

Protein kinase G oxidation is a major cause of injury during sepsis

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Sepsis is a common life-threatening clinical syndrome involving complications as a result of severe infection. A cardinal feature of sepsis is inflammation that results in oxidative stress. Sepsis in wild-type mice induced oxidative activation of cGMP-dependent protein kinase 1 alpha (PKG α), which increased blood vessel dilation and permeability, and also lowered cardiac output. These responses are typical features of sepsis and their combined effect is a lowering of blood pressure. This hypotension, a hallmark of sepsis, resulted in underperfusion of end organs, resulting in their damage. A central role for PKG α oxidative activation in injury is supported by oxidation-resistant Cys42Ser PKG α knock-in mice being markedly protected from these clinical indices of injury during sepsis. We conclude that oxidative activation of PKG α is a key mediator of hypotension and consequential organ injury during sepsis.

redox | cardiovascular function | endotoxin

Sepsis, a prevalent medical condition caused by severe infection with systemic inflammation, causes substantial morbidity and mortality (1). Prognosis is poor with 85% survival in uncomplicated sepsis, falling to 20% in those with multiorgan failure (2). The cost of acute care is enormous (3), but survivors often suffer long-term cognitive impairment generating a chronic health care burden (4). Sepsis is characterized by systemic inflammation (5), decreased peripheral vascular resistance (1), microvascular leak (6), and decreased cardiac output (1). The combined effect of these alterations is low blood pressure (hypotension), a major clinical feature of sepsis (1). This hypotension results in underperfusion of end organs that leads to their functional failure and too often patient death (1).

Oxidative stress is a hallmark of sepsis, consistent with the inflammatory respiratory burst by neutrophils generating high levels of oxidants (5). However, multiple oxidant-generating systems, including nicotinamide adenine dinucleotide phosphate oxidase, uncoupled nitric oxide synthase (NOS) (7), lysozyme-c (8), and mitochondria (9) are activated during sepsis. Consistent with this, the levels of superoxide and hydroxyl radicals, hydrogen peroxide, peroxynitrite, nitrogen dioxide (7), nitroxyl (10), and nitrosothiols (11) can increase during sepsis. Because oxidants can activate cGMP-dependent protein kinase 1 alpha (PKG α) to lower blood pressure (12–14), we hypothesized this process underlies sepsis-induced hypotension and consequential organ injury. Because PKG couples to enhance endothelial permeability (6, 15), oxidative activation would also account for the enhanced microvascular leak that would further exacerbate the hypotension. PKG is also negatively inotropic (13, 16); therefore, oxidative activation might also explain the attenuated cardiac output characteristic of sepsis, further exacerbating the hypotension.

Results and Discussion

To investigate the hypothesis that PKG α oxidation mediates septic injury, we used a “redox-dead” Cys42Ser PKG α knock-in (KI) mouse that cannot be oxidant-activated (17). Two experimental

models were used, comparing the responses of KI mice with wild-type (WT) littermate controls to endotoxemia or sepsis. Intraperitoneal injection of the bacterial endotoxin LPS caused a peak hypotension at ~6–7 h that was threefold greater in WT than in KI (Fig. 1A). Thus, the peak mean arterial pressure (MAP) drop was 19 ± 4 mmHg in WT, whereas KI was substantially resistant and declined only 6 ± 3 mmHg (Fig. 1A, *Inset*). Lipopolysaccharide (LPS) increased PKG α disulfide dimerization in WT mesenteric vessels at the time of maximal hypotension (~6 h after LPS injection) compared with vehicle-treated controls (Fig. 1B). However, by 16 h, the MAP in WT had recovered toward basal, as had the redox state of PKG α in mesenteric vessels. Cecal ligation and puncture (CLP), a widely used model exhibiting many clinical features of sepsis, partially reflecting ruptured appendicitis or perforated diverticulitis in patients, were next studied. First, we validated the CLP model in WT mice in our laboratory, observing a peak hypotension at ~7–8 h with reduced locomotor activity compared with sham-operated controls (Fig. 2A and B). As anticipated, CLP decreased kidney function, evidenced by an eightfold increase in blood urea nitrogen (BUN, Fig. 2C). A global marker of tissue injury, plasma lactate dehydrogenase (LDH), was also markedly increased 6 and 24 h after CLP, but not in sham-operated controls (Fig. 2D). Having validated the CLP model, we compared the responses of KI and WT littermates in this model. There was an initial 22 ± 5 mmHg peak decline in MAP in WT after CLP, whereas the KI was protected from this hypotension and only declined by 10 ± 3 mmHg (Fig. 1C, *Inset*). CLP-induced a sustained hypotension over the entire 24-h observation period, not just the initial phase as occurred in the endotoxemia model. The KI mice were resistant to the hypotension induced by CLP compared with WT. CLP, as in LPS-treated mice, also increased PKG α disulfide dimerization in WT mesenteries compared with controls by 6 and 24 h (Fig. 1D). There is a temporal correlation between PKG α disulfide levels and sepsis-induced hypotension, which is consistent with kinase oxidation significantly mediating the blood pressure lowering. This helps substantiate the cause-and-effect relationship between kinase oxidation and hypotension and is consistent with previous studies demonstrating that PKG α oxidation lowers blood pressure (17, 18). We compared the dose–response of mesenteric vessels from septic or nonseptic mice to the vasoconstrictor drug U46619 using myography. The maximal U46619-induced vasoconstriction in WT nonseptic vessels was 10.0 ± 0.9 mN, whereas sepsis reduced this to 8.3 ± 0.2 mN (Fig. 3A). This is consistent with the widely acknowledged fact that sepsis generates a potent “vasodilatory

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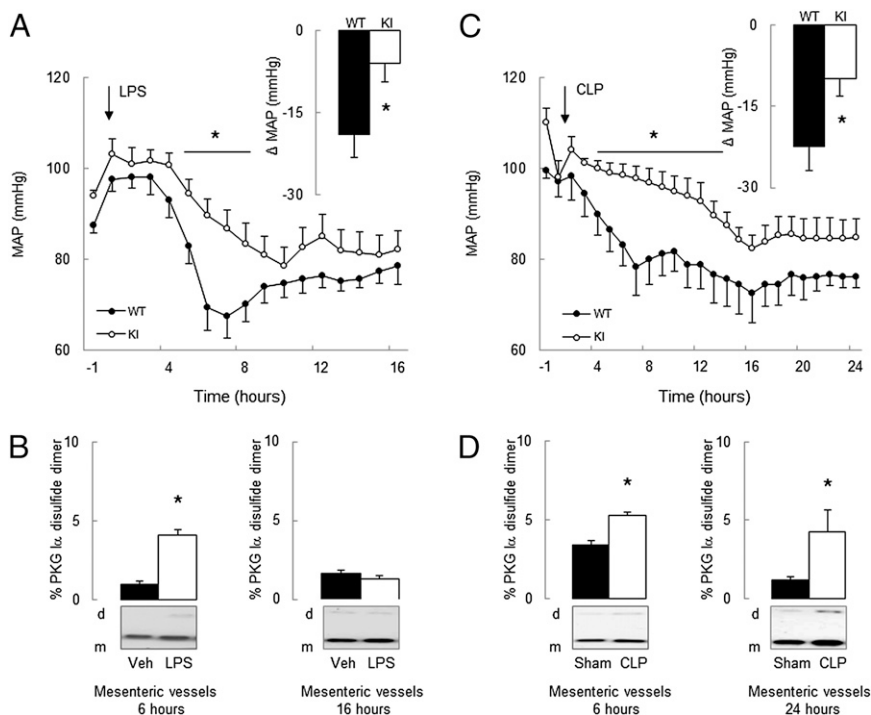


Fig. 1. Differential responses of WT and KI mice to sepsis-induced hypotension. (A) In vivo telemetric blood pressure monitoring comparing KI mice with WT controls before and during LPS-induced sepsis. (Inset) The difference between baseline MAP and the initial hypotensive response to LPS. (B) Comparison of PKG α disulfide formation in mesenteric vessels at 6 and 16 h time points from control vehicle- or LPS-treated mice. (C) Telemetric blood pressure monitoring comparing KI mice with WT controls before and during CLP-induced sepsis. (Inset) The difference between baseline MAP and initial hypotension in response to CLP. (D) Comparison of PKG α disulfide formation in mesenteric vessels at 6 and 24 h time points from sham-operated or CLP-treated mice. Δ MAP, change in MAP. * P < 0.05, comparing WT to KI by repeated measures analysis of variance (RM ANOVA) or t test. Data are mean \pm SEM.

signal” to cause hypotension, which our observations suggest are mediated by PKG α disulfide activation. Indeed, sepsis did not lower the constriction induced by U46619 in KI, which is consistent with their protection from hypotension in vivo. This supports the premise that PKG α oxidation underlies the injurious hypotension during sepsis.

Evans blue staining of blood vessels was used to index vascular permeability. CLP prominently increased vascular leak eight- and fourfold in WT mesenteries and aorta respectively compared with vessels from identically treated KI littermates (Fig. 3B). This concurs with recent work showing PKG α disulfide formation increased kidney podocyte permeability (15), further supporting the idea that oxidative activation is a crucial, causative mediator of this classical feature of sepsis. CLP resulted in PKG α disulfide dimerization in cardiac tissue at 6 and 24 h (Fig. 3C). Cardiac output was also compared between genotypes before and after CLP using echocardiography. Again the KI was protected, with a better preserved stroke volume and cardiac output relative to WT (Fig. 3D). This is consistent with our previous finding that PKG α oxidation in isolated heart preparations is negatively inotropic (13). Mice without the nitrosothiol-reducing enzyme S-nitrosoglutathione reductase have increased systemic S-nitrosylation, tissue damage, and mortality following septic shock (11). This is notable in the context of this study because nitrosothiols induce PKG α disulfide formation (13).

It is evident that PKG α oxidation during sepsis reduces blood pressure. This hypotension during sepsis ensues because oxidant-activated PKG increases the dilation and permeability of blood vessels as well as attenuating cardiac output. All three of these effected processes are not only prominent clinical features of sepsis, but each of them is also known to be regulated by PKG, further supporting the cause-and-effect nature of our observations

evident from the “protected phenotype” of the KI. The consequent hypotension and resulting organ underperfusion is anticipated to be systemically injurious. Indeed, KI mice subjected to CLP have less renal damage (indexed by plasma BUN levels, Fig. 3E) as well as less global tissue damage (plasma LDH levels, Fig. 3F) compared with WT. Both BUN and LDH negatively correlate with animal survival after sepsis (19), consistent with our synopsis that KI mice are protected compared with WT. Indeed, KI mice subjected to CLP have attenuated hypothermia (Fig. 3G) and preserved locomotor activity (Fig. 3H), indicating better systemic well being compared with WT. The KI locomotor activity compared with WT is markedly preserved during the initial 8 h after CLP. However, between 9 and 24 h, the protection in the KI is relatively small. These data are significantly complicated by the diurnal variation, but at the later phase perhaps the protection is lost because it is mediated by different mechanisms, such as cognitive impairment, which is a major consequence of sepsis in human (4), and mice (20).

We examined the ability of N-acetylcysteine (NAC) to prevent sepsis-induced PKG α oxidation. The antioxidant capacity of NAC is due to it containing a thiol, which can react with (scavenge) oxidants such as hydrogen peroxide and nitrosothiols. Because PKG α oxidation is also thiol-dependent, NAC is considered a rationale choice of antioxidant because it will potentially scavenge the same molecular species that oxidize Cys42 in the kinase. However, we found NAC did not prevent CLP-induced disulfide formation (Fig. 4A) and also failed to reduce organ damage as indexed by plasma LDH levels (Fig. 4B).

Although antioxidants historically have been considered a panacea for disease, there are several factors that may limit their effectiveness. Although compounds such as NAC can scavenge thiol-oxidizing species such as hydrogen peroxide or nitrosothiols to prevent PKG α oxidation, one limitation is that thiol

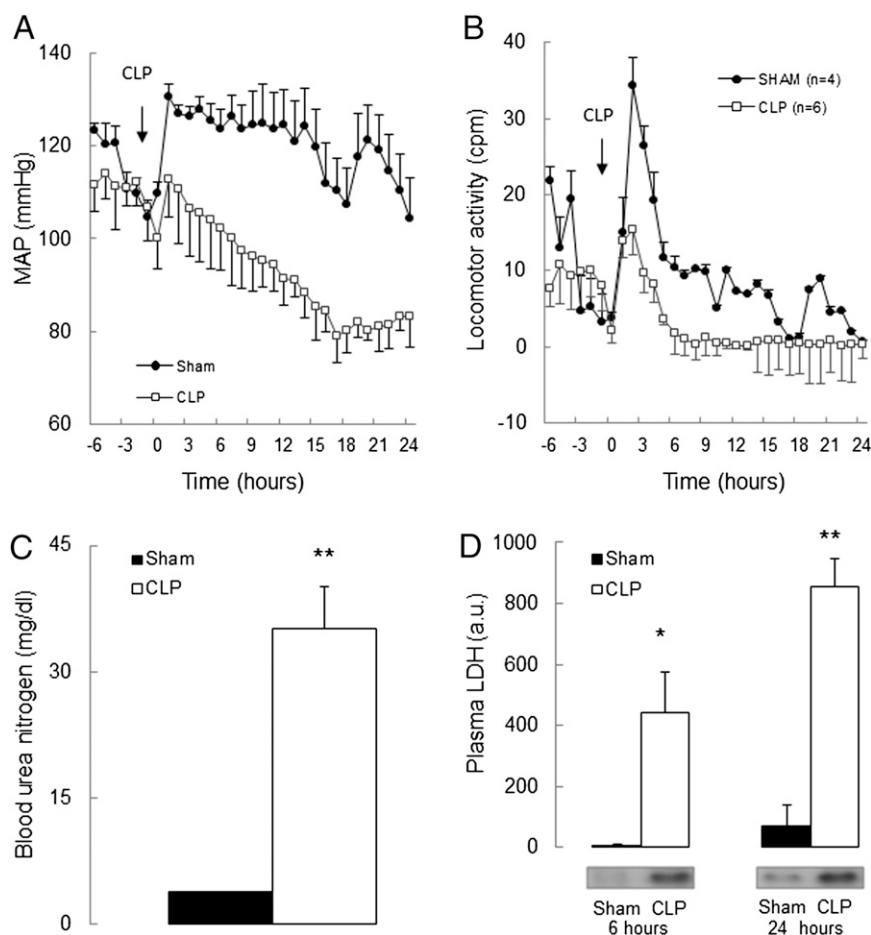


Fig. 2. Validation of sepsis-induced hypotension and organ damage in WT mice after CLP. (A and B) Comparison of in vivo telemetric blood pressure and locomotor activity monitoring in WT mice before and during 24 h of CLP-induced sepsis versus sham-operated WT mice. (C) Plasma level of LDH as an indirect measure of multiple organ injury in WT mice in 6 and 24 h after CLP-induced sepsis or sham operation. (D) Comparison of BUN level in WT mice after 24 h CLP-induced sepsis or sham-operated WT mice. * $P < 0.05$, ** $P < 0.01$ comparing WT CLP with WT sham-operated mice by *t* test. Data are mean \pm SEM.

oxidant sensors may be significantly more reactive. So a very high concentration of an antioxidant may not efficiently compete with a low pK_a protein thiol to prevent its oxidation. Even if the antioxidant did compete, at some point it will become exhausted (i.e., fully oxidized). Unlike endogenous antioxidant thiols (e.g., glutathione, thioredoxin), reduced NAC may not be regenerated by cellular reducing enzymes. Even if NAC can be recycled to the antioxidant reduced form by the cell, during sepsis the cellular reducing capacity of the cell is compromised—hence the oxidative stress. A further consideration is that antioxidants may also prevent oxidation of sensor proteins that couple to protective responses. In this scenario, antioxidants could be harmful by preventing appropriate adaptive cellular responses. Indeed, it is now understood that antioxidants are injurious in many disease scenarios (21, 22). Furthermore, because antioxidants are reducing molecules, they could directly or indirectly serve as electron donors for cellular oxidases. Because these enzymes generate oxidant species, antioxidants can therefore counterintuitively have pro-oxidant effects and exacerbate target oxidation and worsen disease progression. Indeed, a meta-analysis of *N*-acetylcysteine during sepsis concluded that it certainly did not reduce mortality and cautioned that it could worsen the outcome (23).

We conclude that sepsis, long associated with oxidative stress, induces disulfide-activated PKG $I\alpha$ to lower blood pressure. This hypotension results in underperfusion of organs and systemic

dysfunction. These findings provide insight for rational therapy design, for example drugs that limit PKG $I\alpha$ disulfide formation may be protective. Although antioxidant therapies can be envisaged as a potential therapeutic strategy to attenuate sepsis-induced injury by limiting PKG $I\alpha$ oxidation, as discussed previously, this approach may not be viable.

Materials and Methods

Animal Study. All procedures were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 in the United Kingdom and were approved by the King's College Animal Welfare and Ethical Review Body. Mice constitutively expressing PKG $I\alpha$ Cys42Ser were generated on a pure C57BL/6 background by TaconicArtemis as described elsewhere (17). Only male mice were used in this study. C57BL/6 mice were used for sepsis validation studies. Age- and body weight-matched WT and PKG $I\alpha$ Cys42Ser KI mice were used in all other studies. All studies were performed at the same time of day to obviate circadian influences in pair-matched littermates (i.e., WT and KIs). All animals had ad libitum access to standard chow and water. Mice were kept under specific pathogen-free conditions and under a 12-h day/night cycle.

Systemic Blood Pressure Measurements. Mean arterial pressure and the locomotor activity were assessed by telemetry in conscious, freely moving mice as described elsewhere (14, 24). Briefly, mice were anesthetized with 2% (vol/vol) isoflurane (Centaur Services) in 1 L of oxygen per minute with pre- and postoperative analgesia (buprenorphine, 0.1 mg per kilogram of body weight, Abbot Laboratories), and a TA11PA-C10 probe catheter (Data Science International) was implanted into the aortic arch via the left carotid artery.

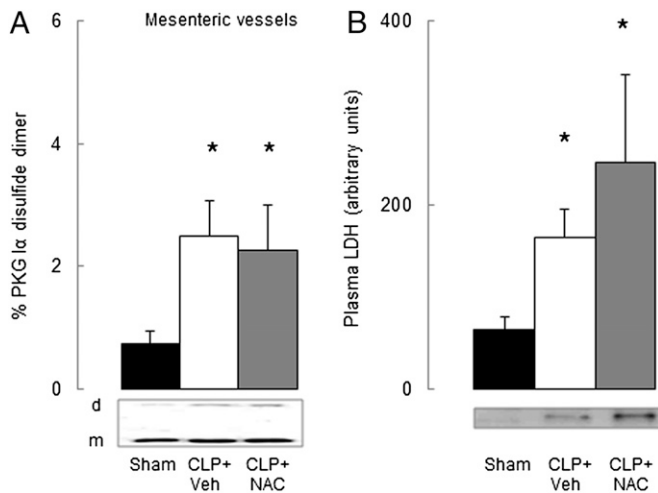


Fig. 4. Effect of *N*-acetylcysteine (NAC) treatment on PKG α disulfide formation or tissue LDH release. (A) Comparison of PKG α disulfide formation in WT mesenteric vessels 6 h after a sham operation, or CLP in the presence of NAC or a vehicle control. (B) Comparison of plasma LDH levels in WT mice 6 h after a sham operation, or CLP in the presence of NAC or a vehicle control. * $P < 0.05$, comparing WT CLP with WT sham operated by *t* test. Data are mean \pm SEM.

anesthetic overdose and perfused through the left ventricle with 4% (wt/vol) formaldehyde in PBS; the main mesenteric vessel with small branches and a segment containing the aortic arch, the brachiocephalic and carotid arteries were isolated, dried, and weighed. EBD was extracted by incubation

in formamide for 24 h at 60 °C, and the absorbance was measured at 620 nm. Standard curves for pure EBD were used to calculate the total amount of dye in the tissue ($n = 7$ per group).

Echocardiography. Twenty-four hours after sepsis induction by CLP, mice were anesthetized and examined by echocardiography at the body temperature 36 °C using a high resolution Vevo 770 echocardiography system (VisualSonics) with a RMV-707B transducer running at 30 MHz. High-resolution, 2D B-mode and M-mode images at the level of the papillary muscles were obtained for offline stroke volume and cardiac output measurements with Vevo Software (VisualSonics) ($n = 10$ per group).

Plasma Analysis and Immunoblotting. After completion of echocardiography imaging, abdominal dissection was performed; blood was sampled from inferior vena cava. Fresh blood was used for rapid biochemical analysis of BUN by using an iSTAT blood biochemistry analyzer (courtesy of Manasi Nandi, King's College London) ($n = 10$ per group). The rest of the blood was centrifuged and plasma was frozen for measurement of LDH level. Immunoblotting for PKG α disulfide dimer was performed as described previously (14), with maleimide (100 mmol/L) used in preparation buffers to alkylate thiols and prevent thiol disulfide exchange. Antibodies used in these studies included cGK1 α (ADI-KAP-PK005; Enzo Life Science) and LDH (ab7638, Abcam). HRP-linked secondary antibody (Cell Signaling) and ECL reagent (GE Healthcare) were used. Digitized immunoblots were analyzed quantitatively with Gel-Pro Analyzer 3.1 software.

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