Inactivation of Host Akt/Protein Kinase B Signaling by Bacterial Pore-forming Toxins

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Uropathogenic *Escherichia coli* (UPEC) are the major cause of urinary tract infections (UTIs), and they have the capacity to induce the death and exfoliation of target uroepithelial cells. This process can be facilitated by the pore-forming toxin α -hemolysin (HlyA), which is expressed and secreted by many UPEC isolates. Here, we demonstrate that HlyA can potentially inhibit activation of Akt (protein kinase B), a key regulator of host cell survival, inflammatory responses, proliferation, and metabolism. HlyA ablates Akt activation via an extracellular calcium-dependent, potassium-independent process requiring HlyA insertion into the host plasma membrane and subsequent pore formation. Inhibitor studies indicate that Akt inactivation by HlyA involves aberrant stimulation of host protein phosphatases. We found that two other bacterial pore-forming toxins (aerolysin from *Aeromonas* species and α -toxin from *Staphylococcus aureus*) can also markedly attenuate Akt activation in a dose-dependent manner. These data suggest a novel mechanism by which sublytic concentrations of HlyA and other pore-forming toxins can modulate host cell survival and inflammatory pathways during the course of a bacterial infection.

INTRODUCTION

Strains of uropathogenic *Escherichia coli* (UPEC) are the leading cause of urinary tract infections (UTIs), which currently rank among the most common of infectious diseases worldwide (Foxman, 2003; Marrs *et al.*, 2005). During the course of an infection, UPEC can stimulate a number of antimicrobial, proinflammatory, prodifferentiation, proliferation, and host cell death pathways (Mulvey, 2002; Mysorekar *et al.*, 2002). In some cases, UPEC can reportedly modulate these signaling events, causing attenuation of host inflammatory responses and potentiating host apoptotic cascades (Klumpp *et al.*, 2001, 2006; Hunstad *et al.*, 2005; Billips *et al.*, 2007). These phenomena have been linked in part to the suppression of nuclear factor- κ B (NF κ B) activation and downstream signaling by unknown factors associated with UPEC (Klumpp *et al.*, 2001; Hunstad *et al.*, 2005). By hindering host cytokine expression and ensuing inflammatory responses, UPEC may be better able to establish itself and multiply within the cells and tissues of the urinary tract. At the same time, UPEC-induced death of bladder and renal epithelial cells can compromise mucosal barriers, and it may thereby facilitate bacterial dissemination and persistence within the urinary tract (Mulvey *et al.*, 1998, 2001; Chen *et al.*, 2003a; Bower *et al.*, 2005; Eto *et al.*, 2006; Mansson *et al.*, 2007b).

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Abbreviations used: CNF1, cytotoxic necrotizing factor 1; EGF, epidermal growth factor; LPS, lipopolysaccharide; MOI, multiplicity of infection; PKB, protein kinase B; PMB, polymyxin B; PtdIns, phosphatidylinositol; TNF, tumor necrosis factor; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

A key regulator of host cell survival pathways is Akt (also known as protein kinase B, PKB; for recent reviews, see Fayard *et al.*, 2005; Song *et al.*, 2005; Manning and Cantley, 2007). This serine/threonine kinase is able to inhibit apoptosis, and it can help control cell cycle and metabolic pathways, endocytosis and vesicular trafficking, and host inflammatory responses, including the activation of NF κ B. Akt is activated downstream of phosphoinositide 3-kinase (PI3-kinase), which itself is activated by integrin signaling, the engagement of G protein-coupled receptors, or the stimulation of receptor tyrosine kinases by insulin or other growth factors (Vanhaesebroeck *et al.*, 2001). Activated PI3-kinase converts phosphatidylinositol-(4,5)-diphosphate [PtdIns(4,5)P₂] into the lipid second messenger phosphatidylinositol-(3,4,5)-triphosphate [PtdIns(3,4,5)P₃], which is recognized by the N-terminal pleckstrin homology (PH) domain of Akt. Interactions with PtdIns(3,4,5)P₃ recruit Akt to the plasma membrane, inducing a conformational change in Akt that exposes a phosphorylation site at threonine 308 (T308) within the activation loop of the central kinase domain (Milburn *et al.*, 2003). T308 is phosphorylated by PI-dependent kinase (PDK) 1, and then at a second site, serine 473 (S473), located within the C-terminal regulatory domain of Akt by an as-yet unidentified kinase designated as PDK2 (Alessi *et al.*, 1997; Stephens *et al.*, 1998; Song *et al.*, 2005). Phosphorylation of both T308 and S473 is required for full activation of Akt (Alessi *et al.*, 1996).

Due to its ability to affect cell fate and activities by influencing survival and other central regulatory pathways, Akt has become a prominent point of focus in various fields of research ranging from tumorigenesis to diabetes and Alzheimer's disease (Testa and Bellacosa, 2001; Lu *et al.*, 2003; Griffin *et al.*, 2005; Robertson, 2005; Zdychova and Komers, 2005). In recent years, Akt has also been shown to impact microbial pathogenesis. A number of bacterial pathogens are able to modulate host cell survival via effects on Akt. Among these are *Salmonella enterica*, *Shigella flexneri*, and *Neisseria*

Table 1. Bacterial strains and plasmids

Strain or plasmid	Plasmid genotype or relevant phenotype	Hemolytic activity ^a	Source or reference
<i>E. coli</i> strains			
UTI89	Cystitis isolate	+	Mulvey <i>et al.</i> (2001)
NU14	Cystitis isolate	+	Mulvey <i>et al.</i> (1998); Johnson <i>et al.</i> (2001)
B218	Cystitis isolate	+	MedImmune (Gaithersburg, MD)
B223	Cystitis isolate	+	MedImmune
B217	Cystitis isolate	–	MedImmune
EC42	Cystitis isolate	–	MedImmune
EC56	Cystitis isolate	–	MedImmune
DS17	Pyelonephritis isolate	+	Roberts <i>et al.</i> (1994)
CFT073	Pyelonephritis isolate	+	Mobley <i>et al.</i> (1990)
A8	Pyelonephritis isolate	+	O'Hanley <i>et al.</i> (1985)
W3110	K-12 strain	–	Mansson <i>et al.</i> (2007a)
<i>Salmonella</i> strain			
<i>S. typhimurium</i> LT2		–	John Roth lab
Recombinant plasmids			
pSH2	Type 1 pilus gene cluster		Blomfield <i>et al.</i> (1991)
pANN202-812	<i>hlyCABD</i>		Mansson <i>et al.</i> (2007a)
pSF4000	<i>hlyCABD</i> in pACYC184		Bauer and Welch (1996)
pSF4000 Δ BamHI	<i>hlyABD</i> Δ <i>hlyC</i>		Bauer and Welch (1996)
pCMV5-HA-PKB α	HA-tagged wild-type Akt1		Alessi <i>et al.</i> (1996)
pCMV5-m/p-HA-PKB α	HA-tagged constitutively active Akt1		Andjolkovic <i>et al.</i> (1997)
pCMV5- Δ PH-HA-PKB α	Pleckstrin homology domain null HA-tagged Akt1		Dario Alessi lab, Alessi <i>et al.</i> (1997)
Recombinant strains			
MM642 (Δ <i>fimH</i> UTI89)	UTI89 <i>fimH::clm</i>	+	This study
MM746 (Δ <i>hlyA</i> UTI89)	UTI89 <i>hlyA::kan (sw)</i>	–	This study
MM487 (Δ <i>cnf1</i> UTI89)	UTI89 <i>cnf1::clm (sw)</i>	+	This study
AAEC185/pSH2	K-12 lab strain AAEC185 with pSH2 plasmid	–	Blomfield <i>et al.</i> (1991)
W3110/pANN202-812	K-12 lab strain W3110 with pANN202-812 plasmid	+	Mansson <i>et al.</i> (2007a)
WAM582	K-12 lab strain DH1 with pSF4000 plasmid	+	Bauer and Welch (1996)
WAM783	K-12 lab strain DH1 with pSF4000 Δ BamHI plasmid	–	Bauer and Welch (1996)
UTI89 16C1	LPS core polysaccharide mutant	+	Hunstad <i>et al.</i> (2005)
UTI89 11F5	O-antigen mutant	+	Hunstad <i>et al.</i> (2005)

^a Hemolytic strains were identified by growth on blood agar plates at 37°C, where clear zones developed around hemolysin-positive bacterial colonies.

gonorrhoeae, which inject into their host cells effector molecules that can activate Akt and thereby inhibit apoptosis during infection (Knodler *et al.*, 2005; Edwards and Apicella, 2006; Pendaries *et al.*, 2006). Similarly, cytotoxic necrotizing factor 1 (CNF1), a secreted toxin encoded by a number of *E. coli* pathogens, including some UPEC isolates, can prevent host cell apoptosis by the stimulation of Akt and subsequent NF κ B activation (Miraglia *et al.*, 2007). *Pseudomonas aeruginosa* can also protect target host cells from death, apparently via stabilization of the X-chromosome-linked inhibitor of apoptosis protein, downstream of Akt activation by as-yet unidentified bacterial factor(s) (Ashare *et al.*, 2007). Interestingly, *P. aeruginosa* not only stimulates Akt phosphorylation but also requires Akt activation to effectively invade target host cells, as does *N. gonorrhoeae* (Kierbel *et al.*, 2005; Edwards and Apicella, 2006). Stimulation of Akt by another invasive pathogen, *Francisella novicida*, promotes the expression of proinflammatory cytokines by infected macrophages via Akt-dependent activation of NF κ B (Rajaram *et al.*, 2006). Finally, the bovine respiratory pathogen *Mannheimia hemolytica* has been shown to produce a leukotoxin that can promote apoptosis of leukocytes, possibly via inhibition of Akt activation (Atapattu and Czuprynski, 2005).

In light of these various findings, we were interested in the effects that UPEC might have on Akt activation in host bladder epithelial cells. The results presented here reveal a novel function for pore-forming toxins secreted by UPEC and other pathogens as potent inducers of Akt inactivation.

MATERIALS AND METHODS

Bacterial Strains and Constructs

All bacterial strains and plasmids used in this study are listed in Table 1. Before infection, bacteria were grown at 37°C for 48 h in 20-ml static M9 minimal medium (6 g/l Na₂HPO₄, 3 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.5 g/l NaCl, 1 mM MgSO₄, 0.1 mM CaCl₂, 0.1% glucose, 0.0025% nicotinic acid, 0.2% casein amino acids, and 16.5 μ g/ml thiamine in H₂O). Antibiotics (chloramphenicol, kanamycin, or ampicillin) were added to the growth medium when necessary to maintain recombinant plasmids. Targeted knockouts of *fimH*, *hlyA*, and *cnf1* were created in UTI89 using the lambda Red-mediated linear transformation system (Datsenko and Wanner, 2000; Murphy and Campbell, 2003). Primer sequences used for generating the *fimH*, *hlyA*, and *cnf1* knockouts were fimHFOR (5'-AGTGATTAGCATCAC CTATACCTACAGCTGAACCCAAAAGAGCAACCAACACCCCAAAAC-3'), fimHREV (5'-TAATATTGCGTACCTGCATTAGCAATGCCCTGTGATTCTCACACAAC-CACACCACACCAC-3'), hlyAFOR (5'-CAGATTTCATTTTTCATTAA CAGGTAAAGAGGTAATTAACACCAACACCCCAAAAC-3'), hlyAREV (5'-GGCACAGCCAGTAAGA TTGCTATCATTTAAATTAATATACACAAACCAACCAACCAAC-3'), cnf1FOR (5'-ATGGGTAAACCAATG-GCAACAAAATATCTTCTTGAGTACACCAACCAACACCCCAAAAC-3'), and cnf1REV (5'-TCAAAATTTTGTGAAAATACCTTCAATACC-GATA TTTCGCACACACCAACCAACCAACCAAC-3'). Disruption of the *fimH*, *hlyA*, and *cnf1* genes was verified by polymerase chain reaction (PCR). Hemolytic strains were identified and confirmed by growth on blood agar plates at 37°C, where clear zones developed around hemolysin-positive bacterial colonies.

Inhibitors and Other Reagents

Tautomycin, calyculin A (CA), 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), EGTA, nocodazole, and human recombinant epidermal growth factor (EGF) were obtained from Calbiochem (San Diego, CA). Okadaic acid (OA), FK506, U-73122, cytochalasin D,

valinomycin, and α -toxin were purchased from Sigma-Aldrich (St. Louis, MO). Human recombinant tumor necrosis factor (TNF)- α was obtained from eBioscience (San Diego, CA), and aerolysin was from ProtoX Biotech (Victoria, BC, Canada).

Cell Culture and Infections

5637 human bladder epithelial cells (ATCC HTB-9; American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) at 37°C in 5% CO₂. After reaching confluence, 5637 cells grown in 12-well plates (Corning Life Science, Acton, MA) were serum starved overnight. These monolayers were then treated with the indicated drugs or with carrier alone for the specified times. For infected samples, bacteria were added using a multiplicity of infection (MOI) of 25–30 microbes per host cell. M9 medium was added to mock-infected control samples. Plates were spun at 600 $\times$ g for 5 min to expedite and synchronize bacterial contact with the host cell monolayers.

To obtain crude bacteria-free pore-forming toxin α -hemolysin (HlyA) preparations, overnight cultures of the WAM582 and WAM783 strains were diluted 1:20 into Luria-Bertani (LB) broth and grown shaking at 37°C for 3.5 h. Bacteria were then pelleted at 10,000 $\times$ g for 8 min, and the supernatants were filtered through 0.45- μ m HT Tuffryn Acrodisc filters (Pall Corporation, East Hills, NY). Fresh HlyA-containing supernatants were diluted 1:10 in RPMI 1640 medium and added to 5637 cell monolayers in the presence or absence of 10% dextran (M_r ~150,000, Spectrum). Alternately, HlyA-containing supernatants were treated on ice for 15 min with 1 μ g/ml polymyxin B sulfate (PMB; Sigma-Aldrich) before dilution into RPMI 1640 medium.

Expression of Recombinant Akt Proteins in Bladder Epithelial Cells

5637 bladder cells at 60–70% confluence were transfected with pCMV5-hemagglutinin (HA)-PKB α (wt-Akt), pCMV5-HA-membrane targeted-PKB α (m/p-Akt), or pCMV5- Δ PH-HA-PKB α (Δ PH-Akt) (kindly provided by D. Alessi, University of Dundee, Dundee, United Kingdom) by using TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI). Plasmids were purified using the Endofree Plasmid Maxi kit (QIAGEN, Madison, WI), and 2–3 μ g/ml each plasmid was used per well of a 12-well plate. At 24 h after transfection, monolayers were serum starved overnight followed by infection with either UTI89 or UTI89 Δ hlyA as described above. Mock-infected controls received only M9 medium.

Western Blot Analysis

After the indicated infection and/or treatment protocols, 5637 cells were washed three times with phosphate-buffered saline (PBS) containing Mg²⁺/Ca²⁺. Cells were then lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 $\times$ Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN), 105 μ g/ml phenylmethylsulfonyl fluoride (Sigma-Aldrich), 1 mM NaF, and 0.4 mM Na₃VO₄. Proteins (20 μ g) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-FL polyvinylidene fluoride membranes (Millipore, Billerica, MA) by using a Trans Blot SD semidry transfer cell (Bio-Rad, Hercules, CA). Membranes were blocked at room temperature for 20–30 min by using SuperBlock buffer (Pierce Chemical, Rockford, IL) and Tris-buffered saline (TBS; 20 mM Tris base, pH 7.6, and 150 mM NaCl). Blocked membranes were subsequently probed with primary antibodies diluted 1:1000 in incubation buffer, a 1:1 mix of SuperBlock and TBS-T (TBS containing 0.1% Tween 20). Antibodies used included anti-Akt1 (2H10), anti-pAkt S473 (193H12), anti-pAkt T308 (244F9), anti-pGSK-3 α / β S21/9, and anti-pFOXO1 S256 (all from Cell Signaling Technology, Danvers, MA). Additional antibodies included anti- β actin (ACTN05 [C4]; Abcam, Cambridge, MA), anti-PP1 (E-9; Santa Cruz Biotechnology, Santa Cruz, CA) anti-PP2Ac (a gift from Dr. David Virshup, University of Utah, Salt Lake City, UT), and anti- β -tubulin (Sigma-Aldrich). After overnight incubations at 4°C, blots were washed five times in TBS-T and probed with Alexa 680-conjugated goat anti-rabbit (Invitrogen) and/or goat anti-mouse IRDYE 800-labeled secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA) diluted 1:12,000 in SuperBlock containing 0.1% Tween and 0.02% SDS. After 30-min incubations at room temperature, membranes were washed four times in TBS-T and three times in TBS, and then they were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band quantification was normalized by dividing the integrated intensity of the signal from phosphorylated Akt1 by the corresponding total Akt1 signal. All assays were repeated three or more times with similar results.

Microscopy

Phase-contrast images of live toxin-treated 5637 cells and untreated controls were acquired using an Olympus IX51 microscope equipped with a 40 $\times$ objective (Olympus LUCPlanFLN NA 0.60 Ph2; Olympus America, Melville, NY) and an Infinity 2 charge-coupled device camera (Lumenera, Ottawa, ON, Canada).

Gene Silencing

ON-TARGETplus SMARTpool small interfering RNA (siRNA) oligonucleotides specific for the α -catalytic subunits of PP2A and PP1, and nonspecific control oligonucleotides, were obtained from Dharmacon RNA Technologies (Lafayette, CO). 5637 cells were seeded to 80% confluence in T-25 flasks, and, after an overnight incubation at 37°C, they were transfected with the siRNA oligonucleotides (final concentration of 80 nM) using Dharmafect1 transfection reagent (Dharmacon RNA Technologies). After a 6- to 10-h incubation, fresh media were added to the cells. The next day cells were split into 12-well plates and serum starved overnight before infection (or mock infection). To silence expression of both PP2Ac and PP1c together, two consecutive transfections were conducted using each PP2Ac- and PP1c-specific oligo pool at a final concentration of 40 nM (total oligo concentration of 80 nM). Efficiency of PP2Ac and PP1c knockdown was assessed by Western blot with labeling of β -tubulin in each sample used as a loading control.

RESULTS

UPEC Stimulates Dephosphorylation of Akt in Bladder Epithelial Cells

To assess the effects that UPEC might have on Akt activation, we infected bladder epithelial cells (designated 5637 cells) with either the human cystitis isolate UTI89 or a recombinant laboratory K-12 *E. coli* strain, AAEC185/pSH2. Both of these strains express filamentous adhesive organelles called type 1 pili (Blomfield *et al.*, 1991; Langermann *et al.*, 1997). These fibers mediate both bacterial adherence to and invasion of host epithelial cells, and they are major virulence factors encoded by virtually all UPEC isolates (Martinez *et al.*, 2000). Akt activation status in infected 5637 cells was determined by Western blot analysis of host cell lysates by using antibodies to detect total Akt (specifically Akt1, which is the prominent Akt isoform found in bladder epithelial cells; unpublished observation) or activated Akt that has been phosphorylated at S473 (pAkt S473). UTI89 caused a marked decline in S473 phosphorylation by 90 min after infection, whereas infection with AAEC185/pSH2 did not (Figure 1A). These results were quantified and normalized by dividing pAkt S473 levels by total Akt1 levels, as shown in the graph in Figure 1A.

Some bacterial pathogens are known to actively modulate Akt signaling by secreting effector proteins into target host cells (Edwards and Apicella, 2006; Knodler *et al.*, 2005; Penardies *et al.*, 2006; Ashare *et al.*, 2007). To address this possibility with UPEC, we infected 5637 bladder cells with either live or gentamicin-killed UTI89. Killed (nonviable; nv) UTI89 had no effect on Akt phosphorylation status relative to mock-infected samples, whereas viable UTI89 caused substantial dephosphorylation of Akt S473 by 2 h after infection (Figure 1B, left). Because UPEC act as opportunistic intracellular pathogens (Mulvey, 2002), we were interested whether host cell invasion was required for UPEC-mediated dephosphorylation of Akt S473. Type 1 pilus-mediated invasion of bladder epithelial cells by UPEC requires functional actin and microtubule cytoskeletal networks (unpublished data; Martinez *et al.*, 2000). Treatment of 5637 cells with either the actin-disrupting drug cytochalasin D or the microtubule-disrupting agent nocodazole inhibit UPEC invasion, but neither drug prevented UTI89-mediated dephosphorylation of Akt S473 (Figure 1B, right). UTI89 thus affects Akt activation status in bladder epithelial cells by an active, invasion-independent mechanism.

HlyA Stimulates Akt Dephosphorylation

The effect of UTI89 infection on Akt dephosphorylation was not limited to this specific UPEC isolate. Assays using additional cystitis (NU14, B218, and B223) and pyelonephritis (DS17, CFT073, and A8) isolates showed that other UPEC strains could also induce Akt dephosphorylation within 2 h

Figure 1. Kinetics of UPEC-induced dephosphorylation of Akt and the role of bacterial viability and host cell invasion. (A) Shown is a representative immunoblot of 5637 bladder cell lysates that was probed with antibodies specific for either total Akt or Akt that is phosphorylated at S473. Lysates were collected 15, 30, 60, 90, or 120 min after infection with AAEC185/pSH2 or UTI89, and bands were quantified (as shown in the graph) by normalizing pAkt S473 to total Akt levels. (B) Left, 5637 bladder cells were infected with viable UTI89 or nonviable (nv, gentamicin-killed) UTI89 for 2 h. Right, 5637 cells were pretreated with dimethyl sulfoxide (DMSO) (solvent control), 2 μ g/ml cytochalasin D (actin polymerization inhibitor), or 10 μ g/ml nocodazole (microtubule inhibitor) for 1 h. After pretreatment, cells were infected with UTI89 for 2 h in the continued presence of the inhibitors or DMSO. M9 medium was added as a control to uninfected samples. Blots of cell lysates were probed as described in A.

after infection of bladder epithelial cells (Figure 2A). These data supported the possibility that a secreted factor, common to many UPEC isolates, negatively impacts Akt activation. Two secreted toxins often associated with UPEC strains are cytotoxic necrotizing factor 1 (CNF1) and HlyA (Marrs et al., 2005; Bonacorsi et al., 2006). Targeted disruption of each of these toxin-encoding genes in UTI89 revealed that *hlyA*, but not *cnf1*, was required for UTI89-mediated inactivation of Akt (Figure 2B). Indeed, the Δ *hlyA* UTI89 mutant actually boosted pAkt S473 levels relative to mock-infected samples, whereas the Δ *cnf1* mutant behaved like wild-type. *Salmonella enterica* serovar Typhimurium, a pathogen that has been shown previously to sustain activated Akt (Marcus et al., 2001; Knodler et al., 2005), served as a control and it did not result in Akt dephosphorylation when used to infect 5637 cells (Figure 2B).

A role for α -hemolysin as a negative regulator of Akt activation was further tested by analysis of UPEC isolates that naturally lack a hemolytic phenotype. The hemolytic negative cystitis isolates B217, EC42, and EC56 were unable to induce Akt dephosphorylation relative to the hemolytic positive strains UTI89, B218, and B223 (Figure 2C). In addition, we found that infection of 5637 cells with the recombinant K-12 *E. coli* strain W3110/pANN202-812, which expresses and secretes wild-type HlyA, effectively induced

Figure 2. HlyA stimulates Akt dephosphorylation. Blots show total and pAkt S473 levels in 5637 cells 2 h after infection with various bacterial isolates relative to uninfected controls that were treated with only M9 medium. (A) Cells were infected with the UPEC cystitis isolates NU14, B218, and B223 or the pyelonephritis isolates DS17, CFT073, and A8. (B) 5637 cells were infected with wild-type UTI89, UTI89 Δ *hlyA*, UTI89 Δ *cnf1*, or *S. enterica*. (C) Blots show the effects of hemolytic cystitis isolates UTI89, B218, and B223 versus the nonhemolytic isolates B217, EC42, and EC56 on total Akt and pAkt S473 levels. (D) Alternatively, 5637 cells were infected with the K-12 lab strains W3110, W3110/pANN202-812 (encoding the entire *hly* operon), WAM582 (DH1/pSF4000, encoding the entire *hly* operon), or WAM783 (DH1/pSF4000 Δ *Bam*HI, a *hlyC*-negative variant of pSF4000). (E) Blot shows pAkt S473 and total Akt levels in 5637 cells after infection with wild-type UTI89 versus the Δ *fimH* mutant, an O-antigen mutant carrying an insertion within the *rfb* operon (11F5), and an LPS core polysaccharide mutant harboring an insertion within the *rfa* operon (16C1). (F) Phospho-Akt status was assessed in 5637 cells after 2-h incubations with LB broth alone or with crude bacteria-free HlyA preparations from either WAM582 (HlyA⁵⁸²) or WAM783 (HlyA⁷⁸³), $\pm$ dextran and PMB treatments as indicated.

Akt dephosphorylation (Figure 2D). In contrast, W3110 lacking the hemolysin-expressing plasmid pANN202-812 had no inhibitory effect.

The pore-forming activity of HlyA has been shown to depend on HlyC, an acyltransferase that acylates HlyA (Stanley et al., 1998). Previous findings revealed that HlyA produced in the absence of HlyC can insert into host membranes, but it is unable to form pores (Bauer and Welch, 1996; Schindel et al., 2001). Infection of 5637 cells with WAM582, a K-12 strain that encodes the wild-type α -hemo-

lysine operon (Bauer and Welch, 1996), effectively inactivated Akt, similar to W3110/pANN202-812 and the hemolytic positive UPEC isolates (Figure 2D). In contrast, infection with WAM783, which carries the α -hemolysin operon lacking *hlyC*, had no inhibitory effect on Akt activation. These results indicate that pore formation by HlyA, after HlyA insertion into host membranes, is required to stimulate Akt dephosphorylation.

Recently, it has been suggested that the FimH adhesin localized at the tips of type 1 pili can function as a tethered toxin (Klump *et al.*, 2006), raising the possibility that FimH functions synergistically with HlyA in promoting Akt dephosphorylation. However, we found that a UTI89 Δ fimH mutant is able to inactivate Akt like the wild-type isolate, indicating that HlyA-mediated dephosphorylation of Akt does not require functional type 1 pili (Figure 2E). Furthermore, we found that disruption of LPS (lipopolysaccharide) biosynthetic genes within the *rfa* and *rfb* operons, which were recently shown to contribute to UPEC-mediated suppression of host cytokine responses (Hunstad *et al.*, 2005), did not prevent UTI89-mediated dephosphorylation of Akt (Figure 2E). LPS promotes hemolysin interactions with host cells and mutations within the *rfa* operon, which affect the generation of the LPS core polysaccharide, result in decreased levels of hemolysin secretion (Bauer and Welch, 1997; Mansson *et al.*, 2007a). Interestingly, in our experiments the *rfa* mutant was reproducibly less effective than wild-type UTI89 at inducing Akt dephosphorylation (Figure 2E), possibly reflecting either decreased HlyA secretion by the *rfa* mutant and/or impaired LPS-modulated HlyA interactions with the target host cells.

To more specifically address the role of LPS in HlyA-mediated inactivation of Akt, we first isolated wild-type (HlyA⁵⁸²) and inactive (HlyA⁷⁸³) HlyA toxins from WAM582 and WAM783 cultures, respectively, as described in *Materials and Methods*. Crude supernatants containing HlyA⁵⁸² effectively inactivated Akt in 5637 bladder cells, whereas HlyA⁷⁸³ had no effect (Figure 2F). The addition of 10% dextran (150,000 mol. wt.), which has been shown to block HlyA pores (Bhakdi *et al.*, 1986), prevented HlyA⁵⁸²-induced dephosphorylation of Akt, verifying a role for pore formation by HlyA⁵⁸² upstream of Akt inactivation (Figure 2F). Preincubation of HlyA⁵⁸² supernatants with polymyxin B (PMB), which binds LPS and inhibits its stimulatory activities (Schilling *et al.*, 2001), abrogated HlyA⁵⁸²-mediated Akt dephosphorylation. Notably, PMB also prevented the host cell cytotoxicity normally observed in HlyA⁵⁸²-treated cells. These data indicate a role for LPS in facilitating HlyA activity, but whether LPS contributes more directly to signaling events required for HlyA-mediated Akt inactivation is not clear.

HlyA Does Not Inactivate Akt by Altering PtdIns(3,4,5)P₃ Levels

Our results indicate that HlyA decreases basal levels of Akt phosphorylation compared with mock-infected controls, whereas most *hlyA*-negative bacteria tested enhance Akt phosphorylation (Figure 2). To further investigate the inactivating effect of HlyA on Akt, we treated UTI89-infected 5637 cells with either EGF or TNF- α . EGF stimulates the PI3-kinase pathway, resulting in the generation of PtdIns(3,4,5)P₃ and subsequent activation of Akt, whereas TNF- α activates Akt via an alternative pathway mediated by NF κ B (Halse *et al.*, 1999; Meng *et al.*, 2002). As expected, 15-min treatments of uninfected bladder cells with either EGF or TNF- α notably increase pAkt S473 levels (Figure 3A). However, neither EGF nor TNF- α was able to overcome the

Figure 3. HlyA negates the effects of TNF- α , EGF, and activating mutations on the phosphorylation status of Akt. (A) 5637 cells were infected with UTI89 for 105 min before the addition of 15 ng/ml TNF- α or 100 nM EGF for 15 min. Immunoblots show total Akt and pAkt S473 levels. (B) 5637 cells were transfected with pCMV5 plasmids encoding wild-type Akt (wt-Akt), membrane targeted Akt (m/p-Akt), or Δ PH-Akt. After reaching confluence, transfected cells were treated with M9 alone or with infected with UTI89 Δ hlyA or wild-type UTI89 for 2 h. Blots of cell lysates were probed to determine total Akt, pAkt S473, and pAkt T308 levels. These results were quantified by normalizing either pAkt S473 or pAkt T308 signals to total relative Akt1 signals, with values presented relative to the uninfected wt-Akt control (M9, set to a value of 1).

inhibitory effect of HlyA in bladder cells that had been infected with UTI89 for 105 min. These results show that HlyA has a very disruptive effect on both canonical and noncanonical Akt signaling pathways.

To address the possibility that HlyA blocks the normal translocation of Akt to the plasma membrane, and thereby prevents Akt activation, we used two constitutively active Akt variants, m/p-Akt and Δ PH-Akt. The m/p-Akt mutant contains a myristoylation signal, which causes PtdIns(3,4,5)P₃-independent translocation of Akt to the plasma membrane resulting in Akt hyperactivation (Andjelkovic *et al.*, 1997). The Δ PH-Akt mutant, which lacks a PH domain and is unable to bind PtdIns(3,4,5)P₃, has an altered conformation that keeps T308 exposed (Milburn *et al.*, 2003). Consequently, cytosolic Δ PH-Akt can be readily phosphorylated by PDK1, allowing for full activation of Akt after S473 phosphorylation in the absence of PtdIns(3,4,5)P₃ generation (Alessi *et al.*, 1997). 5637 bladder cells were transfected with expression constructs encoding these Akt variants or wild-type Akt (wt-Akt). These cells were subsequently mock infected (M9 samples) or infected with either Δ hlyA or wild-type UTI89. In contrast to experiments where endogenous levels of Akt were probed, overexpression of recombinant Akt allowed us to detect phosphorylation of Akt at both S473 and T308. UTI89 infection induced substantial dephosphorylation of Akt at both S473 and T308, whereas Δ hlyA UTI89 had no inhibitory effect (Figure 3B). Bladder cells expressing m/p-Akt showed substantially elevated levels of Akt phosphorylation at both S473 and T308, reflective of the hyperactivated phenotype of this mutant. Cells expressing the Δ PH-Akt mutant, however, showed more modest effects

on Akt phosphorylation compared with cells expressing the m/p-Akt construct. Interestingly, infection with wild-type UTI89, but not the $\Delta hlyA$ mutant, effectively inactivated both m/p-Akt and ΔPH -Akt variants. To better assess the effects of infection on the phosphorylation status of the recombinant Akt proteins, levels of pAkt S473 and pAkt T308 were normalized to total Akt amounts and are presented relative to wt-Akt levels in mock-infected samples below each blot. These data indicate that HlyA does not interfere with Akt phosphorylation by depleting PtdIns(3,4,5)P₃ levels or accessibility and suggest that the toxin instead inactivates Akt by an alternate route.

Protein Phosphatases Facilitate HlyA-mediated Akt Dephosphorylation

Akt can be negatively regulated by activated protein phosphatases (Fayard *et al.*, 2005), some of which can be modulated by intracellular Ca²⁺ levels. For example, the protein phosphatase PP2B, also called calcineurin, is regulated by Ca²⁺, and it is able to dephosphorylate Akt (Rusnak and Mertz, 2000; Wu *et al.*, 2006). Increases in intracellular Ca²⁺ levels result in PP2B association with calmodulin and subsequent activation of PP2B phosphatase activity (Rusnak and Mertz, 2000). Pore formation by HlyA triggers the influx

of extracellular Ca²⁺, and the release of intracellular Ca²⁺ stores (Grimminger *et al.*, 1991; Uhlen *et al.*, 2000; Koschinski *et al.*, 2006). It has been shown that HlyA can elicit phosphoinositide hydrolysis in host cells (Grimminger *et al.*, 1991) and that phospholipase (PLC)- γ has a partial role in causing HlyA-induced calcium oscillations (Uhlen *et al.*, 2000). PLC- γ activity results in the hydrolysis of membrane-bound PtdIns(4,5)P₂ to inositol-(1,4,5)-triphosphate (InsP₃) and diacylglycerol (Patterson *et al.*, 2005). Increasing levels of InsP₃ activate InsP₃ receptors that release intracellular calcium stores from the smooth endoplasmic reticulum. To investigate possible roles for PLC- γ and calcium-regulated PP2B in HlyA-mediated inactivation of Akt, bladder cells were treated with either the PLC γ inhibitor U-73122 or the PP2B inhibitor FK506. Neither drug interfered with the ability of UTI89 to inactivate Akt (Figure 4A). The ineffectiveness of U-73122 suggested that the release of intracellular Ca²⁺ stores stimulated by HlyA was inconsequential to HlyA-mediated inactivation of Akt. To further address this possibility, 5637 cells were treated with either BAPTA-AM, a host cell-permeable Ca²⁺ chelator, or with EGTA, which chelates extracellular Ca²⁺. BAPTA-AM, like U-73122, was unable to prevent Akt inactivation by HlyA (Figure 4B). EGTA, however, blocked HlyA-mediated dephosphorylation of Akt.

Figure 4. Role of calcium and host protein phosphatases in HlyA-mediated Akt dephosphorylation. 5637 cell monolayers were infected with UTI89 for 2 h in the presence of FK506 (a PP2B inhibitor) or U-73122 (a PLC- γ inhibitor) (A); BAPTA-AM (an intracellular calcium chelator) and EGTA (an extracellular calcium chelator) (B); and CA (a nonspecific protein phosphatase inhibitor), OA (a PP2A inhibitor), or TM (a PP1 inhibitor) (C). Drugs were used at the indicated concentrations. Blots of cell lysates were probed to assess total Akt and pAkt S473 levels. For the graph shown in C, the ratio of pAkt S473 to total Akt1 for each sample was normalized to the uninfected M9-treated sample. (D and E) siRNA oligonucleotides were used to silence either PP2Ac or PP1c, both individually (D) and together (E). D and E, top, show total and pAkt S473 levels, with β -tubulin used as an additional loading control. Blots showing levels of PP2Ac, PP1c, and β -tubulin in the specific and control siRNA-treated samples are shown in D and E, bottom.

This result supports previous work showing that pore formation by HlyA requires extracellular Ca^{2+} (Ostolaza and Goni, 1995), and it is consistent with the observation that nonacylated HlyA, which cannot form pores (Bauer and Welch, 1996), also does not inactivate Akt (Figure 2D).

Two other host phosphatases that can dephosphorylate Akt are PP2A and PP1 (Andjelkovic *et al.*, 1996; Chen *et al.*, 2005). Both of these phosphatases are inhibited by CA, whereas PP2A is more specifically inhibited by 100 nM OA, and PP1 is more specifically inhibited by tautomycin (TM) (Connor *et al.*, 1999; Mitsuhashi *et al.*, 2003; Chen *et al.*, 2005). Calyculin A treatment of 5637 cells led to substantial increases in pAkt S473 levels, whereas okadaic acid and tautomycin had less dramatic effects (Figure 4C). All three drugs effectively inhibited the ability of UTI89 to inactivate Akt in a dose-dependent manner. Normalization of pAkt S473 signals from drug-treated and infected samples relative to an uninfected control (M9) verified these results (Figure 4C, graph), suggesting that both PP1 and PP2A are able to dephosphorylate Akt downstream of HlyA pore formation. Neither okadaic acid nor tautomycin inhibited bacterial viability or growth during the course of these assays (unpublished data).

To more specifically assess the role of PP2A and PP1 in HlyA-mediated dephosphorylation of Akt, we used siRNA to knockdown expression of the α -catalytic subunits of each phosphatase, PP2Ac and PP1c. Silencing of either subunit alone had no effect on the ability of UTI89 to mediate Akt dephosphorylation (Figure 4D). Western blot analysis confirmed that expression of both PP2Ac and PP1c were effectively silenced, with each subunit knocked down 75–86% relative to control siRNA-treated samples. To address the possibility that PP2A and PP1 have redundant roles in HlyA-mediated Akt dephosphorylation, 5637 bladder cells were cotransfected with siRNA specific for both PP2Ac and PP1c. Although expression of both subunits was effectively silenced in these samples, no effect on HlyA-mediated Akt dephosphorylation was observed (Figure 4E). These data suggest that either very low levels of the PP2Ac or PP1c α subunits are sufficient to inactivate Akt in response to HlyA exposure, or other host phosphatases/catalytic subunits are involved.

Downstream Targets of Akt Are Dephosphorylated in a HlyA-dependent Manner

Akt has several downstream targets, two of which are glycogen synthase kinase (GSK)-3 β and FOXO1 (Manning and Cantley, 2007). GSK-3 β has been implicated in cell proliferation, metabolism, and survival pathways and in host inflammatory responses. This kinase is inactivated by Akt-mediated phosphorylation at serine 9 (S9), a modification that is often used as an indicator to assess Akt activity. We observed a decline in phosphorylated GSK-3 β at S9 (pGSK-3 β S9) starting at 2 h after infection with UTI89, paralleling the effects seen with Akt (Figure 5A). Similarly, phosphorylation of the proapoptotic Forkhead transcription factor FOXO1 at serine 256 (S256) was also markedly diminished by 2 h after infection of 5637 cells with UTI89 (Figure 5B). Notably, UTI89 Δ hlyA had no effect on the phosphorylation status of either GSK-3 β or FOXO1 (Figure 5, A and B). Thus, HlyA-mediated inactivation of Akt correlates with decreased phosphorylation of downstream Akt targets.

Inactivation of Akt by Other Pore-forming Toxins

Many bacterial pathogens, in addition to UPEC, express pore-forming toxins (Parker and Feil, 2005). Among the most studied of these are α -toxin produced by *Staphylococcus*

Figure 5. HlyA-mediated inactivation of Akt leads to decreased phosphorylation of GSK-3 β and FOXO1. Blots show levels of pGSK-3 β S9 (A) or pFOXO1 S256 (B) in 5637 cells at the indicated times after infection with either wild-type UTI89 or UTI89 Δ hlyA. Mock-infected samples were treated with M9 alone. Staining for β -actin levels in each sample served as a loading control. In A, after the initial 2-h infection period, the antibiotic gentamicin (100 μ g/ml) was added to wells to prevent replicating bacteria from overtaking and destroying the host cell monolayers.

aureus and aerolysin made by *Aeromonas hydrophila*. These toxins create selectively permeable pores that are ~1–4 nm in diameter, similar in size to those formed by HlyA (Bhakdi *et al.*, 1988; Moayeri and Welch, 1994; Parker *et al.*, 1994; Song *et al.*, 1996). To determine whether other pore-forming toxins could function like HlyA and inactivate Akt, 5637 bladder epithelial cells were treated with increasing concentrations of purified α -toxin or aerolysin. Western blot analysis of lysates from treated cells revealed that within 2 h both toxins, like HlyA from UTI89, could induce Akt dephosphorylation (Figure 6A). Aerolysin was particularly potent, whereas substantially higher concentrations of α -toxin were required to promote Akt inactivation. During these assays, even at the highest concentrations used, neither α -toxin nor aerolysin compromised the integrity of the host cells as determined by trypan blue exclusion assays, although both toxins did cause some cytotoxic effects relative to mock-infected or Δ hlyA UTI89-infected samples (Figure 6B). Wild-type UTI89, in contrast, had more notable cytotoxic effects, although still the majority (>75%) of UTI89-infected bladder cells remained intact and viable 2 h after infection. These results indicate that Akt dephosphorylation by sublytic concentrations of pore-forming toxins may be a common theme during the course of many bacterial infections.

Figure 6. Aerolysin and α -toxin mimic the effects of UPEC α -hemolysin on Akt inactivation. 5637 bladder cells were infected with wild-type UTI89, or treated with α -toxin or aerolysin for 2 h at the indicated concentrations. (A) Immunoblots show total Akt and pAkt S473 levels after UTI89 infection or toxin treatments relative to M9-treated samples. (B) Confluent monolayers of 5637 cells were imaged by phase contrast microscopy 2 h after infection with UTI89 or UTI89 Δ hlyA, or after treatment with α -toxin (30 μ g/ml), aerolysin (10 ng/ml), or M9 medium. Bar, 10 μ m.

DISCUSSION

The pore-forming toxin HlyA can enhance UPEC virulence in mouse UTI models, and HlyA expression correlates with increased clinical severity in UTI patients (Johnson, 1991; O'Hanley *et al.*, 1991; Nagy *et al.*, 2006). Although <15% of all commensal *E. coli* isolates encode genes for HlyA expression, this value is substantially higher among strains that cause cystitis (31–48%), pyelonephritis (44–49%), or bacteraemia (39%) (Johnson, 1991; Marrs *et al.*, 2002, 2005). At high concentrations, HlyA is able to lyse erythrocytes and nucleated host cells, a process that may enable extraintestinal pathogens like UPEC to better cross mucosal barriers, damage effector immune cells, and gain enhanced access to host nutrients and iron stores (Cavaliere *et al.*, 1984; Johnson, 1991). At sublytic concentrations, HlyA can induce the apoptosis of target host cells (like neutrophils, T lymphocytes, and renal cells) and promote the exfoliation of bladder epithelial cells, although the signaling pathways triggered by HlyA during such events have not been elucidated (Jonas *et al.*, 1993; Weinrauch and Zychlinsky, 1999; Russo *et al.*, 2005; Chen *et al.*, 2006; Smith *et al.*, 2006).

Here, we have shown that sublytic concentrations of HlyA produced by UPEC within the first few hours of an infection induce the dephosphorylation of the host serine/threonine kinase Akt, a key regulator of survival and inflammatory signaling pathways. This effect was apparent by 90 min after infection, and it was seen using initial MOIs as low as 7 bacteria per host cell (Figure 1A; unpublished data). Our results indicate that pore formation by HlyA in the membrane of target bladder epithelial cells is required to inactivate Akt. Pores formed by HlyA stimulate both Ca^{2+} fluxes and decreases in cytosolic K^{+} levels, events that can significantly affect cellular signaling processes (Uhlen *et al.*, 2000; Gurcel *et al.*, 2006). However, changes in intracellular Ca^{2+} or K^{+} concentrations did not seem to affect HlyA-mediated inactivation of Akt. Specifically, the treatment of bladder cells with the K^{+} ionophore valinomycin (1–40 μM for 2 h) did not alter the phosphorylation status of Akt and neither chelation of intracellular Ca^{2+} pools nor the use of 5 mM KCl in the cell culture medium (to prevent K^{+} release during infection with UPEC) had any inhibitory effect on HlyA-mediated dephosphorylation of Akt (Figure 4B; unpublished data). Moreover, the mechanism by which HlyA induces Ca^{2+} oscillations has recently been shown to be dependent on the small GTPase RhoA (Mansson *et al.*, 2007a). By using a cell-permeable variant of the RhoA inhibitor C3 transferase (4 $\mu\text{g}/\text{ml}$), which effectively inactivates RhoA in 5637 bladder cells, we again observed no abrogation of HlyA-mediated dephosphorylation of Akt (unpublished data). In total, these data indicate that the stimulation of intracellular Ca^{2+} fluxes or K^{+} leakage by HlyA is not required for HlyA-mediated inactivation of Akt.

Akt dephosphorylation by HlyA was prevented by the use of the phosphatase inhibitors calyculin A, tautomycin, or okadaic acid, suggesting a role for both PP2A and PP1 in this process. PP2A and PP1 are among ~40 serine/threonine phosphatases that regulate nearly two thirds of the 500 or so kinases encoded by the human genome (Cohen, 2002). The regulation of these phosphatases is exceptionally complex (Aggen *et al.*, 2000; Janssens and Goris, 2001; Lechward *et al.*, 2001; Cohen, 2002; Sim and Ludowyke, 2002). PP2A exists as a cytosolic heterodimer consisting of either an α or β scaffold (A) subunit in association with either an α - or β -catalytic (C) subunit (Lechward *et al.*, 2001). Interactions between PP2A heterodimers and so-called B regulatory subunits modulate phosphatase catalytic activity, substrate affinity, and cellular

localization (Sim and Ludowyke, 2002). Greater than 75 distinct multimeric configurations of PP2A are possible (Janssens and Goris, 2001). The composition and regulation of PP1 is similarly complex, with α , α_2 , β , γ_1 , or γ_2 PP1 catalytic subunits being capable of interacting with >50 regulatory subunits (Cohen, 2002). Using siRNA, we attempted to verify a role for PP2A and PP1 in HlyA-mediated dephosphorylation of Akt by knocking down expression of the PP2A and PP1 α -catalytic subunits, both of which have been previously implicated in the regulation of Akt (Andjelkovic *et al.*, 1996; Xu *et al.*, 2003; Basu *et al.*, 2007). However, siRNA-mediated knockdown of the α -catalytic subunits, either individually or together, had no effect on HlyA-mediated Akt dephosphorylation in our assays. These results suggest that 1) residual levels of the PP1 and/or PP2A α -catalytic subunits are sufficient to inactivate Akt in response to HlyA treatment, 2) other PP1 or PP2A catalytic subunits are involved, or 3) other host phosphatases can mediate HlyA-induced dephosphorylation of Akt. Recently, the PP2C phosphatase family member PHLPP was found to dephosphorylate Akt at S743 (Gao *et al.*, 2005; Brognard *et al.*, 2007; Mendoza and Blenis, 2007). However, PHLPP is not sensitive to okadaic acid, and it does not normally act on T308 in Akt, suggesting that this particular phosphatase is not involved in HlyA-mediated inactivation of Akt.

Our experiments using purified preparations of aerolysin and α -toxin, in addition to a recent study using leukotoxin from *Mannheimia hemolytica* (Atapattu and Czuprynski, 2005), indicate that the effects of HlyA on Akt inactivation are not unique. These toxins all form selectively permeable pores of similar sizes, from ~0.9 to 4 nm in diameter (Bhakdi *et al.*, 1988; Clinkenbeard *et al.*, 1989; Parker *et al.*, 1994; Song *et al.*, 1996). The common effect of all four of these toxins on the inactivation of Akt suggests that sublytic concentrations of other pore-forming factors, including perforin and the complement membrane attack complex used by the host immune system, may similarly affect Akt. By inactivating Akt, these pore-forming complexes may be able to fine-tune various host responses, including inflammatory and apoptotic signaling cascades that are initiated during the course of an infection.

Here, we found that HlyA-induced inactivation of Akt correlates with reduced phosphorylation of two key downstream targets of Akt, GSK-3 β , and FOXO1. Phosphorylation by Akt inhibits the activities of both of these host factors. A recent study demonstrated that GSK-3 β overexpression in human endothelial cells decreases the half-life of the proinflammatory transcription factor NF κ B, coordinate with reduced production of interleukin (IL)-6 and monocyte chemoattractant protein-1 in response to TNF- α or IL-1 β stimulation (Vines *et al.*, 2006). These observations suggest a means by which pore-forming toxins such as HlyA may attenuate NF κ B-dependent host inflammatory responses via enhanced GSK-3 β activation as an indirect consequence of Akt dephosphorylation. Toxin-induced dephosphorylation of Akt may also temper NF κ B-mediated transcriptional responses by preventing transient formation of complexes between Akt and inhibitor of nuclear factor- κ B kinase, an Akt-stimulated host factor that is directly involved in NF κ B activation (Romashkova and Makarov, 1999).

Inactivation of Akt by pore-forming toxins may adversely affect host inflammatory responses by other means as well. For example, Akt promotes the activation of endothelial nitric-oxide synthase (eNOS) and p47^{phox}, two key host factors involved in, respectively, the generation of reactive nitrogen and oxygen species that can disable UPEC and

other invading pathogens. In response to lipopolysaccharide, eNOS within the bladder is rapidly induced and activated, presumably as part of the host defense against UTIs (Kang *et al.*, 2004). Akt-dependent phosphorylation and subsequent activation of eNOS stimulates the production of nitric oxide, which can have considerable bacteriostatic effects on UPEC (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999; Bower and Mulvey, 2006). Analogously, Akt-mediated phosphorylation of p47^{phox} facilitates the generation of reactive oxygen species during respiratory burst by neutrophils in response to bacterial pathogens such as UPEC (Chen *et al.*, 2003b; Hoyal *et al.*, 2003; Bedard and Krause, 2007). By indirectly interfering with both eNOS and p47^{phox} activation via dephosphorylation of Akt, HlyA may help reduce the levels of nitrosative and oxidative stress encountered by UPEC during the course of a UTI.

Along with inhibitory effects on host inflammatory responses, pore-forming toxins may also promote host cell apoptotic death via indirect activation of FOXO1 and/or other proapoptotic factors. FOXO1 is a member of the Forkhead family of transcription factors that have been implicated in the expression of proapoptotic genes such as Fas ligand and the Bcl-2 family member Bim (Tran *et al.*, 2003). More specifically, activated FOXO1 is required for TNF- α -induced apoptosis in cultured primary human adult dermal fibroblast cells (Alikhani *et al.*, 2005). In our bladder cell culture infection model, we did not observe any significant difference among mock-infected, UTI89 Δ hlyA-infected, or wild-type UTI89-infected 5637 host cells in apoptosis levels (as determined using quantitative assays for activated caspase-3 carried out 4 h after infection; unpublished data). However, the effects of Akt inactivation on apoptotic pathways are variable depending on cell type and environmental cues (Yang *et al.*, 2004; Manning and Cantley, 2007). In vivo, HlyA-mediated inactivation of Akt and subsequent effects on downstream factors such as FOXO1 may stimulate host cell apoptotic death, promoting bacterial penetration into deeper tissue and clearing immune effector cells. For example, the HlyA-positive UPEC isolate CP9 was recently shown to induce rapid activation of proapoptotic executioner caspases in neutrophils much more efficiently than a HlyA-negative mutant (Russo *et al.*, 2005). Relative to an isogenic hlyA mutant, the wild-type CP9 strain also induces more robust exfoliation of bladder epithelial cells (Smith *et al.*, 2006), a phenomenon that seems to involve activation of apoptotic pathways (Mulvey *et al.*, 1998; Schaeffer *et al.*, 2004; Klumpp *et al.*, 2006).

Cumulatively, the data presented in this report indicate a novel role for sublytic concentrations of bacterial pore-forming toxins such as HlyA as negative regulators of Akt and its downstream targets. This process requires toxin-mediated pore formation and likely involves aberrant activation of host phosphatases. Of note, the use of purified aerolysin and α -toxin (which is derived from a Gram-positive organism lacking LPS) indicates that LPS is not a required cofactor for pore-forming toxin-mediated inactivation of Akt. In addition, generic membrane damage does not seem to be sufficient to induce Akt dephosphorylation, because the treatment of bladder cells with 0.0025 or 0.025% saponin, which forms relatively large pores in host cell membranes, had no effect on Akt (unpublished data). Interestingly, a recent report indicated that sublytic concentrations of pore-forming toxins can trigger osmotic stress in target epithelial cells (Ratner *et al.*, 2006), and osmotic stress has been shown to modulate Akt activation via effects on phosphatases (Meier *et al.*, 1998). These studies suggest a scenario in which osmotic stress induced by small pore-forming toxins may stim-

ulate host protein phosphatase activation and subsequent dephosphorylation of Akt, a possibility that is supported by our results using dextran ($M_r \sim 150,000$), which blocks HlyA pores and limits osmotic stress.

Independently of their effects on the inactivation of Akt, it seems that pore-forming toxins like HlyA can impact additional host factors and signaling events. Specifically, sublytic concentrations of α -toxin from *S. aureus*, streptolysin O from *Streptococcus pyogenes*, anthrolysin O from *Bacillus anthracis*, and pneumolysin from *Streptococcus pneumoniae*, have been shown to stimulate MAP kinase signaling (Ratner *et al.*, 2006). In addition, pneumolysin, as well listeriolysin O from *Listeria monocytogenes* and perfringolysin from *Clostridium perfringens*, have been shown to induce histone modifications within target host cells (Hamon *et al.*, 2007). Our results, coupled with these findings, indicate that modulation of host signaling cascades, rather than host cell lysis, may be the major physiological role for HlyA and other pore-forming toxins during the course of an infection.

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