Recognition of intestinal epithelial HIF-1α activation by
Pseudomonas aeruginosa


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There is now ample evidence to show that opportunistic pathogens that colonize the intestinal epithelial surface are fully capable of recognizing and responding to a variety of host-derived compounds encompassing multiple host cell functions such as immune activation (22), cellular cytprotection (7), and mucosal defense function (1). The notion that microbial organisms have evolved a “sense and respond” system to recognize elements within their hosts is logical, not only as a means to develop an effective countermeasure to an attack by the host immune system but also because such a contingency-based system allows bacteria to be more cost effective with regard to virulence gene activation. Multiple lines of evidence are beginning to uncover the mechanistic details of this molecular dialogue in the direction between the host and pathogen (4, 7, 11, 14, 20, 22), and there are likely additional, yet to be identified, cell-to-cell communication molecules that allow bidirectional communication. Elucidation of the various compounds and pathways involved in this complex molecular interaction has the potential to provide a unique opportunity to interdict in the infectious process at its most proximate point.

In this regard, we have previously shown that the human opportunistic pathogen Pseudomonas aeruginosa is able to activate its virulence circuitry in response to soluble elements of epithelial hypoxia and reoxygenation (7). Using the human intestinal epithelial cell line Caco-2-BBE, our group (7) demonstrated that soluble compounds released into the apical but not basolateral medium by hypoxic and/or reoxygenated cultured intestinal epithelial cells induced the expression of a key virulence protein in P. aeruginosa, the PA-I lectin/adhesin. Our group (9) previously showed that the PA-I lectin of P. aeruginosa induces a defect in intestinal barrier function that allows exotoxin A to cross the epithelium, resulting in lethal gut-derived sepsis in mice. PA-I is regulated by the well-described quorum-sensing signaling system (QS), a hierarchical system of virulence regulation in P. aeruginosa and other bacteria (21). Activation of QS-dependent virulence factors in P. aeruginosa in response to medium from hypoxic Caco-2-BBE cells provides a novel example by which bacterial cell-cell communication networks can recognize eukaryotic paracrine signals. The precise signals released into the apical medium of Caco-2 cells in response to hypoxia and reoxygenation that activate P. aeruginosa to express a virulent phenotype are unknown. Furthermore, the pathways within intestinal epithelial cells that regulate the release of host-derived bacterial signaling compounds are also unknown. In the present study, we sought to define the role of hypoxia-inducible factor 1α (HIF-1α), a molecule central to hypoxic signaling, on the ability of epithelial cells to release soluble compounds that activate P. aeruginosa virulence. We used the PA-I as a biologically and clinically relevant marker of virulence activation. In addition, we sought to define the role of adenosine in...
this response because adenosine is released by hypoxic epithelial cells (16, 17), and our group (7) previously identified adenosine as a compound with PA-I-inducing activity.

MATERIALS AND METHODS

Reagents. Adenosine, inosine, hypoxanthine, AMP, ADP, and ATP were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine deaminase obtained from bovine spleen was also purchased from Sigma-Aldrich.

Bacterial and human epithelial cells. Four bacterial strains of *Pseudomonas aeruginosa* were used in these studies. Strain PA27853 is a prototype clinical strain used in our previous studies and was obtained from the American Type Culture Collection (Manassas, VA) (23). Strain PAO1 is a completely genomically sequenced strain with a comprehensive transposon mutant library available from the University of Washington Genome Center and previously used in our laboratory (22). An adenosine deaminase mutant derivative of the PAO1 strain was obtained from the transposon mutant library at the University of Washington. Strain PA27853/PLL-EGFP is a PA-I green fluorescence protein (GFP) reporter strain previously constructed and validated in our laboratory (7). The high-resistance human intestinal epithelial cell line Caco-2/8bc and a stably transfected Caco-2/8bc cell line overexpressing HIF-1α were constructed as described previously (3). Verification of HIF-1α overexpression in the HIF-1α-transfected cells was confirmed by Western blot analysis (data not shown). Caco-2/8bc cells were maintained in high-glucose DMEM (HDMEM) with 10% FCS, 15 mM HEPES, pH 7.4, and 0.25 mg/ml genetin, as previously described (19). Cells were plated on six-well plates (Corning-Costar, Acton, MA) with identical medium without genetin and grown to confluence.

Mouse model of segmental intestinal ischemia-reperfusion. To determine whether soluble factors released into the intestinal lumen during ischemia and reperfusion could induce PA-I expression in *P. aeruginosa*, intestinal contents were collected from segments of small bowel exposed to segmental vascular occlusion and reperfusion. Balb/c mice weighing between 25 and 35 g were used for all studies. Animals were kept non per os except for water 24 h before the procedure. All studies were approved by the Animal Care and Use Committee at the University of Chicago. Mice were anesthetized with xylazine (10 mg/kg) and ketamine (80 mg/kg). A midline laparotomy was made, and a constant 10-cm segment of mid-small bowel was isolated. The proximal end of the 10-cm segment was ligated with 3-0 silk suture, and the lumen was cannulated with Silastic tubing and flushed with Ringer solution to evacuate all luminal contents. After the sample was flushed, the distal end of the 10-cm segment was ligated with a silk suture, and the feeding mesenteric vessels were identified and isolated. Upon 8 , the lumen was flushed with 1 ml of Ringer solution; 1 min later, the lumen was collected via the tubing from the distal end. Luminal flushes and collections were then repeated after 10 min of mesenteric vessel occlusion with a vascular clamp and again repeated after 10 min of reperfusion. Luminal flushes were then filtered through a 0.22-μm filter, placed on ice, and immediately used in PA-I expression assays.

Role of HIF-1α on the extracellular release of soluble mediators from Caco-2/8bc cells that induce PA-I expression in *P. aeruginosa*. Medium from HIF-1α-overexpressing (experimental) and parental Caco-2/8bc cells (control) was passed through 0.22-μm filters and tested for its ability to induce PA-I expression in the PA-I GFP reporter strain of *P. aeruginosa*, PA27853/PLL-EGFP. To identify potential compounds released by HIF-1α-overexpressing cells responsible for the PA-I-inducing effect, media from control and experimental groups were collected, filtered, and fractionated by molecular weight with the use of Millipore centrifugal filter units. In parallel, Caco-2/8bc parental cells were subjected to 2 h of hypoxia (<0.3% O₂) as previously described (7), and the apical medium from the hypoxic cells (experimental) was collected and subjected to filtration and fractionation as described above.

GFP fluorescence assay to detect PA-I expression in *P. aeruginosa*. The PA27853/PLL-EGFP reporter strain was used to measure the expression of PA-I lectin as previously described (7), with modifications. The PA27853/PLL-EGFP was cultured overnight in LB medium containing 50 μg/ml gentamicin at 37°C under shaking conditions. Twenty microliters of the bacterial suspension were added to the 96-well plates containing 180 μl of the experimental or control extracellular fractions from epithelial cells. GFP fluorescence (485/528 nm) and optical density (600 nm) measurements were recorded with the use of a 96-well microplate fluorometer (Synergy HT; Biotek, Winooski, VT) immediately after bacterial inoculation and then hourly thereafter for 7 h. Between measurements, plates were maintained in an incubator shaker set at 37°C and 100 rpm. The relative fluorescence unit (RFU) of the reporter strain for each time point was divided by its corresponding optical density to control for small variations in bacterial cell density. Fluorescence values were calculated as follows, where RFUx refers to experimental and RFUc refers to control at time t; percent of control = 100 × (RFUx – RFUc)/RFUc.

Measurement of adenosine concentration by LC-MS-MS. In preliminary experiments, molecular mass fractions of <3 kDa induced a significant increase in PA-I expression. On the basis of database searches and our previously published data showing adenosine to be a potent inducer of PA-I expression (7), extracellular adenosine concentration was measured in the <3-kDa filtered medium samples obtained from parental Caco-2/8bc cells, HIF-1α-overexpressing cells, and parental Caco-2/8bc cells exposed to hypoxia (<0.3% O₂, 2 h). Adenosine was measured by liquid chromatography-coupled mass spectrometry (LC-MS-MS). Briefly, adenosine was quantified by LC-MS-MS (Agilent 1100 series LC/MSD Trap XCT). Samples were chromatographed on a 3.0 × 150-mm column packed with 5-μm particles of C18 (4.6 mm × 5 μm). The mobile phase consisted of solution A (97.5% H₂O, 2.5% methanol, 0.1% formic acid) and solution B (99.9% acetonitrile, 0.1% formic acid). Adenosine was eluted with a solvent B gradient of 0–60% for 9–23 min. The solvent flow rate was 400 μl/min. The data-dependent MS/MS-MS was run with the MS-MS trigger for any ion >10,000 intensity. The scan range was 90–400 Da. Adenosine was identified and quantified by comparison of the MS-MS fragmentation of the samples to adenosine standards. Adenosine was measured at various time periods of hypoxia (0–5 h), and its concentration was found to be greatest at the 2-h time point (data not shown). Inosine was also quantified with the above method.

Role of adenosine and its precursors and metabolites on PA-I expression in *P. aeruginosa*. Adenosine alone was tested for its ability to induce PA-I expression. A 100 mM stock solution of adenosine was prepared in HDMEM acidified with HCl to dissolve the adenosine. An equal amount of HCl was added to the HDMEM alone to control for the effect of the acid. The 100 mM adenosine solution was serially diluted in HDMEM to make 10, 5, 1, and 0.5 mM concentrations. AMP, ADP, and ATP, and inosine were dissolved in HDMEM to make 10, 5, 1, and 0.5 mM concentrations. All of the above compounds were tested for their effect on PA-I expression in *P. aeruginosa* using the GFP fluorescence assay.

Depletion of Caco-2/8bc cell medium of adenosine by treatment with adenosine deaminase. To determine the putative role of adenosine in PA-I expression within the medium of HIF-1α-overexpressing cells, medium was treated with the adenosine deaminase to deplete adenosine. Briefly, 25 U of adenosine deaminase was added to medium from HIF-1α-overexpressing cells and, as a control, to a solution containing 10 mM adenosine. An equal concentration of adenosine deaminase was also added to the parental Caco-2/8bc cell medium and HDMEM free of adenosine to control for potential impurities in the enzyme solution that could affect PA-I expression. Preliminary data demonstrated that the addition of adenosine deaminase to either
medium from Caco-2\textsubscript{HBe} cells overexpressing HIF-1\textalpha or to stock solutions of adenosine increased PA-I expression. We therefore tested the ability of inosine, the immediate downstream metabolite of adenosine, to induce PA-I expression. We therefore tested the ability of inosine, the immediate downstream metabolite of adenosine, hypoxanthine, could induce PA-I expression. A 100 mM stock solution of hypoxanthine was prepared in HDMEM and NaOH to dissolve the hypoxanthine. An equal amount of NaOH was added to the HDMEM alone to control for the possible pH effect. The 100 mM hypoxanthine solution was serially diluted in HDMEM to make 10, 5, 1, and 0.5 mM concentrations and tested in the GFP fluorescence assay.

**Determination of inosine concentration in adenosine solution treated with P. aeruginosa.** Preliminary experiments indicated no detectable levels of inosine in HIF-1\textalpha-overexpressing and hypoxic cell medium as measured by LC-MS-MS (data not shown). We therefore speculated that P. aeruginosa may convert adenosine to inosine. From the annotation of the PAO1 genome sequence (15), PA0148 was found to have a 46% similarity to adenosine deaminase from *Escherichia coli* and 62% similarity to adenosine deaminase (putative) from *Saccharomyces cerevisiae* and therefore was characterized as probable adenosine deaminase (http://www.pseudomonas.com/). To test the ability of *P. aeruginosa* to metabolize adenosine to inosine, the PAO1 strain of *P. aeruginosa* was cultured overnight in tryptic soy broth (TSB) at 37°C under shaking conditions. The PAO1 strain was then added in a 1-to-10 ratio to TSB and 10 mM adenosine solution. After bacterial inoculation, samples were placed at 37°C and 300 rpm for 6 h. The four groups, 1) TSB, 2) TSB with PAO1, 3) 10 mM adenosine, and 4) 10 mM adenosine with PAO1, were analyzed by LC-MS-MS and thin-layer chromatography (TLC) for adenosine and inosine.

Does *P. aeruginosa* change its metabolism of adenosine in the presence of medium from HIF-1\textalpha-overexpressing or hypoxic Caco-2\textsubscript{HBe} cells? Next, we sought to determine whether the dynamics by which *P. aeruginosa* metabolizes adenosine are altered by the medium from Caco-2\textsubscript{HBe} cells overexpressing HIF-1\textalpha or Caco-2\textsubscript{HBe} cells subjected to hypoxia, speculating that conditioned medium might itself change the kinetics of *P. aeruginosa* adenosine deaminase. The PAO1 strain of *P. aeruginosa* was grown overnight in TBS at 37°C under shaking conditions. The overnight culture was added at 1:10 to samples containing 1) HDMEM (control), 2) medium from parental Caco-2\textsubscript{HBe} cells, 3) medium from Caco-2\textsubscript{HBe} cells overexpressing HIF-1\textalpha, and 4) medium from parental Caco-2\textsubscript{HBe} cells subjected to hypoxia.

**Determination that *P. aeruginosa* metabolizes adenosine to inosine via its potential adenosine deaminase.** To further confirm that adenosine deaminase originating from *P. aeruginosa* was responsible for the conversion of adenosine to inosine, we used wild-type PAO1 and its derivative strain, ID35276, with an adenosine deaminase (PA0148) knockout mutation (5). The derivative strain was confirmed to be a knockout by PCR analysis (data not shown). PAO1 and its adenosine deaminase mutant strain of *P. aeruginosa* were cultured overnight in TBS at 37°C under shaking conditions. Mutant and PAO1 strains were added in a 1-to-10 ratio to 10 mM adenosine solution. After bacterial inoculation, samples were placed at 37°C and 300 rpm for 12 h. Samples were then centrifuged at 5,000 rpm for 3 min to separate the bacteria, and the supernatant was collected. The two groups, 1) 10 mM adenosine with PAO1 and 2) 10 mM adenosine with adenosine deaminase mutant strain of *P. aeruginosa*, were analyzed by TLC for adenosine and inosine.

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**Fig. 1.** Mouse model of segmental intestinal ischemia-reperfusion injury (I/R). Soluble factors from the lumen of mouse intestine subjected to I/R injury induce the expression of the PA-I lectin in *Pseudomonas aeruginosa*. After 10 min (10\textsuperscript{th} of vascular occlusion of a 10-cm portion of small bowel or after 10 min of reperfusion, filtered perfusates of the intestinal lumen induced a significant increase in PA-I expression (**\( P < 0.01 \), ANOVA). Systemic blood collected immediately after I/R experiment (exp) did not induce PA-I expression in *P. aeruginosa* (\( P = \) not significant).
2 h of hypoxia. All samples were placed at 37°C under shaking conditions for 8 h. After 8 h, all samples were inoculated with adenosine. Samples were kept at 37°C under shaking conditions and evaluated for adenosine and inosine via TLC at 0, 5, and 15 h.

Western blot analysis. In selected experiments, PA-I expression was confirmed by Western blot analyses as previously described (23).

Statistical analysis. Data analysis and statistical significance calculations were performed with Prism 4.0 (GraphPad Software, San Diego, CA). Statistical significance was defined as \( P < 0.05 \) by two-way ANOVA or Student’s \( t \)-test as appropriate.

RESULTS

Soluble factors from the lumen of mouse intestine subjected to segmental ischemia and reperfusion induce PA-I expression in \( P. \) aeruginosa. As seen in Fig. 1, filtered perfusates from the lumen of mouse intestine subjected to 10 min of vascular occlusion induced the expression of PA-I in \( P. \) aeruginosa strain PA27853/PLL-EGFP (\( P < 0.01 \), ANOVA, \( n = 6 \)). This finding was also observed when PA27853/PLL-EGFP was exposed to normoxia (21% for 2 h), 2 h of hypoxia (0.3% \( \mathrm{O}_2 \)), and 2 h of hypoxia and 2 h of normoxia. Results demonstrate a time-dependent increase in PA-I expression in \( P. \) aeruginosa strain PA27853/PLL-EGFP overexpressing HIF-1\( \alpha \) (\( P < 0.001 \), ANOVA). Results demonstrate a significant increase in extracellular adenosine concentration in Caco-2 cells exposed to hypoxia and Caco-2 cells overexpressing HIF-1\( \alpha \) (\( P < 0.001 \), ANOVA).

Fig. 2. A: effect of medium from Caco-2 cells overexpressing hypoxia-inducible factor (HIF)-1\( \alpha \) and their parental controls on PA-I expression in \( P. \) aeruginosa. Results demonstrate a time-dependent increase in PA-I expression in PA27853/PLL-EGFP reporter strains exposed to medium from Caco-2 cells overexpressing HIF-1\( \alpha \) (\( *P < 0.001 \), ANOVA). B: results were confirmed by Western blot. C: effect of \(<3\)-kDa medium fraction on PA-I expression \( P. \) aeruginosa from 1) parental Caco-2 cells exposed to normoxia (21% for 2 h), 2) Caco-2 cells overexpressing HIF-1\( \alpha \), and 3) parental Caco-2 cells exposed to 2 h of hypoxia (0.3% \( \mathrm{O}_2 \)). Results demonstrate a significant increase in PA-I expression in Caco-2 cells exposed to hypoxia and Caco-2 cells overexpressing HIF-1\( \alpha \) (\( *P < 0.001 \), ANOVA).

Fig. 3. A: effect of adenosine on PA-I expression in \( P. \) aeruginosa. PA-I expression was significantly increased in the PA27853/PLL-EGFP reporter strain in response to 10 mM adenosine (\( *P < 0.001 \), ANOVA). B: increased expression was confirmed by Western blot analysis (***\( P < 0.05 \), Student’s \( t \)-test).
exposed to luminal perfusates and after 10 min of reperfusion (Fig. 1). In contrast, blood components did not induce PA-I (*P < 0.05, ANOVA, n = 6), raising the possibility that the factors responsible for PA-I expression are released from the intestinal tissue itself. To rule out the possibility that the in vivo expression of the PA-I expression was not due to secondary effects of surgical stress such as physicochemical changes in the local microenvironment, stock strain PA27853 and reporter strain PA27853/PLL-EGFP were exposed to ambient hypoxia (0.3% O₂), pH changes (6–8), and 80% CO₂. None of these conditions induced PA-I expression (data not shown).

Medium from Caco-2BBc cells overexpressing HIF-1α induces PA-I expression in P. aeruginosa. The PA27853/PLL-EGFP reporter strain of P. aeruginosa exposed to medium from HIF-1α-overexpressing cells demonstrated significant time-dependent induction of PA-I as measured by fluorescence (*P < 0.001, ANOVA, n = 3) (Fig. 2A). Results were confirmed by Western blot analysis (*P < 0.05, Student's t-test, n = 3) (Fig. 2B).

Medium fractions <3 kDa induce PA-I expression in P. aeruginosa. To identify specific molecular mass fractions from the medium of Caco-2BBc cells that induce PA-I expression, media from parental Caco-2BBc cells, Caco-2BBc cells exposed to hypoxia (2 h < 0.3% O₂), and Caco-2BBc cells with forced expression of HIF-1α was fractionated into four molecular mass fractions and tested for their ability to induce PA-I expression by the GFP fluorescence assay. Results demonstrated that medium fractions with a molecular mass of <3 kDa in both HIF-1α-overexpressing and hypoxic cell media significantly induced PA-I expression (*P < 0.001, ANOVA, n = 6) (Fig. 2C). The remaining fractions had no effect on PA-I expression (data not shown).

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Fig. 4. A: metabolites and precursors of adenosine and their rate-limiting enzymes. B: effect of medium from Caco-2 cells overexpressing HIF-1α treated with adenosine deaminase (Ado Deam) to deplete samples of adenosine. Treatment of samples with adenosine deaminase resulted in a significant increase in PA-I expression compared with medium from HIF-1α-overexpressing cells alone (*P < 0.001, ANOVA). C: effect of adenosine plus adenosine deaminase on PA-I expression in P. aeruginosa. Similar to results in B, addition of adenosine deaminase to samples with adenosine induced an increase in PA-I expression greater than that with adenosine alone (**P < 0.05, ANOVA), raising the possibility that metabolism of adenosine to inosine induces PA-I expression. D: effect of inosine on PA-I expression. Exposure of P. aeruginosa to inosine resulted in a dose-dependent increase PA-I expression at a concentration 10-fold less than adenosine (*P < 0.001, ANOVA).

Fig. 5. Effect of hypoxanthine, the next downstream metabolite of inosine, on PA-I expression in P. aeruginosa. PA-I expression was significantly increased in the PA27853/PLL-EGFP reporter strain in response to a 10 mM hypoxanthine (**P < 0.01, ANOVA).
Extracellular adenosine concentration is ~10³-fold increased in Caco-2BBe cells overexpressing HIF-1α or after exposure to hypoxia. Potential candidate compounds in the <3-kDa range that could induce PA-I expression in *P. aeruginosa* included adenosine, a nucleoside known to extracellularly accumulate in high concentration following intestinal epithelial cell hypoxia and HIF-1α activation. To confirm the presence of extracellular adenosine in the <3-kDa range fraction of Caco-2BBe cells, adenosine concentration was measured in the <3-kDa fraction from parental Caco-2BBe cells, Caco-2BBe cells exposed to hypoxia (2 h at <0.3% O₂), and Caco-2BBe cells overexpressing HIF-1α by the LC-MS-MS assay. Adenosine concentration was found to be significantly higher in the medium from HIF-1α-overexpressing and hypoxic cells than in medium from parental Caco-2BBe cells under normal conditions (*P* < 0.001, Student’s *t*-test, *n* = 3) (Fig. 2D).

Adenosine alone induces PA-I expression in *P. aeruginosa.* To determine whether adenosine alone could induce PA-I expression in *P. aeruginosa,* varying concentrations of adenosine were tested in our GFP fluorescence assay. Results demonstrated that PA27853/PLL-EGFP responded to 10 mM adenosine with a significant increase in PA-I expression (*P* < 0.001, ANOVA, *n* = 6) (Fig. 3A). Results were confirmed by Western blot (*P* < 0.05, Student’s *t*-test, *n* = 3) (Fig. 3B). The effect of ATP, ADP, and AMP at similar concentrations were also tested; however, no inducing effect was found (data not shown).

Depletion of adenosine in the cell medium from Caco-2BBe cells overexpressing HIF-1α with adenosine deaminase significantly increases PA-I expression. To determine whether adenosine was the putative component within the medium of HIF-1α-overexpressing cells that induces the expression of PA-I, the enzyme adenosine deaminase was added to deplete the medium of adenosine. Remarkably, the addition of adenosine deaminase to the medium resulted in a significant increase in PA-I expression (*P* < 0.001, ANOVA, *n* = 6) (Fig. 4B). Adenosine alone treated with adenosine deaminase also resulted in a significant increase in PA-I expression (*P* < 0.05, ANOVA, *n* = 6) (Fig. 4C), raising the possibility that inosine, the immediate downstream metabolite of adenosine, might play a role in PA-I expression. We next determined whether inosine alone could induce PA-I expression by testing varying concentrations of inosine in our GFP fluorescence assay and found that inosine significantly induced PA-I expression at 1 mM (*P* < 0.001, ANOVA, *n* = 12), a concentration of 10-fold less than that of adenosine (Fig. 4D). Next, we determined whether the next downstream metabolite of inosine, hypoxanthine, could also induce PA-I expression using the GFP fluorescence assay. Hypoxanthine significantly induced PA-I expression at 10 mM (*P* < 0.01, ANOVA, *n* = 8), the same concentration required for adenosine to induce PA-I (Fig. 5).

*P. aeruginosa* metabolizes adenosine to inosine via its adenosine deaminase, PA0148. Given that inosine is a more potent inducer of PA-I expression than adenosine, its concentration...
was measured in HIF-1α-overexpressing and hypoxic cell media via LC-MS-MS. No detectable levels of inosine were found (data not shown). Therefore we speculated that P. aeruginosa itself might convert adenosine to inosine. When adenosine solutions were inoculated with overnight cultures of wild-type PAO1, significant levels of inosine were detected by LC-MS-MS ($P < 0.001$, Student’s t-test) (lane 1: adenosine; lane 2: inosine; lane 3: P. aeruginosa cultured in the presence of high-glucose DMEM (HDMEM; control); lane 4: P. aeruginosa cultured in the presence of parental control Caco-2 cell media; lane 5: P. aeruginosa cultured in the presence of medium from Caco-2 cells overexpressing HIF-1α; lane 6: P. aeruginosa cultured in the presence of Caco-2 cells exposed to hypoxia).

To determine whether conditioned medium from Caco-2BBe cells exposed to hypoxia could shift the metabolism of P. aeruginosa to convert adenosine to inosine, P. aeruginosa was cultured in the presence of HDMEM (control), medium from parental Caco-2BBe cells, medium from HIF-1α-overexpressing Caco-2BBe cells, and medium from Caco-2BBe cells exposed to hypoxia. After 8 h of growth, all samples were inoculated with adenosine and assayed for adenosine and inosine using TLC for 0, 5, and 15 h. Results demonstrated that all conditioned medium converted adenosine to inosine (Fig. 7B); however, at 15 h, significant accumulation of inosine was seen only in P. aeruginosa exposed to medium from either HIF-1α-overexpressing Caco-2 cells or Caco-2 cells exposed to hypoxia ($*P < 0.001$, Student’s t-test) (lane 1: adenosine; lane 2: inosine; lane 3: P. aeruginosa cultured in the presence of high-glucose DMEM (HDMEM; control); lane 4: P. aeruginosa cultured in the presence of parental control Caco-2 cell media; lane 5: P. aeruginosa cultured in the presence of medium from Caco-2BBe cells overexpressing HIF-1α; lane 6: P. aeruginosa cultured in the presence of Caco-2 cells exposed to hypoxia).

DISCUSSION

Data from the present study add to the small but growing body of evidence demonstrating that certain bacteria are fully capable of recognizing and responding to host-derived elements released during physiological stress. Under conditions of physiological stress or immune activation, soluble compounds released by the host, such as epinephrine and IFN-γ, have been shown to activate the virulence of important intestinal bacteria such as E. coli and P. aeruginosa (14, 22). That intestinal bacteria are signaled to upregulate their virulence by host-derived compounds released during physiological stress may have important implications in the pathogenesis by which intestinal bacteria cause sepsis in critically ill patients. This
may be particularly relevant for the human opportunistic pathogen *P. aeruginosa* whose prevalence within the intestinal tract of critically ill patients approaches 50% and whose mere presence in this site is associated with a fourfold increase in mortality (10).

Intestinal ischemia and hypoxia are physiological disturbances that invariably complicate the course of critically ill patients as blood flow is redistributed away from the intestinal tract to more vital organs. As a compensatory response, HIF-1α, a highly conserved global transcriptional regulator, is activated in direct response to both hypoxia and inflammation. As previously reported, hypoxia and HIF-1α expression result in the extracellular accumulation of the cytoprotective compound adenosine that develops as a result of 5′-eptonucleosidase (CD73), which accelerates the conversion of AMP to adenosine; 2) downregulation of adenosine deaminase, which prevents adenosine metabolism to inosine; and 3) downregulation of adenosine kinase, which prevents recycling of adenosine back to AMP (6, 17). Upregulation of CD73 with resultant accumulation of adenosine has been shown to be cytoprotective by enhancing tight junctional barrier function via mechanisms that involve adenosine activation of the 28-kDa adenosine receptor (2, 13, 18). We have previously reported that hypoxic intestinal epithelial cells remain resistant to the barrier-dysregulating effect of *P. aeruginosa*, whereas, in the absence of hypoxia, *P. aeruginosa* induces a profound and rapid effect on the barrier function of cultured intestinal epithelial cells (Caco-2b) via expression of the PA-I lectin (7). However, over time, the medium of hypoxic intestinal epithelial cells directly upregulates the expression of the potent barrier-dysregulating PA-I protein in *P. aeruginosa*, eventually leading to a decrease in transepithelial electrical resistance of cultured intestinal epithelial cell monolayers (7). Taken together, data from the present study, in conjunction with our previous data and data from others, suggest that, during intestinal epithelial hypoxia, eukaryotic cells activate a cytoprotective barrier-enhancing response to invading pathogens in association with HIF-1α expression and extracellular adenosine release. However, at the same time, prokaryotic cells (*P. aeruginosa*) can interpret and use these signals to develop a countermeasure to this response by expressing potent barrier dysregulating proteins such as the PA-I lectin.

Data from the present study suggest that *P. aeruginosa* may have developed a system to not only recognize and respond to end products of intestinal epithelial hypoxia but also to metabolize these products into molecules that can participate in bacterial cell-cell communication networks such as QSL. The observation in the present study that medium from HIF-1α-expressing Caco-2 cells or medium from hypoxic Caco-2 cells alters the metabolism of *P. aeruginosa* such that inosine accumulates may provide an example whereby *P. aeruginosa* itself uses eukaryotic signals as its own QSL molecule. Whether inosine can act as a surrogate QSL molecule in *P. aeruginosa* similar to that described for epinephrine in *E. coli* remains to be clarified (14). The precise mechanisms by which adenosine and inosine activate *P. aeruginosa* to express the QSL-dependent virulence factor PA-I will require further studies.

It has been recently shown that apical exposure of Caco-2 cells to *P. aeruginosa* results in HIF-1α expression (8). When Caco-2 cells were cocultured with *P. aeruginosa*, even during the reoxygenation phase, HIF-1α protein levels remained elevated, unlike cells exposed to hypoxia and reoxygenation in the absence of *P. aeruginosa*, whereby HIF-1α expression is rapidly degraded (8). The fact that the HIF-1 response is potentiated by *P. aeruginosa* is interesting, given that results of the present study, and provides further evidence that the molecular dialogue between intestinal pathogens and the intestinal epithelium is bidirectional and highly dynamic. During severe critical illness where intestinal ischemia is often present and where intestinal colonization with *P. aeruginosa* is highly prevalent, the final interplay of this dynamic interaction may be highly predictive of the development of severe sepsis and a systemic inflammatory response (12). The observation that intestinal ischemia is lethal when accompanied by intestinal colonization with *P. aeruginosa* could be explained in part by the findings in the present study that show that the virulence circuitry of this pathogen has evolved to recognize and respond to end products of epithelial hypoxia (24). That the enzyme of *P. aeruginosa* that metabolize adenosine (adenosine deaminase) is upregulated in response to medium from hypoxic epithelial cells also suggests that *P. aeruginosa* may have evolved a very clever virulence tactic to deplete epithelial cells of a major cytoprotective compound, rendering them all the more vulnerable to the effects of this highly feared pathogen. In summary, data from the present study suggest that the coevolution of bacteria and epithelial cells, unique to the intestinal tract environment, may have resulted in a bacterial-epithelial molecular dialogue that is much more complex than previously appreciated.

REFERENCES


