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Mitochondrial DNA Damage Initiates Acute Lung Injury and Multi-Organ System Failure Evoked in Rats by Intra-tracheal *Pseudomonas aeruginosa*

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Abstract

Although studies in rat cultured pulmonary artery endothelial cells, perfused lungs, and intact mice support the concept that oxidative mitochondrial (mt) DNA damage triggers acute lung injury (ALI), it has not yet been determined whether enhanced mtDNA repair forestalls development of ALI and its progression to multiple organ system failure (MOSF). Accordingly, here we examined the effect of a fusion protein construct targeting the DNA glycosylase, Ogg1, to mitochondria in a rat model intra-tracheal *P. aeruginosa* (strain 103; PA103)-induced ALI and MOSF. Relative to controls, animals given PA103 displayed increases in lung vascular filtration coefficient accompanied by transient lung tissue oxidative mtDNA damage and variable changes in mtDNA copy number without evidence of nuclear DNA damage. The approximate 40% of animals surviving 24h after bacterial administration exhibited multiple organ dysfunction, manifest as increased serum and tissue-specific indices of kidney and liver failure, along with depressed heart rate and blood pressure. While administration of mt-targeted Ogg1 to control animals was innocuous, the active fusion protein, but not a DNA repair-deficient mutant, prevented bacteria-induced increases in lung tissue oxidative mtDNA damage, failed to alter mtDNA copy number, and attenuated lung endothelial barrier degradation. These changes were associated with suppression of liver, kidney, and cardiovascular dysfunction and with decreased 24h mortality. Collectively, the present findings indicate that oxidative mtDNA damage in lung tissue initiates PA103-induced ALI and MOSF in rats.

Keywords

Oxidant stress; pneumonia; mtDNA repair; *P. aeruginosa*

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Conflicts of Interest: MNG is co-owner of Exscien, Inc., a start-up biotech company engaged in development of mitochondrial DNA repair agents.

Introduction

Despite decades of study, a pharmacologic target suitable for suppressing or reversing the evolution of acute lung injury (ALI) and its more lethal sequel, multiple organ system failure (MOSF), has yet to be identified. One of the second messenger systems that has been intensely scrutinized involves Reactive Oxygen Species (ROS). However, despite experimental evidence for salutary effects of anti-oxidants and ROS scavengers in animal models of ALI and MOSF, the outcomes of over 50 clinical trials on anti-oxidants been disappointing (1). The reason for this disparity remains elusive, but may relate to the interrelated prospects that molecular trigger(s) activating the numerous pathways responding to oxidant stress have not been identified and/or that the non-selective anti-oxidants so far studied disrupt reparative as well as injurious pathways.

Common and related features of ALI and MOSF, regardless of the inciting event, are mitochondrial and bioenergetic dysfunction (2). Transcriptional and metabolomic profiling in both laboratory animals and human patients also implicate bioenergetic abnormalities in clinical outcomes (3, 4). Importantly, redox-sensitive mechanisms seem to initiate a mitochondrial biogenic response that is critical for survival (5). As the central role of mitochondrial dysfunction in critical illness emerges, identification of the specific molecular targets of ROS that initially trigger mitochondrial signaling and progression to end-organ injury becomes increasingly imperative.

Multiple lines of indirect evidence point to the idea that oxidative mitochondrial (mt) DNA damage functions as a molecular sentinel in the setting of oxidant stress. First, studies in cultured cells show demonstrate a conspicuous association between mtDNA damage and ROS-mediated cell death, with the propensity for cytotoxicity inversely related to the efficiency of mtDNA repair (6). Second, modulation of the first and rate-limiting step in mtDNA repair, mediated by Ogg1 - a DNA glycosylase that excises oxidatively damaged purines, coordinately regulates ROS-induced mtDNA damage and cell death in multiple cultured cell populations (7–9). Use of mitochondria-targeted Ogg1 constructs, which eliminate the possibility that biological consequences of Ogg1 over-expression are related to effects on nuclear DNA repair, show that over-expression of mitochondria-targeted Ogg1 in pulmonary artery endothelial cells reduces sensitivities to both oxidative mtDNA damage and cytotoxicity evoked by exogenous ROS stressors (8, 10). Finally, reduction in total cell Ogg1 using siRNA, while not sensitizing nuclear DNA to oxidative damage, leads to persistent ROS-induced damage to the mitochondrial genome and increased apoptosis and cytotoxicity (11).

Equally persuasive evidence that that mtDNA damage is critical for acute lung injury is derived from studies in isolated perfused rat lungs treated with fusion protein constructs targeting Ogg1 to mitochondria. The protein construct displayed rapid uptake into lung tissue mitochondria and suppressed both H₂O₂-induced damaged to lung mtDNA as well as increases in the vascular filtration coefficient (K_f), an index of endothelial barrier dysfunction (12). Also in isolated rat lung preparations, the mt-targeted Ogg1 fusion protein inhibited both mtDNA damage evoked intra-tracheal instillation of *Pseudomonas aeruginosa*

strain 103 (PA103) and the accompanying bacteria-mediated rises in K_f . These findings in isolated lungs appear to be relevant to intact animals; mitochondrially-targeted DNA repair proteins suppress ventilator induced lung injury in intact mice (13). It remains unknown, however, whether enhanced mtDNA repair forestalls progression of ALI to MOSF. Accordingly, here we took advantage of a rat model of PA103-induced lung endothelial injury and distant organ dysfunction recently described by Audia and coworkers (14) to test the hypothesis that pharmacologic enhancement of mtDNA repair prevents and reverses bacteria-related ALI, MOSF, and mortality.

Materials and Methods

Mitochondrially-targeted wild-type and DNA repair-deficient mutant (m) Ogg1 fusion protein preparation

Mitochondrially-targeted Ogg1 and mOgg1 fusion proteins were produced and isolated as previously described (15). In brief, *Escherichia coli* were transfected with plasmids encoding fusion protein constructs consisting of either Ogg1 or mOgg1, a 9 amino acid TAT sequence to facilitate cellular uptake, the mitochondrial targeting sequence from manganese superoxide dismutase to direct the fusion protein to mitochondria, a hemagglutinin (HA) tag for immunodetection, and a polyhistidine tail to isolate the fusion protein from culture media. Bacteria were then sonicated, and lysates incubated with Ni-NTA-agarose for affinity chromatography. After assessment of fusion protein purity using SDS-PAGE and oligonucleotide cleavage assays, respectively, the protein was aliquotted and stored at -80°C until use.

Rat model of *Pseudomonas aeruginosa*-induced ALI and MOSF

Pseudomonas aeruginosa strain 103 was obtained from frozen glycerol stock and streaked onto Vogel-Bonner with 0.5% glucose agar plates. Plates were then incubated at 37°C for 24 h. Colonies were scraped from the plate and, using a vortex mixer, suspended in 10 ml isotonic saline solution, and centrifuged at $4000 \times g$ for 10 min at room temperature. The supernatant was discarded and the pellet re-suspended in 1 ml saline.

Animal experiments were approved by the University of South Alabama Institutional Animal Care and Use Committee (IACUC). Male Sprague-Dawley rats weighing 250–350 g (Charles River Laboratories Inc., MA, USA) were anesthetized to a surgical plane via an intraperitoneal injection of sodium pentobarbital (40–50 mg/kg). After exposing the trachea, the external jugular vein was cannulated with PE50 polyethylene tubing (Becton Dickinson and Company, NJ, USA). Animals were given intravenous bolus injections 1 $\mu\text{g/kg}$ Ogg1 or mOgg1 fusion protein or an equal volume normal saline at either 30 min prior to bacterial instillation, termed the “pre-treatment protocol”, or 30 min after intra-tracheal bacterial administration, termed the “post-treatment protocol”. An approximate LD_{50} dose of PA103 reported previously to be 5×10^7 cfu (14) was injected into the exposed trachea using a tuberculin syringe. Control rats received 0.1 mL of sterile saline solution. Incisions over the trachea and jugular vein were then closed using 4–0 nylon suture. Animals were warmed with a heat lamp, observed until the effects of anesthesia had subsided, and then returned to

the vivarium. During the post bacterial instillation period, animals were neither treated with antibiotics nor resuscitated.

Determination of pulmonary vascular endothelial barrier integrity

Pulmonary vascular endothelial barrier integrity, as determined by the vascular filtration coefficient, K_f , was measured in *ex vivo* lungs isolated and perfused as a function of time after intra-tracheal administration of PA103 to intact rats. Using previously-described methods (16), rats anesthetized with intra-peritoneal sodium pentobarbital were ventilated using a Harvard rodent ventilator (Harvard Apparatus Company) with a humidified gas mixture consisting of 21% O_2 , 5% CO_2 , and N_2 at 60 breaths/min, a tidal volume of 2.5 ml, and a positive end-expiratory pressure of 2.5 cm H_2O . Following administration of 100 U heparin sulfate, the pulmonary artery and left ventricle were cannulated and the pulmonary circulation perfused at a constant flow rate (0.04 ml/g body weight/min) with physiologic salt solution (3.2 mM $CaCl_2$, 119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4 , 22.6 mM $NaHCO_3$, and 5.5 mM D-glucose) containing 4% bovine serum albumin. The heart and lungs were then removed *en bloc* and suspended in a humidified chamber from a force displacement transducer (Grass FT03, Grass Instruments) to record changes in lung weight. Zone III conditions were maintained. Pulmonary artery and venous pressures were monitored with Cobe pressure transducers (Cobe Laboratories) and a Model 7F Grass polygraph. Pulmonary capillary pressure was estimated using the double occlusion method. Values of K_f were calculated as described previously, normalized to 100g predicted lung weight, and expressed in ml/min/cm H_2O /100g lung weight.

Determination of mtDNA damage, mtDNA copy number and nuclear DNA damage

At termination of *ex vivo* perfusion, lungs were snap-frozen in liquid N_2 and stored at $-80^\circ C$. Frozen lungs were then powdered with a mortar and pestle, total DNA was isolated, and oxidative mtDNA damage assessed using previously described methods (6, 12). In brief, purified DNA samples were digested with the restriction enzymes PpuMI and AhdI (New England Biolabs). Digested samples were precipitated, dissolved in TE buffer, and quantified using a VersaFluor fluorometer (Bio-Rad) with a Quant-IT Picogreen dsDNA Assay kit (Molecular Probe). To reveal oxidative base damage, DNA samples were divided into two aliquot parts; one was treated with formamidopyrimidine glycosylase (Fpg), a bacterial DNA repair enzyme that recognizes and cleaves oxidized purines to create apurinic sites and single-strand breaks (New England Biolabs) while the other was left un-treated. Both Fpg-treated and untreated samples were then incubated with 0.1 N NaOH for 15 min at $37^\circ C$ to cleave the strands at abasic sites or sites of damage to the deoxyribose backbone, mixed with loading dye, and resolved in a 0.6% agarose alkaline gel. Following electrophoresis, DNA was vacuum-transferred to a nylon membrane (Roche Diagnostics) and hybridized with a PCR-generated probe to a 13.6kb fragment of mtDNA encompassing the common deletion sequence. The probe was generated using rat mtDNA sequence as template and the following primers: 5'-CCCTACTTACTGGCTTCAATCTAC-3' for the sense strand and 5'-CATACCATACCTATATATCCGAAGG-3' for the anti-sense strand and subsequently labeled with a DIG-labeled kit (Sigma). Hybridization bands were detected with Amersham Hyperfilm ECL (GE Healthcare) and a Gel Logic 1500 Imaging System (Kodak). Changes in equilibrium density of Fpg-detectable lesions were calculated as

negative *ln* of the quotient of hybridization intensities in Fpg-treated and untreated (alkali only) bands and normalized to 10kb, as described previously (12).

The content of mtDNA in rat lung was determined by slot blot analysis (16). In brief, DNA was isolated, digested with restriction enzymes, and precisely quantified as described above. After adjusting to the same concentrations with H₂O, and treatment with 0.4 N NaOH for 10 min at room temperature to denature the DNA, equal amounts of total DNA were then blotted onto a nylon membrane (Roche Diagnostics, Mannheim, Germany) using a slot blot apparatus (Hoefer, San Francisco, CA. Membranes were hybridized with a DIG-labeled mtDNA-specific probe, prepared as described above, after which they were washed and processed according to the manufacturer's suggestions. Mitochondrial DNA content was normalized to the abundance of a sequence of the nuclear VEGF gene, also determined by Slot blot analysis with a probes generated by PCR using the following primers: 5' - TCTGTCTGCCAGCTGTCTCT-3' for the sense strand and 5' - GAGCTCTTGTCTGATCTTCATAC-3' for the anti-sense strand.

Quantitative alkaline gel electrophoresis was used to determine the mean DNA fragment length of ethidium-stained DNA as an index of nuclear DNA damage. Using previously described methods (12), DNA was isolated, purified, and restricted as described above, and precisely quantified samples were subjected to alkaline gel electrophoresis. Line scans of pixel densities of ethidium-stained alkaline gels were used to identify the position of the mean DNA fragments, whose lengths were then determined by relating the mean fragment position to the position of size standards on the same gels.

Evaluation of multiple organ system failure

At the 24 h time point, surviving rats were again anesthetized. For assessment of blood pressure and heart rate, a cannula inserted into the femoral artery was connected to a TruWave™ pressure transducer (Edwards Lifesciences, CA, USA). After hemodynamic measurements, a 0.5 mL blood sample was withdrawn and transferred to Microtainer® tubes with lithium heparin (Becton Dickinson and Company, NJ, USA) for measurements of creatinine and aspartate transaminase.

To assess changes in microvascular permeability in liver and kidney, extravasation of Evans Blue-labeled albumin (EBA) was determined, with the labeled albumin prepared by dialyzing a filter-sterilized solution of 1.5 mg/mL Evans Blue dye in 5% albumin through a 3500 MWCO cassette at 4° C for 24 h. For this measurement, the femoral vein was cannulated, and dialyzed EBA (15 mg/kg Evans Blue dye) was infused over five minutes and allowed to circulate for an additional 25 min. A segment of the right median lobe of liver and the right kidney were excised, gently rinsed with saline, and weighed. Harvested organs were then immersed in 5 mL/g of formamide (Sigma Aldrich, MO, USA) and homogenized using a PowerGen™ 700 homogenizer (Thermo Fisher Scientific Inc., MA, USA). The homogenized mixture was then incubated in a 37°C water bath for 24 hours. The mixture was then centrifuged at 4000 × *g* for 30 minutes at room temperature. A 200 µL aliquot of supernatant was collected into a Costar™ clear polystyrene 96-well plate (Thermo Fisher Scientific Inc., MA, USA) and dual-wavelength spectrophotometry using a Spectramax® M5 microplate reader (Molecular Devices, LLC, CA, USA) at 620 and 740 nm used to

estimate dye content; correction for heme content used the formula $Abs_{corr} = Abs_{620} - (1.426 * Abs_{740}) + 0.03$ as previously described (17).

Statistical Analyses

Data are expressed as either mean $\pm$ standard error (SEM) or as a scatter plot. Determination of significant differences between groups was performed using a one-way analysis of variance and Tuckey's post hoc tests for multiple comparisons. Significance was defined as $p < 0.05$. The Mantel-Cox log-rank test was applied to evaluate survival after bacterial infection. All statistical analyses were performed using GraphPad Prism® software.

Results

Time-dependent effects of PA103 on pulmonary vascular endothelial integrity and oxidative mtDNA damage

Intra-tracheal challenge with PA103 in intact rats has previously been reported to increase lung vascular K_f (16). Our initial experiments sought to confirm these findings and, of particular relevance to the present report, determine the temporal relationship between endothelial barrier degradation and accumulation of lung tissue mtDNA damage. As shown in FIGURE 1, endothelial barrier dysfunction evolved rapidly, with marked increases in K_f apparent within 3 h of bacterial challenge. In the approximate 40% of animals surviving PA103 challenge, increases in K_f persisted for at least 72h. The impairment in lung vascular endothelial barrier function was accompanied by a transient phase of oxidative mtDNA damage. Also as shown in FIGURE 1, the equilibrium level of oxidative mtDNA damage in lung tissue was increased within 3 h of PA103 administration, but declined to control levels over the 72 h observation period despite the persistent elevations in K_f .

Impact of pharmacologic enhancement of mtDNA repair with mt-targeted Ogg1 in lungs challenged with PA103

We next evaluated the effect of enhanced mtDNA repair with Ogg1 on the decrement in lung endothelial barrier function and accumulation in lung oxidative mtDNA damage noted at 3 h after PA103 administration. As depicted in FIGURE 2, although Ogg1 alone was without effect on K_f , the fusion protein inhibited the increase normally associated with intra-tracheal PA103 challenge. Mutant Ogg1 also failed to impact baseline K_f , but unlike the active enzyme, did not abrogate the bacteria-induced increase. The effects of active and mutant Ogg1 fusion proteins on PA103-induced lung mtDNA damage were similar to their actions on endothelial permeability. In this latter context, FIGURE 2 shows that intra-tracheal PA103 given 3 h earlier caused substantial mtDNA damage. Importantly, increased mtDNA damage repair afforded by Ogg1, while not altering the baseline level of oxidative damage, prevented the rise normally evoked by bacterial challenge. Mutant Ogg1 failed to influence either the baseline or the PA103-induced increase in mtDNA damage.

Because mitochondrial biogenesis has been reported to be a determinant of survival in critically ill human patients and in animal models (5) we determined the effect of PA103 in the absence and presence of treatment with active and mutant Ogg1 fusion proteins on mtDNA copy number. Also as shown in FIGURE 2, there were no significant differences in

the mean mtDNA copy number between the experimental groups. However, closer inspection of the data revealed the presence of two differentially-responding subgroups in all but control animals. Regardless of the experimental treatment, one subgroup of animals appeared to display a robust increase in copy number while mtDNA content appeared to decrease or remain unchanged in a second subgroup. This pattern was particularly prominent in PA103-challenged animals, in animals treated with Ogg1 alone, and in animals receiving both bacteria and mtDNA repair enzyme.

To determine if the protective actions of mitochondrially-targeted Ogg1 were specific for the mitochondrial genome, we measured nuclear DNA damage in the same experimental groups noted above, also 3 h after bacterial challenge. As shown in FIGURE 3, there were no inter-group dependent differences in mean DNA fragment length, indicating that nuclear DNA damage in lung tissue is not an acute feature of PA103-induced lung injury or of treatment with mitochondrially-targeted DNA repair protein constructs.

Effect of mt-targeted Ogg1 on PA103-induced MOSF and mortality

We next addressed the issue of whether Ogg1 prevented MOSF. Heart rate and blood pressure, serum indices of liver and kidney function, and liver and kidney albumin extravasation were measured in control rats, PA103-challenged animals, and animals given Ogg1 in the absence and presence of PA103 in animals surviving bacterial challenge for 24 h. Data summarized in TABLE 1 demonstrate that relative to controls, intra-tracheal PA103 caused MOSF characterized by cardiovascular dysfunction (hypotension and bradycardia), renal dysfunction (increased serum creatinine), liver dysfunction (increased serum AST), and widespread endothelial abnormalities manifest as elevated pulmonary vascular K_f and increased liver and kidney albumin extravasation. Administration of Ogg1 in the absence of bacterial challenge was innocuous, apart from elevated serum AST - which had returned to control levels within 48 h (data not shown). Importantly, Ogg1 given prior to intra-tracheal PA103 prevented abnormalities in all indices of organ dysfunction.

Lastly, we determined the effect of Ogg1 on time-dependent mortality in PA103-challenged rats. Survival curves depicted in FIGURE 4 show that about 60 % of animals died within 24 h of intra-tracheal PA103 challenge. However, both pre-treatment with Ogg1 or post-treatment (60 min after bacterial administration) dramatically reduced bacteria-induced lethality to about 10 %. Perhaps most surprisingly, pre-treatment with mutant Ogg1 also was associated with diminished PA103-induced lethality, although its protective effect was roughly half of that observed with the mtDNA repair-competent enzyme.

Discussion

Despite intense efforts spanning decades, a pharmacologic strategy to prevent or reverse evolution of ALI and MODS has yet to be developed. Many explanations for this failure have been advanced, but a recent report describing a “genomic storm” consisting of over 16,000 differentially-regulated transcripts in severely injured or critically ill patients paints a daunting picture for pharmacologic target identification at a molecular level (18). Mitochondrial dysfunction, often characterized as a bioenergetic crisis, has been frequently noted in severely injured or ill human subjects and in relevant animal models (2); indeed, the

evidence that mitochondrial abnormalities, which also has accumulated over decades, drives organ failure is now supported by recent transcriptomic and metabolomic profiling studies (3, 4). Our interest in exploring the utility of a pharmacologic strategy to suppress ALI and MOSF directed against mitochondria was therefore prompted by a straightforward question: Is there a molecular sentinel located in mitochondrial that could explain the complex pathogenesis of ALI and MOSF but that may have remained undiscovered by the multiple experimental approaches previously applied?

The mitochondrial genome emerged as a reasonable point of interest. The molecule is relatively sensitive to damage induced by exogenously-applied ROS (6), and ROS have been incriminated repeatedly in ALI, MOSF and related disorders (19). Mitochondrial DNA resides in close but variable appositions to the inner mitochondrial membrane from which ROS are continuously “leaked” into the mitochondrial matrix. Mitochondrial DNA is also packaged in a complex consisting of about 30+ proteins including all of those required for mt mRNA transcription (20). Damage to the mitochondrial genome activates nuclear transcriptional pathways and rapidly-adapting signaling events with the potential to tip the intracellular context towards cytotoxic or survival modes (21). Mitochondrial biogenesis, likely initiated by mtDNA replication and regulated in part by PGC-1 α a nuclear-encoded factors (5), appears to dictate survival in critical illness (22). And lastly, oxidative damage to the mitochondrial genome leads to its fragmentation (16) into so-called mtDNA Damage Associated Molecular Patterns (DAMPs). The fragments so created can activate both the NLRP3 inflammasome or the Cgas/STING pathways (23, 24). The NLRP3 inflammasome and free mtDNA also can be exported to the extracellular environment where either could propagate injury to distant sites via distinct mechanisms (25).

Certain aspects of these mtDNA-driven signaling pathways seemed to be highly relevant to the genomic storm and pro-inflammatory environments characterizing severe injury- or illness-related ALI and MODS: First, through their actions on the signaling cascades just described, mtDNA fragments formed as a consequence of mtDNA damage could initiate all or part of the genomic and cytokine “storms” associated with severe injury or illness (18). And second, although substantial evidence points to mtDNA damage as an important pathogenic component of ALI and ARDS, and although mtDNA damage has been detected in various inflammatory settings (26), its causal role had yet to be clarified.

Against this background, the present study tests the concept that mtDNA damage triggers ALI and MODS in the setting of bacteria-induced pulmonary insult. In support of this hypothesis, we found that intra-tracheal PA103 induced a transient phase of oxidative mtDNA damage that coincided with deterioration of lung endothelial barrier function. Moreover, mt-targeted Ogg1, but not an inactive mutant, prevented both accumulation of oxidative mtDNA damage in lung tissue and decrements in lung endothelial barrier function. That these bacterial- and mtDNA damage-dependent events in the lung couple ALI to delayed MOSF and lethality are supported by our finding that intravenous administration of mt-targeted Ogg1 suppressed not only ALI but also cardiovascular, hepatic, and renal dysfunction measured in animals surviving for 24 h after intra-tracheal challenge with PA103. Similarly, pre- or post-treatment with Ogg1 reduced bacteria-induced mortality monitored across the 24 h post-challenge period.

Two unexpected findings were prominent in the current study. The first pertained to the effect of PA103 and Ogg1 on mtDNA copy number. Particularly in the case of PA103-challenged lungs, but in the other treatment cohorts as well, mtDNA copy number tended to cluster in two groups; some lungs tended to display robust increases in copy number above control, while in others, copy number was unchanged or tended to decrease. These findings have interesting implications for two reasons. As noted previously, mitochondrial biogenesis has been linked to survival in critically-ill human subjects (22). Survival after PA103-challenge may have identified animals who, for currently unknown reasons, had an intrinsic capacity for robust mtDNA replication after injury. The second and related implication pertains to the reason why Ogg1, in the absence of PA103-induced injury, produced a biogenic phenotype. Appreciating that there is a baseline level of oxidative damage in the mitochondrial genome, it seems reasonable to suspect that increasing DNA glycosylase activity by supplementation with exogenous, mt-targeted Ogg1 could elevate the density of apurinic sites and DNA strand breaks. In light of recent findings that oxidative base damage in the D-loop region stimulates mtDNA replication in hypoxia (27), it seems likely that a mt-targeted Ogg1-induced rise in AP sites and strand break densities could have initiated an increase in mtDNA copy number by a similar mechanism. Additional studies will be required to explore this therapeutically-relevant concept.

A second unexpected observation was that although the inactive Ogg1 mutant failed to suppress either PA103-induced lung tissue mtDNA damage or ALI, the DNA repair-defective mutant attenuated lethality to about 50% of the nearly full protection afforded by the active enzyme. This finding points to the prospect that mtDNA damage and repair could exert more complex actions governing the response to bacterial injury than initially anticipated. In this regard, Boldogh and colleagues demonstrated that free 8-oxoguanine, which is excised from oxidatively damaged DNA by Ogg1, forms a complex with the glycosylase to function as a guanine exchange factor activating *Ras*-dependent signaling, particularly those pathways driving innate immune responses (28). Importantly, 8-oxoguanine binds to Ogg1 at a site distinct from its DNA binding and repair domain of the protein. In light of these considerations, it is tempting to speculate that the mutant Ogg1 used in the present experiments, because it retains the capability to complex with 8-oxoguanine and activate *Ras*-mediated signaling, could potentially stimulate pathways blunting responses to intra-tracheal PA103 and thereby reducing mortality. An alternate for the unexpected actions of the mutant Ogg1 can be found in reports by Kamp and colleagues who showed that both active and mutant Ogg1 prevents oxidant-induced apoptosis in airways epithelial cells by acting as a chaperone for aconitase (9). Obviously, fully understanding the complex mechanisms by which Ogg1 and mitochondrial base excision repair defend against bacteria-induced ALI and MOSF will require experiments of design different than those used herein.

Also left unresolved by the present study is the question of whether mtDNA damage is present in non-pulmonary organs and, if so, whether the protection against MODS and lethality afforded by Ogg1 is attributed to a reduction in mtDNA damage-related events in those organs or rather through attenuation of the initial pulmonary insult and concomitant inflammatory signals. Closely related to this question is the mechanism by which intra-tracheally administered PA103 leads to lung injury that propagates to MOSF. In this context,

previous experiments in isolated, buffer-perfused lungs showed that intra-tracheal administration of PA103 causes oxidative mtDNA damage and release of mtDNA DAMPs (16), the latter of which appear to initiate decrements in lung endothelial barrier function via a TLR9-dependent pathway (16). Using a model of sepsis-induced lung injury, it was also reported that cardiac dysfunction was mediated by mtDNA damage-induced local myocardial inflammation (29). Here, a pathway involving extracellular accumulation of NLRP3 with attendant local synthesis of damaging cytokines was invoked, although it is not known whether extracellular trafficking of this inflammatory scaffold is driven by mtDNA damage. Resolution of these and related hypotheses to explain the mechanism by which lung tissue mtDNA damage and ALI is propagated to distant organs will require additional study.

It does, however, seem clear that injury propagation from the lung to distant organs is not merely a function of circulatory accumulation of mtDNA DAMPs. Damaged mitochondria release multiple DAMPs, including not only mtDNA but formylated and non-formylated peptides as well (30, 31). Neutrophil extracellular traps composed of nuclear DNA and mobilized by resident or itinerant lung inflammatory cells may function to propagate alarm signals (32). The fact that the timing of DNase administration relative to the initial event dictates effectiveness of the intervention – with early administration worsening outcomes, and later administration improving organ function and survival – suggests complex interactions with other pathways of tissue damage (33). Indeed, synergy between mt-derived DAMPs has been demonstrated (34). Despite these vagaries, the present findings that mt-targeted Ogg1 suppresses bacteria-induced lung tissue mtDNA damage and ALI accompanied by attenuation of MOSF and lethality is strong evidence that a loss of mitochondrial genomic integrity is a critical step driving injury propagation.

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FIGURE 1. *P. aeruginosa* (strain 103; PA103) given intra-tracheally to intact rats causes persistent increases in pulmonary vascular permeability measured *ex vivo* accompanied by transient lung tissue mtDNA damage

PA 103 (5×10^7 CFU in 100 μ l physiologic salt solution) was instilled as a bolus into the trachea of intact, anesthetized rats. At the indicated times, lungs were either excised, mechanically ventilated, and perfused at constant flow rate with physiologic salt solution to measure the vascular filtration coefficient (K_f) or reserved for assessment of oxidative mtDNA damage as described in the Methods. As shown in *Panel A*, relative to preparations derived from control animals, lungs from rats challenged with PA103 displayed increases in K_f that persistent for at least 72 h after bacterial instillation. Data shown in *Panel B* indicates that PA103 given as described above caused a transient increase in lung tissue oxidative mtDNA damage, detected at 3 h post bacterial administration, which had subsided to control levels within 18 h. N=4 for all time points and groups. *Different from control at $p < 0.05$.

FIGURE 2. Mitochondria-targeted Ogg1 reduces PA103-induced increases in K_f and lung tissue oxidative mtDNA damage

Rats received intravenous injections of mitochondrial-targeted Ogg1 or inactive mutant Ogg1 (mOgg1) 30 min prior to intra-tracheal instillation of PA103 (5×10^7 CFU in 100 μ l physiologic salt solution). Control animals (CON) were treated in with iv or intra-tracheal saline. At 3 h after bacteria or saline instillation, lungs were excised, mechanically ventilated, and perfused at constant flow rate with physiologic salt solution containing or reserved for mtDNA analyses as described in the Methods. The impact of active or mutant Ogg1 given in the presence or absence of bacterial challenge on K_f is shown *Panel A*. While

neither active nor mutant Ogg1 altered K_f relative to controls, the increase normally evoked by PA103 was suppressed by active Ogg1 but not by the inactive mutant. *Panel B* shows calculated changes in the equilibrium lesion density of oxidative mtDNA damage for the same experimental groups. Here, too, while neither active or mutant Ogg1 altered baseline oxidative mtDNA damage, active Ogg1, but not the mutant, suppressed the increase in mtDNA damage normally evoked by intratracheal instillation of 5×10^7 CFU PA103. $N=4-6$ for all groups, apart from mOgg1 alone, for which $N=3$. *PA103 alone and in the presence of mutant Different from mOgg1 were different from all other groups at $p < 0.05$. *Panel C* displays a scatter diagram along with the mean $\pm$ the S.E. of mtDNA copy number normalized to the nuclear VEGF gene abundance, both measured by slot blot, for the experimental groups noted above. Although there were no differences in mean values, for all groups but control there was a conspicuous tendency for data to segregate on the basis of high or low mtDNA copy numbers. See text for details.

FIGURE 3. Impact of mt-targeted Ogg1 and mutant Ogg1 on nuclear DNA integrity in intact rats challenged with intra-tracheal PA103

Rats received intravenous injections of mitochondrial-targeted Ogg1 or inactive mutant Ogg1 (mOgg1) 30 min prior to intra-tracheal instillation of PA103 (5×10^7 CFU in 100 μ l physiologic salt solution). Control animals (CON) were treated in with iv or intra-tracheal saline. At 3 h after bacteria or saline instillation, lungs were excised and reserved for quantitative gel electrophoresis to determine nuclear DNA integrity. There were no significant differences between experimental groups indicating that neither PA103 alone or treated with active or mutant Ogg1 altered integrity of the nuclear genome in lung tissue. N=4–6 for all groups, apart from mOgg1 alone, for which N=3.

FIGURE 4. Kaplan-Meier curve demonstrating impact of mt-targeted Ogg1 and mutant Ogg1 on survival in intact rats challenged with intra-tracheal PA103

Survival was determined at the indicated times with the following numbers of rats in each experimental group. Active or mutant Ogg1 was administered intravenously either 30 min before or 30 after intra-tracheal instillation of PA103 (5×10^7 CFU in 100 μ l physiologic salt solution). Numbers of animals/group: Control; 17, PA103; 32, PA103 + Ogg; 14, Ogg alone; 4, mOgg alone; 2, PA + mOgg; 11, PA103 + Ogg reversal; 8.

Table 1

Indices of organ dysfunction depicted as mean $\pm$ SEM or % survival for animals surviving 24 hours after intra-tracheal instillation of PA103

	Control	Ogg1 alone	PA103 alone	PA103+Ogg1
Kf (mL/min/cmH ₂ O/100g)	0.22 $\pm$ 0.003	0.22 $\pm$ 0.02	1.33 $\pm$ 0.02 ^{*,**}	0.32 $\pm$ 0.05 [†]
Heart Rate (beats/min)	402.8 $\pm$ 5.36	388.7 $\pm$ 8.09	326.3 $\pm$ 25.20 ^{*,**}	381.3 $\pm$ 6.84 [†]
Mean Arterial Pressure (mmHg)	107.6 $\pm$ 4.20	103.3 $\pm$ 2.60	84.50 $\pm$ 6.70 ^{*,**}	100.8 $\pm$ 2.53
Creatinine (mg/dL)	0.73 $\pm$ 0.08	0.57 $\pm$ 0.09	1.70 $\pm$ 0.32 ^{*,**}	0.59 $\pm$ 0.04 [†]
Renal Absorbance (AU)	0.070 $\pm$ 0.004	0.062 $\pm$ 0.003	0.19 $\pm$ 0.03 ^{*,**}	0.14 $\pm$ 0.03
Aspartate Transaminase (mg/dL)	139.9 $\pm$ 18.04	262.7 $\pm$ 53.38 [*]	265.8 $\pm$ 25.62 [*]	181.8 $\pm$ 20.18
Hepatic Absorbance (AU)	0.052 $\pm$ 0.005	0.045 $\pm$ 0.014	0.092 $\pm$ 0.006 ^{*,**}	0.073 $\pm$ 0.006 [†]
Survival	100%	100%	41.9% ^{*,**}	92.9% [†]

* Significantly different from control

** Significantly different from Ogg1 alone

† Significantly different from PA103 alone