Activation of a bacterial flow sensor by hypochlorous acid from stimulated neutrophils

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ABSTRACT

Bacteria sense environmental stressors and activate responses to improve their survival in harsh growth conditions. Neutrophils respond to the presence of bacteria by producing oxidative antibacterial species including hypochlorous acid (HOCl). However, the extent that bacteria detect activated neutrophils or HOCl has not been known. Here, we report that the opportunistic bacterial pathogen *Pseudomonas aeruginosa* responds to activated neutrophils by activating the *fro* system, which regulates the expression of antioxidative factors. We show that this response is specific to HOCl and that other oxidative factors including H₂O₂, do not trigger a *fro* response. The *fro* system has been previously shown to detect flow that is present in host vasculature, such as in animal circulatory systems. Our data thus suggest a model in which *fro* serves as an early host detection system in *P. aeruginosa* that improves its survival against neutrophil-mediated defenses, which could promote colonization in human tissue and increase pathogenicity.

One Sentence Summary: *P. aeruginosa* primes its defenses against neutrophils by sensing flow and hypochlorous acid via the *fro* system.

INTRODUCTION

P. aeruginosa is a Gram-negative bacterium that is a major cause of acute hospital-acquired infections and a significant threat for antibiotic-resistant infections (1). To survive in a broad range of conditions, this bacterium has evolved signaling pathways that relay information about changes in nutrients, chemical gradients, and mechanical forces such as those generated by flow, and stimulates responses that promote survival (2, 3). Many of these signaling pathways improve the bacterium's survival in hosts in particular. Understanding how such environmental-sensing pathways detect and respond to host cues is critical for improving treatments of bacterial infections.

Mammalian host immune systems respond to the presence of bacterial pathogens by recruiting immune cells to sites of infection. Neutrophils are among the first and most abundant immune cells recruited to inflamed or damaged tissues (4). Neutrophils are stimulated into an activated state by the presence of the bacterial components, including the membrane constituent lipopolysaccharide. Activated neutrophils kill bacteria through a number of mechanisms, including producing respiratory burst products such as reactive oxygen species (ROS) and reactive chlorine species (RCS) (5, 6). While the toxic effects of ROS on bacteria are understood, far less is known about how bacteria are affected by RCS (7, 8). Hypochlorous acid (HOCl) is a major neutrophil RCS that has strong bactericidal activity towards bacteria (9, 10). HOCl is formed through the activity of myeloperoxidase with H₂O₂ and chlorine ions (5, 6, 11). *P. aeruginosa* is a potent activator of neutrophil respiratory bursts (12). However, whether and how *P. aeruginosa* detects and responds to neutrophil respiratory bursts is not understood.

Recently, it was reported that *P. aeruginosa* detects the flow of fluids via the flow-regulated operon *fro* (3). Flow is ubiquitous in the lung and in the circulatory system, which

neutrophils and other immune cells must travel through to reach infected sites. The *fro* system could thus enable *P. aeruginosa* to detect the presence of flow-associated host environments and immune system components. *P. aeruginosa* induces *fro* operon expression during infections in humans (3, 14) (Fig. 1A). The operon consists of the genes *froABCD* (*PA14_21570*, *PA14_21580*, *PA14_21590*, *PA14_21600*). FroB is predicted to localize in cytoplasm while FroC and FroD are predicted to localize to the inner membrane (15). The *fro* operon is regulated by two upstream genes, *froR* (*PA14_21550*) and *froI* (*PA14_21560*), which are sigma and anti-sigma factors, respectively (16). Overexpression of *froR* increases *fro* expression whereas overexpression of *froI* eliminates *fro* induction (3). Flow activates FroR-dependent transcription of *froABCD* through an unknown mechanism.

We investigated whether the *fro* system directly responds to components of the circulatory system. Here, we show that the expression of the *fro* system is upregulated by activated neutrophils. We determine that HOCl is a critical component of the neutrophil activation that is responsible for the *fro* upregulation and that its upregulation reduces the bactericidal effects of HOCl. These data suggest a novel mechanism by which a bacterial defense system is primed to detect host immune system activity through both flow and the presence of the immune cell effector HOCl, and responds by producing protective antioxidant proteins.

Figure 1

Fig. 1 Activation of *fro* transcription by flow and during infection of human tissue. (A) RNA-seq heatmap of *P. aeruginosa* genes that are significantly upregulated at least 3-fold both by flow and during infections in human wounds. RNA-seq datasets are from (3, 14). **(B)** Top: schematic of a *P. aeruginosa* fluorescent reporter strain that contains a transcriptional fusion of *yfp* to *fro* and constitutively expresses *mCherry*. Representative phase contrast, YFP, and mCherry images the reporter under flow (right) or non-flow (left) conditions for 30 minutes. **(C)** Representative phase contrast, YFP, mCherry, and merged images of the *P. aeruginosa* reporter strain co-incubated with neutrophils, macrophages, fibroblasts, or host cell-free media for 30 minutes. Dotted lines outline the boundaries of the host cells. White arrows in merged images indicate *P. aeruginosa* with the high ratios of YFP/mCherry intensities. Images are representative of three independent repetitions. Scale bars represent 5 μ m.

RESULTS

Host cells stimulate *fro* expression in *P. aeruginosa* under non-flow conditions

We investigated if the *fro* operon is activated directly by immune cells. To quantify *fro* expression, we used a *P. aeruginosa* strain that co-expresses the yellow fluorescent protein (YFP) under the transcriptional control of the *fro* promoter and mCherry under the control of a constitutive promoter (Fig. 1B). Neutrophils and macrophages are among the first immune cells that are recruited to sites of infection. We co-incubated *P. aeruginosa* with these cells for 30 minutes to determine whether they would elicit a response in the *fro* operon. Experiments were performed in the absence of flow in order to deconvolve the effects of flow from potential activation of *fro* by host cells. Relative to *P. aeruginosa* that were incubated in host cell-free media, *P. aeruginosa* in close proximity to neutrophils or that were engulfed in macrophages had higher levels of *fro* expression (Fig. 1C). Furthermore, increases in *fro* expression were observed in some *P. aeruginosa* that were internalized in fibroblasts (Fig. 1C). These data suggest direct interaction or close proximity with host cells stimulates *fro* expression in *P. aeruginosa*.

fro expression is induced by activated neutrophils

We focused our efforts on understanding how neutrophils activate *fro* expression, as neutrophils are one of the first immune cells that are recruited to bacterial infection sites. *P. aeruginosa* triggers respiratory bursts in neutrophils (12), which generates ROS and RCS. We hypothesized that the observed *fro* activation could be a response to respiratory burst products. To test this, we determined whether molecules released by activated neutrophils could induce *fro* expression. We isolated cell-free conditioned medium from neutrophils that were stimulated to induce respiratory bursts using 100 ng/mL phorbol myristate acetate (PMA) in neutrophil

isolation medium (PGBT) (17). *P. aeruginosa* were cultured for 3 hours in the conditioned medium (Fig. 2A). The expression of *fro* was quantified by computing the ratio of YFP to mCherry fluorescence in individual *P. aeruginosa* and averaging over at least one hundred *P. aeruginosa*. Conditioned medium from PMA-stimulated neutrophils increased the expression of *fro* by 30-fold compared to medium from unstimulated neutrophils (Fig. 2B). The upregulation in *fro* was not due to PMA, as the addition of PMA without neutrophils had no effect on *fro* expression (Fig. 2B). The conditioned media from unstimulated neutrophils had no significant effect on *fro* expression compared to PGBT medium alone (Fig. 2B).

Figure 2

Fig. 2 Conditioned medium from stimulated neutrophils induces *fro* expression. (A) Representative phase-contrast and fluorescence images and (B) quantification of *fro* expression levels of *P. aeruginosa* that were cultured for 3 hours in PGBT medium with or without 100 ng/mL of PMA, conditioned medium from unstimulated neutrophils, or conditioned medium from neutrophils that were stimulated with 100 ng/mL of PMA. Bars indicate the mean and error bars represent the standard error of the mean (SEM) of three independent experiments. P-values were calculated using a two-tailed, unequal variance t-test. ns indicates $p > 0.05$. Scale bars represent 5 μ m.

***fro* expression is induced by HOCl but not by H₂O₂ or HNO₃**

We assessed the ability of specific respiratory burst products to drive *fro* activation. Activated neutrophils generate multiple ROS and RCS oxidants, which have an inhibitory effect on bacterial growth (5–7, 9, 10). We hypothesized that these oxidants induce *fro* expression and tested the impact of these species on *fro* expression at near-inhibitory concentrations (Fig. 3A). We established that 1 μM of NaOCl or 8 μM of H₂O₂ were at the threshold of growth inhibition for *P. aeruginosa* (Fig. 3B), and subsequently measured *fro* expression in cultures up to these concentration thresholds. H₂O₂ in a range of concentrations below, at, or above 8 μM had no significant impact on *fro* expression (Fig. 3C-D). In contrast, 1 μM NaOCl increased *fro* expression by up 78-fold compared to untreated cultures. This activation is not due to sodium or chloride ions alone, as neither NaCl at the same concentration or HCl up to the highest concentration without changing pH of the minimal medium did not induce *fro* expression (Fig. 3D). In addition, the *fro* response to NaOCl is not due to basic pH alone, as treatment with 6 mM NaOH, which adjusted the medium to the same pH as 1 μM NaOCl (pH 7.1), had no significant impact on *fro* activation (Fig. 3D). NaOCl-induced *fro* expression occurred at concentrations below those associated with growth inhibition (Fig. 3B-D). The activation of *fro* by NaOCl is consistent with a recent transcriptional study that identified *fro* as a gene that is upregulated by HOCl (18). We note that a higher concentration of 2 μM NaOCl did not increase *fro* expression. However, this concentration caused a significant decrease in cell size (Fig. S1 in Supplementary Materials), indicative of cell stress that may have interfered with transcription.

Figure 3

Fig. 3 The expression of *fro* is induced by HOCl but not by H₂O₂ or HNO₃. (A) Schematic of model indicating that PMA-stimulated neutrophils produce respiratory burst products that could induce *fro* expression. (B) Growth profiles, measured by optical density (OD₆₀₀), of *P. aeruginosa* that were treated with sublethal and lethal concentrations of H₂O₂, NaOCl, HNO₃, or untreated. Data points represent the mean and error bars indicate the SEM of at least two independent experiments. (C) The expression of *fro* in *P. aeruginosa* that were treated for 3 hours with sublethal concentrations of H₂O₂, NaOCl, HNO₃, or were untreated. (D) The expression of *fro* in *P. aeruginosa* that were treated for 3 hours with 8 μM H₂O₂, 1 μM NaOCl, 25 mM HNO₃, 6 mM NaOH, 1 μM NaCl, 200 μM HCl, or untreated. Bars represent the mean and error bars indicate the SEM of at least two independent experiments. Boxplots indicate the mean (black line) and interquartile range (yellow) of three independent experiments. P-values were calculated using a two-tailed, unequal variance t-test. ns indicates $p > 0.05$.

We further assessed whether *fro* expression could be activated by the strong oxidant HNO₃. The effects of this oxidizer were measured up to inhibitory concentrations (Fig. 3B-D). However, no impact on *fro* expression was detected. These results suggest that *fro* responds specifically to RCS stress from HOCl and supports the hypothesis that HOCl produced by stimulated neutrophils activates *fro* expression.

Antioxidant supplements inhibit *fro* activation

HOCl oxidizes amine and sulfur groups within amino acids (19, 22). We posited that blocking the oxidative activity of HOCl could inhibit *fro* activation. Sulfur-containing amino acids (e.g., methionine and cysteine) and beta-mercaptoethanol (βME) are antioxidants and major targets for HOCl-mediated oxidation (21, 23). We hypothesized that supplementing 1 μM NaOCl treatments with methionine, cysteine, or βME reduces *fro* expression. Indeed, we observed that supplementing these antioxidants at equal or lower concentrations as NaOCl (1 μM methionine, 0.1 μM cysteine, or 1 μM βME) significantly reduced the NaOCl-induced *fro* expression (Fig. 4A, Fig. S2 in Supplementary Materials) indicating that the oxidative activity of NaOCl activates *fro*. Concentrations of antioxidants that were higher than 1 μM also inhibited

fro expression (Fig. S2 in Supplementary Materials). Treatments of cysteine, methionine, and β ME alone had no significant impact on *fro* expression (Fig. S3 in Supplementary Materials). Based on these data, we hypothesized that the activation of *fro* expression by PMA-stimulated neutrophils is suppressed by antioxidants. Indeed, we found that supplementing conditioned media from PMA-stimulated neutrophils with 100 μ M of methionine inhibited *fro* activation (Fig. 4B) comparable to that observed in PBGT media alone and in unstimulated neutrophils (Figs. 2B and 4B). Together, these results suggest that stimulated neutrophils activate *fro* expression through the oxidative activity of HOCl.

Figure 4

Fig. 4 Antioxidants inhibit *fro* activation that is induced by HOCl and conditioned media from PMA-stimulated neutrophils. (A) The expression of *fro* in *P. aeruginosa* that are untreated, treated with 1 μ M NaOCl, or treated with 1 μ M NaOCl and supplemented with 1 μ M methionine, 0.1 μ M cysteine, or 1 μ M beta-mercaptoethanol (BME), for 3 hours. (B) Quantification of *fro* expression levels in *P. aeruginosa* that were cultured in PBGT medium or in PBGT medium from PMA-stimulated neutrophils (replotted from Fig. 2B) that was untreated or supplemented with

100 μ M methionine. Bars represent the mean and error bars indicate the SEM of three independent experiments. P-values were calculated using a two-tailed, unequal variance t-test. ns indicates $p > 0.05$.

***fro* enhances *P. aeruginosa* survival against HOCl**

We investigated whether *fro* activation has a beneficial impact on *P. aeruginosa* survival in response to HOCl-induced stress. We measured the growth of wild-type *P. aeruginosa* and the mutant strain Δ *froR*, which lacks the sigma factor that is required for *fro* activation. In the absence of NaOCl, both strains grew comparably. Treatment with 16 μ M NaOCl significantly inhibited the growth of the wild-type strain (Fig. 5A). This treatment resulted in even greater growth inhibition in the Δ *froR* strain. This result suggests that activation of the *fro* operon increases *P. aeruginosa* survival against oxidative stress induced by HOCl.

We assessed the transcriptional pathways induced by *fro* activation to understand how *fro* activation promotes the survival of *P. aeruginosa* during oxidative stress. We performed a Gene Ontology (GO) analysis of RNA-seq data from the wild-type strain and Δ *froR* mutant (3). This analysis revealed that 128 genes with oxidoreductase activity were upregulated by at least 2-fold in the wild-type strain compared to the Δ *froR* mutant (Supplemental Spreadsheet 1 in Supplementary Materials). The most upregulated genes included *ahpC*, *ahpF*, and *katB*, which encode proteins with oxidoreductase or antioxidant activity (Fig. 5B and Supplemental Spreadsheet 2 in Supplementary Materials). These results suggest that *fro* activation promotes *P. aeruginosa* survival against HOCl stress by activating transcriptional responses that mitigate oxidative damage.

Fig. 5 FroR improves *P. aeruginosa* survival against HOCl stress and regulates the transcription of genes involved in antioxidant defense. (A) Growth profiles of wild-type (WT) *P. aeruginosa* and Δ *froR* mutants that were treated with 16 μ M NaOCl or untreated. Data points represent the mean and the error bars indicate the SEM of three independent experiments. (B) Volcano plot indicating the fold-changes in transcripts and p-values of the wild-type (WT) strain relative to the Δ *froR* mutant using RNA-seq dataset from (3). The colors reflect genes that are downregulated (blue), upregulated (red), or unchanged (grey) in WT strains compared to the Δ *froR* mutant. False discovery rate < 0.05.

Discussion

Neutrophils are activated by the presence of bacteria during the infection of host tissues. In this activated state, neutrophils produce respiratory bursts that contain high concentrations of HOCl. Whether and how bacteria respond to activated neutrophils and the RCS that the neutrophils produce has not been clear. In this study, we have demonstrated that *P. aeruginosa* responds to activated neutrophils by upregulating the transcriptional expression of the *fro* operon, which relieves HOCl-induced stress. Our data suggest that *fro* activation could function as a bacterial defense mechanism against a neutrophil attack mechanism. The *fro* response may

thus improve *P. aeruginosa* survival against neutrophils and enhance the colonization of human tissues during infection.

One of the striking features of the *fro* system is that it is finely tuned to activate at sub-lethal concentrations (1-2 μ M) of NaOCl. Activated neutrophils produce HOCl as high as 50 μ M HOCl (24). The activation of *fro* at relatively low concentrations suggests that the system functions as a sensitive early detector of HOCl. In host environments, *fro* may activate in environments where HOCl is present at low concentrations, such as during early stages of neutrophil stimulation or when *P. aeruginosa* is spatially distant from activated neutrophils. The early activation of *fro* could benefit *P. aeruginosa* by triggering a protective mechanism that relieves the impact of HOCl stress before HOCl reaches lethal concentrations. This mechanism would ensure that the system is already activated when *P. aeruginosa* encounters neutrophils. Indeed, we observed that *fro* expression is strongly upregulated in *P. aeruginosa* that are in close proximity to activated neutrophils (Fig. 1C). Additional evidence supports the function of *fro* as a defensive early detection mechanism. The transcription of *fro* is partially activated by moving fluids (3) that generate wall shear stresses in animal circulatory systems. Neutrophils move in the fluidic networks of the circulatory system in response to tissue injury. *P. aeruginosa* that are present in the circulatory system could prime its defenses via flowing sensing by *fro* against potential HOCl produced by recruited neutrophils.

How does *fro* relieve HOCl stress in bacteria? The *fro* operon activates over 100 genes, including *ahpC*, *ahpF*, and *katB*, that encode proteins that reduce the activity of ROS and are critical for bacterial survival against oxidative stress (Fig. 5B) (25, 26). While the effect of these antioxidant proteins on RCS activity is unclear, our data suggest that *fro* activation mitigates the toxic effects of HOCl (Fig. 5A). In addition, our results indicate that the presence of antioxidant

agents, including methionine, cysteine, or BME, reduces the activity of HOCl (Figs. 3-4). Together, our data suggest a model in which *P. aeruginosa* senses HOCl via the *fro* system, which upregulates the production of antioxidant proteins that suppress the toxic effects of HOCl. This would constitute an early detection mechanism that defends against neutrophils. A recent finding identified the RclR transcriptional regulator as a sensor of HOCl (27). Further work is necessary to determine how antioxidative proteins and antioxidative agents reduce the toxic activity of HOCl and the extent that RclR and *fro* are co-regulated by HOCl.

In addition to promoting colonization of the lung and gastrointestinal tract (13, 28), the *fro* system may have additional impacts on pathogenicity, such as increasing antibiotic resistance. Bacteria that are exposed to low doses of HOCl have higher rates of horizontal gene transfer and can acquire antibiotic resistance genes more rapidly (29, 30). The tolerance of *P. aeruginosa* to HOCl via *fro* may thus provide a mechanism to facilitate antibiotic resistance. The findings here thus suggest that the *fro* pathway could be an important defense mechanism to target. Inhibition of this pathway could increase *P. aeruginosa* susceptibility to neutrophil-mediated killing while minimizing antibiotic resistance.

MATERIALS AND METHODS

Strains and Growth Conditions

Experiments were performed using the *P. aeruginosa* strain AL143, which contains the *yfp* gene integrated between *froA* and *froB* and a constitutively-expressed *mCherry* gene (3). For experiments with response of *P. aeruginosa* against NaOCl Δ *froR* and Δ *froI* mutant strains were used (3). Strains were streaked onto LB-Miller (BD Biosciences, Franklin Lakes, NJ) petri dishes containing 2% Bacto agar (BD Biosciences, Franklin Lakes, NJ), incubated at 37 °C to obtain single colonies, and inoculated into MinA minimal medium, which contains per 1 L of water: 4.5 g KH₂PO₄, 10.5 g K₂HPO₄, 1 g (NH₄)₂SO₄, 1 mM MgSO₄ and 0.2 % (w/v) sodium citrate (31). Strains were cultured in minimal medium for 18 hours in a roller drum spinning at 200 rpm at 37 °C, diluted 1:1000 into the same medium supplemented with oxidative or antioxidative agents, conditioned medium from neutrophils, or were not supplemented, cultured for 1 hour at 37 °C, and imaged using microscopy.

Phase Contrast and Fluorescence Microscopy

Bacteria were immobilized on 1% agarose pads containing minimal medium and imaged immediately. Images were acquired using a Nikon Eclipse Ti-E microscope (Nikon, Melville, NY) containing a Nikon 100X Plan Apo (1.45 N.A.) objective, a Nikon Ph3 phase contrast condenser annulus, a Sola light engine (Lumencor, Beaverton, OR), an LED-DA/FI/TX filter set (Semrock, Rochester, NY) for visualizing the mCherry fluorescence spectrum containing a 409/493/596 dichroic and 575/25 nm filters for excitation and 641/75 nm filters for emission, an LED-CFP/YFP/MCHERRY filter set (Semrock) for visualizing YFP fluorescence containing a

459/526/596 dichroic and 509/22 nm filters for excitation and 544/24 nm filters for emission, and a Hamamatsu Orca Flash 4.0 V2 camera (Hamamatsu, Bridgewater, NJ).

Images were acquired using Nikon NIS-Elements and analyzed using custom built software written previously (32) in Matlab (Mathworks, Natick, MA). See the “Code availability” section below to download code. Briefly, cell masks were determined from phase contrast images using an edge-detection algorithm. The YFP to mCherry fluorescence ratios in the masked cell areas were computed. The expression of *fro* is quantified as the ratio of YFP to mCherry fluorescence in individual *P. aeruginosa*, which is averaged over at least one hundred *P. aeruginosa*. A minimum of 100 cells were imaged and analyzed in each experiment.

Imaging *P. aeruginosa* under flow

Microfluidic devices were fabricated using standard soft lithography techniques as described previously (33) with channel dimensions of 100 μm x 50 μm (width x height) and a flow rate of approximately 5 $\mu\text{L}/\text{min}$.

Growth Curves

Growth curve experiments were performed using a Synergy HTX multi-mode plate reader and sterile, tissue-culture treated, clear bottom, black polystyrene 96-well microplates (Corning, Corning, NY) containing 200 μL of culture in each well. The temperature set point was 37 $^{\circ}\text{C}$ and preheated before beginning measurements. For experiments performed with stationary phase bacteria, overnight cultures of bacteria were grown for 18 hours at 37 $^{\circ}\text{C}$ in the roller drum at 200 rpm to saturation, diluted into minimal medium to an optical density at 600 nm (OD_{600}) of 0.01 containing NaOCl , H_2O_2 , HNO_3 or no supplement. Plates were incubated at

37 °C with continuous orbital shaking with an amplitude of 3 mm at the frequency of 180 cycles per minute and measured for OD₆₀₀ every 20 minutes.

Cell size analysis

P. aeruginosa were cultured for 18 hours at 37 °C in a roller drum spinning at 200 rpm, diluted 1:1000 into fresh minimal medium, treated 1 μM NaCl, 200 μM HCl or with serial dilutions of NaOCl, H₂O₂, HNO₃ or media only, and incubated at 37 °C in 250 mL flasks for 3 hours with shaking at 225 rpm. Cells were immobilized on 1% agarose pads containing minimal medium, imaged using phase contrast microscopy, and analyzed using the edge detection image analysis tools in Matlab described above. The total pixel area of each individual cell was determined by computing the mask area and converting from pixels to μm² by multiplying the mask area by a factor of 0.004225 μm²/pixel to account for the microscope camera pixel size and objective magnification.

Neutrophil isolation

Blood samples from healthy donors between 18 to 65 years old were collected at the Institute for Clinical and Translational Science at the University of California, Irvine, through approved protocols with the UCI Institutional Review Board, and informed consent was obtained from all donors. Neutrophils were prepared essentially as described in (34). Briefly, 3% dextran (Leuconostoc spp, Mr 450,000-650,000, Sigma-Aldrich, St. Louis, MO) in PBS (Gibco, ThermoFisher, Waltham, MA) was used to separate red blood cells (RBCs) from whole blood. Neutrophils were purified from the remaining cells by overlaying on a Ficoll density gradient

(GE Healthcare, Chicago, IL) following centrifugation in Ficoll-Paque Centrifugation Media (GE Healthcare, Chicago, IL) for 25 minutes at $500 \times g$.

The remaining RBCs were lysed using RBC Lysis Buffer (Fisher Scientific, Hampton, NH), and neutrophils were resuspended in RPMI 1640 medium (ATCC, Manassas, VA) and purity was assessed by flow cytometry using the ACEA NovoCyte Flow Cytometer and fluorescent antibodies APC-CD11b, FITC-CD16 and PE-CD66b (eBioscience, San Diego, CA). Neutrophils were immediately used for neutrophil stimulation or co-incubation experiments with *P. aeruginosa*. This procedure routinely yielded greater than 95% CD11B-positive neutrophil populations.

Tissue culturing

J774.1 mouse macrophages (ATCC) or primary neonatal normal human dermal fibroblasts (Neo-NHDF) (ATCC) were cultured in 10 mL DMEM (Gibco, ThermoFisher) in T75 flasks (VWR, Radnor, PA) at 37 °C with 5% CO₂. Macrophages were passaged every three days through scraping and Neo-NHDF cells were passaged every 5 days through trypsinization.

Co-incubation of mammalian cells with P. aeruginosa

P. aeruginosa were cultured for 18 hours in minimal medium, centrifuged at $4600 \times g$ for 2 minutes and resuspended in DPBS (Gibco, ThermoFisher) three times, and diluted to a final OD₆₀₀ of 0.2. Macrophages or Neo-NHDF cells that were harvested from growth flasks, or freshly prepared neutrophils, were washed three times by centrifuging at $300 \times g$ for 5 minutes and resuspending in DPBS, and 1 mL aliquots containing 4×10^5 cells/mL were transferred into flat-bottom dishes. *P. aeruginosa* culture was added to the cells at a multiplicity of infection of

10 and incubated at 37 °C with CO₂ for 30 minutes. Dishes were aspirated until approximately 20 µL of media remained and pads consisting of 1 % agarose containing DPBS were placed on top of the samples in the dishes to immobilize bacteria and cells. Samples were imaged immediately using microscopy.

Preparation of neutrophil-stimulated conditioned medium

Approximately 2 million/mL of freshly harvested neutrophils were incubated at 37 °C in a roller drum spinning at a speed of 4 rotations per minute in PBGT medium (10 mM phosphate buffer pH 7.4 (2.46 mM monobasic with 7.54 mM dibasic sodium phosphate), 140 mM sodium chloride, 10 mM potassium chloride, 0.5 mM magnesium chloride, 1 mM calcium chloride, 1 mg/mL glucose, and 5 mM taurine (24)). Neutrophils were supplemented with 100 ng/mL of PMA or left untreated for 1 hour, and were separated from the supernatant by 5 minute centrifugation at 4,600 x g at 25 °C. The supernatant was isolated and used as conditioned medium.

P. aeruginosa were cultured in minimal medium for 18 hours at 37 °C in a roller drum spinning at 200 rpm, diluted 1:1000 in conditioned media with or without the addition of 100 µM methionine, and incubated for 3 hours. Bacterial culture from each sample was concentrated using cellulose syringe filters with a 0.22 µm pore size (Millipore, Burlington, MA). *P. aeruginosa* cells were immobilized on 1% agarose pads and imaged. Data analysis was performed as described in the “cell size analysis” methods section.

Antioxidant treatments

P. aeruginosa cultured for 18 hours at 37 °C in a roller drum spinning at 200 rpm, diluted 1:1000 into fresh minimal medium were diluted 1:1000 into minimal medium alone or

supplemented with 1 μ M NaOCl with an addition of methionine (1 μ M, 10 μ M, 100 μ M), β -mercaptoethanol (β ME) (1 μ M, 10 μ M, 100 μ M) or cysteine (0.1 μ M, 1 μ M, 10 μ M). 50 mL of each bacterial culture was incubated at 37 °C in 250 mL flasks for 3 hours with shaking at 225 rpm. Data analyses were performed as described in the “cell size analysis” methods section showed that the visible decrease of *fro* response to 1 μ M NaOCl was observed when using 0.1 μ M cysteine, 1 μ M methionine and 1 μ M β ME. While *fro* response was not elevated at higher concentrations of these antioxidants.

Code Availability

The custom Matlab scripts used to process and analyze the fluorescence microscopy data have been previously described (32) and are freely available from Zenodo at <https://doi.org/10.5281/zenodo.3898289>. Gene Ontology enrichment analysis was performed using GOEnrichment version 2.0.1 (35). The analysis of RNA-seq data was performed using the limma Bioconductor package in Galaxy version 3.48.0 (36).

Supplementary Material

Supplemental Figures S1-S3

Supplemental Spreadsheet 1

Supplemental Spreadsheet 2

Source Data file

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Supplementary Material

Supplemental Figures S1-S3

Supplemental Spreadsheet 1 (Microsoft Excel format)

Supplemental Spreadsheet 2 (Microsoft Excel format)

Source Data file (Microsoft Excel format). Contains raw data and statistics for main and supplemental figures

Supplemental Figure S1

Fig. S1 Effects on cell size and global transcription in *P. aeruginosa* treated with NaOCl, H₂O₂, or HNO₃. Top: average area of individual *P. aeruginosa* cells, Middle: average mCherry/area, Bottom: average YFP/area in *P. aeruginosa* treated with dilutions of H₂O₂, NaOCl, HNO₃, or untreated for 3 hours. Boxplots show the mean (black line) and the interquartile range of three independent experiments. Blue boxes indicate the datasets that were used to compute the values for Fig. 3D.

Supplemental Figure S2

Fig. S2. Dose-dependent effects of antioxidant supplements on *fro* expression induced by HOCl. Quantification of *fro* expression levels in *P. aeruginosa* cultures treated with 1 μ M NaOCl and supplemented with methionine, cysteine, or beta-mercaptoethanol (BME). Plots show the mean and SEM for three independent experiments. P-values were calculated using two-tailed, unequal variance t-test. ns indicates $p > 0.05$.

Supplemental Figure S3

Fig. S3. Effects of antioxidants alone on *fro* expression. Quantification of *fro* expression levels in *P. aeruginosa* cultures treated with 1 μ M methionine, 0.1 μ M cysteine, 1 μ M beta-mercaptoethanol (BME), or untreated (MinA data replotted from Fig. 3C). Plots show the mean and the standard error of the mean (SEM) for three independent experiments. P-values were calculated using two-tailed, unequal variance t-test. ns indicates $p > 0.05$.