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Anthrax Edema Toxin Impairs Clearance in Mice

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The anthrax edema toxin (ET) of *Bacillus anthracis* is composed of the receptor-binding component protective antigen (PA) and of the adenylyl cyclase catalytic moiety, edema factor (EF). Uptake of ET into cells raises intracellular concentrations of the secondary messenger cyclic AMP, thereby impairing or activating host cell functions. We report here on a new consequence of ET action *in vivo*. We show that in mouse models of toxemia and infection, serum PA concentrations were significantly higher in the presence of enzymatically active EF. These higher concentrations were not caused by ET-induced inhibition of PA endocytosis; on the contrary, ET induced increased PA binding and uptake of the PA oligomer *in vitro* and *in vivo* through upregulation of the PA receptors TEM8 and CMG2 in both myeloid and nonmyeloid cells. ET effects on protein clearance from circulation appeared to be global and were not limited to PA. ET also impaired the clearance of ovalbumin, green fluorescent protein, and EF itself, as well as the small molecule biotin when these molecules were coinjected with the toxin. Effects on injected protein levels were not a result of general increase in protein concentrations due to fluid loss. Functional markers for liver and kidney were altered in response to ET. Concomitantly, ET caused phosphorylation and activation of the aquaporin-2 water channel present in the principal cells of the collecting ducts of the kidneys that are responsible for fluid homeostasis. Our data suggest that *in vivo*, ET alters circulatory protein and small molecule pharmacokinetics by an as-yet-undefined mechanism, thereby potentially allowing a prolonged circulation of anthrax virulence factors such as EF during infection.

The *Bacillus anthracis* virulence plasmid pXO1 encodes the three anthrax toxin proteins linked most directly to the symptoms and lethality associated with infection. The protective antigen protein (PA) interacts with the cellular receptor capillary morphogenesis gene 2 (CMG2) and tumor endothelial marker 8 (TEM8) and combines with either lethal factor (LF) or edema factor (EF) to form two toxins, lethal toxin (LT) and edema toxin (ET). PA, a secreted 83-kDa polypeptide, is cleaved by the host protease furin upon binding to its receptors, allowing the 63-kDa receptor-bound moiety to form oligomers. Secreted LF and/or EF bound to oligomerized PA are shuttled into the cell via clathrin-mediated endocytosis, and subsequent acidification of the endocytic compartment induces channel formation by PA and release of the toxins into the cytosol. While LF is a zinc-dependent protease that cleaves cellular mitogen-activated protein kinase kinases (MAPKKs), EF functions as a highly active calmodulin-dependent adenylyl cyclase, raising cellular concentrations of the secondary messenger cyclic AMP (cAMP). The enzymatic activities of both toxins greatly affect cellular functions by modulation of signaling pathways. LF cleavage of the MAPKK proteins causes effects as varied as cell cycle arrest, apoptosis, modulation of immune responses, cytoskeletal changes, and a rapid Nlrp1-inflammasome-dependent cell death in some inbred mouse and rat macrophages (for a review, see reference 29). The cAMP produced by EF activates protein kinase A (PKA) and the exchange protein directly activated by cAMP (EPAC), often resulting in downstream activation of the transcription factor CREB (cAMP response element binding protein). cAMP signaling influences a vast range of cellular functions and mediates most, if not all, of this toxin's effects. These effects include inhibition of the phagocytic function of monocytes, dendritic cells, and neutrophils, inhibition of cytokine production and response, and increase of transendothelial resistance (for a review, see reference 52). Furthermore, EF has also been reported to cause upregulation of the PA receptors in monocytic cells (6, 25).

Most studies to date have focused on the toxic effects of LF *in vitro* and *in vivo*; however, less is known about how EF contributes to anthrax disease. *B. anthracis* mutants defective in EF production are attenuated for virulence in mice in some infection models (22, 40) but not in others (4), and EF has been reported to influence bacterial dissemination (8). ET injection into mice causes symptoms typical of anthrax disease, including massive edema, hemorrhages in organs, hypotension, bradycardia, and death (12). In rats, ET induces an increased heart rate in addition to hypotension (7). In the present study we report on a novel *in vivo* function of anthrax ET. Using several proteins and one small molecule as markers, we found that ET alters the kinetics of clearance from circulation, resulting in higher levels of circulating molecules during toxemia and infection. These effects were accompanied by ET-induced changes in markers of liver and kidney function, as well as alterations of the activation state of the aquaporin-2 (AQP-2) channel in kidneys.

MATERIALS AND METHODS

Toxins and chemicals. PA and LF expressed in *Bacillus anthracis* and EF and EF K346R expressed in *Escherichia coli* were purified as previously

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described (39, 49). EF K346R is a variant of EF that has 10,000-fold decreased enzymatic activity (12). Diphtheria toxin (DT) of *Corynebacterium diphtheriae* was purified in our laboratory as previously described (3). Forskolin and chicken ovalbumin (OVA) were purchased from Sigma (St. Louis, MO). Biotin and horseradish peroxidase (HRP)-conjugated biotin were purchased from Invitrogen (Invitrogen, Carlsbad, CA). Polyglutamic acid (PGA) and green fluorescent protein (GFP) were kindly provided by Rachel Schneerson (NICHD, NIH) and Robert Liddington (Sanford-Burnham Medical Research Institute), respectively.

Animals and infections. Male and female 10- to 16-week-old BALB/cJ mice (Jackson Laboratories, ME) were used for toxin studies. Sterne-sensitive C57BL/6J mice (Jackson Laboratories) were used for infections with *B. anthracis* (28). CMG2 and TEM8 receptor knockout mice used for *in vivo* endocytosis studies were described previously (20). Animals were injected intravenously (200 μl) with different concentrations of toxins combined with OVA, GFP, PGA, and biotin (all prepared in phosphate-buffered saline [PBS]) or forskolin (prepared in 75% dimethyl sulfoxide [DMSO]) followed by terminal bleeds via cardiac puncture. ET concentrations correspond to the concentration of each toxin component (i.e., 50 μg of ET is 50 μg of PA + 50 μg of EF). For infections with *B. anthracis*, mice were injected intravenously with 10^6 vegetative bacteria grown to early exponential growth phase. The wild-type and EF knockout bacterial strains were described previously (22). All animal protocols were approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

ELISAs. PA in the serum of toxin-treated animals was detected by a previously described europium nanoparticle-based immunoassay (51) or standard PA enzyme-linked immunosorbent assay (ELISA) (30). For detection of OVA in serum of animals, Immulon 96-well plates (Thermo Scientific, Rockford, IL) were coated overnight with mouse monoclonal anti-chicken OVA antibody (10 μg/ml; Sigma), washed with PBS-Tween 20 (0.05% [vol/vol]) (PBST), and blocked with 1% gelatin (Bio-Rad, Hercules, CA) in PBS at 37°C for 45 min. OVA standard (10 ng/ml to 10 μg/ml) or serum from toxin-treated animals (diluted 1:10 and 1:100) was added. Plates were incubated for 2 h, washed with PBST, and incubated for an additional 90 min with rabbit polyclonal anti-OVA (2 μg/ml; Millipore, Billerica, MA). To detect EF in serum of mice, a protocol described by Mabry et al. (23) was used. Briefly, Immulon 96-well plates were coated overnight with PA63 (5 μg/ml, PBS [pH 8.0]), washed with PBST, and incubated with EF (0.1 ng/ml-2 μg/ml) or serially diluted mouse sera for 2 h. Plates were washed, and rabbit polyclonal anti-EF serum (#5900, developed in our laboratory; 1:2,000) was added. For both OVA and EF ELISAs, an HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA; 1:2,000, 90 min) and tetramethylbenzidine (TMB) substrate (R&D Systems, Minneapolis, MN) were used to assess levels of bound rabbit antibody. A competitive ELISA was used for biotin detection. Dilutions of D-Biotin (Invitrogen) or serum samples were mixed with 25 ng of biotin-HRP (Invitrogen)/ml and added to streptavidin-coated ELISA plates (Pierce Thermo Scientific, Rockford, IL). The plates were incubated for 2 h at room temperature, washed with PBST, and processed with TMB substrate (R&D Systems). For detection of PGA levels, Immulon 96-well plates (Thermo Scientific) were coated for 2 h with anti-PGA horse serum (1:1,000 in PBS, kindly provided by Ikuo Uchida) (53), washed with PBST, and blocked with 1% bovine serum albumin in PBS for 1 h. PGA standard (0.3 ng/ml to 2 μg/ml) or sera from toxin-treated animals in a dilution curve were added. The plates were incubated for 90 min, washed with PBST, and incubated for an additional 60 min with rabbit polyclonal anti-PGA serum (obtained from rabbit immunizations of PGA conjugated to tetanus toxoid) at 1:1,000 in PBS. An HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnologies; 1:2,000, 60 min) and TMB were used for assessment of bound rabbit antibody. All ELISAs were read at 450 nm in a Victor3 Reader (Perkin-Elmer, Inc., Waltham, MA).

Western blotting for OVA and GFP detection. Serum samples were diluted 1:10 in sterile water and mixed in equal parts with sodium dodecyl sulfate (SDS)-sample loading buffer prior to separation on a 4 to 20% Tris-glycine gel (Invitrogen) and blotted onto nitrocellulose membrane. All blocking and antibody incubation steps were carried out in LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE) supplemented with 0.05% Tween 20. For detection of OVA, a rabbit anti-OVA antibody (Millipore, Billerica, MA; 1:1,000) was used. GFP was detected with a polyclonal rabbit anti-GFP (Rockland Immunochemicals, Inc., Gilbertsville, PA) at 1:2,000. Infrared dye (IR700) conjugated anti-rabbit IgG (Rockland Immunochemicals) at 1:5,000 was used as a secondary antibody. Blots were imaged on a LI-COR Odyssey infrared imager (LI-COR Biosciences).

Cytotoxicity assays. Chinese hamster ovary (CHO) WTP4 cells (21) were cultured in α-modified Eagle medium supplemented with Glutamax (AMEM), 5% fetal bovine serum, 10 mM HEPES, and 100 μg of gentamicin/ml (all from Invitrogen) at 37°C in 5% CO_2. For toxicity studies, cells were subcultured in 96-well plates and grown to 75 to 80% confluence before treatment with PA (40 ng/ml) or PA in combination with either EF or EF K346R (both at 100 ng/ml) for 2 h. Cells were repeatedly washed with AMEM to remove toxins, and DT was subsequently added for 2 h. The cells were washed again and incubated for an additional 24 h at 37°C before assessment of cell viability as previously described (31).

Endocytosis studies. CHO WTP4 cells were cultured to >90% confluence in six-well plates, and PA (40 ng/ml) in combination with EF or EF K346R (100 ng/ml) was added. Cells were evaluated microscopically for the characteristic cell shape changes in response to ET. After 2.5 h, a higher concentration of PA (2 μg/ml) was added for 40 min. Cells were washed with AMEM and treated with 0.05% trypsin-EDTA (Invitrogen) to remove cell surface-bound PA, followed by lysis in radioimmunoprecipitation assay (RIPA) buffer containing EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). For detection of PA oligomer in organ lysates, animals were intravenously injected with PA (50 μg) in combination with EF or EF K346R (50 μg) and, after 2 h, perfused with PBS to remove the blood present in the organs. Liver and spleen lysates were made in RIPA buffer containing protease inhibitor as described above using a Tissue Tearor (Biospec Products, Bartlesville, OK). PA was detected by Western blotting with polyclonal rabbit anti-PA antibodies (#5308; 1:5,000) (30) and infrared dye (IR800)-conjugated anti-rabbit IgG (Rockland Immunochemicals) at 1:10,000 as described above.

Serum chemistry and histopathology. Mice were intravenously injected with 100 μl of PA (50 μg), PA in combination with EF (50 μg), forskolin (3.75 mg/kg), or vehicle (75% DMSO). Sera were analyzed for blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using the VetACE clinical chemistry system (Alfa Wassermann, Inc., West Caldwell, NJ).

For histopathology, toxin-injected or control mice were euthanized and kidneys were perfusion fixed with 10% formalin in 0.1 M sodium cacodylate (pH 7.4) through the abdominal aorta and postfixed for 24 h in the same buffer before paraffin embedding, sectioning, and staining. AQP-2 staining was performed either using a polyclonal anti-rat, serine-264 specific AQP-2 antibody (Phospho Solutions, Aurora, CO) (1:7,500), or polyclonal anti-human AQP-2 antibody recognizing a C-terminal cytoplasmic domain (H-40; Santa Cruz) at 1:100. All slide preparations and stainings were performed by Histoserv, Inc. (Germantown, MD). Slides were evaluated by a board-certified veterinary pathologist.

RESULTS

Treatment of mice with ET increases circulating PA concentrations. In studies aimed at detecting low concentrations of anthrax toxins in the blood using a highly sensitive europium nanoparticle-based immunoassay (ENIA) (51), we consistently observed that animals injected with ET had increased concentrations of PA in circulation. Mice were injected with either 50 μg of PA alone, or in combination with different amounts of EF (2 to 50 μg), followed by quantification of circulating PA present in the serum at 2 or 6 h postinjection using ENIA. We found

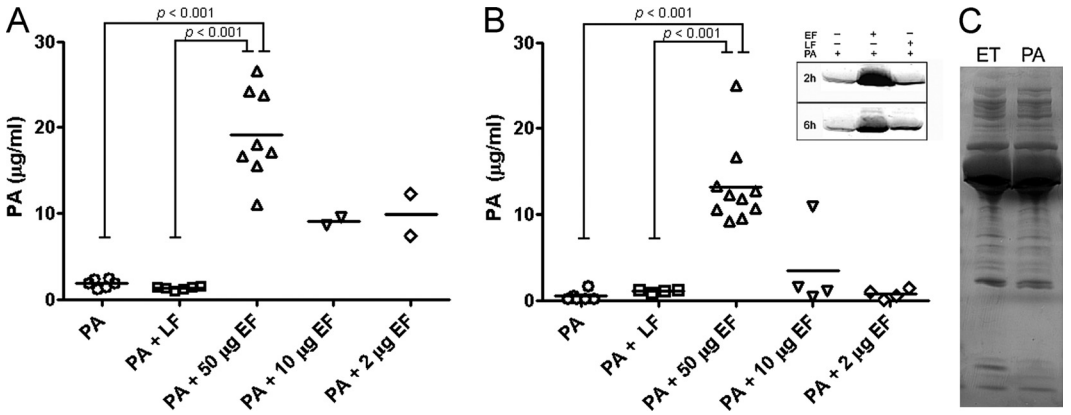

FIG 1 Treatment of animals with PA+EF leads to increased circulating PA concentrations. ENIA and Western blot (inset) analysis of PA levels in serum of BALB/cJ mice after challenge with toxin were performed. (A) PA serum levels at 2 h in mice treated with PA (50 μg), PA+LF (50 μg of each), or 50 μg of PA+EF at the indicated amounts. (B) PA levels at 6 h in mice treated with PA (50 μg), PA+LF (50 μg of each), or 50 μg of PA+EF at the indicated amounts. Each open symbol represents one animal. The results represent pooled data of four independent experiments. The inset shows a Western blot of PA levels in the serum of one representative animal per group after 6 h of toxin treatment. Significant differences between control and EF groups were determined by one-way analyses of variance and Dunnett's post test. (C) Proteins present in diluted sera from mice treated with PA or PA+EF (50 μg) for 2 h.

a 10-fold (10.56 $\pm$ 4.9) increase of PA in animals receiving 50 μg of ET in comparison to control animals receiving PA alone, and this effect was dependent on the amount of EF administered (Fig. 1A and B). In contrast, the administration of PA+LF resulted in circulating PA concentrations that were similar to those detected in sera of animals treated with equivalent amounts of PA alone, indicating that the observed effect was not a consequence of general binding of an enzymatic moiety to cleaved PA in circulation (Fig. 1A and B). Furthermore, an enzymatically inactive EF mutant (EF K346R), which does not exhibit adenylyl cyclase activity but still binds to PA (18), did not induce increased levels of PA, confirming that intracellular ET-mediated effects were required (data not shown). These findings were confirmed by Western blot analyses of PA (Fig. 1B, inset). We also verified that general effects of ET on vasculature and fluid loss did not result in an overall increase of serum proteins as all animals had similar protein concentrations (Fig. 1C) and hematocrits irrespective of toxin treatment. These results demonstrate that the enzymatic activity of EF but not LF causes an increase in the concentration of PA in circulation.

EF causes increases in circulating PA concentrations during infection with *B. anthracis*. To explore whether EF-induced increases in circulating PA concentrations occurred during *B. anthracis* infection, we determined serum PA concentrations in C57BL/6J mice challenged intravenously with 10^6 of *B. anthracis* Sterne-type (fully toxigenic) bacteria or mutant bacteria with the EF gene, *cya*, deleted (22). The intravenous route was chosen in order to circumvent any role EF plays in dissemination (8). The EF knockout strain injected into mice intravenously at this dose had the same virulence as wild-type bacteria (data not shown), indicating that any measured PA concentration differences are not due to differences in establishing infection and thus a lower number of mutant bacteria. We first detected PA in the serum of infected animals 20 h postinfection (Fig. 2), whereas PA was undetectable at 6 h (data not shown). We found that in infections with the mutant bacterium lacking EF, circulating PA concentrations were significantly lower in comparison to those found in wild-type strain infections (Fig. 2). Infections with an isogenic LF deletion strain that expresses both PA and EF did not lead to PA detection in sera of the infected mice (data not shown), likely due to the avirulent nature of this strain and its rapid clearance from animals (22). These results demonstrate that an EF-induced increase in circulating PA concentrations also occurs during infection.

EF-mediated increases in circulating PA are not due to decreased endocytosis. Endocytosis and receptor recycling are events that are highly dependent on a functional cytoskeleton. Since treatment of cells with ET can cause characteristic cell shape changes indicative of cytoskeletal alterations (15, 19) and because ET was shown to inhibit the Rab11/Sec15 exocyst formation, which contributes to the recycling of cellular receptors (14), we considered that EF might affect endocytosis and/or receptor recycling, ultimately inhibiting the uptake and removal of PA from circulation. We first tested the effect of ET on endocytosis in the Chinese hamster ovary (CHO) cell line WTP4 using a diph-

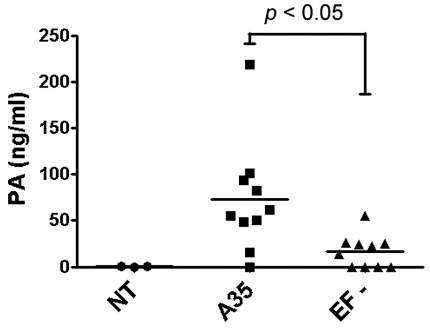

FIG 2 ET causes increase in circulating PA concentrations during *B. anthracis* infection. PA concentrations in sera of C57BL/6J mice challenged with A35 wild-type (n = 10) or an isogenic EF knockout strain (n = 10) were determined by ELISA. Graph shows pooled data from two independent experiments at 20 h postinfection, with each closed symbol representing one animal. Significant differences were determined by two-tailed, unpaired Student t test comparing the indicated groups. NT, noninfected animals.

theria toxin (DT) induced cell death assay. DT is a well-characterized toxin that binds to the cellular receptor heparin-binding epidermal growth factor precursor (35), enters cells via clathrin-mediated endocytosis (47), and ADP-ribosylates cellular elongation factor 2, thereby inhibiting protein synthesis (5). The cells were treated for 2.5 h with a low concentration of ET (40 ng of PA/ml + 100 ng of EF/ml) until the characteristic cell shape change indicating toxin uptake and action was observed, followed by the addition of different concentrations of diphtheria toxin. We hypothesized that any inhibition of general endocytosis by ET should partially rescue DT-treated cells from cell death. However, treatment of cells with PA in combination with either EF or with the enzymatically inactive EF variant (K346R) did not provide any protection against DT (Fig. 3A). Previous comparisons of DT and anthrax toxin showed that they use different adapter proteins for endocytic cell entry (1). Thus, to exclude differences in the endocytic pathways used by DT and PA as the cause for the lack of effect of ET on DT intoxication, we tested the hypothesis that ET inhibits endocytosis by assessing PA uptake. The amount of intracellular, endocytosed PA can be visualized as a high-molecular-weight, heat- and SDS-resistant oligomer that forms only in intracellular acidic compartments (27). Cells were treated with a low concentration of ET established to be sufficient to induce morphological changes induced by the toxin (data not shown), followed by a single, high concentration of PA. The amount of intracellular, endocytosed PA was quantified by Western blotting. Surprisingly, instead of detecting less endocytosed PA in cells treated with ET (as hypothesized), the amount of SDS/heat-stable PA oligomer was increased upon ET treatment, indicating a higher uptake of PA in those cells (Fig. 3B). In contrast, treatment with the enzymatic inactive EF protein K346R did not cause increased PA uptake.

To test whether endocytosis in response to ET was affected in a more complex *in vivo* situation, we challenged mice with PA alone (50 μg), or ET (50 μg) and analyzed the formation of intracellular PA oligomer in perfused liver and spleen lysates after 2 h. Again, we found the amounts of heat-stable oligomer to be higher in ET-injected animals than in animals receiving PA alone (Fig. 3C). These results indicate that more PA oligomer is endocytosed in the presence of EF, both *in vivo* and in CHO WTP4 cells. These findings are likely due to the reported upregulation of anthrax toxin receptors in response to ET (6, 22), although in our studies increases were not limited to monocytic cells. From these results we concluded that the higher concentrations of circulating PA observed in ET-challenged animals (Fig. 1) were not caused by EF-induced inhibition of PA endocytosis.

CMG2 and TEM8 are upregulated in response to ET in nonmyeloid cells in mice. It was previously reported that ET induces the upregulation of both anthrax toxin receptors *in vitro* but that this effect is limited to monocytic cells (6, 25). To determine whether this receptor upregulation occurs *in vivo* and in other cell types, we used recently characterized anthrax receptor knockout mice (20). TEM8 and CMG2 knockout mice were treated with PA or ET for 2 h, and the amounts of PA heptamer in perfused spleen lysates were analyzed by Western blotting. Similar to what was seen in wild-type mice (Fig. 3C), we detected very little PA oligomer in mice that had received PA alone. In contrast, coinjection of EF resulted in higher amounts of intracellular, heat-stable PA in spleen lysates (Fig. 4). This increase could be observed in both types of knockout mice, indicating that in the spleen, both receptors are likely upregulated in response to ET. However, in concordance with previously published work that showed CMG2 to be the major anthrax toxin receptor *in vivo* (22), there was less PA oligomer formation in mice expressing TEM8 alone (Fig. 4). We also found PA oligomer formation in mice in which CMG2 was knocked out only in myeloid cells (22), indicating that the ET-mediated increase in PA oligomer (Fig. 4) and presumably receptor levels were likely not limited to myeloid/monocytic populations as previously reported (25).

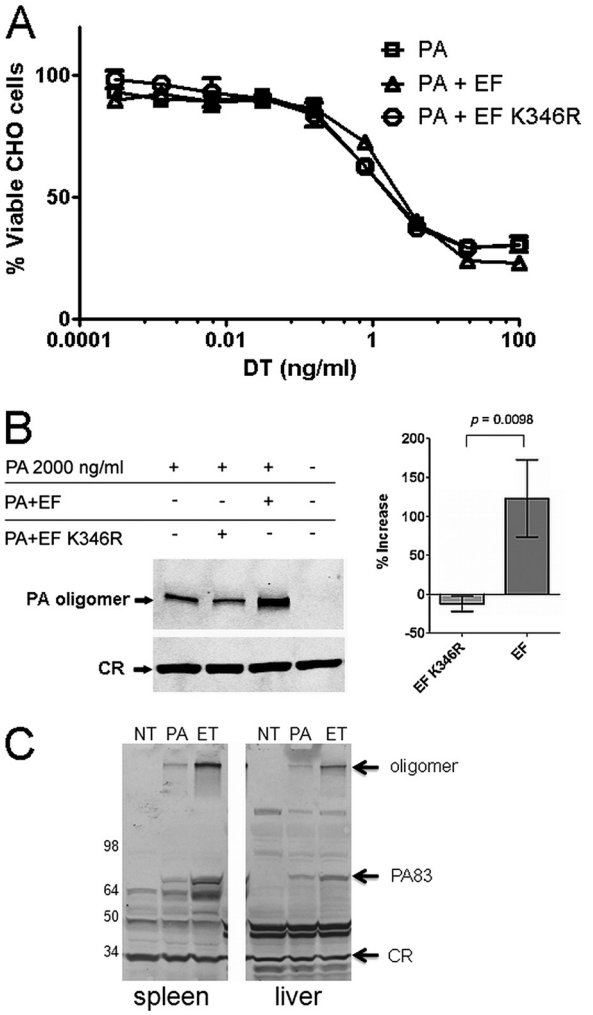

FIG 3 ET causes upregulation of PA receptors in nonmyeloid cells and *in vivo*. (A) Chinese hamster ovary (CHO) cells were pretreated with enzymatically active or inactive ET prior to 24 h treatment with diphtheria toxin (DT), and the cell viability was determined. (B) CHO cells were pretreated with a low concentration of ET (40 ng of PA/ml + 100 ng of EF/ml), followed by a 2.5-h treatment with a high concentration (2 μg/ml) of PA, and were analyzed for the formation of SDS/heat-resistant PA oligomer. Oligomer bands from three gels were quantified (mean ± the standard deviation), normalized to the indicated antibody cross-reactive control band (CR), and displayed as percentage increase relative to PA only controls (graph inset). Statistical differences were determined by two-tailed, unpaired Student *t* test. (C) Internalized SDS/heat-resistant PA oligomer in lysates of spleen and liver cells from mice treated with PA (50 μg) or ET (50 μg) by Western blotting. PA83 represents the uncleaved form of PA prior to internalization. "CR" indicates a cellular protein which cross-reacts with anti-PA antibody and provides an equal protein loading control. The results are representative of two independent studies with n = 2 mice per treatment. NT, nontreated control animals.

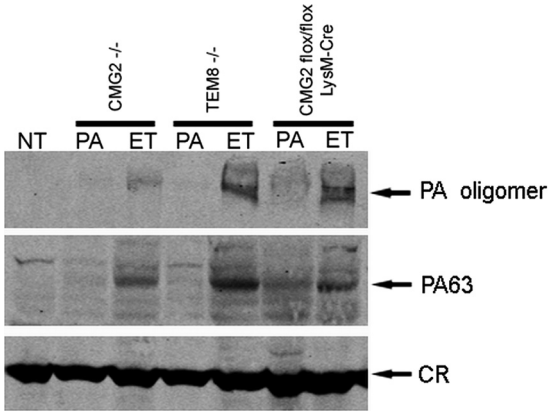

FIG 4 ET causes upregulation of CMG2 and TEM8 *in vivo*. (A) Western blot showing internalized SDS/heat-stable PA oligomer in lysates of spleen cells from CMG2$^{-/-}$, TEM8$^{-/-}$, or myeloid-specific CMG2$^{flox/flox}$ Lys-Cre mice which were treated with PA (50 μg) or ET (50 μg) for 2 h. PA63 represents the noninternalized, furin-cleaved portion of PA. NT, nontreated control animals. CR indicates a cellular protein that cross-reacts with the anti-PA antibody, providing an equal protein loading control.

ET impairs protein and small molecule pharmacokinetics. The fact that ET treatment raised circulating PA concentrations suggested that EF activity might negatively influence general protein clearance. Thus, we analyzed the pharmacokinetics of two unrelated proteins, chicken ovalbumin (OVA) and GFP, in mice. We injected mice with a single dose of either OVA (100 μg/mouse) or GFP (50 μg/mouse) in addition to PA, PA+EF (ET), or PA+EF K346R (50 μg/mouse for each protein), and analyzed OVA or GFP clearance by measuring the concentrations remaining in circulation. We found significantly elevated OVA concentrations in sera of mice injected with OVA in addition to ET compared to sera of animals receiving the control proteins (Fig. 5A). These differences were not caused by differential degradation of OVA in the serum, as determined by Western blotting, or by an ET-induced general increase in concentration of serum proteins, due to fluid loss, as evidenced by similar levels of serum proteins showing cross-reactivity to the anti-OVA antibody. (Fig. 5B). Interestingly, while nearly all of the OVA was cleared in animals receiving PA or inactive ET after 5 h, the OVA concentrations in ET-treated groups remained at the same high concentrations as seen at 2 h. Higher levels of GFP were also seen in ET-treated mice compared to mice in which PA or enzymatically inactive ET were injected (Fig. 5C). Similar to what was seen in OVA experiments, nonspecific serum proteins which were anti-GFP antibody reactive were not increased with ET-treated animals (Fig. 5C).

Next, we wanted to analyze whether the clearance of other bacterial proteins involved in anthrax pathogenesis was impaired. Thus, we tested clearance rates of EF itself in mice having received active ET or inactive ET (PA+EF K346R). At 2 h postinjection, the mice were bled, and the EF levels in serum were analyzed by ELISA using immobilized PA63 as the capture moiety. We found that EF concentrations were significantly higher in mice injected with active ET compared to mice that received inactive ET (Fig. 5D). Interestingly, when we tested the clearance of purified polyglutamic acid (PGA), which represents the capsule material of fully virulent *B. anthracis*, we did not observe differences in the clearance. Injection of 100 μg of purified PGA in combination with ET or inactive ET resulted in detection of similar levels (1 to 2 μg/ml) of PGA in all mice (Fig. 5E). This result was likely reflective of the unique surface binding characteristics of PGA, which may result in its exclusion from general clearance mechanisms, or of its high molecular mass, which can reach >100 kDa (24). Unfortunately, acid-cleaved PGA loses ELISA reactivity because the antibody used to detect PGA likely recognizes the backbone structure of this molecule. Thus, clearance of smaller-molecular-weight PGA could not be assessed (data not shown).

During clearance, xenobiotics are generally processed in the liver before smaller peptides or drugs are filtered by the kidneys and excreted through the urine. Thus, to test whether impaired removal of xenobiotics from circulation was restricted to proteins, we tested the clearance of the vitamin biotin, a water-soluble small-molecule. Coinjection of biotin with ET resulted in detectable amounts of biotin in the serum of mice, and these amounts were significantly higher compared to biotin levels of animals having received PA or inactive ET (Fig. 5F).

Taken together, these data show that ET profoundly affects protein and small molecule pharmacokinetics in mice, leading to a longer half-life of bacterially expressed virulence factors crucial for pathogenesis and disease progression.

ET increases the concentration of liver and renal function markers. Altered protein pharmacokinetics are often associated with altered renal and hepatic function (41, 44, 45). Thus, we assessed serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as standard measures of liver function at 6 h after ET injection. We found both enzymes to be significantly elevated relative to PA-treated control animals (7.6- and 5.2-fold, respectively) (Fig. 6A and B). However, despite these increases, the absence of significant histopathological changes at this time point (data not shown) suggested that the levels of these enzymes were not indicative of liver failure but possibly of the initial stages of toxin-induced dysfunction.

The kidney is another organ site associated with rapid clearance of small molecules from circulation. BUN and creatinine are metabolic waste products that are cleared by the kidneys, and their increased levels in blood are indicators of renal insufficiency. Both BUN and creatinine concentrations were moderately but significantly higher (3.2- and 2.2-fold, respectively) 6 h after ET injection compared to control animals receiving PA alone (Fig. 6C and D). However, the levels for these markers were under the thresholds associated with renal disease and thus likely secondary to other ET-mediated changes, such as dehydration and necrosis. As a comparison, we also tested the effect of forskolin, a drug that activates endogenous adenylyl cyclases (46) and raises cellular cAMP concentrations. We found that BUN concentrations in forskolin-treated animals in comparison to the DMSO treated controls were significantly higher. Interestingly, in contrast to ET-treated groups, creatinine concentrations did not change in forskolin-treated animals compared to nontreated controls. Taken together, these results show that treatment of animals with ET rapidly induces altered levels of liver and kidney markers in circulation.

ET mediates activation of the renal water channel AQP-2. Aquaporin-2 (AQP-2) is a water channel present in the principal cells of the kidney collecting ducts (CDs). In response to the hormone vasopressin, cAMP concentrations within the cells increase and act via a PKA-dependent pathway to induce phosphorylation and translocation of AQP-2 from intracellular compartments to

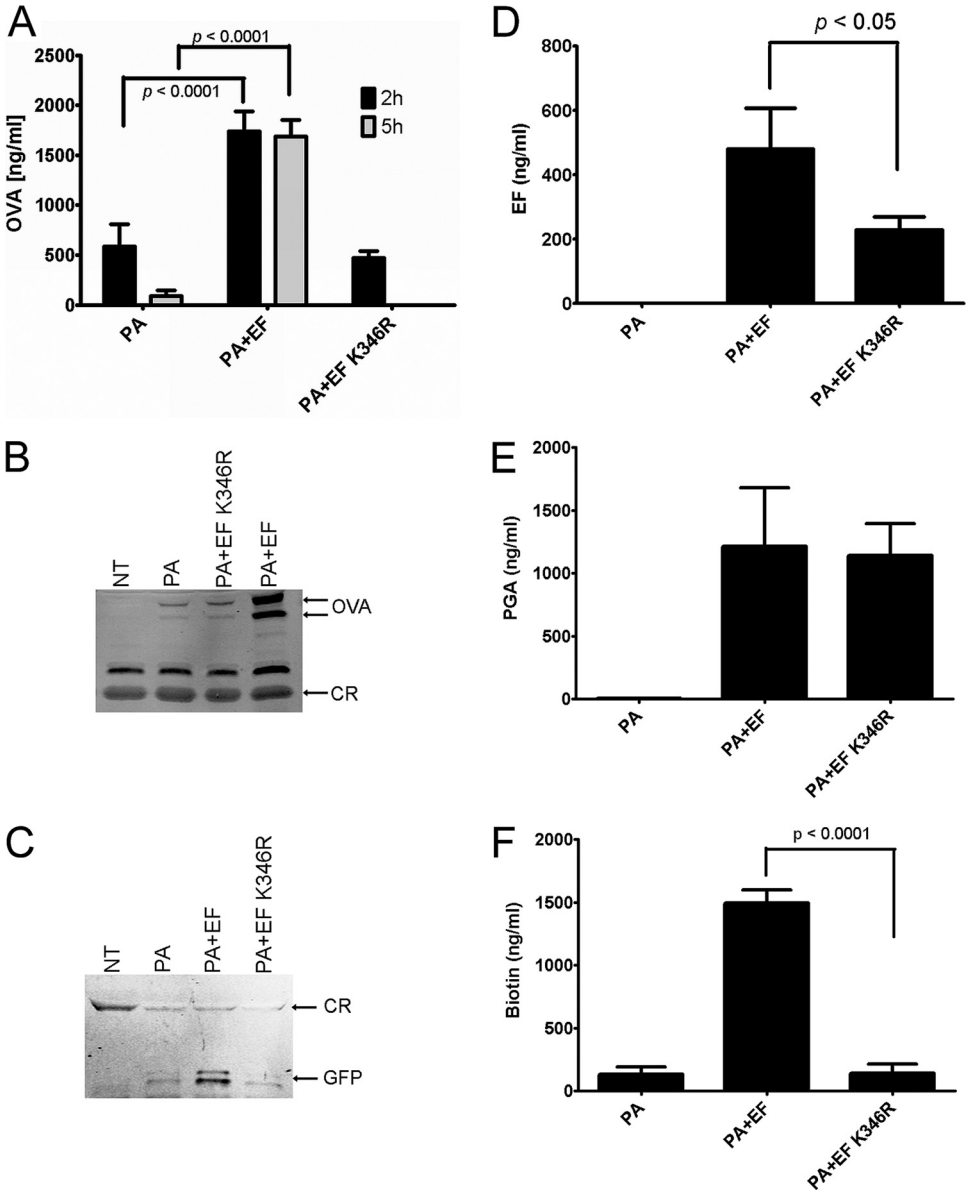

FIG 5 ET impairs protein and small molecule clearance *in vivo*. (A) Ovalbumin (OVA) levels in sera of mice ($n = 3$ per group) treated with 100 μg of OVA combined with PA, ET, or inactive ET (50 μg each) at 2 or 5 h postinjection. (B) Western analysis of OVA present in serum of toxin-treated animals after 5 h. NT, serum of animals not having received toxin and OVA treatment. "CR" indicates a resident serum protein which is cross-reactive with the anti-OVA antibody. (C) Western blot showing GFP levels in serum 6 h after injection of GFP (50 μg) in combination with PA, ET, or inactive ET (50 μg). (D) EF levels in sera of mice treated with ET or enzymatically inactive ET (50 μg) at 2 h ($n = 3$ per group) (E) Levels of polyglutamic acid (PGA) at 6 h in serum of mice treated with PA alone or with ET or inactive ET (50 μg each) in combination with 50 μg of PGA ($n = 3$ per group). (F) Biotin levels in sera of mice 6 h after injection of biotin (50 μg) in combination with either PA, ET, or enzymatically inactive ET (50 μg each) ($n = 3$ per group). All statistical differences were determined by two-tailed, unpaired Student *t* test between indicated groups.

the apical membrane, thereby allowing water to be reabsorbed (10, 37). Both anthrax toxin receptors, CMG2 and TEM8, are highly expressed in kidney cells (22), and therefore this organ is likely to be a direct target for ET. Thus, we considered that water regulation in kidneys of ET-treated mice could be impacted as a result of differential translocation of AQP-2. Animals were treated with PA, ET, or forskolin for 6 h, and the kidneys were removed and immunohistologically analyzed. Kidney sections were stained either with antibodies recognizing the C terminus of AQP-2, which stains AQP-2 present in the cytoplasm and the apical membrane of cells equally well, or with antibodies specific to the S246-phosphorylated form of AQP-2 (p-AQP-2) (11) which, if used at a proper dilution, only recognizes activated p-AQP-2. Analyses of the pelvic area of mouse kidneys showed high expression of total AQP-2 in cells of the CDs of both control and ET-treated animals (Fig. 7, upper panels). The activated, phosphorylated AQP-2 was strongly stained in both longitudinal and cross-sections of the kidneys of ET-treated animals, demonstrating a clear translocation of p-AQP-2 to the apical membrane in cells of the pelvic CD, with some staining also present in the cytoplasm (Fig. 7, lower

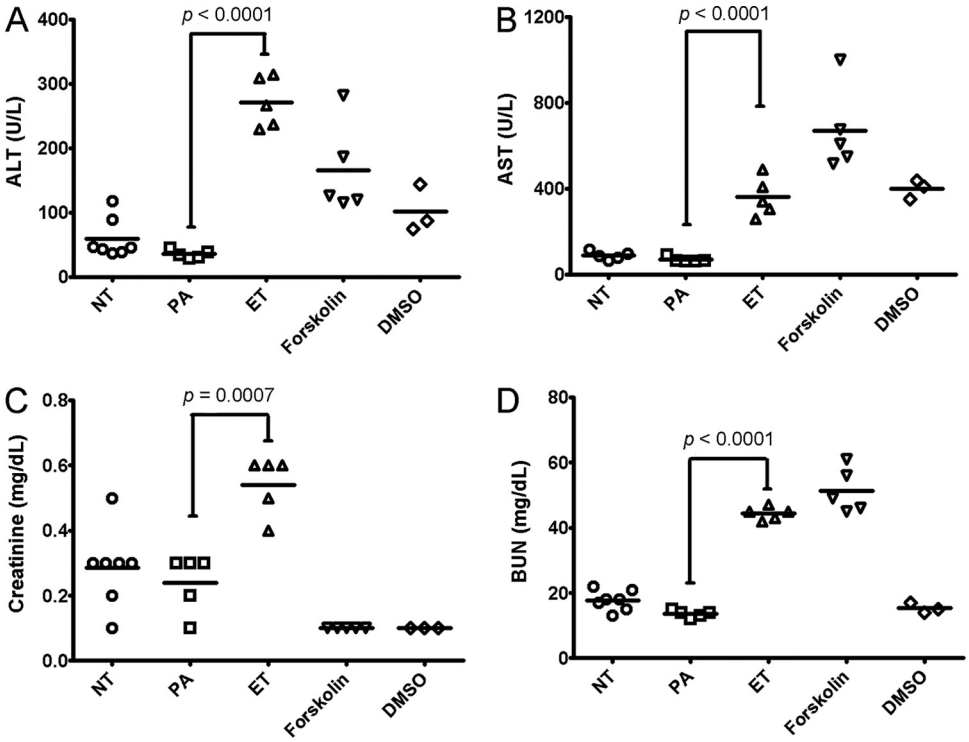

FIG 6 Measures of liver and kidney function markers increase in response to edema toxin *in vivo*. (A) Alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) creatinine, and (D) blood urea nitrogen (BUN) were measured in mice treated for 6 h with 50 μg of PA, 50 μg of ET, 75 μg of forskolin, or vehicle (75% DMSO). NT represent animals not treated. The results show pooled data of two independent experiments, and each open symbol represents one animal. Statistical differences were determined by two-tailed unpaired Student *t* test between the indicated groups.

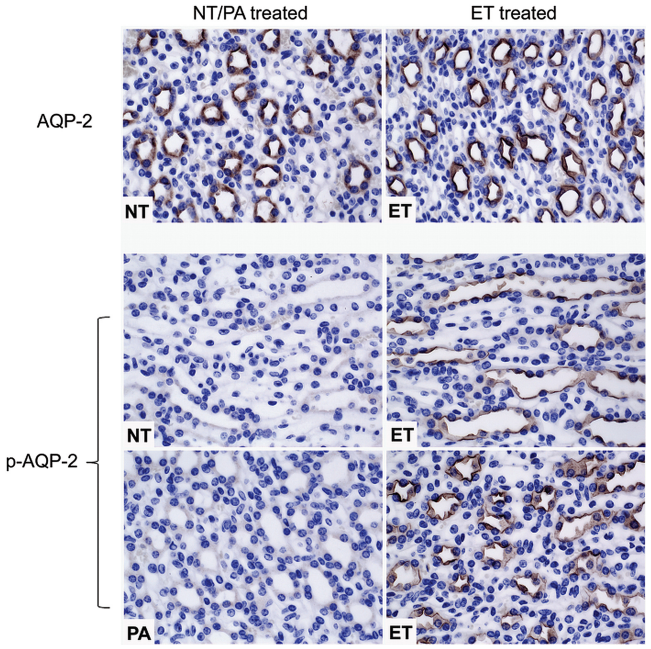

FIG 7 ET induces activation and translocation of the vasopressin-inducible water channel AQP-2. Histological analysis of the pelvic areas of kidneys retrieved from mice either not treated (NT, upper left and middle left panel), treated with either 50 μg of PA (lower left panel) or with 50 μg of ET (right panels) for 6 h. Sections were incubated with an antibody recognizing total AQP-2 (upper panel) or AQP-2 phosphorylated at serine 264 (p-AQP-2, middle panels and lower panels). Longitudinal sections are shown in the central two panels, while the upper and lower two panels show cross-sections. Magnification, ×400.

right panels). Little to no p-AQP-2 could be detected in the same cells of PA-treated or control animals compared to ET-treated animals (Fig. 7, lower left panels). Low-level staining with p-AQP-2 was observed in cells of the CDs in the cortex area of the kidney for untreated control animals (data not shown). Analysis of the renal medulla also showed distinct, but weaker staining for the activated protein (data not shown). These results show that the ET-induced increase of cellular cAMP can circumvent the vasopressin-dependent signaling pathway within the principal cells of the CD, thus resulting in translocation of the AQP-2 water channels to the apical side of the membrane, inducing water dysregulation and affecting renal function.

DISCUSSION

Most anthrax toxin studies focus on the role of LT as the major toxin causing lethality in mice (29). However, studies using EF deletion strains have shown that this toxin also contributes to virulence and lethality of spore infections; the 50% lethal dose (LD_{50}) values for EF mutants are ~10-fold higher than those of toxigenic bacteria (22, 40), and EF mutants are impaired in dissemination efficiency (8). Previous studies of ET have shown that higher doses of this toxin can induce lethality in mice, inducing both edema and hemorrhaging in a wide range of organs (12). At lower nonlethal doses, ET alters adrenal gland function, with potential consequence for responses to LT (13).

In the present study we report on a new ET effect in animals which may contribute to toxin-induced pathology in mice. Our observations that challenge of mice with ET results in higher circulating concentrations of PA both in a toxemia model and during

infection indicate that EF has additional actions other than its known contributions to the development of edema (48) and inhibition of the innate immune system (38, 52).

We considered two hypotheses that could explain the effect EF has on PA retention in circulation. First, we hypothesized that the endocytic function of cells exposed to EF was altered due to the toxin's established effects on the cytoskeleton (15, 54), thereby causing an impaired uptake and delayed removal of PA from circulation. In endothelial cells, it was shown that EF targets the Rab11/Sec15 exocyst, thereby possibly effecting receptor recycling (14), which could also contribute to a delayed PA removal from circulation. However, three types of experiments indicated that altered endocytosis was not involved in this response to ET. First, nonmyeloid cells pretreated with ET *in vitro* were unchanged in susceptibility to challenge with DT, a toxin that impairs protein synthesis following its entry into cells via clathrin-mediated endocytosis (34). Second, PA oligomer formation and endocytosis subsequent to ET treatment were not inhibited, but actually increased in nonmyeloid CHO WTP4 cells. This increase was likely due to the reported upregulation of the PA receptors in response to ET, although this upregulation was previously thought to be limited to monocyte-derived cells (25). Given the fact that expression of functional PA receptor(s) occurs on nearly all cells tested to date (29), the finding that nonmyeloid cells respond in a similar fashion to ET treatment was not surprising. Lastly, analysis of PA endocytosis in perfused organs of receptor sufficient and individual PA receptor knockout mice showed that ET-mediated upregulation of receptor binding sites occurred *in vivo* in both CMG2 and TEM8 knockout mice, as well as in myeloid-specific receptor knockout mice. Although it is possible that cAMP diffusion from neighboring cells can result in a bystander effect on the receptors present on myeloid cell populations in spleen and liver, it is unlikely that this small cell subset alone is responsible for the increased PA binding and uptake, especially since the ET-mediated effect was also observed in nonmyeloid CHO WTP4 cells. In summary, the *in vivo* experiments confirmed that impaired PA removal from circulation was not caused by the dysfunctional endocytic ability of EF-intoxicated cells. On the contrary, ET induced increased levels of PA binding and endocytosis by both anthrax receptors in whole organs.

The fact that endocytosis was not negatively affected by EF led us to a second hypothesis that the toxin altered protein clearance in mice. This hypothesis was supported by the impaired clearance of diverse and unrelated proteins OVA and GFP. Furthermore, levels of EF, a factor made by the bacterium during infection were impacted by its own enzymatic activity. Interestingly, we found that not only the clearance of large proteins, but also of a small molecule, biotin, was impaired. Thus, the ET-induced phenomenon is based on a mechanism unrelated to enzymatic function of protein-degrading liver enzymes. Furthermore, the increased levels of injected proteins was independent of general effects on fluid loss or serum concentration, since the concentrations of serum proteins were unaffected by ET in the time course of these experiments.

We also observed that impaired clearance was accompanied by significantly increased concentrations of the metabolic waste products BUN and creatinine. However, the measured levels of these products were still below levels indicative of renal dysfunction and may have been secondary to physiological events induced by ET. Furthermore, the liver, the primary organ responsible for protein metabolism, was also affected by ET as indicated by rapid increases in circulatory liver function markers. Again, the changes in liver markers were not to an extent that would indicate liver failure or dysfunction. Interestingly, injection of forskolin, a drug that raises cAMP concentrations in a nonenzymatic fashion, as a substitute for ET also increased ALT, AST, and BUN concentrations, but not those of creatinine. The differences observed between ET and forskolin might be due to the biochemically different activity; while EF is an enzyme that raises cAMP concentrations over time, forskolin acts faster, but elevated cAMP concentrations drop rapidly (43). The observation that forskolin-injected animals were sick shortly after injection but recovered quickly thereafter (data not shown) suggests that *in vivo*, forskolin acts in such a manner as well.

The fact that BUN and creatinine are clinical markers of kidney function led us to further explore kidney proteins that might be affected by EF's enzymatic activity and contribute to altered fluid homeostasis. Aquaporin water channels are highly abundant in specific kidney cells and function as transporters for water across cellular membranes (42). AQP-2, regulated by the peptide hormone arginine vasopressin, is mainly expressed in the principal cells of the collecting ducts and is important for water readsorption and urine concentration (36). Binding of vasopressin, the body's main antidiuretic hormone, to its receptor in the kidney results in elevated concentrations of cAMP via the GTP-binding protein, G_s, and the resulting cAMP exerts both short and long-term effects on APQ-2. First, cAMP-mediated phosphorylation of AQP-2 via PKA and the exchange factor directly activated by cAMP (EPAC) at four C-terminally located serines leads to insertion of AQP-2 from intracellular compartments into the apical membrane, thereby allowing water to be absorbed (2, 9). Second, cAMP directly interacts with the cAMP response element (CRE) present in the promoter of the AQP-2 gene, thus increasing transcription and ultimately protein expression (16, 26). We hypothesized that EF was likely to affect at least one of these mechanisms. We found striking differences in AQP-2 activation and apical translocation in kidneys of ET-treated animals. This activation was much more prominent than the translocation of AQP-2 induced by forskolin (data not shown), the drug that is generally used as positive control for such studies (32, 50). To our knowledge, this is the first report of a bacterial toxin affecting AQP-2 *in vivo*, but it is likely that other toxins that raise cAMP concentrations, such as CyaA of *Bordetella pertussis*, will have a similar effect. *Clostridium difficile* toxin B, a protein that ADP-ribosylates and therefore inactivates Rho-GTPases involved in organization of the coactin cytoskeleton, has been shown to inhibit AQP-2 activation in an *in vitro* model for rat inner medulla collecting duct cells (17), and this inhibition is likely to be due to the requirement of actin for the translocation process. However, it is unknown whether this toxin also exerts effects on AQP-2 *in vivo*. While the AQP-2-dependent water dysregulation and likely decrease in urine output of ET-challenged mice does not explain the prolonged retention of an 83 or 63-kDa protein (full-length and cleaved PA) in circulation, it is interesting to consider the effects this second level of renal targeting by ET could have on the pathogenesis of anthrax disease.

The mechanism for ET-mediated effects on protein and small molecule pharmacokinetics remains to be determined. Pharma-

cokinetics of all molecules in circulation are directly influenced by hepatic and renal blood flow rates. ET causes rapid hypotension and tachycardia (12), and we hypothesize that changes in the rate of blood flow through cAMP-mediated effects on vasculature may impact the circulation rates of xenomolecules through filtering organs, with consequences for their removal.

The studies presented here suggest that *B. anthracis* (and possibly other bacteria secreting adenylyl cyclase toxins) may have evolved a mechanism by which to increase the half-life of their secreted proteins in circulation. In the case of anthrax, maintaining higher levels of toxin in circulation over longer periods of time could potentially allow a higher efficacy of intoxication by maintaining toxin levels after all receptor binding sites are saturated and over the time periods required for renewed expression of functional receptors (30, 33).

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