Specific inhibition of mitochondrial oxidative stress suppresses inflammation and improves cardiac function in a rat pneumonia-related sepsis model

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Zang QS, Sadek H, Maass DL, Martinez B, Ma L, Kilgore JA, Williams NS, Frantz DE, Wigginton JG, Nwariaku FE, Wolf SE, Minei JP. Specific inhibition of mitochondrial oxidative stress suppresses inflammation and improves cardiac function in a rat pneumonia-related sepsis model. Am J Physiol Heart Circ Physiol 302: H1847–H1859, 2012. First published March 9, 2012; doi:10.1152/ajpheart.00203.2011. — Using a mitochondria-targeted vitamin E (Mito-Vit-E) in a rat pneumonia-related sepsis model, we examined the role of mitochondrial reactive oxygen species in sepsis-mediated myocardial inflammation and subsequent cardiac contractile dysfunction. Sepsis was produced in adult male Sprague-Dawley rats via intratracheal injection of S. pneumoniae (4 × 10⁶ colony formation units per rat). A single dose of Mito-Vit-E, vitamin E, or control vehicle, at 21.5 μmol/kg, was administered 30 min postinoculation. Blood was collected, and heart tissue was harvested at various time points. Mito-Vit-E in vivo distribution was confirmed by mass spectrometry. In cardiac mitochondria, Mito-Vit-E improved total antioxidant capacity and suppressed H₂O₂ generation, whereas vitamin E offered little effect. In cytosol, both antioxidants decreased H₂O₂ levels, but only vitamin E strengthened antioxidant capacity. Mito-Vit-E protected mitochondrial structure and function in the heart during sepsis, demonstrated by reduction in lipid and protein oxidation, preservation of mitochondrial membrane integrity, and recovery of respiratory function. While both Mito-Vit-E and vitamin E suppressed sepsis-induced peripheral and myocardial production of proinflammatory cytokines (tumor necrosis factor-α, interleukin-1β, and interleukin-6), Mito-Vit-E exhibited significantly higher efficacy (P < 0.05). Stronger anti-inflammatory action of Mito-Vit-E was further shown by its near-complete inhibition of sepsis-induced myeloperoxidase accumulation in myocardium, suggesting its effect on neutrophil infiltration. Echocardiography analysis indicated that Mito-Vit-E ameliorated cardiac contractility of sepsis animals, shown by improved fractional shortening and ejection fraction. Together, our data suggest that targeted scavenging of mitochondrial reactive oxygen species protects mitochondrial function, attenuates tissue-level inflammation, and improves whole organ activities in the heart during sepsis.

One such approach covalently links biomolecules to lipophilic triphenylphosphonium cation (TPP⁺). Due to a positive charge, the molecules are driven by the mitochondrial membrane potential to accumulate solely in mitochondria (19, 51, 68). Another group of targeted antioxidants, Szeto-Schiller peptides, are small, positively charged peptides that accumulate in mitochondria independent of membrane potential (70, 71). These novel mitochondria-targeted antioxidants (MTAs) have demonstrated their higher capability in various experimental settings to fight oxidative stress and to protect mitochondrial function (23, 24, 36, 46). Currently, these reagents have not yet been used in humans. Nonetheless, mitochondria-targeted ubiquinone (MitoQ) in clinical tests has showed its benefit in treating liver inflammation (30). A phase IIb human trial has been initiated in the UK to assess the efficacy of MitoQ in nonalcoholic fatty liver disease (http://www.antipodeanpharma.com). To date, the therapeutic potential of MTAs is under intense investigation using preclinical models of mitochondrial abnormality-associated diseases, such as neurodegenerative diseases (31, 47), cardiac dysfunction (16), cardiac ischemia-reperfusion injury (2), hypertension (25), diabetes (15), and sepsis (45, 69). Current investigations evaluated MitoQ in sepsis animal models, and available data suggested its therapeutic benefits in improving cardiac function in endotoxin-induced sepsis (69) and in preventing liver damage in lipopolysaccharide-induced sepsis (45). Further study of other MTA effects using different sepsis models will promote translating the application of these novel antioxidants into significantly improved clinical outcomes.

Cardiac dysfunction is an important component of multiorgan failure induced by severe sepsis (20, 61, 83). Sepsis patients with cardiac dysfunction have significantly higher innovative and effective treatments for this threatening disease is a dire necessity.

Among many intracellular players that contribute to the pathogenesis of sepsis, oxidative stress is well recognized as a major promoter (5, 59). Accordingly, antioxidants are expected to attenuate inflammation and improve survival following sepsis. However, although this expectation has been met in animal sepsis models (43, 60), clinical trials of antioxidant therapies have led to inconsistent results (6, 11, 28). One limitation of the conventional antioxidants is that they are globally acting agents, and insufficient dosage and/or lower efficacy are possible reasons for the failures (44). Because mitochondria are the main organelles that produce reactive oxygen species (ROS), more effective therapeutic benefits may be achieved by concentrating antioxidant activities specifically in mitochondria. Recently, strategies for mitochondria-targeted delivery of antioxidants have been developed (2, 4, 22, 70).
mortality compared with patients without this condition (70 vs. 20%) (12, 55). The underlying mechanisms of this condition are not fully understood. Current studies suggest that multiple aspects of mitochondrial dysfunction, such as impaired metabolism, altered energy generation, and elevated production of ROS, contribute to sepsis-associated myocardial injury (14, 42, 77). Previously, our laboratory has developed a pneumonia-related sepsis model in rats (32). In this model, rats were infected with S. pneumonia, and sepsis symptoms were confirmed by positive blood cultures, pulmonary inflammation, lactic acidosis, and a fall in mean arterial blood pressure 24 h postinfection (66, 72, 75, 78). Using this model, we demonstrated that sepsis impaired cardiac mitochondria, causing compromised membrane integrity, increased oxidative stress and decreased antioxidant defense (80). Interestingly, time course study revealed that mitochondria impairment occurred before expression of inflammatory responses in the heart, suggesting that the alterations in mitochondria might lead to induction of myocardial inflammation during sepsis. We hypothesized that protecting mitochondria by MTAs will alleviate the induction of myocardial inflammation during sepsis. We hypothesized that protecting mitochondria by MTAs will alleviate the induction of myocardial inflammation during sepsis. We hypothesized that protecting mitochondria by MTAs will alleviate the induction of myocardial inflammation during sepsis. We hypothesized that protecting mitochondria by MTAs will alleviate the induction of myocardial inflammation during sepsis. We hypothesized that protecting mitochondria by MTAs will alleviate the induction of myocardial inflammation during sepsis. We hypothesized that protecting mitochondria by MTAs will alleviate the induction of myocardial inflammation during sepsis. 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tase (Sigma-Aldrich, St. Louis, MO) was added at 50 U/ml to convert all superoxide into H$_2$O$_2$. Mitochondrial respiration substrates (2 mM malate + 20 mM glutamate or 5 mM succinate) were added to start the reaction. During 30-min incubation in the dark at 37°C, H$_2$O$_2$-dependent oxidation of Amplex Red. The end product Resorufin Red was measured by fluorescence reading at excitation/emission = 570/620 (PHERAstar, BMG LABTECH, Cary, NC). Similarly, cytosolic H$_2$O$_2$ levels were measured according to a published method (10) with minor changes. Cytosolic fraction (100 µg) was added to 50 µM Amplex Red, 0.1 U/ml HRP, and 50 U/ml superoxide dismutase in a total of 100 µl reaction (in 50 mM Na$_2$HPO$_4$, pH 7.4). Fluorescent reading was obtained following 15-min incubation in the dark at 37°C. All measurements were performed in at least duplicate. H$_2$O$_2$ concentrations in micromoles were calculated according to individual standard curves in these assays.

**Protein Oxidation**

Protein oxidative modifications were detected by depriving carboxyl groups to 2,4-dinitrophenylhydrazone (DNP-hydrazone), followed by Western blot using a DNP-specific antibody (80). Using OxyBlot protein oxidation detection assay (Chemicon, Temecula, CA), 20 µg protein of each sample were applied to a derivatization reaction and loaded on 4–15% SDS-PAGE gel (BioRad, Hercules, CA), followed by Western blot using an anti-DNP antibody. Results were quantified by densitometry.

**Lipid Peroxidation**

Levels of malondialdehyde (MDA) were measured using a lipid peroxidation assay kit (Enzo Life Sciences, Plymouth Meeting, PA). Briefly, all mitochondrial extracts were preadjusted to 1–1.5 mg/ml in a resuspension buffer containing 5 mM butylated hydroxyl toluene. For each reaction, 200-µl sample was added to 650 µl chromogenic reagent and 150 µl 12 N HCl. After incubation at 45°C for 60 min, the sample was cooled at 4°C and centrifuged at 10,000 g for 5 min. The supernatant was collected, and absorbance at 586 nm was recorded. MDA concentration was calculated using a standard curve. All measurements were performed in triplicate, and values were normalized by amount of protein per reaction.

**Mitochondrial Outer Membrane Damage**

As previously described (81), mitochondrial outer membrane integrity was evaluated by the measurement of cytochrome-c oxidase (COX) activity in mitochondrial fractions in the presence and absence of detergent n-dodecyl β-D-maltoside. According to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO), 20 µg freshly isolated mitochondrial fraction were used for each reaction. COX activity was measured by its oxidation of substrate ferrocytochrome c. Mitochondrial outer membrane damage was assessed from the ratio between the mitochondrial fraction were used for each reaction. COX activity was measured spectrophotometrically following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm. Results were expressed as changes of absorbance per minute per milligram protein.

**Western Blots**

Protein samples were subjected to SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk-PBS at room temperature for 1 h and subsequently probed with primary antibodies [anti-cytochrome c, anti-glyceraldehyde-3-phosphate dehydrogenase, and anti-DNP were from Millipore, Denver, MA; anti-adenine nucleotide translocase (ANT) was from Santa Cruz Biotechnology, Santa Cruz, CA; Mitochondrial outer membrane damage was assessed from the ratio between the mitochondrial fraction were used for each reaction. COX activity was measured spectrophotometrically following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm. Results were expressed as changes of absorbance per minute per milligram protein.

**Cytokine Levels**

The concentrations of tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in serum and in heart tissue lysates were examined using enzyme-linked immunosorbent assay kits (Biosource, Camarillo, CA). Results in the tissue lysates were normalized by protein amount, and the results in serum were normalized by volume. All measurements were performed in triplicate.

**Myeloperoxidase Activities**

Myeloperoxidase (MPO) activities in heart lysates were measured according to published methods (54, 64) with minor modifications. Each MPO assay reaction contained 10- to 50-µg tissue lysate with 2 mM 3,3′,5′-tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO) and 0.0003% (vol/vol) H$_2$O$_2$ in phosphate buffer (pH 5.5) at a final volume of 500 µl. After 3-min incubation at room temperature, the reaction was terminated by the addition of 500 µl cold 2 M acetic acid. Optical density was determined at 650 nm. The number of MPO unit per reaction was calculated according to a standard curve prepared using purified MPO (Sigma-Aldrich, St. Louis, MO). All measurements were performed in triplicate, and values were normalized by amount of protein per reaction.

**Immunohistochemistry**

Fresh heart tissues were embedded in cryoprotective OCT media, snap-frozen, and stored at −80°C until used. Samples were sectioned at 7 µm, mounted on slides, and fixed with 4% paraformaldehyde for 20 min at room temperature. After blockage with 10% mouse serum and 10% goat serum in PBS, these slides were co-stained with propidium iodide (Invitrogen, Grand Island, NY) and FITC-conjugated mouse anti-MPO (Hycult Biotech, Plymouth Meeting, PA) or FITC-conjugated goat anti-mouse IgG (Invitrogen) for 1 h at room temperature. To reduce autofluorescence, the slides were treated with 0.1% Sudan black B for 30 min. Individual coverslips were mounted on slides with Fluoromount-G slide mounting medium (Electron Microscopy Sciences, Hatfield, PA) to reduce fluorochrome quenching. The slides were then analyzed under an inverted fluorescent microscope (Nikon TE2000-U) at ×40 magnification. All other chemicals were from Sigma-Aldrich, St. Louis, MO).

**Echocardiography**

Noninvasive transthoracic echocardiograms were performed in conscious unsedated rats in a random and blind manner to assess systolic function using HP agilent Sonos 5500 Ultrasound Machine equipped with a 15-MHz linear array transducer (Conquest Imaging, Stockton, CA). For each animal, M-mode images were obtained at baseline and at specific time periods after inoculation. Left ventricular end-diastolic diameter (EDD) and left ventricular end-systolic diameter (ESD) were measured. End-diastolic volume (EDV) and end-systolic volume (ESV) were calculated according to Teichholz's formula. Fractional shortening (FS) and ejection fraction (EF) were calculated according to formulas, $FS\% = (EDD - ESD)/EDD \times 100\%$ and $EF\% = (EDV − ESV)/EDV \times 100\%$. All results were obtained at stable heart rates (HRs), and measurements were averaged over five consecutive cardiac cycles.
Mito-Vitamin-E Synthesis And In Vivo Distribution

We synthesized Mito-Vitamin-E to >90% purity, according to published method (68). Mito-Vitamin-E was given orally to rats at a single 21.5 μmol/kg dose. No gross toxic effects were observed, as determined by appearance and physical activities. To confirm its in vivo distribution 24 h postadministration (Day 1), Mito-Vitamin-E levels in the blood serum and in the tissue lysates of heart and liver were measured by mass spectrometry. Heart and liver were chosen for this experiment because both organs are high in mitochondria content. As expected, significant accumulation of Mito-Vitamin-E was detected in the heart and liver, but not in the serum, indicating that this compound was preferentially loaded to organs instead of circulating in the blood (Fig. 1B). We also monitored Mito-Vitamin-E concentrations in the heart on days 1, 2, and 3 postadministration. Once delivered, heart level of Mito-Vitamin-E was maintained during the first 48 h (days 1–2) and started declining at day 3 (Fig. 1C). These data are consistent with reported in vivo distribution of this group of MTAs (2, 18, 68).

Mito-Vitamin-E Protected Cardiac Mitochondria During Sepsis

Mitochondrial antioxidant defense. We first compared the differences between Mito-Vitamin-E and untargeted vitamin E on mitochondria-specific antioxidant activity in the heart 24 h after sepsis by measuring total antioxidant capacities and levels of H2O2, the most stable form of ROS. As shown in Fig. 2A, sepsis decreased antioxidant capacity to nearly 50% in mitochondria and to 75% in cytosol. Mito-Vitamin-E significantly improved antioxidant capacity in mitochondria, whereas vitamin E provided more protection in the cytosol. Mitochondrial H2O2 production was quantified by a standard Amplex Red assay (17, 26, 50). As expected, sepsis triggered H2O2 overproduction in mitochondria: an ~35% increase in the presence of respiration substrates malate plus glutamate and an ~15% increase in the presence of succinate (Fig. 2B). Mito-Vitamin-E treatment suppressed mitochondrial H2O2 to levels no higher than shams. However, vitamin E had little effect on mitochondrial H2O2. H2O2 levels in cytosolic fractions were compared in parallel (Fig. 2C). Mito-Vitamin-E and vitamin E provided comparable inhibitory effects on sepsis-induced intracellular H2O2. It was shown that part of the intracellular H2O2 molecules were generated from superoxide anion released from mitochondria (34). Our data suggest that mitochondria are the main source of intracellular H2O2 in myocardium during sepsis. This finding is consistent with our recent observation of Mito-Vitamin-E effect on ROS production in endothelial cells (76).

Next, we confirmed that administration of either Mito-Vitamin-E or vitamin E did not affect the purity of mitochondrial preparations. As shown in Fig. 2D, hearts were harvested from rats given Mito-Vitamin-E, vitamin E, or vehicle and subjected to subcellular fractionation. Separation of mitochondria from cytosol was complete, indicated by Western blot using antibodies against mitochondrial marker ANT and cytosolic marker glyceraldehyde-3-phosphate dehydrogenase. Furthermore, neither Mito-Vitamin-E nor vitamin E caused changes in levels of certain key components of mitochondrial respiratory complex proteins in all mitochondria preparations.

Oxidation of mitochondrial molecules. We examined whether Mito-Vitamin-E inhibited or alleviated mitochondrial oxidative injury in the heart after sepsis, which were determined by levels of lipid and protein oxidation. As shown in Fig. 3A, MDA content, a marker of lipid peroxidation, was measured at ~50 nmol/mg of mitochondrial protein in the sham group. Sepsis resulted in a significant rise of MDA levels to 80–100 nmol/mg of mitochondrial protein. Remarkably, in response to the treatment of Mito-Vitamin-E, mitochondrial MDA content in the septic animals was reduced to the levels not different than sham controls. Mitochondrial protein oxidation was detected in parallel. It is known that oxidative modifications to proteins introduce carbonyl groups into protein side chains. Detection of protein oxidation can be achieved by depriving those car-
bonyl groups to form DNP-hydrozone, which is then detected by a specific antibody against DNP (21, 80). As shown in Fig. 3B, protein oxidation in cardiac mitochondria was induced in response to sepsis. Densitometry analysis showed that the level of total mitochondrial protein oxidation steadily increased during sepsis and eventually increased by fivefold with severe sepsis, day 4 after inoculation. However, this difference was not detectable in animals given Mito-Vit-E. As a control, expression of mitochondrial marker protein ANT was not changed in any sample. These results indicate that treating septic rats with Mito-Vit-E alleviates sepsis-mediated oxidative modification on mitochondrial molecules in the heart.

Mitochondrial membrane integrity. We analyzed whether Mito-Vit-E protected mitochondrial structure in the heart after sepsis. Mitochondria membrane integrity was determined using two parameters: cytochrome c in cytosol and the percent changes of mitochondrial outer membrane damage. Leaking of mitochondrial protein cytochrome c to the cytosolic compartment has been used as a well-recognized measurement of impaired mitochondrial membrane (3, 33, 63). We compared cytosol levels of cytochrome c in the heart of sham and septic rats treated with either Mito-Vit-E or vehicle. As the Western blot analysis shown in Fig. 4A, cytosolic cytochrome c increased twofold in early sepsis (day 1) and further elevated over threefold in severe sepsis (day 4). In response to Mito-Vit-E treatment, cytosolic cytochrome c levels in sepsis groups were comparable with those of shams, suggesting that Mito-Vit-E prevented mitochondrial cytochrome c leakage. As a control, total expression of cytochrome c in the heart was not altered in all groups.

In these experimental groups, mitochondrial outer membrane integrity was assessed by the measurement of COX activity in mitochondrial fractions in the presence and absence of detergent n-dodecyl β-D-maltoside. This detergent is one of the few that allows COX dimerization in solution at low detergent concentrations (52). Because mitochondrial membrane is a barrier for cytochrome c entering into the organelle, the ratio between the activity with and without the present of detergent is a well-recognized measurement of membrane integrity (52). We compared COX activities in mitochondrial fractions in the presence and absence of detergent with the activity of sham groups. As shown in Fig. 4B, COX activity in mitochondrial fractions in the presence of detergent was significantly lower than that in sham groups. In contrast, COX activity in mitochondrial fractions in the presence of vehicle was comparable with that in sham groups. These results indicate that Mito-Vit-E prevented mitochondrial membrane damage in response to sepsis.

In summary, we demonstrated that Mito-Vit-E improved mitochondrial antioxidant defense and alleviated mitochondrial damage in response to sepsis. These results suggest that Mito-Vit-E is a potential therapeutic agent for sepsis-induced mitochondrial dysfunction.
n-dodecyl β-D-maltoside is a relative measurement of mitochondrial outer membrane integrity (79). As shown in Fig. 4B, in vehicle-treated animals, cardiac mitochondrial outer membrane damage reached 40% in early sepsis (day 1) and increased to over 60% in severe sepsis (day 4). However, this difference was no longer detectable in response to Mito-Vit-E treatment, indicating that Mito-Vit-E protected mitochondrial membrane integrity in the heart during sepsis.

Mitochondrial respiratory function. We further examined whether Mito-Vit-E protected mitochondrial function in the heart after sepsis. Using heart tissues harvested at different time points from early to severe sepsis, mitochondrial respiratory complex IV (COX) activities were measured in the mitochondrial preparations. As shown in Fig. 5, sepsis caused a significant ~30% loss of complex IV activity, which was, however, rescued by treatment with Mito-Vit-E.

Mito-Vit-E Effectively Suppressed Sepsis-induced Inflammation

Systemic and myocardial proinflammatory cytokines. To determine whether preventing mitochondrial oxidative stress inhibits sepsis-mediated inflammation, we compared the effects of Mito-Vit-E with vitamin E on systemic and myocardial levels of proinflammatory cytokines in septic animals. Rats were inoculated with S. pneumoniae or given PBS sham control. 21.5 μmol/kg of Mito-Vit-E or vehicle were administered orally 30 min postinoculation (day 0). Mito fractions were isolated from the heart tissue harvested at indicated time points. A: levels of malondialdehyde (MDA) were determined by a lipid peroxidation assay. Results were normalized by amount of Mito protein per reaction. B: protein oxidation was evaluated using an OxyBlot protein oxidation detection assay. Oxidative modified proteins were detected by anti-2,4-dinitrophenyl (DNP) Western blot using Mito marker ANT as a control. By densitometry analysis, levels of DNP signals were normalized by levels of ANT, and results are expressed as fold changes relative to shams. All values are means ± SE; n = 6 per group. Statistical significances: *difference between sham and sepsis subjects, and ¶difference between vehicle-treated and Mito-Vit-E-treated groups (P < 0.05).

Neutrophil accumulation in the heart. Tissue level MPO activity has been used as a standard quantitative method to evaluate the extent of neutrophil accumulation in organs (9). In the heart tissue lysates, we detected an over 80% increase of MPO content in severe septic rats (day 4 postinoculation), compared with sham controls (Fig. 7A). At the same dose, Mito-Vit-E more efficiently decreased sepsis-induced MPO levels in myocardium than regular vitamin E.

To further confirm the above observation, we performed immunohistochemistry analysis of the hearts harvested from sham and day 4 postinoculation rats given Mito-Vit-E, vitamin E, or vehicle. Costaining heart tissue sections with a FITC-conjugated MPO antibody and propidium iodide, a fluorescent dye for positive cell staining, confirmed the advantage of Mito-Vit-E over vitamin E on suppressing MPO accumulation (Fig. 7B). Taken together, our data suggest that MTAs may function as stronger anti-inflammatory reagents in the heart during sepsis.

Mito-Vit-E Provided Cardiac Protection from Sepsis

Echocardiogram analysis has been commonly used to characterize sepsis-related cardiac dysfunction (35, 39, 48). Using this application, we examined the heart function of rats receiving S. pneumoniae infection, followed by treatment of Mito-Vit-E or control vehicle. As summarized in Fig. 8, significant
differences between the pre- and postsepsis measurements of left ventricular EDD, ESD, EDV, and ESV were obtained on day 4 postinoculation in vehicle-treated group (Fig. 8). Sepsis substantially reduced %FS from 94.492 ± 1.569 to 84.145 ± 0.661% and %EF from 99.674 ± 0.197 to 96.539 ± 4.267% ($P < 0.05$). Statistically significant changes were not detectable in rats receiving Mito-Vit-E. In addition, sepsis-caused increase of HR due to compensation of failing contractility was also alleviated by Mito-Vit-E. Taken together, our data indicate that treatment with Mito-Vit-E improves cardiac performance during sepsis.

**DISCUSSION**

Using a rat pneumonia-related sepsis model, we examined the effects of a MTA, Mito-Vit-E, on sepsis-induced myocardial mitochondrial damage, inflammation, and cardiac dysfunction. We verified the in vivo tissue accumulation of this targeted antioxidant (Fig. 1). Our results showed that a single-dose postinfection administration of Mito-Vit-E provided mitochondria-specific antioxidant defense (Fig. 2), protected mitochondria from oxidative damage (Fig. 3), prevented mitochondrial membrane damage (Fig. 4), and improved mitochondrial respiratory function (Fig. 5) in the heart over time with sepsis. At the same dose, Mito-Vit-E showed stronger anti-inflammatory effects than conventional vitamin E, indicated by suppression of cytokine production (Fig. 6) and neutrophil infiltration in myocardium (Fig. 7). Lastly, treatment with Mito-Vit-E improved cardiac function in septic animals (Fig. 8). These results provide direct evidence to support the hypothesis that mitochondrial damage contributes to inflamma-

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**Fig. 4.** Mito-Vit-E protected Mito membrane integrity in the heart during early to severe sepsis. Rats were infected by *S. pneumoniae* or given PBS sham control. 21.5 μmol/kg of Mito-Vit-E or vehicle were administered orally 30 min postinoculation (day 0). Total heart tissue lysates and cellular fractions were prepared from rats killed at indicated time points. A: cytochrome c levels were determined in cytosol and in total tissue lysates by Western blot using marker protein GAPDH as a control. Sham group is labeled as S. Using densitometry analysis, levels of cytochrome c signals were normalized by the levels of GAPDH, and results are presented as fold changes relative to shams. B: Mito outer membrane damage was measured using the Mito fractions. All values are means ± SE; $n = 6$ per group. Statistical significances: *difference between sham and sepsis subjects, and ¶difference between vehicle-treated and Mito-Vit-E-treated groups ($P < 0.05$).

**Fig. 5.** Mito-Vit-E protected Mito respiration in the heart after sepsis. Rats were infected by *S. pneumoniae* or given PBS sham control. 21.5 μmol/kg of Mito-Vit-E or vehicle were administered orally 30 min postinoculation (day 0). Mito fractions were prepared from the heart tissue harvested at indicated time points and subjected to complex IV activity assay. Relative complex IV activities were expressed as changes of absorbance [optical density (OD) 550 nm] per minute per milligram protein. All values are means ± SE; $n = 6$ per group. Statistical significances: *difference between sham and sepsis subjects, and ¶difference between vehicle-treated and Mito-Vit-E-treated groups ($P < 0.05$).
tory responses in the heart during sepsis, and they also suggest MTAs are a potential novel therapeutic intervention for sepsis.

Mitochondrial accumulation of this category of targeted antioxidants has previously been shown in cells and in animal models using electron spin resonance spectroscopy(25) and an antibody against lipophilic TPP$^+$ cation, the mitochondrial-targeting moiety (57). These methods are not established in our laboratory. Instead, we confirmed substantial tissue accumulation of Mito-Vit-E in vivo by mass spectrometry (Fig. 1). Because the subcellular fractionation procedures cause mitochondrial depolarization and leak of this category of compounds (68), this method is not suitable to detect Mito-Vit-E in isolated mitochondrial fractions. Nonetheless, tissue accumulation of Mito-Vit-E measured is consistent with the notion that the targeting mechanism is functional. Additional supporting evidence came from functional studies. Our results showed that, in the heart tissue of septic animals, Mito-Vit-E increased total antioxidant capacity and suppressed H$_2$O$_2$ production in mitochondria (Fig. 2). On the contrary, the same dose of vitamin E limited its protection, mainly in cytosol. It was not a surprise to find that Mito-Vit-E also blocked cytosolic H$_2$O$_2$, since it is known that part of mitochondrial generated superoxide anion diffuses toward intermembrane space and contributes to the formation of H$_2$O$_2$ in cytosol (34). In fact, this observation is consistent with previous reports from others and ours that scavenging mtROS with MTAs decreases cellular ROS levels (25, 76). The similar inhibitory effects of Mito-Vit-E and vitamin E on cytosolic H$_2$O$_2$ suggest that mitochondria are a dominant source of cellular H$_2$O$_2$ in the heart during sepsis. As expected, administration of Mito-Vit-E in septic rats provided sufficient protection on mitochondrial structure and function in the heart (Figs. 3–5). Whether or not Mito-Vit-E has any effects on mitochondrial biogenesis, such as the expression and formation of mitochondrial respiratory complexes, remains to be determined.

We compared the anti-inflammatory effects of Mito-Vit-E with untargeted vitamin E in the rat sepsis model. As summarized in Figs. 6 and 7, both types of antioxidants exhibited significant anti-inflammatory effects in serum and in heart tissue of septic animals. The results are consistent with previously reported effects of untargeted antioxidants in animal sepsis models (11, 56, 60) and targeted MitoQ in LPS-challenged endothelial cells (45). We further observed that, at the same dose, Mito-Vit-E provided higher efficacy to reduce cytokine production and to impede neutrophil infiltration in myocardium. This advantage of Mito-Vit-E over vitamin E is likely caused by the fact that vitamin E is distributed globally, and its protection of mitochondria against oxidative damage is less efficient, especially in mitochondria-enriched organs, such as the heart, where mitochondria comprise ~30% of myocardial volume (38). Recent discoveries have revealed that regulation of inflammatory responses through mitochondria is multifactorial. In innate immunity, mtROS are essential for the activation of inflammasome NLRP3 in macrophages (87). Mitochondrial matrix protein MAVS is part of the mitoxosome to activate NF-κB during antiviral responses (65). In the plasma from trauma patients, circulating mtDNA fragments released from damaged mitochondria were identified as mitochondrial-derived, danger-associated molecular patterns to trigger peripheral inflammation (84, 85). These mitochondria-
involved mechanisms are all related to oxidative stress, since imbalanced mtROS cause mitochondrial structural and functional damage via direct oxidation of mitochondrial molecules (8, 73). In this case, due to distribution reasons, untargeted antioxidants, such as vitamin E, may not be sufficiently effective to inhibit mtROS-mediated impairment inside mitochondria and, therefore, produce weaker anti-inflammatory effects compared with targeted MTAs. However, whether these mitochondria-mediated inflammation pathways are utilized to induce tissue-level inflammation and the exact mechanisms of MTAs actions on inflammation during sepsis still remain to be elucidated.

The time course study presented in Figs. 3–7 showed that the Mito-Vit-E effects on mitochondrial protection and anti-inflammation were detected immediately following administration and maintained to later time points, days 3 and 4 postinoculation, when its in vivo bioavailability started to decrease (Fig. 1). This observation suggests that sepsis-induced mtROS and inflammation in myocardium may be linked through a positive feedback-signaling network. In this scenario, in response to septic challenge, mtROS participate in inciting inflammation that further triggers additional increases of mitochondrial damage and mtROS overproduction, leading to downstream exacerbation of inflammatory responses. In fact, myocardial mtROS increase and mitochondrial damage induced through inflammatory mediators have been previously reported using sepsis (58) and nonsepsis models (27). Thus we hypothesize that suppression of mtROS by MTAs, such as Mito-Vit-E, in early sepsis stage provides a therapeutic potential to control the progression of mitochondrial dysfunction and inflammation in severe sepsis stage. However, we cannot rule out the possi-
bility that this prolonged protection by Mito-Vit-E is a yet-unknown, drug-specific effect. Investigation using different type(s) of MTAs, such as MTA peptides (86), in sepsis models is needed to further test this hypothesis.

Using echocardiography, a clinically relevant measurement, we have demonstrated that treatment with Mito-Vit-E improves cardiac function during sepsis. Compared with the vehicle-treated sepsis group, Mito-Vit-E lessened sepsis-associated changes of echocardiographic measurements, such as EDD, ESD, EDV, and ESV (Fig. 8). Mito-Vit-E’s improvement of cardiac contractility in the septic animals is shown by the increase of %FS and %EF. The %FS values presented here appeared high compared with most of the values reported in the literature. This discrepancy may be due to the fact that we used unsedated animals. Our laboratory previously found that restraining and performing the echocardiography on awake animals provided a more accurate assessment of left ventricular function, because light anesthesia is a potent negative inotrope (62). In fact, our present findings with baseline FS ranging from 90–95% were within the range of our laboratory’s previously reported echocardiography performed on awake athymic nude rats (with FS 85–90%) (62), given the difference in rat strains (Sprague-Dawley vs. athymic nude). Unlike its substantial improvement on %FS, Mito-Vit-E provided a modest, yet significant, correction on sepsis-mediated increase of HR. Because HR is affected by a wide range of noncardiomyocyte factors, such as sympathetic and parasympathetic tone (37), the timing and severity of sepsis (1, 35), types of fluid resuscitation (82), and levels of circulating hormonal substances (40), Mito-Vit-E as the only drug administered may have limited control over the combination. Nevertheless, our data of Mito-Vit-E effects on %FS and %EF indicate that Mito-Vit-E improves myocardial contractility in sepsis. This finding is consistent with a previously reported MitoQ application in an endotoxin-induced sepsis model, as MitoQ was shown to rescue cardiac function estimated by in vitro Langendorff technique (69).

In conclusion, our study of Mito-Vit-E in the rat pneumonia-related sepsis model suggests that mtROS are causative factors that induce myocardial inflammation and cardiac dysfunction in sepsis, and targeting mtROS by MTAs presents a novel potential to control sepsis-mediated organ dysfunction. We speculate that, with further progress of preclinical studies to assess MTA effects on vital organ functions and survival, to determine their optimal doses and administrative routes, these new antioxidants will potentially become an effective therapeutic approach to control sepsis clinically.

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