Paraoxonase-2 deficiency enhances *Pseudomonas aeruginosa* quorum sensing in murine tracheal epithelia

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1Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa; 2Department of Medicine and 3Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at University of California, Los Angeles, California; and 4Department of Microbiology, University of Washington, Seattle, Washington

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Paraoxon/2 deficiency enhances *P. aeruginosa* quorum sensing in murine tracheal epithelia. Am J Physiol Lung Cell Mol Physiol 292: L852–L860, 2007. First published November 22, 2006; doi:10.1152/ajplung.00370.2006.—*Pseudomonas aeruginosa* is an important cause of nosocomial infections and is frequently present in the airways of cystic fibrosis patients. Quorum sensing mediates *P. aeruginosa*’s virulence and biofilm formation through density-dependent interbacterial signaling with autoinducers. *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL) is the major autoinducer in *P. aeruginosa*. We have previously shown that human airway epithelia and paraoxonases (PONs) degrade 3OC12-HSL. This study investigated the role of PON1, PON2, and PON3 in airway epithelial cell inactivation of 3OC12-HSL. All three PONs were present in murine tracheal epithelial cells, with PON2 and PON3 expressed at the highest levels. Lysates of tracheal epithelial cells from PON2, but not PON1 or PON3, knockout mice had impaired 3OC12-HSL inactivation compared with wild-type mice. In contrast, PON1-, PON2-, or PON3-targeted deletions did not affect 3OC12-HSL degradation by intact epithelia. Overexpression of PON2 enhanced 3OC12-HSL degradation by human airway epithelial cells lysates but not by intact epithelia. Finally, using a quorum-sensing reporter strain of *P. aeruginosa*, we found that quorum sensing was enhanced in PON2-deficient airway epithelia. In summary, these results show that loss of PON2 impairs 3OC12-HSL degradation by airway epithelial cells and suggests that diffusion of 3OC12-HSL into the airway cells can be the rate-limiting step for degradation of the molecule.

*N*-3-oxododecanoyl homoserine lactone; cystic fibrosis; innate immunity; paraoxonase

The lung is continuously exposed to a variety of infectious pathogens and foreign substances. Airway epithelial cells are uniquely designed for monitoring and clearing invading microbes through a number of mechanisms including mucociliary clearance, proinflammatory cytokine and chemokine production, and release of antimicrobial peptides such as defensins, lactoferrin, and lysozyme (2). In the absence of impaired innate immune function or underlying comorbid medical conditions, these mechanisms are generally effective at preventing the development of pulmonary infections.

An uncommon, but serious, cause of respiratory tract infections is *Pseudomonas aeruginosa*, a gram-negative bacterial pathogen that is ubiquitously present in the environment. Under suitable conditions and in a susceptible host this microorganism is a frequent cause of acute and chronic infections. *P. aeruginosa* is a common cause of hospital-acquired infections including cystitis, burn wound infections, and ventilator-associated pneumonia. In addition, cystic fibrosis (CF) patients are frequently colonized with *P. aeruginosa* and pulmonary decline in CF is directly correlated to infection (22, 27). *P. aeruginosa*’s ability to produce chronic infections is, in part, explained by its capacity to produce and exist in biofilms. Biofilms are communities of bacteria that are characterized by a structured architecture consisting of bacteria contained within an extracellular matrix. This surrounding matrix is composed of both bacterial and host components. Biofilms typically display a marked resistance to antibiotic killing and therefore infections associated with biofilm-forming bacteria are difficult to eradicate (20).

Many bacteria, including *P. aeruginosa*, utilize a cell-to-cell communication system termed quorum sensing to regulate biofilm formation, under certain conditions, and virulence factor production (12). Quorum sensing is dependent on environmental concentrations of small quorum-sensing signaling molecules produced by bacteria and therefore bacterial cell density. Two well-studied quorum-sensing systems in *P. aeruginosa*, las and rhl, utilize molecules that are acylhomoserine lactones (AHLs). LasI is responsible for the production of *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL), and LasR is the transcription factor that responds to 3OC12-HSL. RhlI is an enzyme that synthesizes *N*-butanoyl homoserine lactone (C4-HSL), and RhlR is the transcription factor that responds to C4-HSL. Increasing concentrations of 3OC12-HSL lead to production of C4-HSL. These two systems constitute a regulatory cascade, with the *las* system controlling expression of the *rhl* system. Importantly, as many as 6% of the ~6,000 genes expressed by *P. aeruginosa* are influenced by the *las* or *rhl* quorum-sensing systems (10, 28, 33).

Paraoxonases (PONs) are enzymes originally described based on their ability to hydrolyze organophosphates. Since the initial descriptions of PON’s enzymatic activity, three members of the PON family have been described: PON1, PON2, and PON3 (6). The three genes encoding the enzymes are located next to each other on the long arm of human chromosome 7. Human PON1 and PON3 are primarily produced in the...
liver and found circulating in serum bound to HDL particles. The majority of research related to PONs has been directed at the proposed protective role of PON1 in cardiovascular disease. PON2 is more ubiquitously expressed and is found in many tissues including placenta, brain, lung, and kidney (19). More recent studies have suggested that the native substrates for PONs are lactones (14). We have shown that human airway epithelial (HAE) cells can inactivate AHLs, including 3OC12-HSL (4). This ability to interfere with quorum sensing may represent an additional function of airway epithelial cells in host defense. In support of this concept, PON1 is known to inhibit biofilm formation by *P. aeruginosa* (21). Our hypothesis is that PON activity in airway epithelial cells is important in quorum-sensing modulation and functions as part of the host defense response to *P. aeruginosa*. In this report we describe the functional consequences of targeted deletion of PON1, PON2, and PON3 on quorum-sensing regulation by murine airway epithelial cells.

**MATERIALS AND METHODS**

**Primary airway epithelial cell culture model.** Primary HAE cells were isolated from trachea and bronchi of human donor lungs. The isolated cells were seeded onto collagen-coated, semipermeable membranes (0.6-cm² Millicell-HA; Millipore, Bedford, MA) and grown at the air-liquid interface as previously described (13, 40). All experiments were performed on well-differentiated human airway epithelia (HAE) (>14 days after seeding of cells). Samples were collected with approval of the University of Iowa Institutional Review Board.

Murine tracheal epithelial cells were isolated and grown based on the method previously described by You et al. (38). Mice tracheas were isolated following euthanasia. Tracheas were cut lengthwise and immersed in dissociation medium (15 mg of Pronase per 10 ml of Ham’s F-12 medium at 4°C). Twenty-four hours later, epithelial cell clumps were harvested and resuspended in medium containing a 50:50 ratio of DMEM/Ham’s F-12 supplemented with 4 mM glutamine, 10 µg/ml insulin, 5 µg/ml transferrin, 62 µg/ml chola toxin, 5 mg/ml epidermal growth factor, 0.03 mg/ml bovine pituitary extract, 10⁻⁸ M retinoic acid, 5% fetal bovine serum, and 20 U µg⁻¹ ml⁻¹ Pen-Strep. Harvested cells were preincubated in a culture dish at 37°C in 5% CO₂ for 2–3 h to remove fibroblasts. The epithelial cells were then seeded onto rat tail collagen-precoated transwell membranes (0.33-cm² insert membrane growth area; Corning, NY). Four days postseeding, an apical air interface was attempted daily until a confluent culture epithelium was formed. Once formed, the polarized cultures were established at 4°C in Ham’s F-12 medium supplemented with 3% fetal bovine serum and 10 µg/ml insulin. After reaching confluence, epithelial cell cultures were maintained in medium containing a 50:50 ratio of DMEM/Ham’s F-12 supplemented with 4 mM glutamine, 10⁻⁸ M retinoic acid, 5% fetal bovine serum, and 20 U µg⁻¹ ml⁻¹ Pen-Strep. Culture epithelia were viable for 3–4 mo.

**Microscopic analysis of cultured epithelium.** For light microscopy, epithelial cell cultures were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Samples were prepared for transmission electron microscopy (TEM) imaging using routine methods. Briefly, the intact filters were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed in 1% osmium tetroxide, stained en bloc in 2.5% uranyl acetate, and dehydrated in a graded series of ethanol. After infiltration with Eponate 12 (Ted Pella, Redding, CA), the samples were cured, sectioned at 70 nm, and poststained with uranyl acetate and lead citrate. Sections on formvar-coated and uncoated copper grids were imaged in a JEOL 1230 TEM (Peabody, MA) using a Gatan 2K × 2K UltraScan 1000 camera (Pleasanton, CA).

**3OC12-HSL degradation by intact epithelium and lystate preparations.** For intact epithelium experiments, the apical and basolateral surfaces of cultured airway epithelia were washed twice with PBS containing calcium and magnesium (PBS +/–, pH 7.1). 3OC12-HSL in acidified ethyl acetate (EtAc) was dried under a nitrogen gas stream and then dissolved in PBS +/– to achieve a final concentration of 10 µM 3OC12-HSL. Dissolved 3OC12-HSL was added to the apical (150 µl) and basolateral (250 µl) surface of human airway epithelia. For murine tracheal epithelial cultures, 75 and 250 µl were added to the apical and basolateral surfaces, respectively. Treated epithelia were incubated at 37°C in 5% CO₂, and 6-µl samples of the apical media were collected at 0, 15, 30, and 60 min for determination of remaining 3OC12-HSL using a bioassay. These samples were added to 100 µl of EtAc and stored in air tight glass vials at −20°C.

For epithelial cell lystate experiments, epithelia were first washed with cold PBS +/– and then 50 µl of lysis buffer (50 mM Tris·HCl, pH 6.9, 150 mM NaCl, 10 µM leupeptin, 10 µM aprotinin, 1 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml benzamidine) was added to the apical surface. After 20 min of rocking in lysis buffer at 4°C, the cells were scraped free from the membrane with a pipette tip and lysed by sonication (10 pulses) (Branson Sonifier 250; Danbury, CT). The cellular debris was cleared from the lysate by centrifugation (4,500 g, 30 s, 4°C). Relative protein concentrations were determined by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Lysate preparations (10%) were diluted in PBS and incubated in the presence of 10 µM 3OC12-HSL (in PBS +/–) at 37°C. At various time points, 6-µl aliquots were collected and stored as described above.

**3OC12-HSL and 3OC12-HSL quantitative assay.** All AHLs were obtained from Sigma-Aldrich Chemicals (St. Louis, MO), except for 3OC12-HSL, which was obtained from Vertex Pharmaceuticals (Coralville, IA). 3OC12-HSL was measured in a quantitative bioassay as previously described using *Escherichia coli* MG4 (pKD17) (23).

**CHO and epithelial cell culture gene transfer experiments.** Chinese hamster ovary (CHO) cells were cultured as monolayers in 24-well plates. Recombinant adenosinereceptor type 5 (Ad5) expressing hPON1, hPON2, hPON3, or green fluorescent protein (GFP) were produced by the University of Iowa Vector and Gene Targeting Core (34). CHO cells were infected with recombinant Ad5 by CaP, transfection (8) and allowed to express for 48 h. Synthetic AHLs were reconstituted in PBS +/– to 20 µM and incubated for 1 h on transfected CHO cells. 3OC12-HSL lactone ring hydrolysis was determined as described below.

Primary cultures of human airway epithelia were infected basolaterally with 100 MOI (multiplicity of infection) of Ad5 expressing hPON2 or GFP. The Millicells were returned to fresh medium and incubated at 37°C for 48 h and used for intact epithelia or lystate experiments as previously described above.

**Western blot analysis of CHO cells.** CHO cell lysates were made by incubation in lysis buffer (50 mM Tris·HCl, pH 7.5, 138 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin) with 1% Triton X-100 for 20 min at 4°C on a rocker. Cells were scraped from the tissue culture plates and transferred to a microfuge tube. Sonication was performed for 2.5% uranyl acetate and cell debris was cleared by centrifugation at 4,500 g for 30 s at 4°C. The lysates were then combined with loading buffer and separated by SDS-PAGE. Protein was then transferred to polyvinylidene difluoride membranes (PVDF) (Immobilon-P; Millipore, Billerica, MA). PVDF membranes were blocked overnight at 4°C in 5% bovine serum albumin in PBS. Membranes were then incubated with the appropriate primary antibody for 2 h at room temperature and then washed three times in 1× TTBS (137 mM NaCl, 2.7 mM KCl, 2.5 mM Tris, 0.05% Tween 20). Primary antibodies were PON1, monoclonal anti-human PON1 at 1:1,000 (Abcam, Cambridge, MA); PON2, rabbit polyclonal anti-human PON2 (Orbigen, San Diego, CA); and PON3, rabbit polyclonal anti-human PON3 (generated by Affinity BioReagents, Golden, CO). Secondary antibodies were conjugated to horseradish peroxidase (Amersham Biosciences, Amersham, UK) and used at 1:20,000 for 1 h. Following three washes with PBS.
for these studies. The cells become well differentiated and the polarized epithelium grown at the air-liquid interface used P. aeruginosa and tracheal epithelial cells at the air-liquid interface. Figure 1, PON3 in murine airway epithelium, we first cultured murine sensing signals. P. aeruginosa infection is the lung, and PONs are capable of inactivate 3OC12-HSL (21). Since a common site of degrading 3OC12-HSL (4) and that PONs are also able to inactivate 3OC12-HSL (21). PON2 specificity for 3OC12-HSL supports the hypothesis that this molecule may represent a natural substrate for PON2. 21). PON2 preferentially degrades 3OC12-HSL, PON is a promiscuous enzyme with multiple enzymatic activities including paraoxonase, aryl esterase, and lactonase activity (6). Recent studies have suggested that lactone molecules are likely to be PONs’ natural substrates (7, 14, 21). Since all three members of the PON family are expressed in airway epithelia, we first examined whether substrate specificity was different among the three PONs. CHO cells were transduced with adenoviruses encoding either PON1, PON2, or PON3 (Fig. 3). Forty-eight hours later, the cells were exposed to AHLS of varying side chain lengths and substitution status. The active or closed-ring AHL forms in the cell media were determined by HPLC following a 60-min incubation. Figure 3 shows that all three PONs have similar patterns of inactivating AHLs, with PON2 having the greatest lactonase activity specifically for 3OC12-HSL. Prior studies have shown that PON2 has much greater lactonase activity for AHLS compared with PON1 or PON3 (7, 21). PON2 specificity for 3OC12-HSL supports the hypothesis that this molecule may represent a natural substrate for PON2. We also found that only AHLS with acyl side chains eight carbons in length or longer were susceptible to degradation by the PONs, and the extent of lactonolysis following incubation with the PON-expressing cells was proportional to the side chain length. Side chain substitution also contributed to the amount of degradation in that 3OC12-HSL was more extensively degraded than C12-HSL, indicating that hydroxylation form a pseudostratified epithelium with ciliated cells present and an intact air-liquid interface. Transcripts for all three PONs were present. However, PON2 and PON3 were expressed at the highest level (Fig. 2A). This is in contrast to HAE, which also express PON1, PON2, and PON3 but have much greater expression of PON2 mRNA compared with PON1 or PON3 (Fig. 2B).

**RESULTS**

**Airway epithelial cells express PON1, PON2, and PON3 mRNA.** We have previously shown that HAE cells are capable of degrading 3OC12-HSL (4) and that PONs are also able to inactivate 3OC12-HSL (21). Since a common site of P. aeruginosae infection is the lung, and PONs are capable of inactivating AHLS, we wanted to determine the role of PON1, PON2, and PON3 in the airway epithelia response to quorum-sensing signals.

To investigate the expression profile of PON1, PON2, and PON3 in murine airway epithelium, we first cultured murine tracheal epithelial cells at the air-liquid interface. Figure 1, A and B, show hematoxylin and eosin as well as TEM images of the polarized epithelium grown at the air-liquid interface used for these studies. The cells become well differentiated and

![Fig. 1. Imaging of murine tracheal epithelia. A: hematoxylin and eosin-stained section of well-differentiated murine tracheal epithelial cells on transwell membranes (0.33 cm²). Bar, 10 μm. B: transmission electron microscopy of well-differentiated murine tracheal epithelial cells on transwell membranes (0.33 cm²). Bar, 2 μm.](image-url)
The third carbon of the acyl side chain augments PON’s lactonase activity against the AHLs. Murine tracheal epithelial cell lysates from PON2 knockout mice have impaired 3OC12-HSL inactivating ability. We next asked whether loss of individual PONs would impair the ability of murine tracheal epithelial cell lysates to inactivate 3OC12-HSL. PON1, PON2, and PON3 knockout mice were generated by targeted deletion. Tracheas were harvested from knockout mice and their corresponding wild-type littermates, and then cultures of murine tracheal epithelial cells were established. Figure 4 shows that PON1, PON2, and PON3 knockout murine tracheal epithelia had little to no transcripts present for their respectively deleted PONs, compared with their corresponding wild-type epithelia (Fig. 2A).

To investigate the ability of murine tracheal epithelial cells, individually disrupted for each PON, to degrade 3OC12-HSL, we harvested cell lysates and exposed them to 3OC12-HSL. Wild-type murine tracheal epithelial cell lysates degraded 3OC12-HSL over time with <25% of active 3OC12-HSL remaining after a 60-min incubation. PON1 knockout and PON3 knockout tracheal epithelial cell lysates had preserved 3OC12-HSL degrading capacity compared with littermate controls. However, PON2 knockout epithelial cell lysates had significantly impaired ability to degrade 3OC12-HSL, with >75% of the unhydrolyzed 3OC12-HSL present at the end of a 60-min incubation (Fig. 5). To determine if this defect could be reversed, we added lysates from CHO cells transduced with AdPON2 to tracheal epithelial cell lysates from PON2 knockout mice. 3OC12-HSL inactivation was significantly improved with only 39.6 ± 1.7% of unhydrolyzed 3OC12-HSL remaining after a 60-min incubation, compared with PON2 knockout lysate alone (79.1 ± 5.1%). These data show that loss of PON1 or PON3 activity in murine tracheal epithelial cell lysates does not affect AHL degradation, but the absence of PON2 impairs this activity.

Intact murine tracheal epithelial cell cultures have preserved capacity to degrade 3OC12-HSL despite loss of PON1, PON2, or PON3. To more closely simulate in vivo conditions, 3OC12-HSL hydrolysis by an intact epithelia was determined. We hypothesized that intact epithelia from PON2 knockout mice would display impaired 3OC12-HSL degradation based on our results from the lysate experiments.

We first tested whether airway surface liquid (ASL) contained any AHL degrading activity. Following collection of ASL from 12 transwells (in a total volume of 100 μl), we incubated the collected fluid with 3OC12-HSL. There was no degradation by the ASL (data not shown), suggesting that PONs are either not secreted or not active in the apical...
This is consistent with lack of lactonase activity of human and mouse bronchoalveolar lavage fluid (4).

We then measured the hydrolysis of 10^10 M 3OC12-HSL, following addition to the apical and basolateral compartments of the murine epithelial transwells. Figure 6 shows that intact epithelia from both PON1- and PON3-deficient mice had preserved capacity to degrade 3OC12-HSL. In contrast to our results from the PON2 knockout lysate data, intact epithelia from PON2 knockout mice also degraded 3OC12-HSL at a similar rate as control epithelia. This finding was surprising and raises several possible explanations. First, differing cellular localizations of mouse PON1, PON2, and PON3 may account for the observed differences. Second, the lysate experimental conditions may alter PON activity based on yet undetermined factors including local calcium concentration, redox states, or endogenous inhibitors. Finally, diffusion of 3OC12-HSL across the cell membrane may be the rate-limiting step to degradation at the concentration tested.

Overexpression of PON2 in HAE cell cultures enhances 3OC12-HSL degradation only in lysate preparations. To test whether diffusion of 3OC12-HSL across the cell membrane was the rate-limiting step, we transduced HAE cells with AdGFP or AdPON2. We predicted that if diffusion across the membrane were rate limiting, increasing PON2 levels would result in no change in 3OC12-HSL degradation, by intact epithelia, at the concentration tested. Moreover, increasing concentrations of 3OC12-HSL should result in enhanced degradation in the presence of higher PON2 concentrations. Alternatively, higher 3OC12-HSL concentrations in the setting of impaired lactonase activity (i.e., airway epithelia from PON2-deficient mice) would lead to decreased 3OC12-HSL degradation. We compared the rates of 3OC12-HSL degradation by hPON2 transduced epithelia in lysate and intact epithelia preparations. Figure 7 shows that PON2 transduction significantly enhanced inactivation of 3OC12-HSL in lysates. However, overexpression of PON2 did not influence the rate of 3OC12-HSL degradation by intact epithelium. These data suggest that at 10 µM 3OC12-HSL, diffusion across the cell membrane is rate limiting for inactivation of 3OC12-HSL.

Intact tracheal epithelial cultures from PON2 knockout mice show enhanced quorum-sensing reporting by P. aeruginosa. We next wanted to test the hypothesis that increased concentrations of 3OC12-HSL in the setting of diminished PON activity would result in impaired degradation. However, con-
Data for measurement of inactivation of 3OC12-HSL. Data are expressed as the percentage of active 3OC12-HSL remaining compared with initial levels. A 15-, 30-, or 60-min incubation, samples of apical media were obtained apically and basolaterally. Following varying lengths of incubation, the apical fluid was collected for 3OC12-HSL (final concentration of 10 \(-10^{10}\) M apically and basolaterally). Following a 15-, 30-, or 60-min incubation, samples of apical media were obtained for measurement of inactivation of 3OC12-HSL. Data are expressed as the percentage of active 3OC12-HSL remaining compared with initial levels. Data are means ± SE; n = 2–3 experiments/group performed in quadruplicate.

Fig. 6. Preserved 3OC12-HSL degradation by intact murine tracheal epithelial cell cultures from PON1, PON2, and PON3 knockout mice. Well-differentiated murine tracheal epithelial cell cultures were incubated in the presence of 3OC12-HSL (final concentration of 10 \(\mu\)M apically and basolaterally). Following a 15-, 30-, or 60-min incubation, samples of apical media were obtained for measurement of inactivation of 3OC12-HSL. Data are expressed as the percentage of active 3OC12-HSL remaining compared with initial levels. Data are means ± SE; n = 2–3 experiments/group performed in quadruplicate.

Concentrations of 3OC12-HSL >10–20 \(\mu\)M are difficult to obtain under experimental conditions due to the molecule’s limited solubility in aqueous solutions. Therefore, to expose respiratory epithelium to greater 3OC12-HSL concentrations and more closely mimic in vivo conditions, we developed a \(P.\ aeruginosa\) \(\beta\)-galactosidase (\(\beta\)-gal) quorum-sensing reporter assay. We first developed and tested this assay using serum from wild-type and PON1 knockout mice. We used PON1 knockout sera because PON1 is the predominant PON present in sera. Figure 8A shows that over a 6-h period, \(\beta\)-gal expression increased in serum from both wild-type and PON1 knockout mice. However, PON1 knockout mouse serum had significantly higher \(\beta\)-gal expression compared with control serum. This shows that in the absence of PON1, \(P.\ aeruginosa\) has enhanced quorum sensing as measured by increased \(\beta\)-gal expression.

We next tested this quorum-sensing assay in intact respiratory epithelium. A 2-\(\mu\)l suspension of \(10^{5}\) colony-forming units of PAO1-qsc102-lacZ was applied to the apical surface of murine tracheal epithelial cell cultures. Following varying lengths of incubation, the apical fluid was collected for \(\beta\)-gal measurements (quorum-sensing activity) and bacterial quantification. We hypothesized that PON2 knockout epithelia would show increased \(\beta\)-gal levels compared with controls. At 4 and 6 h following addition of the bacteria, \(\beta\)-gal levels were increased with levels approximately doubled at the 6-h time point compared with control epithelia (Fig. 8B). Interestingly, the bacterial counts were similar between the groups, demonstrating that the observed differences in \(\beta\)-gal expression were caused by changes in quorum sensing and not changes in bacterial replication.

DISCUSSION

The main results of this study show that murine tracheal epithelial cells are capable of degrading the \(P.\ aeruginosa\) quorum-sensing molecule, 3OC12-HSL. In addition, these cells express all three PON enzymes, with PON2 and PON3 being expressed at the highest levels. Although targeted deletion of PON1 and PON3 had no effect on the ability of these cells to degrade 3OC12-HSL, loss of PON2 significantly impaired this ability in lysate preparations. Finally, the absence of PON2 enhanced \(P.\ aeruginosa\) quorum sensing in intact epithelia cultures. This study is the first to demonstrate the individual contributions of PON1, PON2, and PON3 in airway epithelial cell 3OC12-HSL degradation.

We previously reported that HAE cells are capable of inactivating quorum-sensing molecules through an unknown mechanism (4). We, and others, have subsequently shown that PONs can hydrolyze the lactone ring of AHL quorum-sensing molecules, including 3OC12-HSL (7, 21, 37). This raised the question of whether or not PON functions as an inhibitor of quorum-sensing signaling, and therefore as a regulator of \(P.\ aeruginosa\) virulence and, potentially, biofilm formation. In response to this question, we demonstrated that recombinant PON1 and wild-type murine serum reduced biofilm formation, whereas biomass was enhanced in the presence of serum deficient in PON1 (from PON1 knockout mice). Paradoxically, PON1 knockout mice were protected in a peritonitis/sepsis model (21).

The current study advances our knowledge regarding PON’s possible role in airway host defense. The respiratory epithelium plays a very important role in the host response to inhaled toxins and microbes that escape the upper airway clearance mechanisms. These cells are uniquely situated to prevent...
We showed in this study that murine tracheal epithelial lysates from PON2-deficient mice had impaired 3OC12-HSL degrading activity compared with PON1 or PON3 knockout cells. However, intact epithelium showed no differences. Several possible mechanisms may account for this discrepancy. First, although all three mouse PONs are membrane bound (15), they may exhibit different subcellular localization patterns. PON1 is bound to the extracellular membrane and transferred to HDL particles (5). In HeLa cells, PON2 localizes to cytosol and cell membrane fractions but is absent in the supernatant fraction (19). Human PON3 is also found on HDL; however, its subcellular localization is not well understood (25). A global survey study of organ and organelle protein expression in mouse provides evidence that mouse PON1, PON2, and PON3 are all membrane bound without further information on subcellular localization (http://tap.med.utoronto.ca/~mts/) (15). The method of lysate preparation (i.e., clearing centrifugation step) may affect which PONs are present in the reaction mixture and how they individually contribute to 3OC12-HSL degradation. Second, the chemical milieu that the PONs are exposed to may be different in the lysate vs. intact epithelium preparations. A number of conditions have been shown to affect PON activity including redox state, calcium concentration, lipid associations, and pH (1, 11, 17, 32). Finally, diffusion of 3OC12-HSL across the cell membrane may be the rate-determining step for 3OC12-HSL inactivation. Findings from our intact epithelium studies, in which individual deletion of PON1, PON2, or PON3 did not affect 3OC12-HSL inactivation support the latter hypothesis. This suggests that diffusion of the molecule across the cell membrane limits the rate of quorum-sensing molecule inactivation by PON. Also supporting this hypothesis are the results of the experiments in which we overexpressed PON2 in HAE cells. In the PON2 deficiency model, the remaining two PONs, despite having relatively less lactonase activity compared with PON2, are still able to adequately degrade 3OC12-HSL in the intact epithelia studies. The importance of PONs in the regulation of quorum-sensing control will ultimately require a murine model with all three PONs deleted.

One advantage of this study is that with targeted deletion of the individual PONs, the specific contributions of PON1, PON2, and PON3 toward airway epithelial cell degradation of 3OC12-HSL could be examined. However, the true role of PON in airway host defense and regulation of quorum sensing remains to be determined. PON2 may not be required, but redundant, with PON1 and PON3 each contributing to lactonolysis of 3OC12-HSL. Alternatively, PON2 may be required but only for intracellular signaling events or against microbes that become intracellularly located (9). Finally, PON2 may be required, but only when extracellular concentrations of 3OC12-HSL are markedly increased. AHL molecules are thought to be freely diffusible across cell membranes, although to varying degrees based on the carbon side chain length. Very few studies have actually examined transport and kinetics of 3OC12-HSL movement across cell membranes. With the use of a chimeric sensor protein based on LasR, 3OC12-HSL was found to enter COS-1 cells and activate these reporter constructs (35). A study by Pearson et al. (24) reported that although C4-HSL reached steady-state cell concentrations in cell suspensions of P. aeruginosa by 30 s, 3OC12-HSL required ~5 min to reach steady-state levels. Although these

colonization, and ultimately infection, of the distal airways and alveoli. Airway epithelial cells can secrete a number of mediators that are important for mobilization of immune system cells, repair of injured epithelium, and direct pathogen killing with antimicrobial peptides such as β-defensins, lactoferrin, lysozyme, LL-37, and secretory leukocyte proteinase inhibitor (2). We propose that PONs may function as a defense mechanism against bacterial pathogens that utilize lactone molecules to regulate their virulence. PONs are conserved from the nematode Caenorhabditis elegans to mammals, but the endogenous substrate(s) is unknown. Recently, we and others reported findings suggesting that the natural substrates for PONs are in fact lactone molecules (7, 14, 21). Consistent with this hypothesis is that although PON1, PON2, and PON3 all exhibit lactonase activity, there is greater individual variability for other substrates depending on the PON enzyme examined (7, 32). Interestingly, in the human body, naturally occurring lactones are limited and mainly consist of metabolites of fatty acid oxidation. Therefore, the question remains whether exogenous lactones, possibly the homoserine lactone quorum-sensing molecules of P. aeruginosa, represent a natural substrate for PON.

We showed in this study that murine tracheal epithelial lysates from PON2-deficient mice had impaired 3OC12-HSL degrading activity compared with PON1 or PON3 knockout cells. However, intact epithelium showed no differences. Several possible mechanisms may account for this discrepancy. First, although all three mouse PONs are membrane bound (15), they may exhibit different subcellular localization patterns. PON1 is bound to the extracellular membrane and transferred to HDL particles (5). In HeLa cells, PON2 localizes to cytosol and cell membrane fractions but is absent in the supernatant fraction (19). Human PON3 is also found on HDL; however, its subcellular localization is not well understood (25). A global survey study of organ and organelle protein expression in mouse provides evidence that mouse PON1, PON2, and PON3 are all membrane bound without further information on subcellular localization (http://tap.med.utoronto.ca/~mts/) (15). The method of lysate preparation (i.e., clearing centrifugation step) may affect which PONs are present in the reaction mixture and how they individually contribute to 3OC12-HSL degradation. Second, the chemical milieu that the PONs are exposed to may be different in the lysate vs. intact epithelium preparations. A number of conditions have been shown to affect PON activity including redox state, calcium concentration, lipid associations, and pH (1, 11, 17, 32). Finally, diffusion of 3OC12-HSL across the cell membrane may be the rate-determining step for 3OC12-HSL inactivation. Findings from our intact epithelium studies, in which individual deletion of PON1, PON2, or PON3 did not affect 3OC12-HSL inactivation support the latter hypothesis. This suggests that diffusion of the molecule across the cell membrane limits the rate of quorum-sensing molecule inactivation by PON. Also supporting this hypothesis are the results of the experiments in which we overexpressed PON2 in HAE cells. In the PON2 deficiency model, the remaining two PONs, despite having relatively less lactonase activity compared with PON2, are still able to adequately degrade 3OC12-HSL in the intact epithelia studies. The importance of PONs in the regulation of quorum-sensing control will ultimately require a murine model with all three PONs deleted.
experiments were conducted using bacterial cells, it does suggest that 3OC12-HSL is delayed in crossing cell membranes.

Airway epithelial cell paraoxonases may not only function as a component of the innate immune system directly acting at the bacterial level, but these enzymes also may be important for additional control of the inflammatory response by airway epithelial cells responding to these signals. Quorum-sensing molecules have been shown to affect many cells, including fibroblasts and epithelial cells. 3OC12-HSL induces an array of inflammatory molecules including prostaglandin E2, cyclooxygenase-2, interleukin-6, and tumor necrosis factor-α (30, 31). Recently, Kravchenko et al. (16) reported that 3OC12-HSL treatment of epithelial cells and macrophages leads to phosphorylation of p38 and eukaryotic translation initiation factor α (eIF2α) (16), suggesting a possible pathway for 3OC12-HSL’s effects on the epithelial cell’s inflammatory response. PON may regulate these responses through 3OC12-HSL inactivation and inhibit uncontrolled signaling of the inflammatory cascade.

If PON is a naturally occurring inhibitor of quorum sensing, then modulation of its activity or overexpression may represent a potential therapeutic target. P. aeruginosa infection of the lung is common and occurs in the acute setting, such as in nosocomial pneumonia and burn wound infections, as well as in chronic infections such as in cystic fibrosis patients. These infections are oftentimes difficult to treat, and numerous therapeutic options, besides antibiotics, have been proposed. Quorum-sensing inhibitors were recently investigated as potential therapeutic agents with promising results. Experimental data regarding PON’s role in the response to an infection is very limited. Treatment of mice with a synthetic analog of furanone, a naturally occurring quorum-sensing inhibitor, improved bacterial clearance and mortality in a murine model of P. aeruginosa pulmonary infection (36). Similarly, mice treated with garlic extract, another recently described quorum-sensing inhibitor, showed enhanced bacterial clearance in a pneumonia model (3).

In summary, we have shown that murine tracheal epithelial cells express PON1, PON2, and PON3. These cells can degrade 3OC12-HSL, but in the absence of PON2 this activity is impaired. Since PON2 inactivates 3OC12-HSL to the greatest extent and is the most abundant PON in human airway epithelia, it is likely the primary enzyme responsible for quorum-sensing molecule inactivation. The PONs may play a key role in host defense to pathogens such as P. aeruginosa, but additional studies are needed to further address this question.

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