirely ROS-dependent and efficiently prevented with the antioxidant tempol. In addition, ROS-dependent shock specifically relies on  $SK_{Ca}$  channels only. Importantly, ROS and  $SK_{Ca}$ -dependent events are also critical in the progression of shock in the absence of zVAD-fmk, as tempol (shown in this report) and apamin [19] also significantly improve survival in normal TNF- or LPS-induced shock models. Thus, specific inhibitors of vascular/endothelial  $SK_{Ca}$  channels and/or

scavengers of intracellular  $\bullet OH$  radicals and inhibitors of their formation (which may include vascular NOX1 or NOX4 enzymes) could represent interesting new therapeutics for the treatment of hypotension, cardiac dysfunction, and shock associated with inflammation.

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**Conflicts of interest** The authors declare no conflict of interests related to this study.

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