Anthrax Edema Toxin Inhibits Endothelial Cell Chemotaxis via Epac and Rap1*

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Angiogenesis involves the assembly of endothelial cells into capillaries from a pre-existing vasculature. Because abnormal angiogenesis is a hallmark of many cancers, it is critical to find factors that control this process. Endothelial cells are enriched in the anthrax receptor; we therefore determined the effect of anthrax edema toxin (ET), an adenyl cyclase, on chemotaxis. cAMP generated by ET does not block proliferation or survival but causes cytoskeletal changes and inhibits chemotaxis by primary human microvascular endothelial cells (HMVECs). These effects are due to the action of a downstream cAMP effector, Epac, a guanine nucleotide exchange-activating protein for Rap1 (RAP1-GEF). ET induces transcription of Epac-related activators of Rap1, Epac2 (RapGEF4), and MR-GEF/RapGEF5. Similar to ET, activated Epac or Rap1 induces cytoskeletal changes and blocks chemotaxis in human endothelial cells. These results identify Epac and Rap1 as key regulators of signaling cascades leading to endothelial cell chemotaxis.

Angiogenesis, the generation of new blood vessels from previously formed blood vessels, is a process that is critical for normal development and tissue maintenance (reviewed in Ref. 1). Excessive angiogenesis can lead to a variety of disease states, including blindness, rheumatoid arthritis, AIDS complications, psoriasis, and cancer. Conversely, insufficient angiogenesis can cause strokes, heart disease, ulcers, scleroderma, and infertility. Angiogenesis occurs through a discrete series of events including proliferation of endothelial cells and chemotaxis toward pro-angiogenic signals such as vascular endothelial growth factor (VEGF). Identification of the signaling mechanisms that regulate these processes is critical for our understanding of angiogenesis.

Bacterial toxins have evolved to effectively co-opt host cell regulatory functions and have thereby become important pharmacologic tools for probing or targeting eukaryotic signal transduction pathways. Bacillus anthracis is a particularly hardy and deadly spore-forming, Gram-positive bacterium that causes anthrax. Rapidly growing anthrax bacteria secrete three factors, edema factor (EF), lethal factor, and protective antigen (PA), which cause extensive tissue damage. EF is a potent calcium- and calmodulin-dependent adenylyl cyclase that generates cAMP from cellular ATP (2). Lethal factor, a Zn2+-dependent metalloprotease (3), degrades mammalian mitogen-activated protein kinase kinases (4, 5). PA complexes with EF or lethal factor and enables cellular entry by binding to a surface anthrax toxin receptor (TEM-8 or CMG-2) (6–8). Interestingly, vascular endothelial cells are among the tissues enriched in anthrax receptor expression (6, 7). Anthrax lethal toxin has previously been shown to inhibit endothelial cell viability and tumor neo-vascularization (9, 10). However, it is not known whether anthrax edema toxin (ET) similarly acts to suppress the angiogenic process.

Edema toxin raises intracellular levels of cAMP, a second messenger that has multiple signaling targets. These include protein kinase A (PKA), cyclic nucleotide-gated ion channels, and Epac (11), a guanine nucleotide exchange factor (GEF) for Rap1. Rap1 is a member of the Ras GTPase family of signaling proteins that is converted from inactive GDP-bound states to active GTP-bound states by GEFs (reviewed in Ref. 12). Epac has been implicated in a variety of signaling cascades as an activator of Rap1 (reviewed in Ref. 13). The functions of these cascades include modulation of integrins associated with the actin cytoskeleton, promotion of cell junction formation by cadherins, and regulation of actin dynamics.

To understand the pathologic consequences of edema toxin for the endothelium at the molecular level and to realize the potential of edema toxin as a pharmacologic tool, we examined the effect of edema toxin on human microvascular endothelial cells (HMVECs). Our results show that ET or its downstream

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4 The abbreviations and trivial names used are: VEGF, vascular endothelial growth factor; EF, edema factor; Epac, exchange protein directly activated by cAMP; ET, edema toxin; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; PA, protective antigen; PKA, protein kinase A; GFP, green fluorescent protein; HMVEC, human microvascular endothelial cell; CREB, cAMP-response element-binding protein; HA, hemagglutinin; IBMX, isobutylmethylxanthine; BSA, bovine serum albumin; BCPT-2Me-cAMP, 8-(4-chlorophenylthio)-2’-O-methyl-cAMP; 6-Bnz-cAMP, N6-benzoyladenosine-3’,5’-cyclic monophosphate.
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effectors Epac and Rap1 can induce cytoskeletal changes and inhibit chemotaxis in primary human microvascular endothelial cells. These studies identify Epac and Rap1 as pivotal regulators of key steps in angiogenesis.

EXPERIMENTAL PROCEDURES

Reagents—PA was purchased from List Biological Laboratories (Campbell, CA). Alexa Fluor 594 phalloidin was from Molecular Probes (Eugene, Oregon). Epac activator 8-(4-chlorophenylthio)-2’-O-methyl-cAMP (BCEPT-2Me-cAMP) and PKA activator N6-benzoyladenosine-3’,5’-cyclic monophosphate (6-Bnz-cAMP) were from BioLog Life Science Institute (Bremen, Germany). H89 was purchased from Sigma-Aldrich. The myristoylated protein kinase A inhibitor (mPKI) (14–22) (Bremen, Germany). H89 was purchased from Sigma-Aldrich. Rabbit polyclonal antibody against phospho-CREB (Ser-133) was from Cell Signaling Technology (Beverly, MA), and rabbit polyclonal antibody against Rap1 and a mouse monoclonal antibody against α-tubulin were from Santa Cruz Biotechnology. Rat monoclonal antibody against the hemagglutinin (HA) tag (3F10) was from Roche Applied Science. Forskolin and isobutylmethylxanthine (IBMX) were from Sigma-Aldrich. Recombinant human vascular endothelial growth factor was from R & D Systems (Minneapolis, MN). EF was produced in Escherichia coli as previously described (14).

Cell Culture—HMVECs (Cell Systems, Kirkland, WA) were cultured as previously described (15, 16). For treatment with ET, cells were pretreated with PA for 15 min before adding E. coli-produced EF.

Assay of Cellular cAMP—HMVECs were seeded at 0.6 × 10⁵ cells/plate in 35-mm plates. Two days later, cells were treated with or without ET for the indicated times and lysed with 300 µl of 0.1 N HCl. 50 µl of the lysates were applied for measurement of cAMP using the Assay Designs direct cAMP assay kit, non-acetylated version (Ann Arbor, MI). The cAMP level in cells treated with ET for 24 h is a lower limit.

Actin Stress Fiber Staining—HMVECs were seeded directly on 35-mm plates at 30% confluence the day before treatment and starved in 0.1% bovine serum albumin (BSA). Cells were then treated as indicated and actin stress fibers stained with Alexa Fluor 594 phalloidin (3 units/ml) as previously described (17).

Measurement of Perimeter to Area Ratio—Images were obtained using a Nikon microscope (Mellville, NY) and OpenLab software (Improvision Inc., Lexington, MA). Outlines of 30 cells were manually traced using Image J (rsb.info.nih.gov/ij/), and the perimeter and area were calculated using this software as previously described (18).

Cell Proliferation Assay—HMVECs were seeded at 2000 cells/well in 96-well plates the day before treatment. Cells were then treated with or without ET for the indicated times. Cell proliferation was quantified by using the Promega CellTiter 96 Aqueous One Solution cell proliferation assay kit (Madison, WI).

Endothelial Cell Migration Assay—The endothelial cell migration assay was performed as previously described (19). Briefly, HMVECs were starved overnight in medium containing 0.1% BSA, harvested, resuspended into Dulbecco’s modified Eagle’s medium with 0.1% BSA, plated on the bottom side of a modified Boyden chamber (Nucleopore Corporation, MD), and allowed to attach in the inverted chamber for 2 h at 37 °C. The chamber was then reinverted, test substances added to the wells of the upper chamber, and cells were allowed to migrate for 4 h at 37 °C. Membranes were recovered, fixed, and stained and the number of cells that had migrated to the upper chamber/10 high power fields counted. Data is reported as the number of cells migrated/10 high power fields (400×). Dulbecco’s modified Eagle’s medium + 0.1% BSA served as a negative control, and VEGF was used as a positive control at a concentration of 100 pg/ml.

Microarrays—Subconfluent HMVECs (1 × 10⁶ in T25 flask) were starved overnight in EB2 medium containing 0.1% BSA and treated with various reagents as indicated. Total RNA was extracted using the Qiagen RNeasy Minikit (Valencia, CA). Hybridization was performed at the Functional Genomics Facility at the University of Chicago by using the Affymetrix Human Genome U133 plus 2.0 array. Data were analyzed with MAS5 (Applied Biosystems). Thresholds for selecting differentially expressed genes were set at a relative difference of ≥2-fold and an absolute difference of ≥100.

Relative Quantification of Epac1, Epac2, and RapGEF5 mRNA Expression Using Real-time PCR—Reverse transcription was carried out according to the two-step method by RETROscript (Ambion, Austin, TX). Briefly, mRNA was amplified with random decamer primers in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). Assays-on-demand™ for Epac1, Epac2, RapGEF5, and GAPDH were purchased from Applied Biosystems. Reactions were performed in triplicate in an ABI Prism 7300 spectrofluorometric thermocycler according to the protocol from Applied Biosystems. Analysis was performed using RQ Study software, version 1.2.3 (Applied Biosystems, Foster City, CA).

Subcloning of Rap1A63E and Rap1GAP—Rap1A63E and RapGAP cDNAs (20) were PCR-amplified using primers that added an N-terminal HA tag and then were subcloned into pGEM-T-Easy (Promega, Madison, WI). HA-Rap1A63E and HA-Rap1GAP were then subcloned into the pCDH-GFP vector (Systems Biosciences, Mountain View, CA) using the NotI restriction endonuclease (Invitrogen). DNA sequencing was performed at the University of Chicago Cancer Center DNA Sequencing Facility and revealed one synonymous mutation in Rap1GAP (GGC → GGT corresponding to codon 326).

Lentiviral Transduction of HMVECs—Production of lentivirus was performed by co-transfecting pCMV- hsv-V and pCMVΔR8.2 (21) into 293T cells along with the pCDH-GFP (empty vector control), pCDH-Rap1A63E-GFP, or pCDH-Rap1GAP using TransIT®-LT1 (Mirus, Madison, WI). Viral supernatants were collected 48 h after transfection and added to HMVECs in the presence of 8 µg/ml hexadimethrine bromide (Sigma) for 2.5 h at 37 °C. The percentage of cells expressing enhanced GFP was assessed by fluorescence microscopy and flow cytometry.

Cell Lysate Extraction and Western Analysis—Cell lysates were collected, resolved by SDS-PAGE, and analyzed by Western blots using anti-phospho-CREB (Ser-133) antibody or antitubulin antibody at a dilution of 1:1000, or anti-Rap1 at a dilu-
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FIGURE 1. ET increases cellular cAMP concentration, induces cytoskeletal changes in endothelial cells, and blocks VEGF-mediated chemotaxis but does not inhibit proliferation. A, ET increases cellular cAMP concentration. HMVECs were either left untreated or treated for 1 and 24 h with ET. Cellular cAMP was measured as described under “Experimental Procedures.” This result is representative of three independent experiments. B, treatment with ET, forskolin (FSK), or 8CPT-2Me-cAMP (but not 6-Bnz-cAMP) causes endothelial cell spreading. HMVECs were either left untreated or treated with a combination of 5 μg/ml PA + 1 μg/ml EF (ET) or individually with EF (1 μg/ml) or PA (5 μg/ml) for 1 day. HMVECs were alternatively treated with forskolin (50 μM) and IBMX (200 μM), and 6-Bnz-cAMP (100 μM) or 8CPT-2Me-cAMP (100 μM) for 1 h. Images were taken with a phase contrast microscope (400×, upper panels). Actin stress fibers were stained as described under “Experimental Procedures” and visualized by fluorescence microscopy (400×, lower panels). Selected images of single cells are shown. The ratio of perimeter to area was calculated as described under “Experimental Procedures” and plotted with statistical significance as calculated using the Wilcoxon test. Columns, mean (±S.D.); bars, S.D. These results are representative of two independent experiments. C, ET does not inhibit cell proliferation. HMVECs were either left untreated, treated with 5 μg/ml PA, 5 μg/ml PA + 1 μg/ml EF (ET), or 5 μg/ml PA + 1 μg/ml EF (ET) for the indicated time. Cell proliferation was measured by absorbance at 490 nm as described under “Experimental Procedures.” Columns, mean (±S.D.); bars, S.D. These results are representative of two independent experiments. D, ET inhibits endothelial cell chemotaxis. HMVECs were treated with 5 μg/ml PA + EF (ET) at the indicated concentrations in the presence or absence of VEGF. Selected images of single cells are shown. The ratio of perimeter to area was calculated as described under “Experimental Procedures.” Points, mean (±S.D.); bars, S.D. These results are representative of two independent experiments. E, inhibition of chemotaxis by ET is not dependent on activation of PKA. HMVECs were treated with 5 μg/ml PA + EF (ET) with the PKA inhibitors mPKI (25 μM) or H89 (10 μM) in the presence or absence of VEGF as in D. HMVECs were also treated with the PKA inhibitors in the absence of ET or with EF and PA alone as controls. Columns, mean (±S.D.); bars, S.D.

RESULTS

Initially, we determined whether ET, comprising EF and PA, was effectively taken up by endothelial cells and caused physiological changes. Consistent with the role of EF as an adenylyl cyclase, treatment of HMVECs with ET resulted in the rapid intracellular accumulation of cAMP that more than doubled by 1 day following treatment (Fig. 1A). Examination of HMVEC morphology revealed a dramatic change in shape from the normal oval morphology to a flattened morphology (Fig. 1B, upper panels). Similar results were observed upon treatment of human umbilical vein endothelial cells with ET (data not shown). This change in morphology and the underlying actin cytoskeleton is more clearly illustrated by staining actin in ET-treated versus untreated HMVECs with Alexa Fluor 594-labeled phalloidin (Fig. 1B, lower panels). To quantitate the morphologic change, we measured the perimeter to area ratio that decreases with cell spreading (Fig. 1B) (18). Taken together, these results indicate that EF can induce significant morphological and cytoskeletal changes in vascular endothelial cells.

Anthrax Edema Toxin Inhibits Chemotaxis but Not Cell Proliferation in Vascular Endothelial Cells—Given that cAMP is well known for its anti-proliferative effects, the possibility of cell growth blockage was analyzed utilizing a cell proliferation assay. Not only was there no suppression of cell growth, but ET actually appeared to enhance cellular proliferation at a low but statistically significant level (Fig. 1C). These results suggest that the changes in cell shape do not inhibit growth rate.

Following stimulation by pro-angiogenic factors, endothelial cells undergo dramatic morphological changes, including sprouting, migration, and differentiation into hollow tubes (reviewed in Ref. 24). As an in vitro measure of chemotactic migration, HMVECs were placed in a Boyden chamber, and the
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FIGURE 2. ET induces expression of Epac-related RapGEFs in endothelial cells, and EPAC activation inhibits chemotaxis in endothelial cells. A, relative expression of Epac1, Epac2, and RapGEF5 transcripts as assessed by DNA microarray analysis. HMVECs were either left untreated or treated with VEGF or VEGF + ET (5 μg/ml PA + 1 μg/ml EF) for 4 h. Total RNA was hybridized to an Affymetrix human genome U133 plus 2.0 array. The amounts of Epac1, Epac2, and RapGEF5 transcripts isolated from each treatment group were normalized to the untreated control and plotted as fold change. B, relative expression of Epac1, Epac2, and RapGEF5 transcripts isolated from each treatment group were normalized to the untreated control and plotted as fold change. C, relative expression of Epac1, Epac2, and RapGEF5 transcripts isolated from each treatment group were normalized to the untreated control and plotted as fold change. D, transcript expression for each gene was based on cycle threshold, normalized to the untreated control and plotted as fold change. Columns, mean (n = 3); bars, S.D. GADPH served as an internal control for each assay and did not vary significantly with treatment (data not shown). C, relative expression of Epac1, Epac2, and RapGEF5 after treatment with 8CPT-2Me-cAMP by real-time PCR. HMVECs were treated with VEGF, VEGF + 8CPT-2Me-cAMP (100 μM), or 8CPT-2Me-cAMP alone, and the transcript expression of each gene was assayed as in B. D, 8CPT-2Me-cAMP inhibits chemotaxis in endothelial cells. HMVECs were treated with the indicated amount of 8CPT-2Me-cAMP in the presence or absence of VEGF in modified Boyden chambers. The number of migrating cells was counted as described under "Experimental Procedures." Points, mean (n = 4); bars, S.D. These results are representative of two independent experiments. E, 6-Bnz-cAMP does not inhibit endothelial cell chemotaxis. HMVECs were treated with the indicated amounts of 6-Bnz-cAMP as in D.

number of cells that migrated toward a VEGF stimulus were quantitated. When cells were pretreated with ET, the number of cells that underwent VEGF-induced migration decreased in a dose-dependent manner with an ID₅₀ value of 0.07 μg/ml EF (Fig. 1D). All modes of migration were not inhibited by ET, however, as the fraction of cells migrating in the absence of VEGF was the same independent of ET treatment. EF or PA alone did not inhibit VEGF-mediated chemotaxis (Fig. 1E). Thus, ET specifically inhibits the chemotactic response of the endothelial cells to VEGF.

Anthrax Edema Toxin Induces Expression of Rap GEFs—To determine what genes might be involved in mediating the ET effect on VEGF-activated endothelial cell migration, RNA was isolated from HMVECs that were either left untreated, stimulated with VEGF, or stimulated with VEGF in the presence of ET. Because the effects on chemotaxis could be observed by 4 h of ET treatment, we analyzed RNA from cells after treatment for the same 4-h time period. Analysis of the expressed transcripts using oligonucleotide arrays revealed a number of genes whose expression was altered by ET. Most of these changes involved suppression of expression of genes known to be VEGF-induced (data not shown). However, there were a few examples of genes that were induced specifically by ET. Two in particular are of interest (Fig. 2A), because they code for guanine nucleotide exchange factors (GEFs) for Rap1. The cAMP-dependent RapGEF4 (also called Epac2) (25) and the cAMP-independent RapGEF5/MR-GEF (26, 27) transcripts were induced 3–7-fold by ET, as assessed by quantitative real-time PCR (Fig. 2B). This induction was not dependent on the presence of VEGF, as ET alone induced expression of RapGEF4 and RapGEF5. Quantitation of protein expression was not possible due to lack of suitable antibodies (data not shown). Stimulation with a selective agonist for Epac, 8CPT-2Me-cAMP, did not induce expression of these genes (Fig. 2C), indicating that the induction of RapGEF4 and RapGEF5 was mediated by ET but not via Epac/Rap1. This induction was not a generalized effect, as no similar enhancement of RapGEF3 (Epac1) transcription was observed. Because RapGEFs activate Rap isoforms, these results suggest that...
2Me-cAMP (28, 29). Similarly, staining of actin filaments in the HMVECs with Alexa Fluor 594-labeled phalloidin after treatment with 8CPT-2Me-cAMP more clearly depicted these cytoskeletal changes (Fig. 1B).

Because endothelial cell migration toward chemotactic factors is a key feature of angiogenesis, we determined whether Epac activation similarly blocks chemotaxis. 8CPT-2Me-cAMP inhibited VEGF-stimulated HMVEC migration with an ID₅₀ of 0.2 μM with complete inhibition at 2.5 μM (Fig. 2D). Again, no suppression of nonspecific cell migration was observed. In contrast, treatment of HMVECs with 6-benzoyl cAMP, a selective activator of PKA, had no effect on endothelial cell migration (Fig. 2E). If PKA mediates the inhibition of chemotaxis, then PKA inhibitors should rescue the 8CPT-2Me-cAMP-mediated phenotype. Therefore, to address this possibility, we also tested the effect of two PKA inhibitors, a cell-permeable myristoylated PKA inhibitor peptide mPKi and the chemical inhibitor H89, on ET-mediated inhibition of chemotaxis (Fig. 1E). Although both the myristoylated PKA inhibitor peptide and H89 inhibited chemotaxis to some degree in the absence of ET, neither PKA inhibitor was able to rescue the inhibition of chemotaxis by ET. Presumably the inhibition by H89 reflects its inhibition of Rho kinase (30), a kinase required for endothelial cell chemotaxis (data not shown). These results indicate that specific Epac activation (but not PKA) can mimic ET-induced suppression of VEGF-induced chemotaxis.

**Rap1 Is Activated by ET and 8CPT-2Me-cAMP**—If ET and 8CPT-2Me-cAMP promote these cell adhesion-related effects by activation of a Rap1 GEF, then both of these agents should stimulate Rap1 in HMVECs. The Epac activator 8CPT-2Me-cAMP is a robust stimulator of Rap1 as shown by the pulldown assay for GTP-bound Rap1 (Fig. 3A). Stimulation with ET at a concentration that blocks chemotaxis in HMVECs also induces Rap1 activation that is maintained for at least 30 min. Conversely, 6-Bnz-cAMP was not able to activate Rap1, whereas forskolin caused a significant activation of Rap1 (Fig. 3B). To confirm that the Epac activator was not stimulating PKA, we analyzed a substrate of PKA, phosphorylated CREB, in the treated cells. In contrast to forskolin, which robustly stimulated CREB phosphorylation, the 8CPT-2Me-cAMP activator of Epac did not induce significant CREB phosphorylation (Fig. 3C). These results suggest that Rap1 activation can account for the observed physiological effects of ET and 8CPT-2Me-cAMP on vascular endothelial cells.

**Constitutively Activated Rap1 Blocks Chemotaxis**—The previous results suggest that inhibition of chemotaxis is mediated by Epac rather than by PKA or other downstream effectors of cAMP. To test whether activated Rap1 is indeed sufficient to inhibit chemotaxis, HMVECs were transduced with a lentivirus expressing the activated Rap1A63E mutant as well as a GFP marker. Over 95% of the cells expressed GFP by 3 days as assessed by fluorescence microscopy or flow cytometry (data not shown), and the exogenous Rap1 was readily detected by immunoblotting (Fig. 4A). The Rap1-expressing cells also exhibited cell spreading and actin changes (Fig. 4B). Measurement of the perimeter to area ratio in Rap1-expressing cells showed a significant decrease, similar to that seen in cells treated with 8CPT-2Me-cAMP (Fig. 4C). Analysis of the mutant Rap1-expressing HMVECs in a chemotaxis assay showed a potent suppression of VEGF-induced chemotaxis (Fig. 4D). To assess the requirement for Rap1 in 8CPT-2Me-cAMP action, we also transduced cells with lentivirus-expressing Rap1GAP. Consistent with previous results, cells expressing Rap1GAP rounded up (Fig. 4B); however, robust Rap1GAP expression caused loss of matrix attachment, preventing further analysis (data not shown). Taken together, these results demonstrate that Rap1 is sufficient to suppress VEGF-induced chemotaxis in HMVECs and implicates Rap1 in the morphological changes induced by Epac activators.

**DISCUSSION**

The results we have obtained indicate that treatment of endothelial cells with either edema toxin, an adenylyl cyclase, or activators of Epac, a GEF for Rap1, causes cytoskeletal changes and inhibition of VEGF-induced chemotaxis in response to angiogenic factors in human microvascular endothelial cells. We have further demonstrated that activated Epac and Rap1 are effective inhibitors of endothelial cell chemotaxis. The link between ET and Epac is strengthened not only by the fact that Epac is activated by cAMP, the only known product of ET, but also because ET rapidly induces an increase in the transcription of Rap1GEFs, thereby amplifying the signaling through Rap1. These results identify Epac and Rap1 as central mediators of vascular endothelial cell chemotaxis.

The ability of Epac activators to block chemotaxis is consistent with the role of Rap1 in regulating cellular adhesion and...
spreading processes. The results of our chemotaxis assays in HMVECs indicate that Rap1 activation does not inhibit the process of migration per se but instead prevents directed movement toward the angiogenic attractant VEGF. These results contrast with a recent study demonstrating that Rap1 activation via the Rap1-associating molecule RapL is required for directed migration and wound healing in human umbilical vein endothelial cells and human aortic endothelial cells (31). The difference in these results may be due to the different chemoattractants (VEGF versus factors released by wounding in culture) that are reflective of these two different processes as well as the specific cells used. However, it should be noted that the cytoskeletal changes we observed in HMVECs upon treatment with ET or 8CPT-2Me-cAMP were also observed in human umbilical vein endothelial cells (Refs. 23 and 31 and data not shown). Furthermore, we did not detect apoptotic induction by 0.3 mM 8CPT-2Me-cAMP in endothelial cells even in low serum conditions (data not shown), in contrast to some anti-angiogenic agents such as anthrax lethal toxin (9, 10) and thrombospondin-1 (32, 33) that promote endothelial cell death via apoptosis. Activation of the Epac/Rap1 pathway does not appear to inhibit VEGF signaling by inhibiting its receptor. Results from our laboratory have demonstrated that VEGF-mediated phosphorylation of ERK1,2 is not blocked by activation of Epac/Rap1.5 Thus, the inhibitory effects of ET and Epac on vascular endothelial cells appear to be related to cytoskeletal-based processes such as cellular morphology and migration rather than proliferation and cell survival.

The Epac activator appeared to be a more potent inhibitor of chemotaxis than constitutively activated Rap1. Possible explanations relate to the cellular localization of Rap1 and the specific cell type. A recent study by Scott and co-workers identified a complex linking Epac and Rap1 to A-kinase anchoring proteins in muscle cells, and the AKAP acted as a scaffold that bound other interacting signaling molecules such as PKA and cAMP phosphodiesterase (34). The localization of Epac2 can also influence its ability to activate specific pools of Rap1 (35). It is likely that the active Rap1 mutant does not interact as efficiently as wild type Rap1 with its normal targets. Similarly, Stork and co-

5 R. C. Doebele, F. Schulze-Hoepfner, J. Hong, M. W. Lingen, B. Zeitlin, J. E. Nor, Y. Liu, M. Abe, O. Volpert, and M. R. Rosner, manuscript in preparation.
workers (36) noted multiple pools of Rap1 that function differently dependent upon their activators. The cell flattening effect observed with ET and cAMP in HMVECs is distinct from its effects on the morphology of other cell types such as fibroblasts and embryonic kidney cells, which round up via a PKA-dependent mechanism (37). This difference in response is not a function of higher anthrax toxin receptor expression in endothelial cells (Ref. 37 and data not shown) but rather the different signaling systems activated in endothelial versus other cell types.

Previous studies of Epac and Rap1 have implicated a number of downstream signaling targets that regulate actin remodeling (reviewed in Ref. 13) and are undoubtedly contributing factors to the mechanism by which they suppress endothelial cell chemotaxis. Recent reports have indicated that Epac/Rap activation can promote redistribution of cortical actin to endothelial cell junctions, thereby recruiting functional molecules such as vascular endothelial (VE)-cadherin and decreasing endothelial cell permeability (23, 31, 38). We also observed cell aggregation and clustering along with inhibition of long range network formation following ET treatment of endothelial cells (data not shown). Because VEGF promotes increased endothelial cell permeability, these studies provide another means by which Epac and Rap1 could function as angiogenic antagonists.

Because Epac is anti-chemotactic and a target of ET action, our results suggest that suppression of angiogenesis is a normal physiological consequence of anthrax infection. Both anthrax edema and lethal toxins should act rapidly and synergistically to shut down angiogenesis completely. Lethal toxin (by inhibiting mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)) blocks endothelial cell proliferation and survival (9), and edema toxin (by preventing chemotaxis) inhibits the initial stages of the angiogenic process. When injected subcutaneously, edema toxin causes local edema around the injection sites, a phenotype indirectly attributed to the ability of EF to regulate cytokine production in monocytes (39). This edema precluded the ability to determine whether ET suppresses angiogenesis in mice, because administration of the toxin even at lower doses caused extensive edema and swelling in the mouse skin of the implant (data not shown). Edema formation is likely due in part to activation of PKA, ion channels, or other unidentified targets of cAMP action, because mice treated with the Epac activator 8CPT-2Me-cAMP alone did not exhibit swelling at the injection sites (data not shown). The combined loss of the endothelial cell vasculature and the rapid influx of fluid into infected areas could serve to rapidly diffuse anthrax bacteria throughout the tissue.

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