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Anthrax Lethal Toxin Disrupts the Endothelial Permeability Barrier Through Blocking p38 Signaling

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Abstract

Exposure to anthrax causes life-threatening disease through the action of the toxin produced by the *Bacillus anthracis* bacteria. Lethal factor (LF), an anthrax toxin component which causes severe vascular leak and edema, is a protease which specifically degrades MAP kinase kinases (MKK). We have recently shown that p38 MAP kinase activation leading to HSP27 phosphorylation augments the endothelial permeability barrier. We now show that treatment of rat pulmonary microvascular endothelial cells with anthrax lethal toxin (LeTx), which is composed of LF and the protective antigen, increases endothelial barrier permeability and gap formation between endothelial cells through disrupting p38 signaling. LeTx treatment increases MKK3b degradation and in turn decreases p38 activity at baseline as well as after activation of p38 signaling. Consequently, LeTx treatment decreases activation of the p38 substrate kinase, MK2, and the phosphorylation of the latter's substrate, HSP27. LeTx treatment disrupts other signaling pathways leading to suppression of Erk-mediated signaling, but these effects do not correlate with LeTx-induced barrier compromise. Overexpressing phosphomimicking (pm)HSP27, which protects the endothelial permeability barrier against LeTx, blocks LeTx inactivation of p38 and MK2, but it does not block MKK3b degradation or Erk inactivation. Our results suggest that LeTx might cause vascular leak through inactivating p38-MK2-HSP27 signaling and that activating HSP27 phosphorylation specifically restores p38 signaling and blocks anthrax LeTx toxicity. The fact that barrier integrity could be restored by pmHSP27 overexpression without affecting degradation of MKK3b, or inactivation of Erk, suggests a specific and central role for p38-MK2-HSP27 in endothelial barrier permeability regulation.

The mechanism by which exposure to anthrax induces severe vascular leak is not well understood. Anthrax virulence has been ascribed to the toxin produced by the bacteria since antibiotics that eliminate the bacteria are of limited use once sufficient toxin has been produced. Anthrax toxin has been associated with lethal pulmonary edema (Kuo et al., 2008) that is accompanied by vascular leak at multiple organ systems (Moayeri et al., 2003). The toxin is composed of three polypeptides. The first, called edema factor (EF) is an adenylate

cyclase that produces excessive cAMP, the second, called LF is a zinc metalloprotease which is most associated with anthrax virulence (Leppa, 1982; Pezard et al., 1991; Duesbery et al., 1998; Vitale et al., 2000), and the third, called protective antigen (PA), combines with the other components to facilitate their entry into cells. LF proteolyses MAP kinase-activating kinases (MAPKKs or MKKs), which are its only known substrates (Duesbery et al., 1998; Vitale et al., 2000). Injection into animals of LF and PA, known together as LeTx, produces most of the characteristics of anthrax infection (Moayeri et al., 2003). Although EF is associated with edema, it appears to act indirectly through inflammatory mediators (Tessier et al., 2007). LF (or LeTx), on the other hand, is strongly associated with vascular leak which occurs independently of its effects on macrophages and endothelial apoptosis (Moayeri et al., 2003). It has also been reported to directly affect endothelial cells causing endothelial barrier permeability (Moayeri et al., 2003). Yet the signaling pathways that might mediate LeTx effects on endothelial permeability have not been well characterized.

We have recently described how permeability barrier regulation can occur through a tight biological control system where stimuli such as hypoxia activate signaling through the Rho-kinase (ROCK)-myosin light chain (MLC) phosphatase pathway which leads to weakening of the barrier and increased permeability (An et al., 2005; Liu et al., 2009, 2010b). At the same time, and independently of the former signaling pathway, hypoxia activates p38 signaling leading to HSP27 phosphorylation which causes barrier augmentation and decreased permeability (An et al., 2005; Liu et al., 2009, 2010b). In a controlled situation, increasing endothelial permeability allows blood components to enter the site of injury, but the response is limited and reversed through activating barrier augmenting signaling. Identification of LF as a protease with specificity towards MAP kinase-activating kinases (Vitale et al., 2000; Liang et al., 2004) suggested several molecular actions of this component. In particular, its ability to proteolyze p38 MAP-kinase, kinase has been implicated in its ability to induce macrophage apoptosis and inhibit glucocorticoid receptor function (Park et al., 2002; Webster et al., 2003). Moreover, blocking p38-induced HSP27 phosphorylation has been implicated in reducing actin-based motility in human neutrophils (During et al., 2007). Despite evidence that LeTx (LF + PA) causes vascular leak independently of its effects on macrophages and endothelial apoptosis (Moayeri et al., 2003), the role of the p38-MK2-HSP27 pathway has not been investigated thoroughly in endothelial cells. We postulated that LeTx will perturb the permeability control system by blocking the activation of the p38 kinase barrier augmenting pathway. In this article, we describe how LeTx weakens the endothelial permeability barrier properties of pulmonary microvascular endothelial cells. We also link this effect specifically to its ability to block activation of p38 signaling.

Materials and Methods

Materials and reagents

Media and supplements (RPMI), fetal bovine serum (FBS), penicillin G potassium, streptomycin, fungizone, and glutamine were purchased from Invitrogen (Carlsbad, CA). Anthrax lethal factor (LF) and PA were purchased from Calbiochem (La Jolla, CA). The

anthrax lethal toxin (LeTx) used in the experiments was prepared by mixing equal concentrations of LF and PA before treatment. TGF β was purchased from R&D (Minneapolis, MN).

Cell culture

Rat pulmonary microvascular endothelial cells (RPMEC) were cultured in RPMI containing 10% FBS, penicillin, streptomycin, fungizone, and glutamine at 37°C in humidified air containing 5% CO₂. These cells were a gift from Dr. Una Ryan (Avant Immunotherapeutics, Needham, MA) and have been well characterized by us and others (Cote et al., 1996). RPMEC were passaged in 0.25% trypsin—0.02% EDTA solution, and 1 day prior to the experiments, cells were maintained in serum-free media. pmHSP27-RPMEC were generated by transfecting RPMEC with the pcDNA3-HSP27PM (Rogalla et al., 1999) to express HSP27 in which the residues, S15D, S78D, S82D, were mutated to mimic phosphorylated HSP27 (pmHSP27). Stable transfectants were made as we described earlier (Kayyali et al., 2002). For hypoxic exposure, cells were placed in humidified airtight incubation chambers (Billups-Rothenberg, Del Mar, CA) and gassed with 3% O₂, 5% CO₂, balanced N₂. The hypoxic chambers were kept at 37 °C in a tissue culture incubator. For normoxic exposure, cells were maintained at 37°C in humidified air containing 5% CO₂.

Assessment of endothelial permeability

Cells were seeded on Falcon® HTS FluoroBlok™ 1.0 μ m Inserts (Becton Dickinson, Bedford, MA), and cultured to obtain a confluent monolayer. After starvation with serum-free medium for 24 h, apical medium was replaced with 0.35 ml phenol red-free medium containing fluorescent Alexafluor-488-dextran (3 kDa, Molecular Probes, Eugene, OR), and the basolateral chamber was filled with 0.9 ml phenol red-free medium without Alexafluor-488-dextran. The intensity of fluorescence in the basolateral chamber was read from the bottom with treatments of control or LeTx using a Tecan plate reader maintained at 37°C.

Labeling of plasma membrane and nucleus

In order to determine alterations in the gaps between cells under different treatments, RPMECs were grown on collagen-coated glass coverslips. Upon confluence cells were serum-starved overnight before being treated with LeTx. Next, the coverslips were rinsed twice with phosphate-buffered saline (PBS), and fixed for 10 min with 4% formaldehyde. The coverslips were then stained using the Image-iT LIVE Plasma Membrane and Nuclear Labeling Kit (Molecular Probes) according to the manufacturer's instructions. Cells were examined using a Zeiss Fluorescence Microscope and imaging system with 40 $\times$ objective lens.

SDS-PAGE and immunoblotting

Cells were lysed in RIPA or Triton buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1% Triton X-100, 50 mM NaF plus 1% Calbiochem Protease Inhibitor Cocktail) and lysates were assayed for protein using the Bradford protein assay and then diluted with 2 $\times$ Laemmli

loading buffer for SDS-PAGE. Equal amounts of protein were then loaded in 4–20% Tris/glycine gels, and electrophoresed for 90 min at 130 V constant voltage. Next, the gel was blotted onto a PVDF membrane by electrophoretic transfer at 25 V constant voltage overnight. The membrane was then washed, blocked with 5% milk, and probed with primary antibodies. Appropriate secondary antibodies conjugated to horseradish peroxidase (Pierce, Rockford, IL), and a chemiluminescent substrate (SuperSignal; Pierce) were used to visualize immunoreactive bands. The primary antibodies against phospho-p38, total p38, phospho-MK2, total MK2, phospho-ERK, total ERK, phospho-HSP27, and MKK3b were purchased from Cell Signaling (Danvers, MA), antibody against total HSP27 was purchased from Santa Cruz (Santa Cruz, CA). Donkey anti-mouse and donkey anti-rabbit secondary antibodies were purchased from Pierce.

Coimmunoprecipitation

Coimmunoprecipitation experiments involving phospho-p38 and HSP27 were performed using Pierce Crosslink Immunoprecipitation Kit (Pierce) according to the manufacturer's instructions. Briefly, cells were incubated overnight in medium without serum. After treatment, cells were harvested and lysed in buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton, 50 mM NaF, 1 mM Na₃VO₄, 1 mM β -glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.25 mM PMSF, 0.5 g/ml leupeptin) and the lysates were cleared by centrifugation (10,000 g for 10 min) at 4°C. Supernatants were transferred to new tubes for protein concentration determination and normalization. Then samples were transferred into columns filled with antibody-crosslinked Protein A/G Plus Agarose and incubated overnight at 4°C. After elution samples were analyzed with Western blotting as described above.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistically significant differences were determined by ANOVA and Holm-Sidak post hoc analysis. Statistical analysis was carried out using SigmaStat (Systat, San Jose, CA) $P < 0.05$ was considered statistically significant.

Results

Anthrax LeTx weakens the endothelial permeability barrier and induces gap formation in endothelial monolayer

LeTx has been shown to induce vascular leak in animals and has been described to decrease endothelial barrier electrical resistance and increase the flux of labeled albumin across endothelial cell monolayers (Warfel et al., 2005). Since the lung is a major target of anthrax we investigated the effects of LeTx on pulmonary microvascular endothelial cells which are believed to be the major contributors to interstitial and pulmonary edema. The flux of fluorescent-dextran across confluent RPMEC exposed to different treatments was monitored continuously as we described in Materials and Methods section (Liu et al., 2009). As shown in Figure 1A, LeTx treatment (2 μ g/ml) increased the permeability of the endothelial monolayer barrier by 300%. We next examined if LeTx induces gap formation between endothelial cells. Using a membrane stain to delineate the borders of cells as we described earlier (Liu et al., 2009, 2010b), we observed that LeTx treatment induced gap formation

between endothelial cells that were virtually absent in control cells (Fig. 1B). We have observed similar gap formation in response to stimuli that we have previously shown to increase endothelial barrier permeability (Liu et al., 2009, 2010b). Unlike a previous report that described changes in permeability to be most significant at 72 h of treatment (Warfel et al., 2005), the increase in permeability and gap formation in response to LeTx we observed occurred rapidly within an hour of treatment. That time-course corresponded with the signaling changes we describe below.

LeTx disrupts p38 signaling in wild-type RPMEC

Identification of anthrax LF as a protease with specificity towards MAP kinase-activating kinases (Vitale et al., 2000; Liang et al., 2004) suggested several molecular actions of this component. LF is strongly associated with vascular leak (Moayeri et al., 2003), and despite mounting evidence that LeTx targets endothelial cells, the signaling pathways that specifically mediate its actions have not been investigated thoroughly in these cells. Since we have shown that p38-MK2-HSP27 signaling plays an important role in permeability barrier augmentation (Liu et al., 2009, 2010b), we investigated its modulation by LeTx in RPMEC. As shown in Figure 2, LeTx treatment (2 μ g/ml for 30 min) reduced p38 phosphorylation (activation) in RPMEC (Fig. 2A,B). In addition LeTx blocked the activation of p38 by agents that we have previously described to activate p38 signaling in RPMEC (Liu et al., 2009, 2010b) such as hypoxia (3% O₂ for 1 h) or TGF β (1 ng/ml for 15 min). Furthermore, LeTx treatment blocked the phosphorylation (activation) of MK2 (Fig. 2C,D), and its substrate HSP27 (Fig. 2E,F) at baseline, or after activation with hypoxia or TGF β . These results demonstrate that LeTx treatment of RPMEC blocks the p38-MK2-HSP27 pathway which we have previously described to stabilize the actin and vimentin cytoskeleton, increase adhesive forces and augment the endothelial permeability barrier (An et al., 2005; Liu et al., 2009, 2010b).

pmHSP27 overexpression blocks the effects of LeTx on permeability and gap formation and salvages p38 signaling

We have previously described that overexpression of a phosphomimicking form of HSP27 (pmHSP7) in RPMEC stabilizes the actin and vimentin cytoskeleton and augments the endothelial permeability barrier (An et al., 2005; Liu et al., 2009, 2010b). Since we hypothesized that LeTx weakened the permeability barrier via blocking p38-MK2-HSP27 signaling, we tested if RPMEC stably transfected with pmHSP27 (pmHSP27-RPMEC) would be resistant to LeTx. As shown in Figure 3A, treatment of pmHSP27-RPMEC with LeTx (2 μ g/ml) only caused insignificant increase (~25%) over control in the flux of 3 kDa alexafluor-488-dextran. Thus, compared to wild-type RPMEC in which LeTx caused a 300% increase in permeability (Fig. 1A), pmHSP27-RPMEC appeared relatively resistant to its permeability-inducing effect. Consistent with this finding, when we stained pmHSP27-RPMEC with the membrane fluorescent dye, we observed that LeTx did not induce gap formation between endothelial cells (Fig. 3B) as it did in wild-type RPMEC (Fig. 1B). These results suggest that overexpressing pmHSP27 in endothelial cells protects them against LeTx-induced permeability.

We next examined whether pmHSP27 overexpression in RPMEC altered the signaling effects induced by LeTx treatment. First, we examined p38 signaling at baseline and after treatment with hypoxia or TGF β . Unlike in wild-type RPMEC, LeTx treatment (2 μ g/ml for 30 min) did not appear to reduce p38 phosphorylation (activation) in pmHSP27-RPMEC (Fig. 4A,B). In addition LeTx did not block the activation of p38 by hypoxia (3% O₂ for 1 h) or TGF β (1 ng/ml for 15 min) as it did in wild-type RPMEC. Moreover, LeTx treatment did not block the phosphorylation (activation) of MK2 (Fig. 4C,D), and its substrate HSP27 (Fig. 4E,F) at baseline, or after activation with hypoxia or TGF β (LeTx appeared to have some effect on TGF β -induced MK2 phosphorylation, but that inhibition was not statistically significant and not as drastic as the effect on wild-type RPMEC). These results demonstrate that overexpression of pmHSP27 protects p38-MK2 signaling from the effects of LeTx.

pmHSP27 overexpression does not alter MAPKK degradation or Erk inactivation by LeTx

Since LeTx has been described to inactivate MAP kinase signaling by proteolyzing upstream activating kinases, we evaluated whether pmHSP27 overexpression salvages p38-MK2-HSP27 signaling by blocking that step. We also tested if the effects on p38-MK2-HSP27 are specific or pmHSP27 overexpression affected other MAP kinases as well. First, we established that LeTx treatment indeed degrades MAP kinase kinases (MKK). As shown in Figure 5 LeTx treatment (2 μ g/ml for 30 min) reduced the band intensity corresponding MKK3b, a MAPKK that phosphorylates and activates p38 MAP kinase. The reduction in the level of MKK3b was observed after treatment of RPMEC with hypoxia or TGF β (Fig. 5A,B). These results are consistent with the effect of LeTx on p38-MK2-HSP27 signaling in RPMEC signaling being due to its degradation of MAPKKs such as MKK3b.

We next tested if LeTx treatment also affects other MAP kinases, such as Erk. As shown in Figure 5C,D, LeTx treatment (2 μ g/ml for 30 min) reduced the activation (phosphorylation) of Erk, indicating that as expected other MAP kinase signaling pathways are also blocked by LeTx treatment on endothelial cells.

Since pmHSP27 overexpression protected endothelial cells from the LeTx-induced permeability and gap formation and salvaged p38-MK2-HSP27 signaling, we tested if it did the latter by preventing the degradation of p38 MAPKK, MKK3b. Thus, when pmHSP27-RPMEC were treated with LeTx (2 μ g/ml for 30 min) we observed that MKK3b was degraded both at baseline and after stimulation with hypoxia or TGF β (Fig. 6A,B). These results indicate that pmHSP27 overexpression does not interfere with the MAPKK protease activity of LeTx, and that it stabilizes p38-MK2-HSP27 signaling and protects the endothelial permeability barrier via other mechanisms. Consistent with these results we observed that LeTx still caused inactivation of Erk in pmHSP27-RPMEC. As shown in Figure 6C,D, LeTx treatment blocked the activation (phosphorylation) of Erk in pmHSP27-RPMEC at baseline and after stimulation with hypoxia or TGF β . The latter finding also suggests that Erk inactivation by LeTx is not as important to permeability barrier regulation as p38 inactivation since pmHSP27-RPMEC were resistant to permeability-inducing effects of LeTx.

pmHSP27 overexpression does not alter interaction of p38 with HSP27

Since stabilizing of p38-MK2-HSP27 signaling by pmHSP27 over expression does not seem to occur upstream of p38 (e.g., activation of MKK3b or protecting it from proteolysis), we tested if it alters the interaction of p38 and HSP27 by co-immunoprecipitation. Thus, we immunoprecipitated HSP27 and probed the immunoprecipitate with antibodies against total p38 or phospho-p38. As shown in Figure 7, there were no significant differences in the amount of total or phospho p38 co-immunoprecipitating with HSP27.

Discussion

Better understanding of mechanisms by which anthrax toxin acts is important for better management of the disease and the results described in this report explore one mechanism by which that toxin can cause vascular leak. Anthrax is a particularly lethal disease because the infection caused by the *Bacillus anthracis* bacteria can develop asymptotically until severe injury and tissue damage develop. That injury is believed to be mostly due to the effects of the anthrax toxin since antibiotics that eliminate the bacteria are of limited use once sufficient toxin has been produced. Furthermore introducing the anthrax toxin in model animals causes responses that mimic the anthrax infection. The LF component of the anthrax toxin has been most associated with anthrax virulence (Pezard et al., 1991). Unlike the EF component, which is not believed to act directly on endothelial cells (Tessier et al., 2007), LF is strongly associated with vascular leak which occurs independently of its effects on macrophages and its ability to cause endothelial apoptosis (Moayeri et al., 2003). The combination of LF and PA, LeTx, has been shown to produce most of the characteristics of anthrax infection, and has been reported to directly affect endothelial cells leading to endothelial barrier permeability (Moayeri et al., 2003). Yet there are only few studies that link LeTx effects on endothelial barrier permeability to its effects on cell signaling. One study linked LeTx effects on endothelial permeability to redistribution of cadherin and actin, but the reported effects on permeability and the cytoskeleton were mostly at 72 h after treatment of cultured cells (Warfel et al., 2005), a surprisingly long time since LeTx has been reported to cause vascular leak rapidly after injection into animals. Moreover, that study could not find a strong link between the reported effects and MAP kinase signaling. A more recent study focused on cooperative effects of LF and EF on endocytic recycling but this study did not show a direct effect of LF or LeTx on endothelial barrier function (Guichard et al., 2010). Although components of the anthrax toxin are known to target inflammatory cells, recent evidence suggests that this action is important for establishing the infection as animals with impaired myeloid cells or lacking neutrophils were resistant to anthrax infection but not the lethality of the anthrax toxin, which likely results from the toxin's direct action on endothelial cells (Bradley and LeVine, 2010; Liu et al., 2010a). Hence we launched the studies described above to link LeTx-induced signaling to its effects on the endothelial barrier permeability.

Our laboratory has investigated the regulation of the endothelial permeability barrier and pulmonary edema by hypoxia. Using pulmonary microvascular endothelial cells we have found that barrier regulation occurs through a tight biological control system where hypoxia activates signaling through the Rho-kinase-MLC phosphatase-MLC pathway which leads to

increased endothelial cell contractility, weakening of the barrier and increased permeability (An et al., 2005; Liu et al., 2009, 2010b). At the same time, and independently of the former signaling pathway, hypoxia activates p38 MAP kinase signaling leading to HSP27 phosphorylation which causes barrier augmentation and decreased permeability. This type of control is beneficial when biological systems encounter physiological or pathological stimuli (Liu et al., 2009, 2010b). In a properly functioning physiological control system increasing endothelial permeability in response to a stimulus allows blood components to enter the site of injury, but the permeability effect is limited by that stimulus also triggering an endothelial response that ensures reversibility through activating barrier-augmenting signaling. Many studies have focused on the signaling pathways activated by permeability-inducing agents that weaken the barrier and increase permeability, for example, endothelial cell contraction via ROCK. However, increased permeability can also arise in response to disruption of signaling pathways which augment the barrier. Our results indeed demonstrate that LeTx has a direct effect on endothelial barrier permeability that correlates with p38-MK2-HSP27 signaling. First, we demonstrated that LeTx treatment rapidly causes increased flux of fluorescent dextran across an endothelial monolayer and increases intercellular gap formation (Fig. 1). At the same time, LeTx blocked p38 signaling at baseline and in response to hypoxia or TGF β as shown by reduction in phosphorylation of p38, MK2, and HSP27 (Fig. 2). Consistent with its reported MAPKK-specific protease activity we observed that LeTx decreased the amount of detectable MKK3b, the kinase that phosphorylates and activates p38 MAP kinase (Fig. 5). Nevertheless, LeTx destroys other MAPKKs leading to inhibition of other downstream targets such as Erk. Therefore, we tested if the effect on permeability is related to potential LeTx effects on Erk activation. As shown by Figure 5, LeTx treatment indeed blocked Erk activation in endothelial cells. When we tested cells that overexpress pmHSP27 which we have previously described to be resistant to permeability-inducing agents (Liu et al., 2009, 2010b), we observed that they are resistant to LeTx-induced permeability and intercellular gap formation as well (Fig. 3). Moreover, these pmHSP27-RPMEC cells exhibited normal p38-MK2-HSP27 signaling at baseline and after stimulation (Fig. 4). Yet, Erk signaling and MKK3 degradation were similar in pmHSP27-RPMEC and wild-type RPMEC (Fig. 6). These results suggest that: first, pmHSP27 overexpression does not interfere with the protease activity of LeTx on MAPKK, and second, pmHSP27 overexpression salvages p38-MK2-HSP27 specifically and does not salvage Erk signaling. Hence, not only does pmHSP27 overexpression stabilize the endothelial cytoskeleton as we described earlier (Liu et al., 2009, 2010b), it also protects the p38-MK2-HSP27 signaling pathway from inactivation via downregulation of MKK3b. The latter observation is consistent with a major role for p38-MK2-HSP27 signaling in barrier augmentation and its being a target for effecting endothelial permeability and vascular leak associated with anthrax.

As to the mechanism by which pmHSP27 overexpression might stabilize p38-MK2-HSP27 signaling our data suggest that it is not at the level of upstream kinases such as MKK3b since it could not block degradation of MKK3b by LeTx. Indeed HSP27 has been reported to augment p38-MK2 signaling by stabilizing TAK1, a kinase that phosphorylates and activates MKK3 and MKK6 (Alford et al., 2007). However, such a mechanism is not likely based on the significant reduction in MKK3b we observed in LeTx-treated pmHSP27-

RPMEC (Fig. 6) which was even more than in LeTx-treated wild-type RPMEC (Fig. 5). TAK1-binding protein (TAB1) has also been suggested to regulate p38 activity in an MKK3/6-independent manner (Kang et al., 2006) although other reports maintained that MKK3/MKK6 are obligatory for p38 activation by TNF α (Brancho et al., 2003), and TAB1 action appears to be stimulus-dependent in terms of activation or inhibition of p38 (Kang et al., 2006). Another mechanism by which pmHSP27 overexpression might stabilize p38-MK2-HSP7 signaling is by inhibition of p38 phosphatase. While we could not definitively rule out the latter possibility, we do not believe it is likely because MAP kinase phosphatases, such as MKP1, are usually active on both Erk and p38 and hence their inhibition should have salvaged both signaling pathways unlike what we have observed. The most direct explanation for stabilization of p38-MK2-HSP27 signaling would be altered interaction between p38/phospho p38 and HSP27 which would protect the kinase from phosphatases. Co-immunoprecipitation experiments did not reveal differences in p38-HSP27 interactions between wild type and pmHSP27-RPMEC (Fig. 7). Since p38-MK2-HSP27 have been reported to exist in a scaffold, it is possible that pmHSP27 exerts an indirect effect on p38 either by stabilizing the scaffold or its interaction with cytoskeleton structures such as microfilaments or intermediate filaments. We have shown both of these cytoskeletal components to be significantly altered by pmHSP27 overexpression (An et al., 2005; Liu et al., 2009, 2010b). Studies on scaffold structure and localization are underway but are beyond the scope of this report.

In conclusion, we have shown that one mechanism by which exposure to anthrax can lead to the characteristic vascular leak is through blocking the p38-MK2-HSP27 signaling pathway by the proteolysis of p38-activating MKK3b by anthrax toxin LeTx. Moreover, our results suggest that activation of HSP27 phosphorylation salvages p38-MK2-HSP27 signaling and could be a potential target for intervention in the treatment of anthrax and other types of vascular leak.

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Literature Cited

- Alford KA, Glennie S, Turrell BR, Rawlinson L, Saklatvala J, Dean JL. Heat shock protein 27 functions in inflammatory gene expression and transforming growth factor- β -activated kinase-1 (TAK1)-mediated signaling. *J Biol Chem.* 2007; 282:6232–6241. [PubMed: 17202147]
- An SS, Pennella CM, Gonnabathula A, Chen J, Wang N, Gaestel M, Hassoun PM, Fredberg JJ, Kayyali US. Hypoxia alters biophysical properties of endothelial cells via p38 MAPK- and Rho kinase-dependent pathways. *Am J Physiol Cell Physiol.* 2005; 289:C521–C530. [PubMed: 15857906]
- Bradley KA, LeVine SM. Anthrax toxin delivers a one-two punch. *Cell Host Microbe.* 2010; 8:394–395. [PubMed: 21075350]
- Brancho D, Tanaka N, Jaeschke A, Ventura J-J, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ. Mechanism of p38 MAP kinase activation in vivo. *Genes Dev.* 2003; 17:1969–1978. [PubMed: 12893778]
- Cote CG, Yu FS, Zulueta JJ, Vosatka RJ, Hassoun PM. Regulation of intracellular xanthine oxidase by endothelial-derived nitric oxide. *Am J Physiol.* 1996; 271:L869–874. [PubMed: 8944732]

- Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, Copeland TD, Ahn NG, Oskarsson MK, Fukasawa K, Paull KD, Vande Woude GF. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science*. 1998; 280:734–737. [PubMed: 9563949]
- During RL, Gibson BG, Li W, Bishai EA, Sidhu GS, Landry J, Southwick FS. Anthrax lethal toxin paralyzes actin-based motility by blocking Hsp27 phosphorylation. *EMBO J*. 2007; 26:2240–2250. [PubMed: 17446863]
- Guichard A, McGillivray SM, Cruz-Moreno B, van Sorge NM, Nizet V, Bier E. Anthrax toxins cooperatively inhibit endocytic recycling by the Rab11/Sec15 exocyst. *Nature*. 2010; 467:854–858. [PubMed: 20944747]
- Kang YJ, Seit-Nebi A, Davis RJ, Han J. Multiple activation mechanisms of p38 α mitogen-activated protein kinase. *J Biol Chem*. 2006; 281:26225–26234. [PubMed: 16849316]
- Kayyali US, Pennella CM, Trujillo C, Villa O, Gaestel M, Hassoun PM. Cytoskeletal changes in hypoxic pulmonary endothelial cells are dependent on MAPK-activated protein kinase MK2. *J Biol Chem*. 2002; 277:42596–42602. [PubMed: 12202485]
- Kuo S-R, Willingham MC, Bour SH, Andreas EA, Park SK, Jackson C, Duesbery NS, Leppla SH, Tang W-J, Frankel AE. Anthrax toxin-induced shock in rats is associated with pulmonary edema and hemorrhage. *Microb Pathog*. 2008; 44:467–472. [PubMed: 18222626]
- Leppla SH. Anthrax toxin edema factor: A bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc Natl Acad Sci USA*. 1982; 79:3162–3166. [PubMed: 6285339]
- Liang X, Young JJ, Boone SA, Waugh DS, Duesbery NS. Involvement of domain II in toxicity of anthrax lethal factor. *J Biol Chem*. 2004; 279:52473–52478. [PubMed: 15465830]
- Liu T, Guevara OE, Warburton RR, Hill NS, Gaestel M, Kayyali US. Modulation of HSP27 alters hypoxia-induced endothelial permeability and related signaling pathways. *J Cell Physiol*. 2009; 220:600–610. [PubMed: 19373869]
- Liu S, Miller-Randolph S, Crown D, Moayeri M, Sastalla I, Okugawa S, Leppla SH. Anthrax toxin targeting of myeloid cells through the CMG2 receptor is essential for establishment of *Bacillus anthracis* infections in mice. *Cell Host Microbe*. 2010a; 8:455–462. [PubMed: 21075356]
- Liu T, Guevara OE, Warburton RR, Hill NS, Gaestel M, Kayyali US. Regulation of vimentin intermediate filaments in endothelial cells by hypoxia. *Am J Physiol—Cell Physiol*. 2010b; 299:C363–C373. [PubMed: 20427712]
- Moayeri M, Haines D, Young HA, Leppla SH. *Bacillus anthracis* lethal toxin induces TNF- α -independent hypoxia-mediated toxicity in mice. *J Clin Invest*. 2003; 112:670–682. [PubMed: 12952916]
- Park JM, Greten FR, Li Z-W, Karin M. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. *Science*. 2002; 297:2048–2051. [PubMed: 12202685]
- Pezard C, Berche P, Mock M. Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect Immun*. 1991; 59:3472–3477. [PubMed: 1910002]
- Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo AP, Buchner J, Gaestel M. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor α by phosphorylation. *J Biol Chem*. 1999; 274:18947–18956. [PubMed: 10383393]
- Tessier J, Green C, Padgett D, Zhao W, Schwartz L, Hughes M, Hewlett E. Contributions of histamine, prostanoids, and neurokinins to edema elicited by edema toxin from *Bacillus anthracis*. *Infect Immun*. 2007; 75:1895–1903. [PubMed: 17261611]
- Vitale G, Bernardi L, Napolitani G, Mock M, Montecucco C. Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor. *Biochem J*. 2000; 352:739–745. [PubMed: 11104681]
- Warfel JM, Steele AD, D'Agnillo F. Anthrax lethal toxin induces endothelial barrier dysfunction. *Am J Pathol*. 2005; 166:1871–1881. [PubMed: 15920171]
- Webster JI, Tonelli LH, Moayeri M, Simons SS, Leppla SH, Sternberg EM. Anthrax lethal factor represses glucocorticoid and progesterone receptor activity. *Proc Natl Acad Sci USA*. 2003; 100:5706–5711. [PubMed: 12724519]

Fig. 1.

LeTx induces permeability and gap formation in wild-type RPMEC. A: LeTx induces permeability in RPMEC after 45 min of treatment. Monolayers were grown on filter inserts and exposed to LeTx (2 $\mu\text{g}/\text{ml}$). The transfer of Alexa Fluor-dextran (3 kDa) through the monolayer was measured. The bar represents change in fluorescence intensity in the lower chamber compared with control group. Data are presented as mean $\pm$ SD(n=3). B: LeTx promotes gap formation between endothelial cells. RPMEC monolayer was grown on coverslips then treated with LeTx (2 $\mu\text{g}/\text{ml}$) for 30 min. The borders between endothelial cells were visualized by staining plasma membranes and nuclei. Arrows point to regions where gaps formed between cells. This figure is representative of three different experiments. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcp>]

Fig. 2.

LeTx reduces phosphorylation of p38, MK2, and HSP27 in RPMEC. RPMEC were exposed to hypoxia (3% oxygen) or TGFβ with or without pretreatment of LeTx (2 μg/ml) for 30 min. Western blotting was performed with antibodies against phospho-p38 and total p38 (A), phospho-MK2 and total MK2 (C) and phospho-HSP27 and total HSP27 (E). Bar graph shows changes of relative level of phosphorylation of p38 (B), MK2 (D), and HSP27 (F). Data are presented as mean ± SD (n = 3 samples). * Statistically significant difference from normoxia control mean without treatment. *P* < 0.05 in ANOVA and Holm-Sidak post hoc analysis.

Fig. 3.

Overexpressing pmHSP27 protects RPMEC from LeTx-induced permeability and gap formation. A: Overexpressing pmHSP27 attenuated LeTx-induced permeability after 45 min of treatment. Monolayers were grown on filter inserts and exposed to LeTx (2 $\mu\text{g/ml}$). The transfer of AlexaFluor-dextran (3 kDa) through the monolayer was measured. The bar represents change of fluorescence intensity compared with control group. Data are presented as mean $\pm$ SD (n = 3 samples). B: Overexpressing pmHSP27 protects RPMEC from LeTx-induced gap formation. pmHSP27-overexpressing RPMEC monolayers were grown on coverslips then treated with LeTx (2 $\mu\text{g/ml}$) for 30 min. The borders between endothelial cells were visualized by staining plasma membranes and nuclei. This figure is representative of three different experiments. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcp>]

Fig. 4. pmHSP27-Overexpressing RPMEC are resistant to LeTx-induced reduction of phosphorylation of p38, MK2, and HSP27. pmHSP27-RPMEC were exposed to hypoxia (3% oxygen) or TGFβ with or without LeTx pretreatment (2 μg/ml for 30 min). Western blotting was performed with antibodies against phospho-p38 and total p38 (A), phospho-MK2 and total MK2 (C) and phospho-HSP27 and total HSP27 (E). Bar graph shows changes of relative level of phosphorylation of p38 (B), MK2 (D), and HSP27 (F). Data are presented as mean ± SD (n = 3 samples). *Statistically significant difference from normoxia control mean without treatment, #Statistically significant difference from normoxia LeTx treatment. $P < 0.05$ in ANOVA and Holm-Sidak post hoc analysis.

Fig. 5.

LeTx reduces MKK3 level and activation of ERK in RPMEC. RPMEC was exposed to hypoxia (3% oxygen) or TGFβ with or without LeTx pretreatment (2 μg/ml for 30 min). Western blotting was performed with antibodies against MKK3b (A), phospho-ERK and total ERK (C). Bar graph shows changes of relative level of MKK3b (B), and phosphorylation of ERK (D). Data are presented as mean ±SD (n =3 samples). *Statistically significant difference from normoxia control mean without treatment. $P < 0.05$ in ANOVA and Holm-Sidak post hoc analysis.

Fig. 6.

LeTx reduces MKK3 level and activation of ERK in pmHSP27-overexpressing RPMEC. pmHSP27-RPMEC was exposed to hypoxia (3% oxygen) or TGF β with or without LeTx pretreatment (2 μ g/ml for 30 min). Western blotting was performed with antibodies against MKK3b (A), phospho-ERK and total ERK (C). Bar graph shows changes of relative level of MKK3 (B), and phosphorylation of ERK (D). Data are presented as mean $\pm$ SD (n =3 samples). *Statistically significant difference from normoxia control mean without treatment. $P < 0.05$ in ANOVA and Holm-Sidak post hoc analysis.

Fig. 7.

Overexpressing pmHSP27 does not alter interaction of p38 and HSP27. Cell lysates of wild type and pmHSP27-overexpressing RPMEC were immunoprecipitated with anti-HSP27 antibody, and then the precipitates were immunoblotted with anti total or phospho-p38 antibody. Whole cell lysates were analyzed with Western blotting at the same time. This figure is representative of three different experiments.