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CELLULAR MICROBIOLOGY:
PATHOGEN-HOST CELL MOLECULAR INTERACTIONS

Pseudomonas Quinolone Signal Induces Oxidative Stress and Inhibits Heme Oxygenase-1 Expression in Lung Epithelial Cells

Maher Y. Abdalla,[a,b,d] Traci Hoke,[a] Javier Seravalli,[e] Barbara L. Switzer,[a] Melissa Bavitz,[a] Jill D. Fliege,[c] Peter J. Murphy,[c] Bradley E. Britigan[a,b,d]

Departments of Internal Medicine,[a] Pathology and Microbiology,[b] and Pulmonary, Critical Care, Sleep and Allergy Medicine,[c] University of Nebraska Medical Center, Omaha, Nebraska, USA; Research Service VA Medical Center—Nebraska Western Iowa and Department of Pathology and Microbiology, Omaha, Nebraska, USA[d]; Redox Biology Center and Department of Biochemistry, University of Nebraska—Lincoln, Lincoln, Nebraska, USA[e]

ABSTRACT *Pseudomonas aeruginosa* causes lung infections in patients with cystic fibrosis (CF). The *Pseudomonas* quinolone signal (PQS) compound is a secreted *P. aeruginosa* virulence factor that contributes to the pathogenicity of *P. aeruginosa*. We were able to detect PQS in sputum samples from CF patients infected with *P. aeruginosa* but not in samples from uninfected patients. We then tested the hypothesis that PQS induces oxidative stress in host cells by determining the ability of PQS to induce the production of reactive oxygen species (ROS) in lung epithelial cells (A549 and primary normal human bronchial epithelial [NHBE]) cells and macrophages (J774A.1 and THP-1). ROS production induced by PQS was detected with fluorescent probes (dichlorodihydrofluorescein diacetate, dihydroethidium, and MitoSOX Red) in conjunction with confocal microscopy and flow cytometry. PQS induced ROS production in lung epithelial (A549 and NHBE) cells and macrophages (J774A.1 and THP-1 cells). NHBE cells were sensitive to PQS concentrations as low as 500 ng/ml. PQS significantly induced early apoptosis ($P < 0.05$, $n = 6$) in lung epithelial cells, as measured by annexin/propidium iodide detection by flow cytometry. However, no change in apoptosis upon PQS treatment was seen in J774A.1 cells. Heme oxygenase-1 (HO-1) protein is an antioxidant enzyme usually induced by oxidative stress. Interestingly, incubation with PQS significantly reduced HO-1 and NrF2 expression in A549 and NHBE cells but increased HO-1 expression in J774A.1 cells ($P < 0.05$, $n = 3$), as determined by immunoblotting and densitometry. These PQS effects on host cells could play an important role in the pathogenicity of *P. aeruginosa* infections.

KEYWORDS heme oxygenase-1, oxidative stress, PQS, *Pseudomonas* quinolone signal, cystic fibrosis

Pseudomonas aeruginosa causes acute necrotizing pneumonia with a high mortality rate in immunosuppressed and hospitalized patients (1–3). It also causes chronic lung infections in patients with cystic fibrosis (CF) or chronic bronchiectasis (4). Chronic lung injury is the primary cause of death in CF patients and is linked to coexistent *P. aeruginosa* infection. The mechanisms involved in *P. aeruginosa*-mediated tissue damage remain uncertain and are probably multifactorial (5, 6). One important product of *P. aeruginosa* is the *Pseudomonas* quinolone signal (PQS) compound. PQS plays a role in the regulation of multiple genes involved in bacterial quorum sensing (7, 8). Quorum sensing is the regulation of gene expression in response to cell population density, which enables bacteria to coordinate their behavior and facilitate cell-to-cell commu-

Received 6 March 2017 **Returned for modification** 18 April 2017 **Accepted** 13 June 2017

Accepted manuscript posted online 19 June 2017

Citation Abdalla MY, Hoke T, Seravalli J, Switzer BL, Bavitz M, Fliege JD, Murphy PJ, Britigan BE. 2017. *Pseudomonas* quinolone signal induces oxidative stress and inhibits heme oxygenase-1 expression in lung epithelial cells. Infect Immun 85:e00176-17. https://doi.org/10.1128/IAI.00176-17.

Editor Beth McCormick, University of Massachusetts Medical School

Address correspondence to Maher Y. Abdalla, maher.abdalla@unmc.edu.

nication (9, 10). Previously, it was reported that quorum-sensing signaling molecules are detectable in biological samples obtained from CF patients and are positively correlated with pulmonary *P. aeruginosa* levels (11).

Some of the compounds regulated by PQS are virulence factors for *Pseudomonas* infection. Modulation of the production of PQS has been shown to impact *P. aeruginosa* virulence (12–15). Inhibitors of *P. aeruginosa* quorum sensing decreased virulence *in vitro* and *in vivo* (12–15). It was also shown that PQS can affect reactive oxygen species (ROS) production and resultant toxicity in *P. aeruginosa* bacteria (16). When added exogenously, PQS exhibited protective antioxidative behavior, but paradoxically, at higher concentrations, it appeared to function as a pro-oxidant, sensitizing the bacteria to other forms of oxidative stress (16).

Studies of the role of PQS in *P. aeruginosa* pathogenesis have largely focused on the role of this compound in the regulation of *P. aeruginosa* virulence factor production. A few studies have suggested that PQS may have direct effects on host cells (1, 17). With J774A.1 macrophages and human peripheral blood mononuclear cells, it was shown that PQS modulates the expression of multiple genes involved in immune responses and cytokine production (18, 19). However, the extent, magnitude, and mechanism of such changes have only been sparsely investigated. Addressing this gap in knowledge may enable us to develop novel therapeutic strategies and diagnostic tools to detect lung injury and follow up stages of lung diseases.

In this work, we show the ability of PQS to increase ROS production *in vitro* in lung epithelial cells and inhibit heme oxygenase-1 (HO-1) protein expression in lung cell lines, the latter likely via inhibition of the NrF2 pathway. These findings might contribute to the elucidation of some of the pathology associated with *P. aeruginosa* lung infections in CF and other patients.

RESULTS

Detection of PQS in clinical samples. For an *in vitro* study of the effect of PQS on airway cells to have biological relevance, there must be evidence that PQS is generated *in vivo*. Therefore, we assessed whether PQS could be detected in the airways of CF patients infected with *P. aeruginosa*. We collected 22 sputum samples from patients with CF. Half of the samples were from patients whose sputum samples were culture negative for *P. aeruginosa*, and the other half were from patients from whom whose sputum samples *P. aeruginosa* was cultured. These samples were assayed for the presence of PQS by liquid chromatography (LC)-multiple reaction monitoring (MRM)-mass spectrometry (MS) analysis. This technique is a highly sensitive and selective method for the quantitation of small molecules or proteins in biological samples.

Figure 1 shows results from MRM transitions for PQS extracted from a clinical sample. The retention time of 6.3 min agrees with the retention time of the authentic standard compound. As expected, samples obtained from patients culture negative for *P. aeruginosa* had no detectable PQS (data not shown). Table 1 shows the results obtained with sputum from each of the patients with sputum cultures positive for *P. aeruginosa*. The PQS concentrations obtained ranged between 0.09 and 9.4 ng/ml of sputum in these samples. These results show clearly that PQS is present in clinical samples obtained from patients with CF who have airway infections with *P. aeruginosa*. To understand the potential significance of these finding, we tested how PQS may impact airway cells in patients with airway infections with this organism.

Assessment of PQS toxicity. We determined the toxicity of PQS for lung (A549) and normal human bronchial epithelial (NHBE) cells and macrophages (J774A.1 murine and THP-1 human macrophages) after 4, 24, or 48 h of incubation. Cells were exposed to a range of PQS concentrations, and then cytotoxicity was determined with the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; thiazolyl blue] test (Fig. 2). MTT is a water-soluble tetrazolium salt that is converted to insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes of living cells. Dead cells do not cause this change (20).

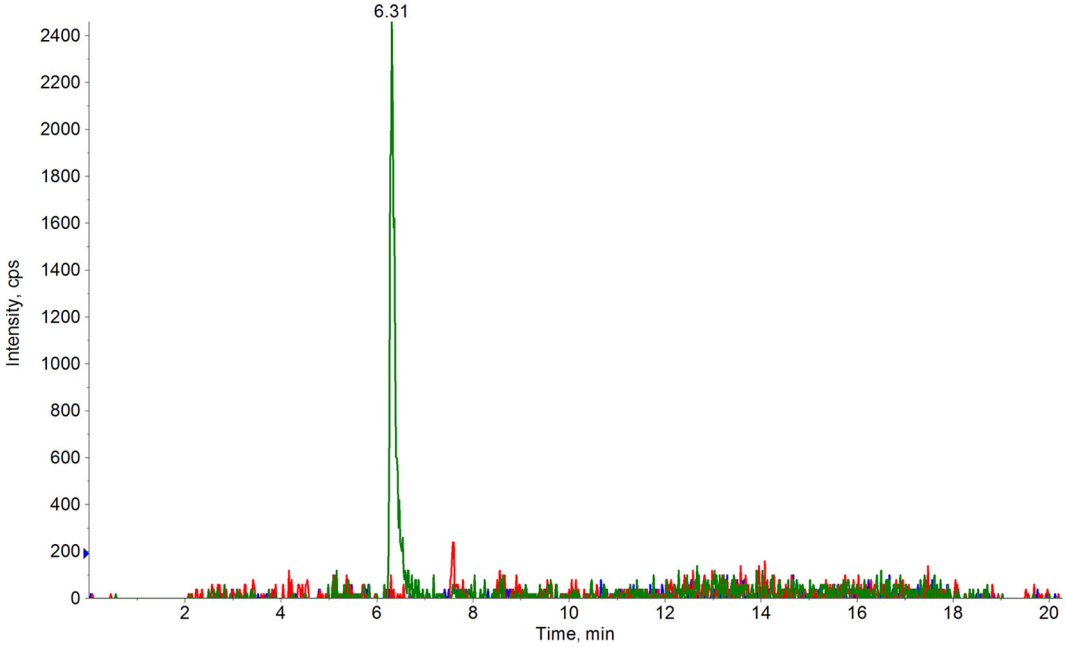

FIG 1 LC-MRM-MS analysis. Sputum samples from CF patients, of whom half had airway infections with *P. aeruginosa* and the other half did not, were prepared as described in Materials and Methods. Extracted chromatograms for PQS monitoring by LC-MRM MS are shown for the 260.4/188, 260.4/175, and 260.4/146 transitions in blue, red, and green, respectively. Peaks for PQS eluted at 6.3 min.

As shown in Fig. 2, PQS produced significant cytotoxicity in a variety of cell types in a concentration-dependent manner. In A549 cells, significant cytotoxicity was seen at concentrations of >5 μg/ml at 4 h of incubation and >2 μg/ml at 24 h of incubation (Fig. 2A and B). Primary (NHBE) cell cytotoxicity was seen beginning at 1.25 μg/ml PQS at both 24 and 48 h (Fig. 2C and D). Although it was not statistically significant, NHBE cells showed some evidence of toxicity following exposure to PQS concentrations as low as 150 ng/ml after 48 h of incubation (Fig. 2D). In J774A.1 cells, PQS produced significant concentration-dependent cytotoxicity at PQS concentrations of >7 μg/ml at 4 h and >1 μg/ml at 24 h (Fig. 2E and F). Human macrophages (THP-1) showed dose-dependent toxicity at PQS concentrations as low as 450 ng/ml when incubated for 24 and 48 h (Fig. 2G and H).

PQS-mediated ROS generation. To test the possible involvement of ROS in PQS-mediated toxicity, we evaluated ROS production in different cell lines by using three different oxidant-sensitive probes: dichlorodihydrofluorescein (DCF), dihydroethidium (DHE), and MitoSOX Red. We treated cells with various concentrations of PQS (20, 40, and 60 μg/ml) for 4 h and measured ROS levels by flow cytometry and confocal

TABLE 1 PQS concentrations in sputum samples from CF patients culture positive for *P. aeruginosa*

Compound or patient no.	PQS concn (ng/ml)
$CHCl_3$ blank	0
1	0.30
2	9.4
3	0.09
4	0.58
5	0.49
6	0.15
7	0.24
8	0.38
9	1.9
10	0.37
11	1.2

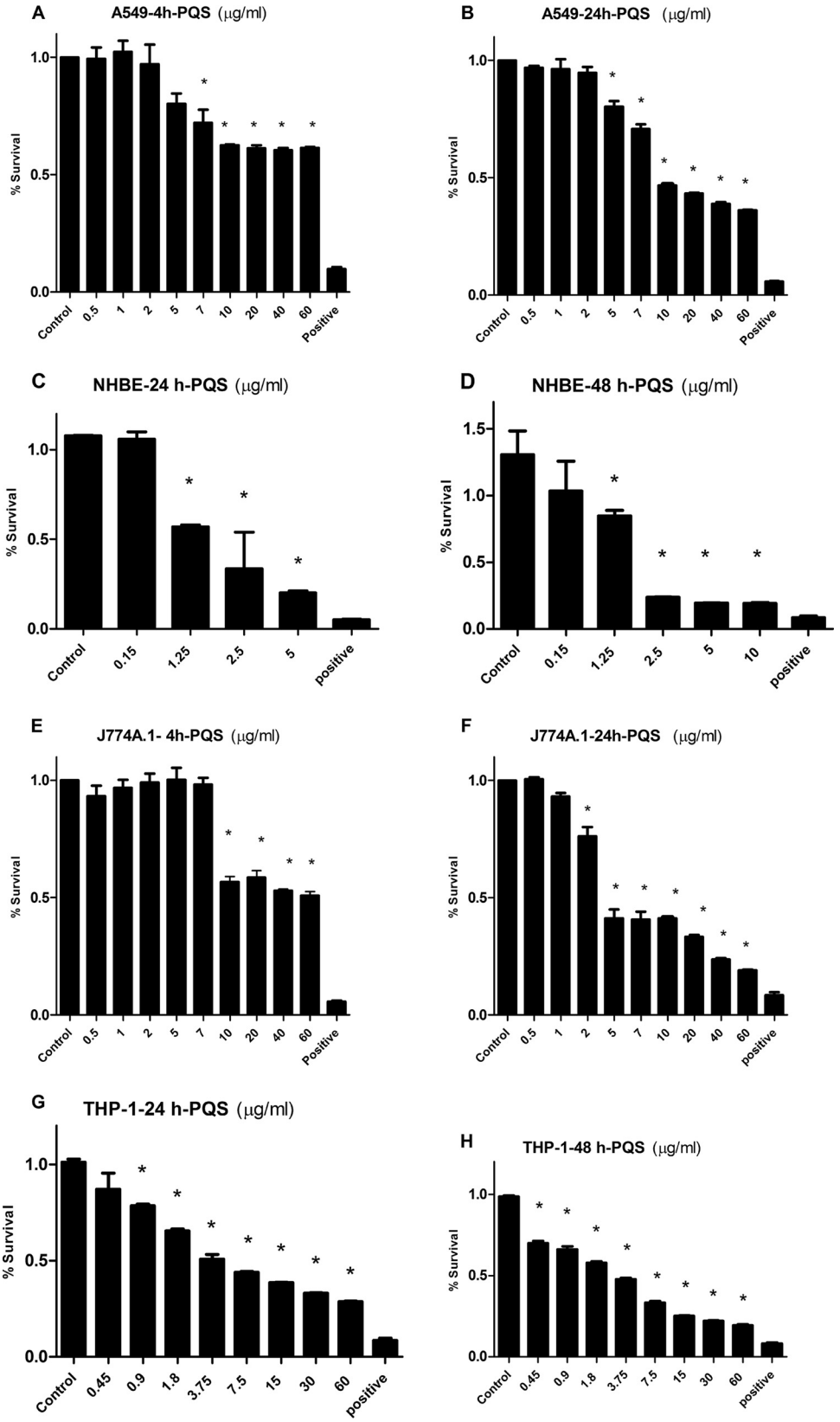

FIG 2 Effect of PQS on cell viability. Human lung epithelial (A549 and NHBE) cells and macrophages (J774A.1 and THP-1) were incubated at 37°C with different concentrations of PQS for 4, 24, and 48 h. Cell viability was assessed at each time point with MTT assays as described in Materials and Methods. Data (percent survival) are the percentages of MTT reduction relative to that of the untreated control and represent the mean ± the SEM of three separate experiments. *, $P < 0.05$ compared with the control group.

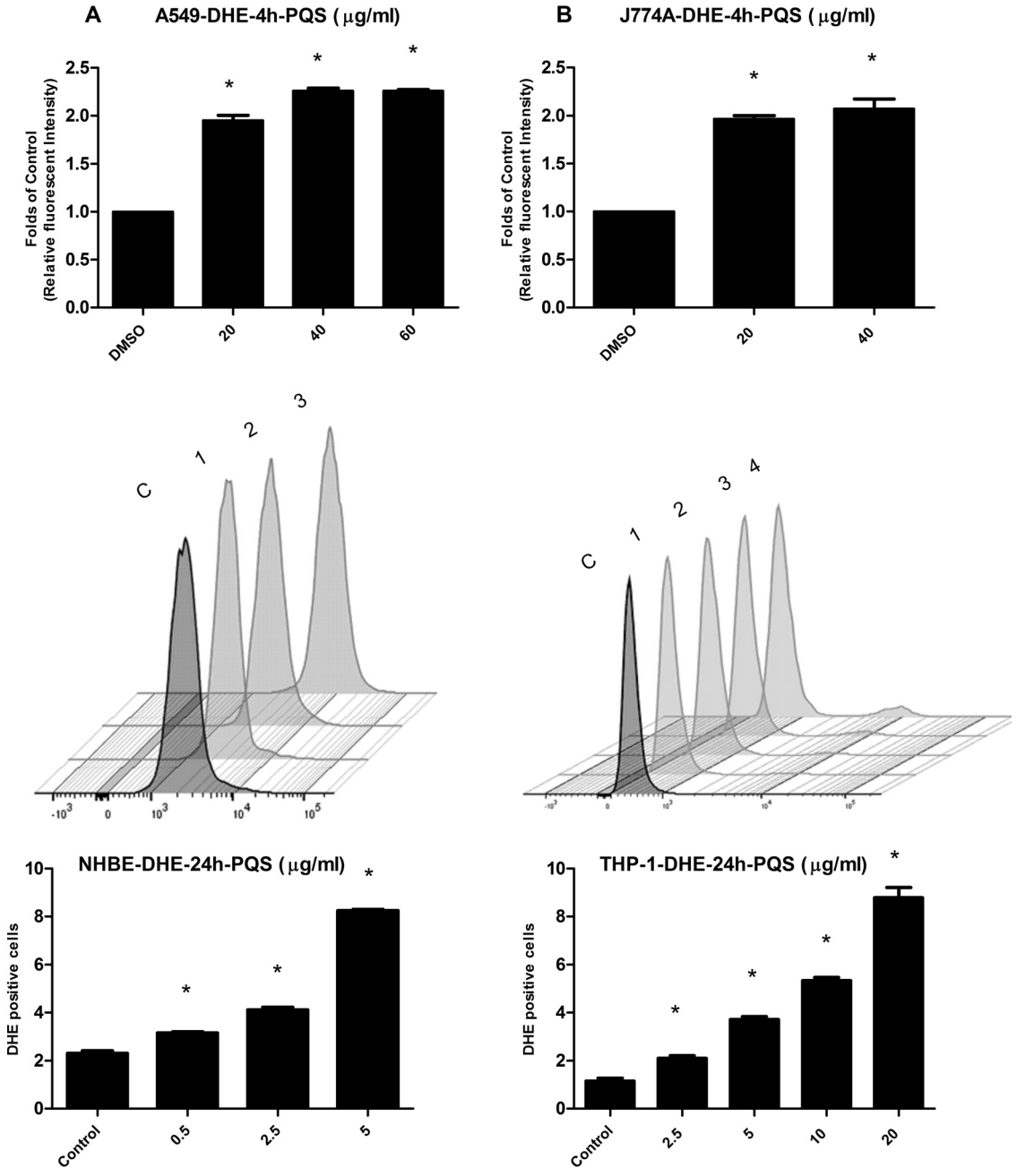

FIG 3 PQS increases ROS production. A549 (A, top), J774A.1 (B, top), NHBE (A, bottom), and THP-1 (B, bottom) cells were cultured in the presence of different concentrations of PQS for 4 or 24 h and then treated with DHE for 30 min as described in Materials and Methods. DHE fluorescence was detected by flow cytometry. Data represent the mean ± the SEM of three separate experiments. *, $P < 0.05$ compared with the control (non-PQS-treated) group. The inserts above the bar graphs at the bottom show the MFI of cells from the control (C) and columns 1 to 4. DMSO, dimethyl sulfoxide.

microscopy. DHE florescence was measured in live cells only, as dead cells were excluded by 7-aminoactinomycin D (7AAD) stain. As shown in Fig. 3A (top), A549 cell cultures treated with 20 μg/ml PQS had a ROS level 1.9-fold higher than that of control cultures, as measured by flow cytometry with DHE. The levels of ROS were further increased in cultures treated with higher concentrations of PQS; there were 2.2- and 2.3-fold increases in cultures treated with 40 and 60 μg/ml, respectively (Fig. 3A, top). Primary bronchial cells demonstrated increased ROS levels when incubated for 24 h with a PQS concentration as low as 500 ng/ml ($P < 0.05$), as shown by the increased number of DHE fluorescence-positive cells (Fig. 3A, bottom) and the increased mean

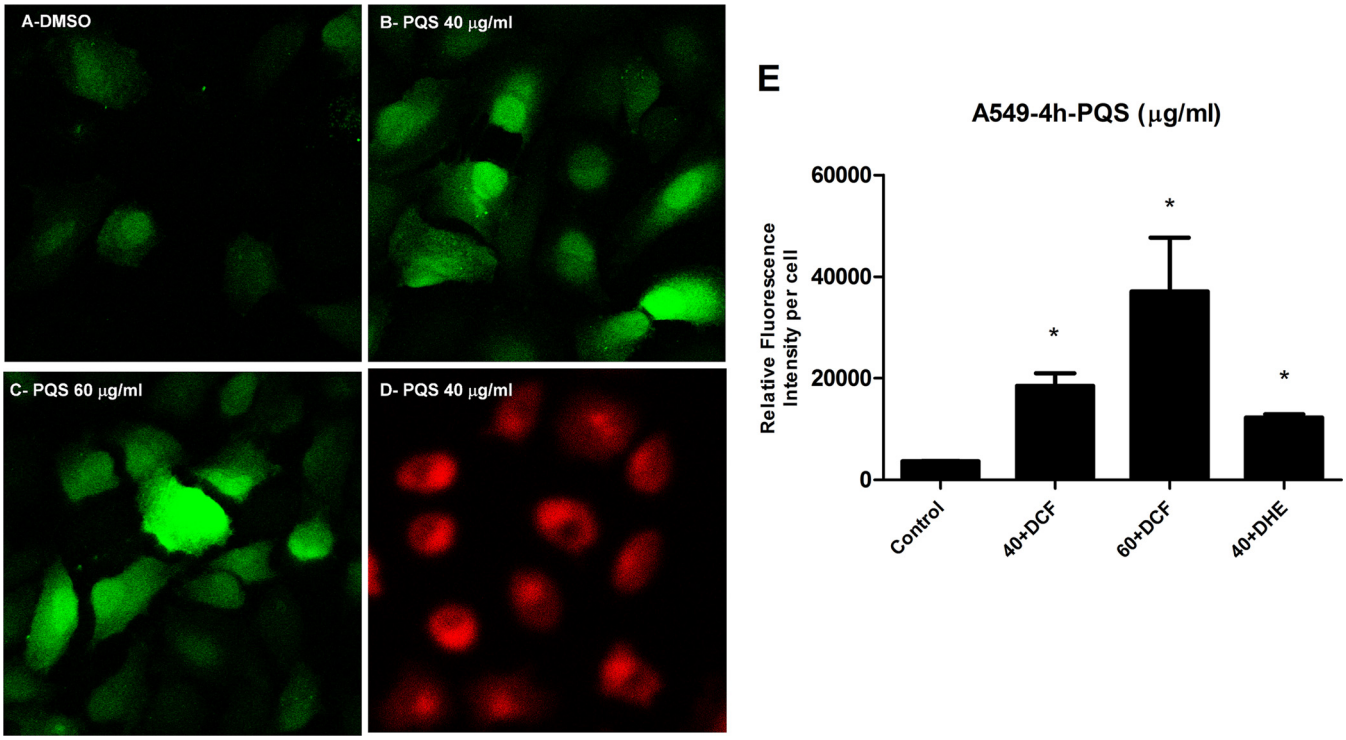

FIG 4 PQS increases the fluorescence of ROS-sensitive probes. A549 cells were cultured in the presence of different concentrations of PQS for 4 h and then treated with DCF for 30 min as described in Materials and Methods. DCF fluorescence was detected by confocal microscopy. Control cells show minimal fluorescence (A), and PQS increased DCF (B and C) and DHE (D) fluorescence, indicating increased production of H_2O_2 and $O_2^{\cdot -}$, respectively. (E) Relative fluorescence intensity per cell of DCF- and DHE-treated cells. Data are the mean ± the SEM of three separate experiments. *, $P < 0.05$ compared with the control (non-PQS-treated) group. DMSO, dimethyl sulfoxide.

fluorescence intensity (MFI) of cells (Fig. 3A, bottom, insert above the graph). Similarly, in J774A.1 cells, ROS production increased 1.9- and 2-fold over the control level when cells were exposed to 20 and 40 μg/ml PQS for 4 h, respectively (Fig. 3B, top). Human THP-1 cells showed a similar significant increase ($P < 0.05$) when exposed to PQS for 24 h (Fig. 3B, bottom, insert above the graph).

To confirm our results, ROS production was assessed with ROS-sensitive probes by confocal microscopy. When ROS was measured with DCF and DHE in A549 cells (Fig. 4), similar changes were observed. Significant 5- and 10-fold increases in DCF fluorescence were seen when these cells were treated with 40 and 60 μg/ml PQS, respectively ($P < 0.05$) (Fig. 4B, C, and E). The DHE assay showed a significant increase in $O_2^{\cdot -}$ when the cells were treated with 40 μg/ml PQS ($P < 0.05$, Fig. 4D and E). In addition, when J774A.1 macrophages were treated with PQS for 4 h and stained with the mitochondrial $O_2^{\cdot -}$ indicator MitoSOX Red, a significant increase ($P < 0.05$) in fluorescence was detected, as shown in Fig. 5. These results indicate that PQS is capable of inducing ROS production. The ROS produced might be involved in tissue damage associated with *P. aeruginosa* infection. Mitochondria could be the target organelle. Further studies are needed.

Effect of PQS on major antioxidant enzymes. Since PQS induced ROS production, we sought to define the effect of PQS treatment on major antioxidant enzymes (MnSOD and catalase) that are often upregulated under conditions of increased ROS production. Cells were cultured in the presence of different concentrations of PQS, and MnSOD and catalase protein expression was detected by immunoblotting. Figure 6 shows that PQS exposure did not result in significant changes in either MnSOD or catalase in A549 epithelial cells (Fig. 6A) or J774A.1 macrophages (Fig. 6B).

PQS reduces HO-1 levels in bronchial epithelial cells. When HO-1 expression was measured in A549 epithelial cells and J774A.1 macrophages upon PQS exposure, significant effects were seen (Fig. 7). As shown in Fig. 7A, the addition of PQS reduced

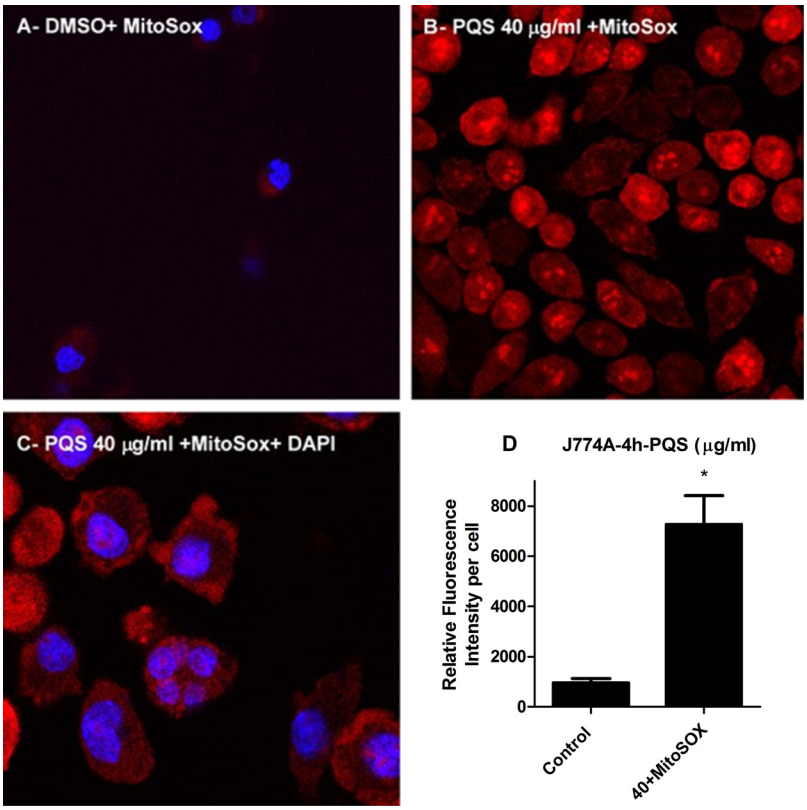

FIG 5 Increased mitochondrial ROS production caused by PQS. J774A.1 macrophages were cultured in the presence of 60 μg/ml PQS for 4 h. Control cells showed minimal fluorescence (A), and PQS increased MitoSOX fluorescence (B). DAPI (blue) stains nuclei in combination with MitoSOX, and cells show increased mitochondrial fluorescence indicating increased production of $O_2^{\cdot -}$ (C). (D) Relative fluorescence intensity per cell of MitoSOX-treated cells. Data represent the mean ± the SEM of three separate experiments. *, $P < 0.05$ compared with the control (non-PQS-treated) group. DMSO, dimethyl sulfoxide.

HO-1 protein expression in A549 airway epithelial cells in a dose-dependent manner. To confirm that the effect of PQS on HO-1 expression in airway cells was not limited to A549 cells, primary NHBE cells were exposed to different concentrations of PQS for 24 h. As shown in Fig. 6A, PQS decreased HO-1 expression in NHBE cells, as it had done in A549 cells. To understand the effect of PQS better, we assessed the effect of PQS on the

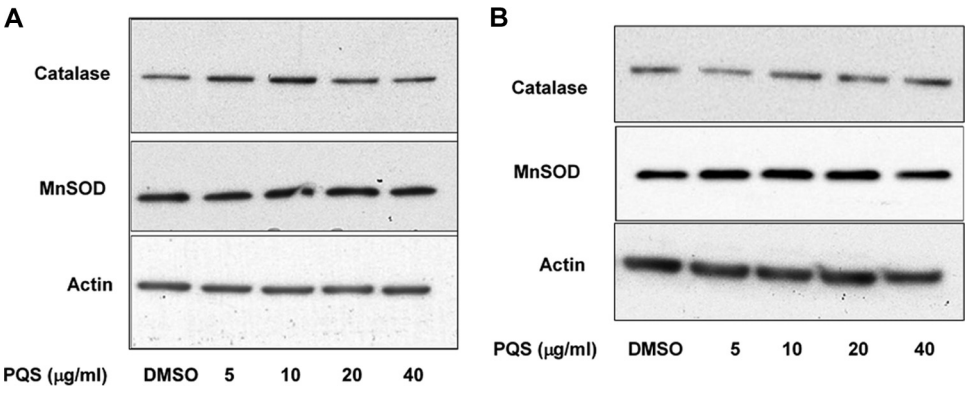

FIG 6 PQS does not change MnSOD or catalase protein expression. A549 lung epithelial cells (A) and J774A.1 macrophages (B) were cultured in the presence of different concentrations of PQS for 24 h. The cells were lysed, and MnSOD, catalase, and actin protein levels were determined by immunoblot analysis as described in Materials and Methods. No significant differences were seen in catalase or MnSOD protein levels in either A549 or J774A.1 cells as a consequence of incubation with PQS. Results are representative of three separate experiments. DMSO, dimethyl sulfoxide.

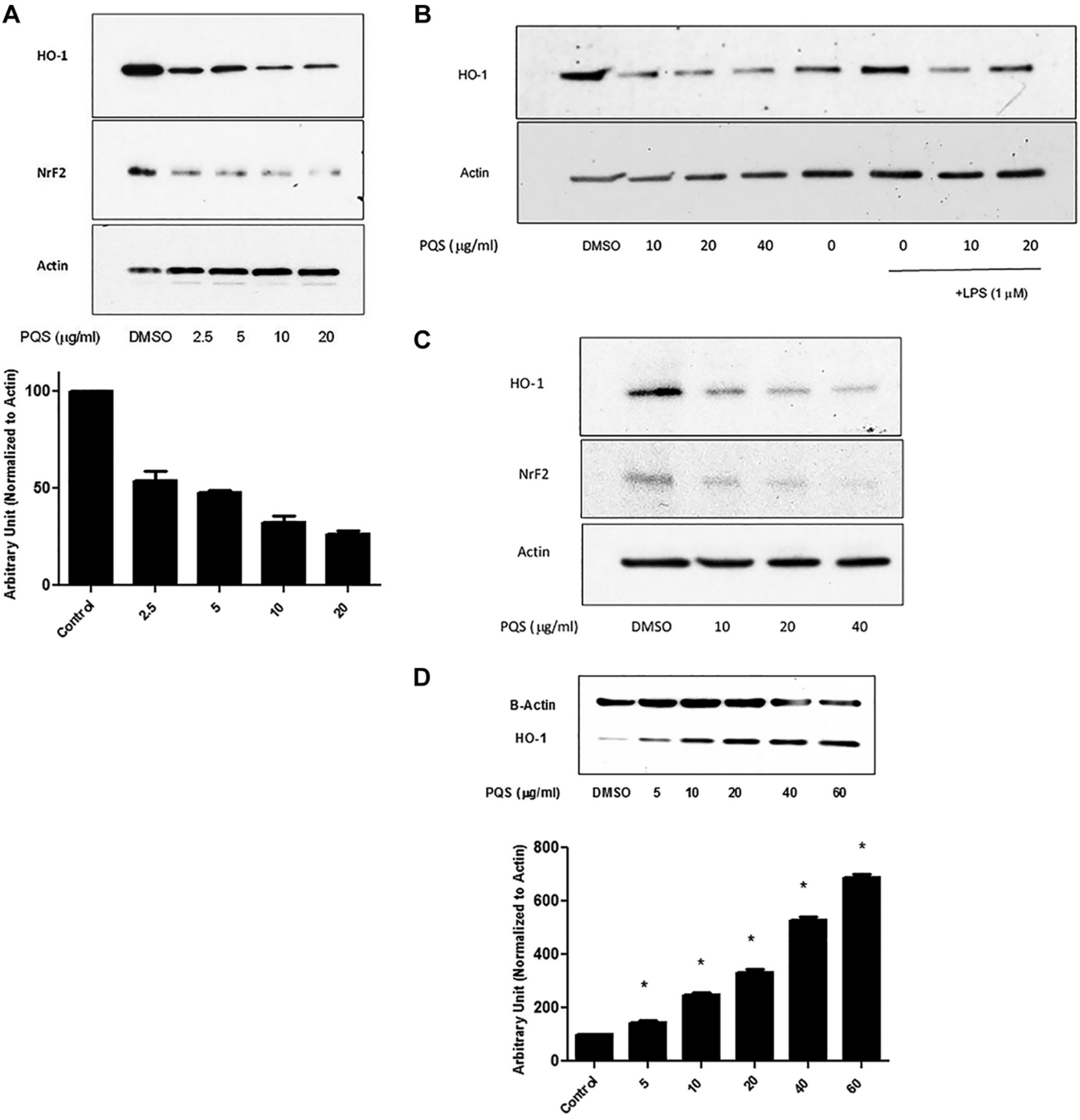

FIG 7 PQS decreases HO-1 and NrF2 protein expression in lung cells. A549 cells (A), primary NHBE cells (B, C), and macrophages (D) were cultured in the presence of different concentrations of PQS for 24 h. The cells were lysed, and the expression of HO-1 and NrF2 was quantitated by immunoblot analysis as described in Materials and Methods. Expression of HO-1 was significantly decreased in lung epithelial cells (A) and primary bronchial cells (B, C) and increased in J774A.1 macrophage cells by incubation with PQS. NrF2 protein expression was also decreased in both groups of epithelial cells (A to C). PQS exposure also decreased LPS-induced HO-1 expression. Bar graph inserts show results of densitometric analyses of HO-1 blot assays. Data represent the mean ± the SEM of three separate experiments. *, $P < 0.05$ compared with the control group. DMSO, dimethyl sulfoxide.

ability of lipopolysaccharide (LPS) to stimulate epithelial cell HO-1 expression (21–23). When primary cells were activated with LPS to induce HO-1 and treated with PQS, lower levels of HO-1 were seen in the PQS-treated cells (Fig. 7B). In marked contrast to the results obtained with epithelial cells, J774A.1 macrophages exposed to PQS showed an increase in HO-1 levels (Fig. 7D). These differential results suggest that different cell types respond differently to PQS with regard to HO-1 expression.

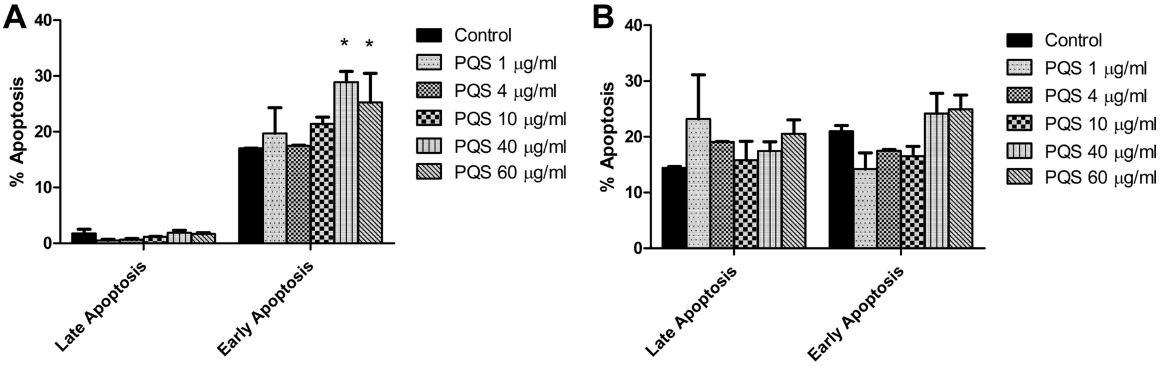

FIG 8 PQS induces apoptosis in lung epithelial cells. Shown is early and late apoptosis in A549 (A) and J774A.1 (B) cells following incubation with PQS or the vehicle for 4 h. (A) A dose-dependent increase in the percentage of early, but not late, apoptotic events was seen in PQS-treated A549 cells compared with that of the untreated group. (B) No significant change in early or late apoptosis was seen in J774A.1 cells as a result of exposure to PQS ($P > 0.05$). The data represent the mean ± the SEM of three independent experiments. *, $P < 0.05$.

The regulation of the expression of the gene for HO-1 occurs at multiple levels (24–26). At the transcriptional level, HO-1 is mediated by the transcription factor NrF2 (24, 26). To understand if HO-1 inhibition occurs at the transcriptional level, we measured NrF2 expression in cells treated with different concentrations of PQS. As shown in Fig. 7A and C, both A549 and primary lung cells showed PQS-associated downregulation of NrF2 by immunoblotting. This is consistent with the idea that suppression of HO-1 expression by PQS is mediated through NrF2 at the transcriptional level (Fig. 7).

PQS induces apoptosis in epithelial cells. Increased production of ROS may lead to apoptosis (27). To define the effect of PQS on cellular apoptosis, we added PQS to cells at different concentrations for 4 h. Apoptosis was measured by the annexin V/propidium iodide (PI) flow cytometry technique. As shown in Fig. 8, epithelial cells exhibited evidence of enhanced early apoptosis, but not late apoptosis changes, in response to PQS (Fig. 8A). In contrast, the pattern of early and late apoptosis in the macrophage cell line was not altered by the presence of PQS (Fig. 8B).

DISCUSSION

P. aeruginosa is an opportunistic pathogen that causes nosocomial pneumonia, particularly in patients with some component of immune deficiency (28). Chronic infection with *P. aeruginosa* also causes serious progressive lung injury in patients with CF (29, 30). The ability of *P. aeruginosa* to regulate gene expression in response to environmental factors is important in the pathogenesis of *P. aeruginosa* lung infection. Among these environmental factors is the density of the bacterial cell population, and its assessment is termed quorum sensing. A previous study found that the presence of quorum sensing signal molecules correlates with the clinical status of patients with CF (11). Critical to the ability of *P. aeruginosa* to quorum sense is its production of the quorum sensing molecule PQS (7, 8).

To fully understand the role of PQS *in vivo*, it is important to consider the potential impact of the molecules on the host, including the effect of PQS secreted by *P. aeruginosa* on surrounding inflammatory cells and lung epithelial cells. Previously, it was shown that PQS has an effect on the immune system by regulating genes involved in immune functions and the NF-κB pathway (18, 31). In addition, it was shown that PQS can inhibit LPS-induced nitric oxide production by J774A.1 activation without affecting apoptosis (18).

Consistent with an earlier study using a different detection technique (32), we detected PQS in sputum samples obtained from patients with CF. This supports the potential importance of this *P. aeruginosa* product in the progressive lung injury that occurs in such patients. The variability in the levels we detected could be due to a variety of factors, including different bacterial loads, *P. aeruginosa* strain differences,

and/or the effect of antibiotic treatment. Further studies are needed to assess these possibilities. One of the limitations of our study is the small number of samples collected. Future studies with more samples will allow comparisons of PQS levels and clinical status. Our work is also consistent with other studies in which PQS could be detected in serum and urine and correlated with systemic biomarkers of inflammation, including proinflammatory cytokines (11). Increasing evidence suggests that *P. aeruginosa* bacterial adherence to respiratory epithelial cells is increased in CF patients (33–37). Impaired bactericidal activity and increased adherence contribute to enhanced *P. aeruginosa* colonization of lung cells (33–37). Therefore, PQS concentrations adjacent to human airway epithelial cells are likely much higher than those detected in these human sputum samples obtained from CF patients.

The current work is the first to show that PQS induces oxidative stress in lung cells. As assessed by fluorescence of intracellular redox sensitive probes, PQS caused oxidative stress in a human lung epithelial cell line, a murine and human macrophage cell lines, and human primary bronchial epithelial cells. Additional work showed that PQS induced mitochondrial ROS production, as demonstrated by increased MitoSOX fluorescence. Further work is needed to understand the mechanism(s) responsible. For example, does PQS alter electron transport inside the mitochondria, thereby increasing local ROS production?

Oxidative damage associated with ROS production can lead to several pathological conditions, including cellular death and apoptosis (38). Consistent with these observations, we found that PQS at concentrations that resulted in enhanced ROS levels also increased early, but not late, apoptosis in A549 cells. In contrast, PQS had no effect on J774A.1 cell apoptosis, which in general had higher levels of late apoptosis than A549 cells not treated with PQS but similar levels of early apoptosis. Thus, there may be a cell type-specific difference in susceptibility to PQS enhancement of early apoptosis. This could be due to the ability of different cell lines to respond to increased ROS production and/or different HO-1 levels. In fact, ROS has been shown to regulate apoptosis under both physiologic and pathological conditions (39, 40). ROS, HO-1, and HO-1-derived products have been shown to be involved in the regulation of apoptosis in multiple cell lines (39–42). ROS also induces Fas receptor and Fas ligand genes in some cells that are linked to apoptosis (43–45).

In most cells, increased production of ROS would be expected to increase the expression of antioxidant enzymes such as MnSOD and catalase, as well as HO-1. HO-1 catalyzes the degradation of heme to produce equimolar quantities of biliverdin, CO, and free iron (46, 47). Cellular protective effects of HO-1 are conferred by its ability to inhibit inflammation and oxidative stress (48). HO-1 is induced by a variety of stimuli, such as ROS, infection, and stress, and it appears to be protective in a variety of inflammatory disease states (24, 49, 50). In addition, induction of HO-1 suppresses apoptotic cell death with the possible involvement of CO (51–54). Therefore, we studied the expression of MnSOD, catalase, and HO-1 in lung epithelial cells exposed to PQS.

No effect of PQS on MnSOD or catalase expression was observed in either the epithelial or the macrophage cell line. We found that PQS did increase HO-1 expression in the J774A.1 macrophage cell line. In contrast, it decreased HO-1 expression in both the airway epithelial cell line and primary lung epithelial cells. Thus, different cells types appear to respond differently to PQS exposure in terms of HO-1 expression. The ability of PQS to downregulate HO-1 in lung epithelial cells and NHBE cells could contribute to some of the cytotoxic effects of PQS on lung cells.

The transcriptional activation of HO-1 is mediated through NrF2, a key regulator of the cellular adaptive response to oxidative stress (24, 26). We found that exposure of A549 and primary lung epithelial cells to PQS led to a decrease in NrF2 levels that paralleled those of HO-1. These results are consistent with the possibility that suppression of HO-1 is mediated at the transcription level through NrF2.

The removal of dying and apoptotic cells and their potentially toxic contents is an important step in the resolution of acute inflammation (55). Different studies have

shown a link between inefficient clearance of apoptotic cells and persistent inflammation in CF patients (56). Among the factors known to disrupt apoptotic cell engulfment are neutrophil elastase, which cleaves the macrophage surface phosphatidylserine receptor (56), and pyocyanin, a toxic metabolite produced by *P. aeruginosa* (57). Previously, it was shown that increased HO-1 activity resulted in macrophage polarization toward an M2 phenotype, leading to an ineffective immune response (58, 59). Thus, it is possible that PQS plays a role in the inefficient clearance of apoptotic cells by manipulating HO-1 levels in epithelial and alveolar macrophages.

This is the first study to show that PQS induces oxidative stress in epithelial and macrophage cell lines and decreases HO-1 expression in lung epithelial cells. Understanding the mechanisms by which virulence factors such as PQS affect tissues may provide new insight into *P. aeruginosa* pathogenesis and enable the development of novel therapeutic strategies and diagnostic tools to detect/prevent lung injury in *P. aeruginosa*-associated lung diseases.

MATERIALS AND METHODS

Cell culture. The human alveolar type II cell line A549 (CL-185; American Type Culture Collection [ATCC], Manassas, VA) and the J774A.1 mouse monocyte/macrophage cell line (ATCC) were cultured and maintained in accordance with ATCC instructions. A549 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin-streptomycin in a 5% CO_2 incubator at 37°C. J774A.1 cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (HyClone, Atlanta, GA) and penicillin-streptomycin (Thermo Fisher, Grand Island, NY).

Primary NHBE cells were a kind gift from Todd Wyatt (University of Nebraska Medical Center [UNMC] College of Public Health Department of Occupational Health). They were cultured in bronchial epithelial cell growth medium (Lonza, Allendale, NJ) supplemented with 2 μg/ml amphotericin (MP Biomedicals LLC, Solon, OH). The cells were plated on type I collagen (Vitrogen 100; Collagen Biomaterials, Palo Alto, CA)-coated tissue culture dishes at 37°C in a humidified 5% CO_2 atmosphere. Cells were passaged once a week at 1:3. Cells between the 2nd and 5th passages were used for experiments.

***In vivo* ROS detection.** *In situ* staining for superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) was performed with three fluorescent probes, 5-(6)-chloromethyl-20, 70-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA), DHE, and MitoSOX Red (Invitrogen, Grand Island, NY). In the presence of $O_2^{\cdot -}$, DHE is oxidized to ethidium bromide (EtBr) and trapped as it intercalates with DNA. EtBr has an excitation wavelength of 488 nm and an emission wavelength of 610 nm (60). After crossing cell plasma membranes and reaching the intracellular space, CM-H_2DCF-DA is deacetylated by cellular esterases and converted to the membrane-impermeable polar derivative DCF, which is then trapped within the cell. In its reduced state, DCF is nonfluorescent, but upon oxidation by intracellular H_2O_2 and other peroxides, it becomes highly fluorescent. The excitation and emission wavelengths of DCF are 495 and 529 nm, respectively (61). MitoSOX Red permeates live cells and selectively targets mitochondria. It is rapidly oxidized by $O_2^{\cdot -}$ but not by other ROS. The oxidized product is highly fluorescent (62). Cells were treated with the compounds of interest for 24 h and washed, and DHE, MitoSOX, and/or DCF were then added to the assigned chambers, and then the cells were incubated for 30 min at 37°C. After this incubation, the medium was removed and the chambers were washed and mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Images were viewed with a Zeiss 510 Meta confocal laser scanning microscope.

Western immunoblotting. To determine the expression of HO-1 and other antioxidant proteins, protein lysates were prepared in radioimmunoprecipitation assay buffer containing protease inhibitors (Thermo Scientific, Rockford, IL) as described previously (63). Lysates were mixed with 2× Laemmli loading dye (Bio-Rad, Hercules, CA) and subjected to SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes, blocked with 5% milk, and probed with primary antibodies to HO-1 (Enzo, Farmingdale, NY), MnSOD (Millipore, Billerica, MA), catalase (Millipore, Billerica, MA), and NrF2 (Cell Signaling, Beverly, MA) overnight at 4°C with constant rocking. Membranes were washed with Tris-buffered saline with Tween 20 (TBST), incubated with horseradish peroxidase-tagged secondary antibodies for 1 h at room temperature, and washed three times with TBST, and then the proteins were visualized with Pierce chemiluminescence reagents (Pierce, Rockford, IL). Densitometry analyses were performed with NIH ImageJ.

Apoptosis assay. Apoptosis was determined with an annexin V-FITC apoptosis detection kit (eBioscience, San Diego, CA) in accordance with the manufacturer's protocol. In brief, the cells were harvested, washed three times with phosphate-buffered saline (PBS), centrifuged at 400 × *g* for 5 min, and resuspended in 200 μl of binding buffer to a final concentration of 1×10^6/ml. After incubation with 5 μl of annexin V-FITC for 10 min and 5 μl of PI for 5 min at room temperature, the cell suspension was subjected to flow cytometry on a FACSCalibur (BD, Franklin Lakes, NJ) and detected at an excitation wavelength of 488 nm. The apoptotic cells were identified as early and late apoptotic cells. In early-stage apoptosis, the plasma membrane excludes viability dyes (PI) and the cells stain positive for only annexin V. However, in late apoptosis, the cell membrane loses integrity and cells stain positive for both annexin V and PI. Thereby, late-stage apoptotic and necrotic cells (annexin V and PI positive) can be differentiated

from early-stage apoptotic cells (annexin V positive, PI negative) (64). The resulting data were analyzed with FlowJo version 10.0 software (TreeStar Inc., Ashland, OR).

Flow cytometric quantification of $O_2^{\cdot-}$. Intracellular production of $O_2^{\cdot-}$ was measured with a DHE probe (Invitrogen/Molecular Probes). The cells were treated with PQS dissolved in dimethyl sulfoxide (obtained from Sigma, St. Louis, MO) for 3 and 24 h. They were then washed, resuspended in PBS, and then incubated with DHE (5 μM). DHE oxidation was monitored by flow cytometry at an excitation wavelength of 485 nm and an emission wavelength of 610 nm. 7AAD was used to exclude dead cells.

Cell toxicity assays. The toxic effects of PQS were evaluated with the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT; Promega, Madison, WI). Cells were seeded into the wells of a 96-well plate at 5×10^3/well. After 24 h of incubation, cells were treated with PQS for 4, 24, or 48 h. MTT (15 μl) was added to each well, and the plate was kept at 37°C for 3 h. The purple formazan so formed in the well was dissolved with 100 μl of solubilizing solution. The absorbance of the product was measured at 540 nm. Cells without PQS treatment served as a vehicle control. Positive controls were generated by adding 1% Triton X-100 or 3% SDS about 45 min before the addition of the MTT solution.

Patient sputum samples. Expectorated sputum samples were obtained from 22 patients with CF in collaboration with the adult CF program at the UNMC. Samples were provided in a deidentified manner to the study team. Eleven of the sputum samples were from patients whose sputum tested negative for *P. aeruginosa* by microbiology culture, and 11 were from patients whose sputum tested positive. All of the patients included were 19 to 45 years old. Pregnant women, prisoners, decisionally impaired persons, and other vulnerable persons were not included. This study was approved by the Institutional Review Board (IRB) of the UNMC, Omaha, NE (IRB number 282-14-EP).

LC-MRM-MS analysis. Human sputum samples were transferred into 2-ml Eppendorf vials and extracted with 1.0 ml of undiluted chloroform by vortexing each mixture for 1 min at room temperature. The samples were then centrifuged at 4°C for 4 min at 15,000 $\times$ *g*. The extracts were removed, evaporated to dryness with a SpeedVac, resuspended with 0.1 ml of chloroform, and then transferred into V vials. The sputum pellets were disrupted with a Bullet Blender cell and tissue disrupter after the addition of 1.0 ml of methanol and ~300 mg of ZrO (0.5-mm) beads to each sample. The samples were disrupted by three 5-min cycles at a power setting of 7 at 4°C. The samples were then centrifuged for 15 min at 17,000 $\times$ *g* and 4°C, and the supernatants were removed for LC-MRM-MS analysis after transfer to vials. The analysis used an LC-MS system that consisted of an Agilent (Santa Clara, CA) LC-1200 high-performance liquid chromatograph coupled to a Sciex 4000QTrap (Sciex, Framingham, MA) mass spectrometer operating in MRM. A Keystone C_{18} BioBasic reversed-phase LC column (2 by 50 mm; Thermo Scientific, Rockford, IL) was run at a flow rate of 0.3 ml/min. The starting mobile phase (A) was 0.1% formic acid in water, and the ending mobile phase (B) was 0.1% formic acid in acetonitrile. The gradient for elution started at 2% B for 1 min increasing to 98% B for 10 min, holding at 98% B for 5 min, decreasing to 2% B at 15.2 min, and then holding at 2% B for 5 min for re-equilibration. The ion source used for electrospray ionization operated at a temperature of 600°C, an ion source voltage of 5,000 V, a GS1 rate of 50 liters/min, a GS2 rate of 30 liters/min, and a curtain gas flow rate of 30 liters/min. For PQS, the transitions monitored were 260.4/188, 260.4/175, and 260.4/14. The PQS standard was obtained from Sigma (St. Louis, MO).

Statistical analysis. All data are presented as the mean $\pm$ the standard error of the mean (SEM). Statistical significance was determined by two-tailed Student *t* test and/or one-way analysis of variance. *P* values of <0.05 were considered statistically significant. Analyses were done with GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

ACKNOWLEDGMENTS

The research reported here was partially supported by the National Institute of General Medical Sciences of the National Institutes of Health under award P30GM103335 and a VA merit review award from the Department of Veterans Affairs. The content of this report is solely the responsibility of its authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Veterans Affairs.

We thank Todd Wyatt and Dawn Katafiasz of the UNMC College of Public Health Department of Environmental, Agricultural and Occupational Health and the UNMC College of Medicine Department of Internal Medicine for providing the primary pulmonary epithelial cell line.

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