Med Microbiol Immunol (2015) 204:673–680
DOI 10.1007/s00430-015-0418-x

ORIGINAL INVESTIGATION

ExoU-induced redox imbalance and oxidative stress in airway epithelial cells during *Pseudomonas aeruginosa* pneumosepsis

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Received: 8 November 2014 / Accepted: 12 April 2015 / Published online: 23 April 2015

Abstract ExoU is a potent proinflammatory toxin produced by *Pseudomonas aeruginosa,* a major agent of severe lung infection and sepsis. Because inflammation is usually associated with oxidative stress, we investigated the effect of ExoU on free radical production and antioxidant defense mechanisms during the course of *P. aeruginosa* infection. In an experimental model of acute pneumonia, ExoU accounted for increased lipid peroxidation in mice lungs as soon as 3 h after intratracheal instillation of PA103 *P. aeruginosa* strain. The contribution of airway cells to the generation of a redox imbalance was assessed by in vitro tests carried out with A549 airway epithelial cells. Cultures infected with the ExoU-producing PA103 *P. aeruginosa* strain produced significantly increased concentrations of lipid hydroperoxides, 8-isoprostane, reactive oxygen intermediates, peroxynitrite and nitric oxide (NO), when compared to cells infected with *exoU*-deficient mutants. Overproduction of NO by PA103-infected cells likely resulted from overexpression of both inducible and endothelial NO synthase isoforms. PA103 infection was also associated with a significantly increased activity of superoxide dismutase (SOD) and decreased levels of reduced glutathione (GSH), a major antioxidant compound. Our findings unveil another potential mechanism of tissue damage during infection by ExoU-producing *P. aeruginosa* strains.

Keywords ExoU · Oxidative stress · Lipid peroxidation · NO synthase · Pneumosepsis · *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa is a major agent of life-threatening pneumonia in hospitalized patients. The poor outcome of these individuals usually correlates with the development of sepsis, a clinical syndrome characterized by the excessive release of proinflammatory mediators, activation of different cell types and increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. In lungs, excessive neutrophil recruitment and accumulation are associated with increased permeability of the capillary/alveolar barrier, lung tissue injury and impaired oxygenation [2].

Under physiological conditions, a homeostatic balance exists between the formation of ROS and RNS, and their removal by endogenous antioxidant scavenging compounds [3]. Oxidative stress occurs when this balance is disrupted by excessive production of oxidative agents and/or by overcoming antioxidant defense mechanisms. ROS and RNS are major mediators of cellular injury via peroxidation of polyunsaturated lipids from biomembranes and subcellular organelles. Breakdown products of lipid peroxides, mostly reactive aldehydes, may serve as oxidative second messengers due to their prolonged half-life and ability to diffuse from their site of formation, compared to free radicals. Lipid breakdown products can also make covalent modifications on nucleic acids and proteins and dramatically alter cell integrity [4]. Among other effects, peroxidation of membrane

lipids represents a major threat to cellular integrity, once protein oxidation can alter metabolic function, and oxidative damage to DNA can impair cell division and repair.

The pathogenesis of *P. aeruginosa* infections involves a number of virulence factors, including ExoU, a potent cytotoxin with phospholipase A_2 (PLA_2) activity that is injected into host cytosol through the type III secretion system. Upon injection, ExoU is activated by host cell cofactors [5–7] and targeted to the plasma membranes, where it cleaves membrane phospholipids, resulting in rapid lysis of a variety of cell types [8]. ExoU PLA_2 activity also accounts for a potent stimulation of inflammatory response and increased release of free arachidonic acid, cytokines, eicosanoids and PAF from infected cells [9–11], as well as for the activation of the NF-κB transcription factor via a PAFR signaling pathway [12, 13]. The proinflammatory effect of ExoU has been extensively confirmed in experimental models of pneumosepsis in which *P. aeruginosa* inoculation into mice airways resulted in a rapid influx of large numbers of inflammatory cells, primarily neutrophils, into the lungs [9, 11–16].

Activated phagocytic cells form the majority of ROS and RNS produced during the inflammatory response. Furthermore, endothelial and airway epithelial cells may also contribute to the generation of a local state of oxidative stress in an inflammatory environment, as documented for the respiratory tract exposed to different stimuli [17–19]. However, there are no studies demonstrating that ExoU is capable to induce oxidative stress in airway epithelial cells.

In the present study, we addressed the question of whether ExoU would induce a state of oxidative stress in lungs of *P. aeruginosa*-infected mice and whether airway cells would contribute to such redox imbalance.

Materials and methods

Bacterial strains and culture conditions

The ExoU-producing PA103 *P. aeruginosa* strain and the *exoU*-deficient PA103Δ*exoU* mutant [9] were used throughout this study. In some assays, the PA103ΔUT/S142A strain was used to confirm the contribution of the PLA_2 activity in the phenomenon under evaluation. PA103ΔUT/S142A, an *exoU*-depleted mutant complemented with an *exoU* gene with a site-specific mutation in the PLA_2 catalytic site, was kindly furnished by Dr. A. Hauser (Northwestern University, USA). Bacteria were grown in Luria-Bertani broth at 37 °C for 14–16 h under moderate agitation, harvested by centrifugation and resuspended in lipopolysaccharide-free saline or in F12 nutrient culture medium (Invitrogen) supplemented with 10 % fetal calf serum and glutamine (complete culture medium).

Mice infection

Female Swiss mice (8–12 weeks old) anesthetized with a mixture of ketamine (65 mg/kg) and xylazine (13 mg/kg) were infected intratracheally with 10^4 colony-forming units of PA103 or PA103Δ*exoU* in 50 μL of lipopolysaccharide-free saline. As control, mice were instilled with saline only. At 3 h post-infection, animals were euthanized and their lungs were removed and frozen in liquid nitrogen. All animal experiments were approved by the Animal Ethics Research Committee of the State University of Rio de Janeiro (CEUA/022/2011) and performed in accordance with the guidelines of this committee.

Assessment of lipid peroxidation in mice lungs

Lipid peroxidation in mice lungs was assessed by the determination of their content of thiobarbituric acid reactive species (TBARS). Briefly, mice lungs were homogenized in cold phosphate buffer, pH 7.4 with 0.2 % BHT (2,6-di-tert-butyl-4-methylphenol). Samples (0.5 ml) were mixed with equal volume of 0.67 % thiobarbituric acid (Sigma-Aldrich) and then heated at 96 °C for 30 min. TBARS were determined by the absorbance at 535 nm.

Cell culture and infection

Human airway epithelial cells from the A549 line (purchased from the Rio de Janeiro Cell Bank) were cultured in F12 nutrient complete culture medium containing antibiotics. Confluent cultures were trypsinized, and cells were suspended in complete medium with antibiotics, seeded in 6-well (8.2×10^5 cells/well), 24-well (1.7×10^5 cells/well), 96-well (3.0×10^4 cells/well) tissue culture plates or 25-cm^2 tissue culture flask (1.12×10^6 cells/flask) and incubated at 37 °C for 48 h. Cells were then infected with *P. aeruginosa* strains at a multiplicity of infection of about 100. Since translocation of effector proteins from the type III secretory system depends on close contact between bacteria and host cells, bacteria were centrifuged (1,000×*g* for 10 min at 4 °C) onto the cultured cells. Control cultures were exposed to culture medium only. After incubation for 1 h at 37 °C, cell cultures were treated with gentamicin at 300 μg/ml in complete culture medium to kill extracellular bacteria and incubated for the remaining time needed to complete 6, 24 or 48 h.

Analysis of lipid peroxidation, catalase activity and SOD activity in cell lysates

Cells were cultured in 6-well or 24-well tissue culture plates or in 25-cm^2 tissue culture flasks to assess lipid peroxidation, catalase and superoxide dismutase (SOD) activity,

respectively, and infected with the different bacterial strains for 6, 24 or 48 h. Infected and noninfected control cells were washed with phosphate-buffered saline pH 7.4 (PBS), scraped from the culture plates, centrifuged at 1260×*g* for 10 min at 15 °C, resuspended in chilled ultrapure water (LPO), SOD buffer (20 mM Hepes pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose) or catalase buffer (50 mM potassium phosphate pH 7.0, 1 mM EDTA) at a final concentration of 3×10^6 cells/ml (LPO) or 1.5×10^7 cells/ml (SOD and catalase) and lysed by 4 short burst of 30 s with a CPX-130 Cole-Parmer sonicator. Cell lysates were centrifuged (10,000×*g* for 15 min at 4 °C), and the supernatants were used for the different analysis using the Calbiochem Lipid Hydroperoxide Assay Kit and the Cayman Chemical commercial kits Superoxide Dismutase Assay and Catalase Assay, according to the manufacturer instructions.

Quantification of 8-isoprostane

Cells cultured in 24-well tissue culture plates were infected for 6, 24 and 48 h. The supernatants from infected and noninfected control cultures were recovered and analyzed with the Cayman Chemical 8-Isoprostane EIA Kit. In parallel, the number of cells excluding the trypan blue dye in infected and noninfected cultures was determined by counting trypsinized cells in Neubauer chambers, in order to normalize the levels of 8-isoprostane by the number of viable cells.

Quantification of glutathione (GSH)

Cells cultured in 96-well tissue culture plates were infected and the GSH levels were measured using a luminescence-based assay (GSH-Glo™ Glutathione Assay, Promega), according to the manufacturer's instructions.

Determination of ROS, NO and peroxynitrite production

Cells were seeded in black 96-well tissue culture plate, cultured overnight in complete F12 medium, washed three times with PBS, incubated with serum-free Hanks balanced salt solution for 1 h and loaded for more 1 h at 37 °C with 5 μM of the Molecular Probes products 5-(6)-chloromethyl-2,7-dichlorodihydrofluorescein (CM-H_2DCF-DA), 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA) or 3′-(p-hydroxyphenyl) fluorescein (HPF) to assess ROS, NO and peroxynitrite production, respectively. After probe load, cells were washed and infected with bacterial suspensions for different periods. Control cells were treated with culture medium only. Fluorescence emitted by each probe was measured in the microplate reader Envision™ (PerkinElmer).

Western blot

Western blot was performed for the detection of iNOS, eNOS and nNOS in extracts from control or infected A549 cells. Briefly, 35 μg of protein from the different cell extracts was separated by SDS-polyacrylamide gel electrophoresis on 10 % acrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5 % fat-free milk at room temperature for 1 h and then incubated overnight at 4 °C with appropriate Santa Cruz Biotechnology primary antibodies against β-actin, iNOS (sc-651), eNOS (sc-654) and nNOS (sc-648) in 5 % milk in Tris-buffered saline (TBS). After being washed with TBS containing 0.05 % Tween 20 (TTBS), membranes were incubated for 1 h with appropriate secondary antibody at room temperature, washed and treated with chemiluminescence reagents (ECL Plus) for 5 min. The target bands were then imaged on a ChemiDoc™ XRS and analyzed with the Image Lab™ software. The expression level of the β-actin housekeeping gene was used for equal loading control in all experiments.

Statistical analysis

The results were expressed as the mean ± standard error of the means of data obtained in at least three different experiments. Statistical analysis was performed using a one-way analysis of variance (ANOVA) for multiple group analysis with a Bonferroni adjustment to determine significant statistical differences between groups. $p < 0.05$ was taken as statistically significant.

Results

ExoU induced a state of oxidative stress in lungs of *P. aeruginosa*-infected mice

Because ROS have extremely short half-lives, they are difficult to measure directly. Therefore, the existence of oxidative stress in mice lungs was assessed by the TBARS assay that measures malondialdehyde, an end product formed during lipid peroxidation [20]. As shown in Fig. 1, as soon as 3 h after bacteria inoculation, malondialdehyde concentration in lungs of PA103-infected mice was significantly increased, when compared to concentrations in lungs of control and PA103Δ*exoU*-infected mice.

Airway A549 cells injected with ExoU exhibited evidences of oxidative stress

Three different approaches were used to ascertain the ability of airway epithelial cells to produce increased amounts of oxidizing agents in response to ExoU: (1) determination

of lipid hydroperoxide concentration in cell lysates; (2) evaluation of 8-isoprostane concentration in cell culture supernatants [21] and (3) evaluation of ROS, NO and peroxynitrite levels in control and infected cell cultures.

PA103-infected cells produced increased concentration of lipid hydroperoxides (Fig. 2a) and 8-isoprostane (Fig. 2b), a prostaglandin-like compound produced by free radical-catalyzed peroxidation of fatty acids, which has been used as an accurate marker of lipid peroxidation in both animal and human models of oxidative stress [21, 22]. These effects were significantly reduced in PA103Δ*exoU*-infected cells.

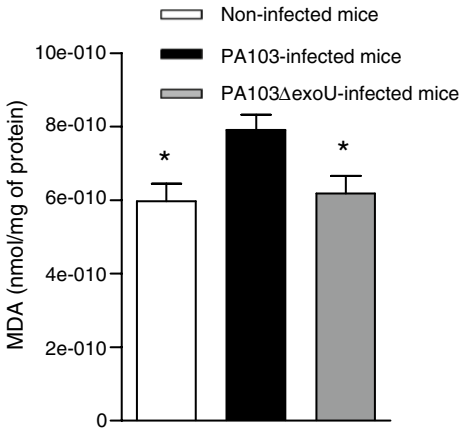

Fig. 1 ExoU induces oxidative stress in lungs of *P. aeruginosa*-infected mice. Malondialdehyde (MDA) concentration in lungs of mice infected for 3 h with the ExoU-producing *P. aeruginosa* strain ($n = 15$) was significantly higher than that in lungs of control ($n = 10$) or PA103Δ*exoU*-infected animals ($n = 15$). *$p < 0.05$ when the values obtained in PA103-infected mice were compared with those obtained in animals from the other two groups

PA103-infected cells also produced increased concentrations of ROS, NO and peroxynitrite, when compared to control noninfected cells (Fig. 3), which were also significantly higher than the increase detected in cells infected with the *exoU*-deficient mutant or with the mutant complemented with ExoU with a site-directed mutated catalytic motif. Significantly increased production of ROS was detected very early, at 10 min after infection, and levels remained elevated for as long as 120 min (data not shown).

NO production by PA103-infected cells is likely to result from overexpression of both eNOS and iNOS. Indeed, the eNOS level in PA103-infected cells was about 150 % the one detected in control cells, while the iNOS level was increased in about 1000 %. Levels of eNOS and iNOS expression in PA103Δ*exoU*-infected cells were similar to those found in control noninfected cells (Fig. 4). No difference was detected in the expression of nNOS in the infected cells, when compared to the expression in control noninfected cells (data not shown).

Effect of ExoU on A549 cell antioxidant defense mechanisms

At 48 h post-infection, PA103-infected cells exhibited a significant increase in SOD activity (Fig. 5a) and

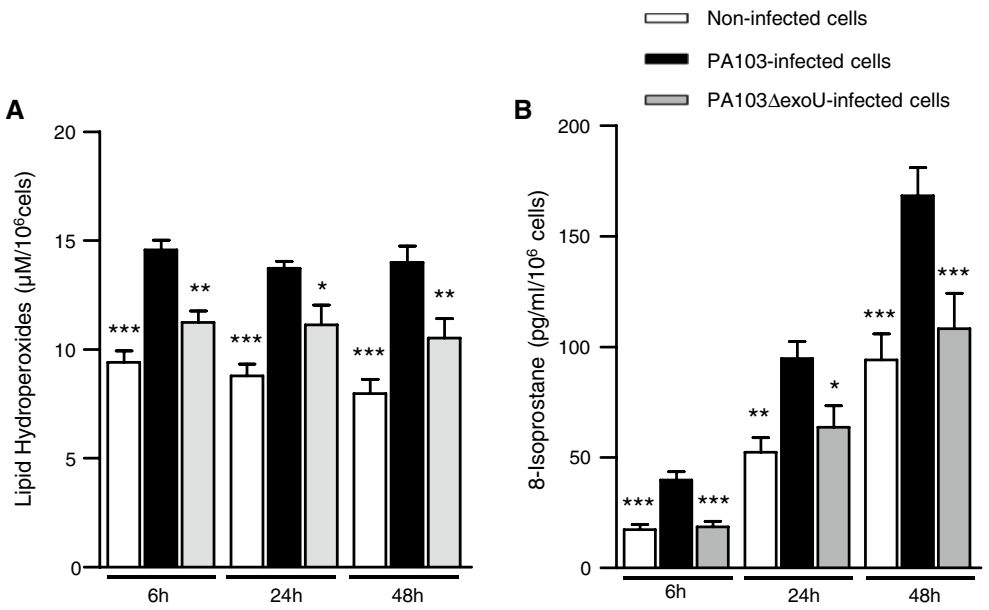

Fig. 2 ExoU induces oxidative stress in PA103-infected A549 cells. Oxidative stress was characterized by enhanced production of lipid hydroperoxides (**a**) and 8-isoprostane (**b**). The *graphs* represent the mean ± SEM from values obtained in three different assays performed in triplicate; *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ when the values obtained in PA103-infected cultures were compared with those obtained in control or in PA103Δ*exoU*-infected cultures

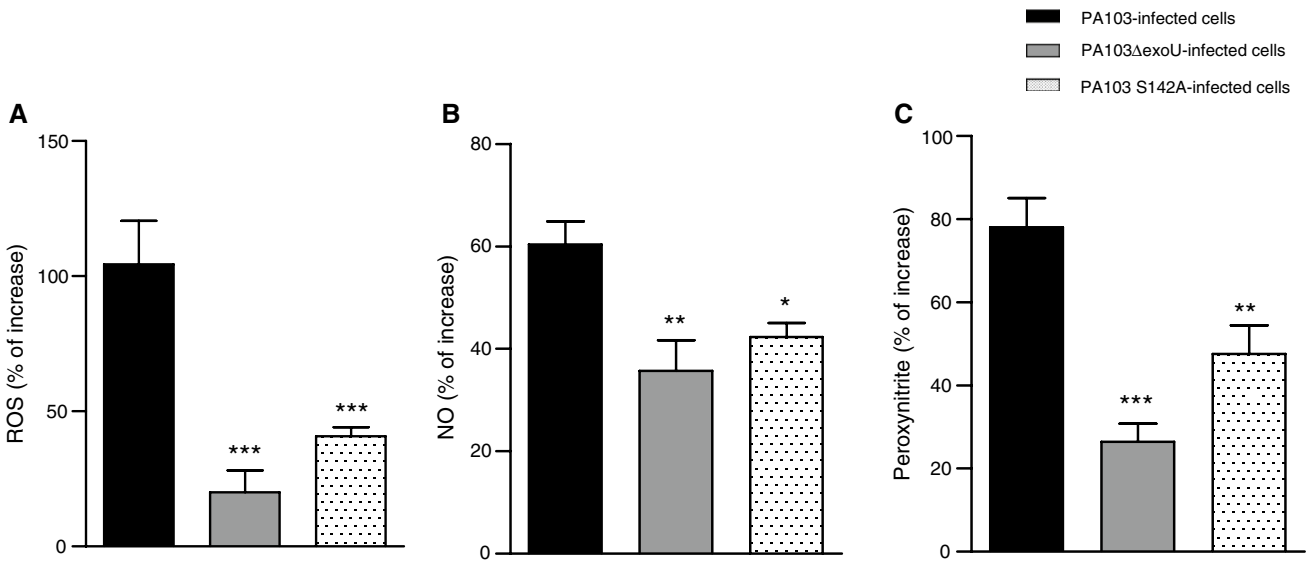

Fig. 3 ExoU-induced oxidative stress results from the enhanced production of ROS, NO and peroxynitrite. At 1 h after infection, the level of ROS (**a**), NO (**b**) and peroxynitrite (**c**) in cells infected with the ExoU-producing PA103 strain was significantly higher than that in cells infected with the PA103Δ*exoU* mutant, which does not produce ExoU, or with the PA103ΔUT/S142A mutant that produces a toxin with impaired PLA_2 activity. The graphs represent the mean ± SEM of the percentual increases, considering the values obtained in control noninfected cultures as 100 %. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ when the values obtained in PA103-infected cells were compared with those obtained in cells infected with the mutants

reduction in GSH levels (Fig. 5b) compared to noninfected or PA103Δ*exoU*-infected cells. No significant difference was detected in catalase activity in control or infected cells (data not shown).

Discussion

Bacterial infection of the lungs is accompanied by an influx of leukocytes, which are stimulated to produce inflammatory mediators, superoxide and hydrogen peroxide, a more stable and membrane-permeable product. Both superoxide and hydrogen peroxide are toxic to cells in the vicinity. Myeloperoxidase is very abundant in activated leukocytes and converts hydrogen peroxide into hypochlorous acid, a more powerful oxidant agent. In this context, oxygen metabolites may lose their physiological role in the killing of pathogens, to turn into toxic effectors responsible for the damage of the pulmonary epithelium, as well as of other components of the lung parenchyma and lining fluids [23]. In consequence, leukocyte overproduction of oxidants within lungs contributes greatly to the pathophysiology of acute respiratory distress syndrome (ARDS), a life-threatening complication of severe pneumonia with high mortality rates [2]. Besides ROS production, neutrophil upregulated expression of iNOS, which accounts for enhanced production of NO, contributes also to exacerbating oxidative stress indices and to ARDS pathophysiology [24].

Lung infection by ExoU-producing *P. aeruginosa* is accompanied by a robust infiltration of inflammatory cells consisting primarily of neutrophils [9, 11–13]. However, this highly cytotoxic toxin kills both resident alveolar macrophages and recruited neutrophils, thereby interfering with the clearance of bacteria from infected lungs [14, 15]. Whether ExoU-injected phagocytic cells could still release reactive oxygen and nitrogen species, and potentially contribute to the damage of lung parenchyma, was yet unknown. In the present study, designed to investigate whether acute pneumonia by ExoU-producing *P. aeruginosa* is associated with oxidative stress in infected lungs, we show that ExoU accounted for increased lipid peroxidation in mice lungs as soon as 3 h after intratracheal instillation of bacteria, testifying the association between this *P. aeruginosa* toxin and the occurrence of oxidative stress.

A described consequence of oxidative stress in lungs is the enhancement of the airspace epithelial permeability, secondary to increased epithelial cell detachment, decreased cell adherence and increased cell lysis [25]. Increased epithelial permeability favors the dissemination of bacterial into host bloodstream [26, 27]. Indeed, in the course of lung infection by a cytotoxic ExoU-producing *P. aeruginosa* strain, bacteria were shown to cause alveolar epithelial injury and progressive dissemination from infected lungs as soon as 4 h after intratracheal instillation [28]. Interestingly, in a more recent study, only relatively few alveolar epithelial cells were shown to be injected with

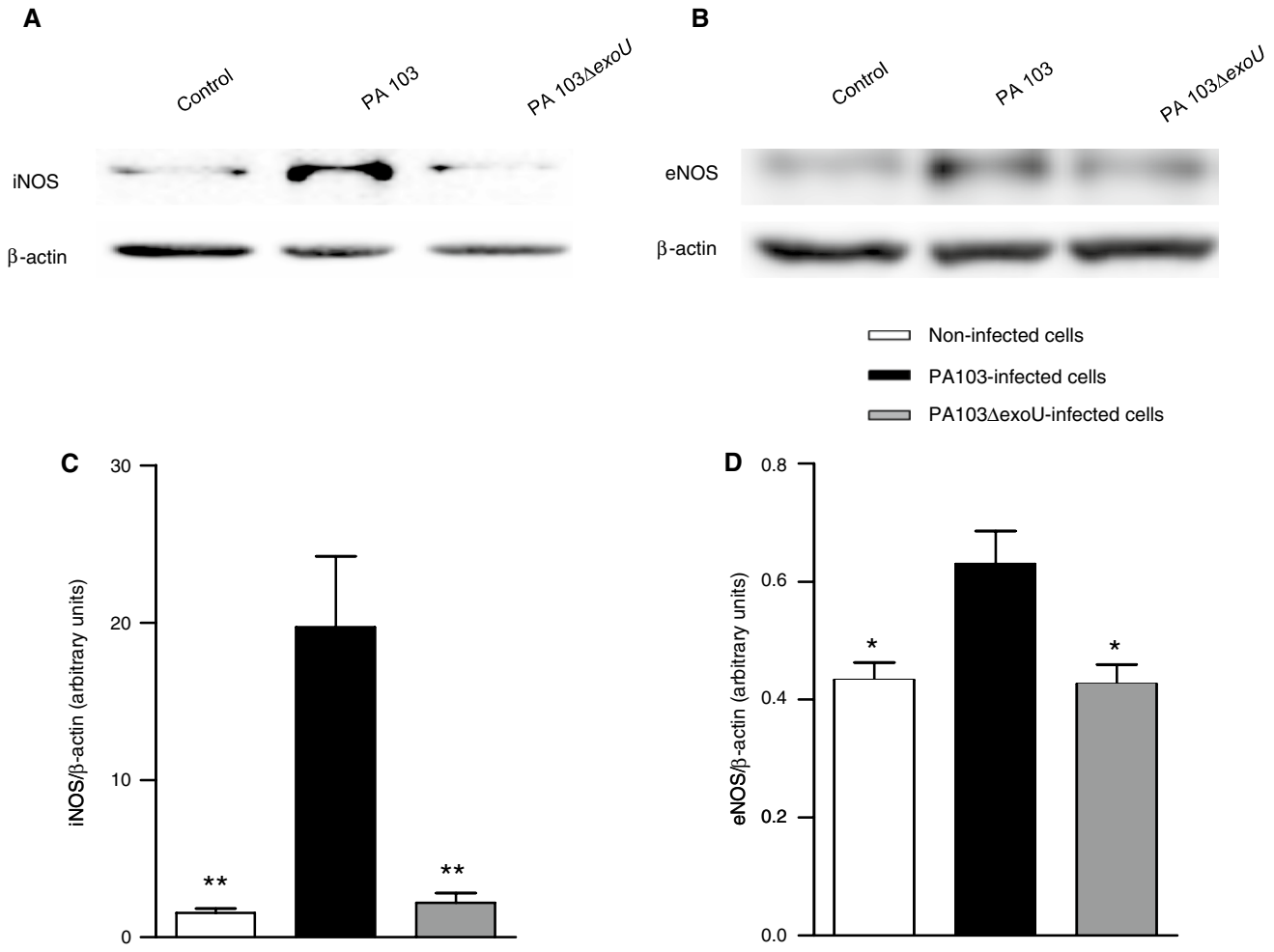

Fig. 4 ExoU increases eNOS and iNOS expression by A549 cells. **a**, **b** Western blot detection of iNOS, eNOS and β-actin in control and infected cells. Graphs in **c** and **d** represents mean ± SEM from values obtained in three different western blot assays. *$p < 0.05$ and **$p < 0.01$ when the values obtained in PA103-infected cells were compared with those obtained in control or in PA103Δ*exoU*-infected cells

ExoU [14]. Therefore, it seems unlikely that alveolar epithelial injury has resulted from a direct effect of ExoU, but it is plausible to assume that the increased epithelial permeability detected in mice with pneumonia by ExoU-producing bacteria has resulted from a disequilibrium between pro-oxidant and antioxidant molecules in infected lungs.

During pulmonary infections, the majority of oxidants in lungs arise from stimulated phagocytic cells that can generate toxic oxygen metabolites from assembly on cell surfaces of NADPH oxidase (also known as NOX2) [23]. Similar pathways leading to oxidant generation can occur in endothelial and alveolar epithelial cells [23]. A recent study revealed that, in later periods of infection, *P. aeruginosa* was able to induce NOX2 and NOX4 expression, but not NOX1 and NOX3, in mice lungs. NF-κB was critical to the expression of both NOX1 and NOX3, but NOX2 and NOX4 seemed to have distinct roles in *P. aeruginosa* pathogenesis, with NOX4 involved in apoptosis and disruption of endothelial barrier integrity and NOX2 in inflammation and oxidative response to the bacteria [29]. Furthermore, it has been shown that, in pulmonary endothelial and phagocytic cells, activation of NOX2 depends on the PLA_2 activity of peroxiredoxin 6 [30, 31]. Since ExoU has PLA_2 activity and activates NF-κB, we hypothesized whether this virulence factor would be able to interfere with the production of oxidants by airway epithelial cells. In agreement with our hypothesis, PA103-infected A549 cells produced increased amounts of lipid hydroperoxide, 8-isoprostane, ROS, NO and peroxynitrite, a strong oxidant that results from the reaction of superoxide and NO.

It is commonly believed that excessive NO production, critically implicated in the pathophysiology of sepsis and ARDS, results from the activity of the iNOS isoform. However, increasing evidence demonstrates the contribution of

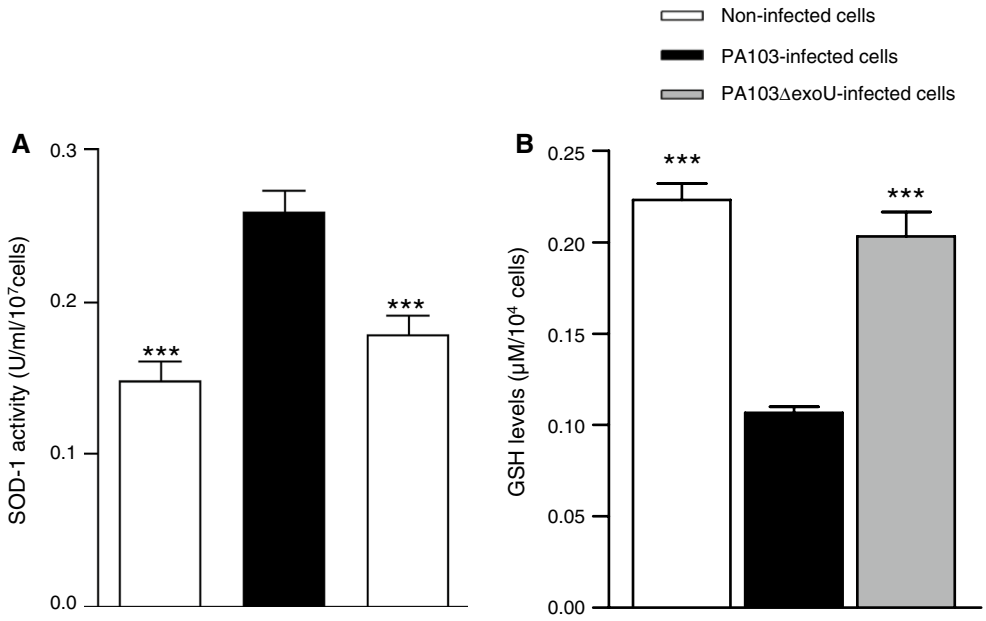

Fig. 5 ExoU impairs the antioxidant defense mechanisms of A549 cells. At 48 h post-infection, ExoU significantly increased SOD activity (**a**) and decreased GSH levels (**b**) in A549 cells. The graphs represent the mean ± SEM from values obtained in three different assays performed in triplicate. ***$p < 0.001$ when the values obtained in PA103-infected cultures were compared with those obtained in the other cultures

constitutively expressed NOS isoforms to NO overproduction [32, 33]. In our study, although airway epithelial cells infected with the ExoU-producing bacteria exhibited enhanced levels of eNOS, expression of iNOS was increased in about 1000 % at as early as 1 h after infection. A similar increase in iNOS and eNOS was reported by Lange et al. [34] in a ovine model of *P. aeruginosa* pneumosepsis. The novelty of our study is the demonstration of the precocity of iNOS expression in cells infected with the ExoU-producing bacteria, which likely stems from the ExoU-triggered NF-κB activation by the canonical pathway [13].

Besides its association with extensive injury of different targets, oxidizing agents can function as cellular signaling molecules influencing a variety of molecular and biochemical processes, including the expression of proinflammatory mediators through the activation of transcription factors [35, 36]. During *P. aeruginosa* pneumonia, *gp91*$^{phox-/-}$ mice showed significantly lower concentrations of H_2O_2, IL-6 and TNF-α in the bronchoalveolar lavage than wild-type mice [29]. Although ExoU activates NF-κB via the PAF–PAFR signaling [13], the increased generation of oxidizing agents is likely to further enhance the NF-kB transcriptional activity and upregulate a number of genes involved in inflammation [37].

Defenses against toxic oxygen metabolites in lung include antioxidant enzymes whose intrapulmonary levels can be greatly elevated when the lung faces a large burden of oxidants that compromise its redox balance. Indeed, enzymes such as SOD can be induced in lung by hyperoxia, bacterial LPS and virus, to name just a few examples [38, 39]. In the present study, infection by ExoU-producing *P. aeruginosa* resulted in significant increase in the activity of SOD, a group of enzymes that convert superoxide anion to H_2O_2. Catalase and glutathione peroxidase exert their antioxidant activity by converting H_2O_2 to water and oxygen. In our study, the increase in SOD activity together with the decreased levels of GSH suggest that infection by ExoU-producing bacteria likely resulted in enhanced intracellular H_2O_2 production that was at least partially detoxified by glutathione peroxidase.

In conclusion, our findings demonstrated the ExoU potential to induce ROS production and disrupt the pro-oxidant–antioxidant balance in favor of the former. The oxidative damage of infected lungs is certainly an important contribution of ExoU to the pathogenesis of *P. aeruginosa* pneumonia.

Acknowledgments We thank Maria Angélica Pereira da Silva for her technical assistance in cell culturing. LG Cunha-Junior and MF Ferreira were supported by CAPES and CNPq, respectively. This work was supported by grants from FAPERJ and CNPq.

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