Anthrax lethal toxin-induced lung injury and treatment by activating MK2

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Endothelial permeability and vascular leak occur in a variety of diseases and can be life-threatening if they occur excessively in organs such as the lung, where they disrupt gas exchange and could lead to acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Permeability is tightly controlled by the vascular endothelial barrier as part of the inflammatory response in which it can be beneficial when it allows the egress of inflammatory mediators and blood components to the site of injury. In certain conditions, such as anthrax or sepsis, vascular permeability is excessive and reflects dysregulation or disruption of the endothelial barrier that can lead to tissue edema, hypovolemia, and shock. Exposure to anthrax is often lethal because the infection can develop asymptomatically until severe injury and tissue damage occur. Anthrax is believed to owe its virulence to the toxin produced by the bacteria since antibiotics that eliminate the bacteria are of limited use once a sufficient toxin has been produced. Moreover, introducing the anthrax toxin in animal models causes responses that mimic the effects of anthrax in humans. Anthrax toxin is composed of three polypeptides. The first, edema factor (EF), is an adenylate cyclase that produces excessive cAMP; the second, called lethal factor (LF), is a metalloprotease that is associated most with anthrax virulence (17); and the third, called protective antigen (PA), combines with the other components to facilitate their entry into cells. Injection of LF and PA, known together as lethal toxin (LeTx), has been shown to produce most of the characteristics of anthrax infection (15). EF appears to act not directly on endothelial cells but indirectly through inflammatory mediators (20). LeTx, on the other hand, is strongly associated with vascular leak, which occurs independently of its effects on macrophages and endothelial apoptosis (15). LeTx has also been reported to directly affect endothelial cells, leading to increased endothelial barrier permeability (15). We have recently linked the effect of LeTx on endothelial barrier permeability to its perturbation of p38 MAP kinase signaling (10). Hence, in developing a treatment against anthrax-induced vascular leak, we have targeted the activation of components downstream of p38 that we have previously linked to barrier regulation.

Increased vascular permeability and pulmonary edema are the most severe manifestations of exposure to anthrax toxin and are often lethal (5). This edema is not responsive to corticosteroids or attempts at fluid regulation and is accompanied by vascular leak in multiple systems (15). Our previous studies on the modulation of endothelial cell signaling and the permeability barrier by hypoxia indicated that barrier regulation occurs through a tight biological control system. In that system, hypoxia activates signaling through the Rho-kinase-myosin phosphatase pathway, which leads to endothelial cell contraction and weakening of the barrier and increased permeability (1, 8). At the same time, and independently of the former signaling pathway, hypoxia activates p38 kinase signaling, leading to phosphorylation of MK2 and its substrate HSP27, which subsequently causes barrier augmentation and decreased permeability (1, 8).

This type of control could be beneficial when biological systems encounter physiological or pathological stimuli. By initially increasing endothelial permeability, the control system allows blood components to enter the site of injury but then limits the response and ensures reversibility through activation of barrier-augmenting signaling. Excessive permeability might develop when signaling pathways leading to endothelial cell contraction become overactive. However, excessive permeability can also arise in response to disruption of signaling pathways that augment the barrier. Our findings have specifically linked the ability of anthrax LeTx to inhibit p38/MK2/HSP27 signaling to its ability to weaken the endothelial permeability barrier (10). This mechanism could explain the severe vascular leak associated with anthrax infection.
Our results further demonstrated that overexpressing a phosphomimicking form of heat shock protein 27 (HSP27) can protect endothelial permeability barriers against anthrax LeTx (10) and other permeability-inducing stimuli such as hypoxia and transforming growth factor-β (TGFβ) (8). Hence, we postulated that drugs designed to activate signaling that leads to HSP27 phosphorylation might be effective against pathogens that cause vascular leak, such as anthrax. Because specific targeted enzyme activators are not typically generated by the drug industry, which focuses on easier to develop enzyme inhibitors, we attempted a novel approach to generate an activator of MK2. In this report, we describe how we designed an MK2-activating peptide (MK2-AP) that reverses anthrax LeTx inhibition of MK2 and blocks anthrax LeTx-induced permeability and vascular leak in endothelial cell culture and in vivo. We expect MK2-AP to be a specific and efficacious agent to treat anthrax-induced vascular leak and edema.

MATERIALS AND METHODS

Reagents. The anthrax LeTx used in the experiments was prepared by mixing equal concentrations of LF and PA (Calbiochem, La Jolla, CA) before treatment. MK2-AP was synthesized by SynthAssist Peptide Synthesis at the Tufts University Core Facility (Boston, MA) with the amino acid sequence of YARAAARQARAHPRNPARRTPG-TRRGAPAA.

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 (4). 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. For siRNA transfection, 80-90% confluent cells were transfected with MK2-siRNA (Cat # J-005286-06 Dharmacon, Chicago, IL), p38-siRNA (s135447 Ambion, TX), or nonspecific control siRNA (D-001810-0x Dharmacon) with Lipofectamine 2000 (Invitrogen, CA) at 100 nM for 24 h according to the manufacturer’s instructions.

Labeling of plasma membrane and nucleus. Gaps between cells were determined using the Image-iT LIVE Plasma Membrane and Nuclear Labeling Kit ( Molecular Probes, Eugene, OR). RPMECs were grown on collagen-coated glass cover slips. Upon confluence, cells were serum-starved overnight before being treated with LeTx and/or MK2-AP. Next, the cover slips were rinsed twice with phosphate-buffered saline (PBS) and fixed for 10 min with 4% formaldehyde. The cover slips 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 objective lens.

SDS-PAGE and immunoblotting. Cells were lysed in Triton buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na2VO4, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1% Triton X-100, and 50 mM NaF plus 1% calf serum protease inhibitor cocktail), and lysates were assayed for protein using the Bradford protein assay and then diluted with 2× 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 electrophotoretic transfer at 300 mA constant current for 2 h. 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, Rockford, IL) were used to visualize immunoreactive bands. The primary antibodies against phospho-p38, total p38, phospho-MK2, total MK2, and phospho-HSP27 were purchased from Cell Signaling Technology (Danvers, MA), and antibody against total HSP27 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-mouse and donkey anti-rabbit secondary antibodies were purchased from Pierce.

MK2 immunoprecipitation kinase assay. MK2 kinase activity was assayed with the MK2 immunoprecipitation kinase assay kit (Upstate, Lake Placid, NY) according to the instructions. Anti-MK2-antibody-agrose resin was incubated with 1 mg of cell lysate for 1.5 h at 4°C. After centrifugation and removal of the supernatant, MK2-antibody-agrose resin was incubated with recombinant HSP27 substrate peptide (Abcam, Cambridge, MA), MK2-AP, or control buffer and reaction buffer for 45 min at 30°C. Reaction samples were analyzed by Western blotting to determine the phosphorylation level of HSP27 recombinant peptide.

Evans blue assay. Fischer 344 rats (150–175 g) were anesthetized, weighed, and injected with 20 mg/kg Evans Blue dye through the right jugular vein. Fifteen minutes later, lungs were excised, weighed, and frozen in liquid nitrogen. Frozen lungs were then homogenized in 1 ml of PBS, and 200 µl of homogenate was aliquoted into another tube for Western blotting. The remaining homogenate was diluted with 2 vol of formamide and incubated at 60°C for 18 h. Then the homogenates were centrifuged at 5,000 g for 30 min. The supernatant was collected, and 200 µl of supernatant was aliquoted into each well of a 96-well plate. The absorbance was measured by spectrophotometer at 620 nm. Concentration of each sample was calculated using a standard curve prepared in formamide.

Statistical analysis. Data are presented as means ± 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

MK2-AP increases HSP27 phosphorylation. Since we demonstrated that p38 and MK2 inhibition and decreased HSP27 phosphorylation can be linked to anthrax LeTx-induced endothelial barrier disruption (10), we aimed to design a drug that activates MK2. Although there are several stimuli that activate the p38 MAP kinase, many of these have other molecular effects beyond activating p38. Moreover, p38 MAP kinase can phosphorylate a variety of substrates besides MK2, and its activation is likely to affect many other targets beyond HSP27 phosphorylation. Since our research suggested that barrier augmentation is mediated by increased HSP27 phosphorylation, we sought a drug that would activate its immediate kinase MK2.

Because targeted peptides offer increased specificity compared with small molecules derived from screening chemical libraries, we designed a peptide that could directly interact with and activate MK2. In designing MK2-AP, we incorporated a sequence of the viral protein Kaposin B that has previously been reported to block the autoinhibitory domain of MK2 (13, 14), with a transduction domain (19) to increase the ability of the peptide to penetrate cells. We next assessed the ability of MK2-AP to activate MK2 in RPMEC, which were treated with increasing concentrations of MK2-AP for 15 min. Cell lysates were then immunoblotted with an antibody against phosphorylated HSP27, an MK2 substrate.

As shown in Fig. 1A, MK2-AP treatment increased HSP27 phosphorylation significantly at 50, 100, and 200 µM concentrations. The MK2-AP treatment had no effect on the level of total HSP27, suggesting that the increased phospho-HSP27...
immunoreactivity in the cell lysates is indeed due to increased HSP27 phosphorylation and not upregulation of the HSP27 protein. Next, RPMEC were treated with 50 μM MK2-AP for different time periods. As shown in Fig. 1B, MK2-AP induced significant HSP27 phosphorylation by 15 min of treatment and disappeared by 240 min of treatment with 50 μM MK2-AP.

MK2-AP blocks LeTx-induced gap formation and HSP27 dephosphorylation. Since our goal was to develop a drug that can reverse the effects of permeability-inducing agents by augmenting the endothelial barrier, we assessed the ability of MK2-AP to protect against anthrax LeTx. We have shown previously that LeTx can induce gap formation between endothelial cells. RPMEC were grown on coverslips and stained with a membrane stain to delineate the borders of cells, as we described earlier (8). As we have reported (10), LeTx treatment (2 μg/ml for 30 min) induced gap formation between endothelial cells that was not observed in control cells (Fig. 2). However, treatment with 50 μM MK2-AP blocked LeTx induction of intercellular gaps (Fig. 2A). These results indicate that MK2-AP at concentrations that induce HSP27 phosphorylation can protect endothelial cells from the effects of anthrax LeTx.

We next tested whether MK2-AP would alter the effects of anthrax LeTx on HSP27 phosphorylation. RPMEC were treated with LeTx (2 μg/ml) for different time periods, followed by treatment with MK2-AP (50 μM) for 15 min. Next, cell lysates were immunoblotted with an antibody against phosphorylated HSP27 and total HSP27. LeTx treatment reduced HSP27 phosphorylation, as we described earlier (10). However, as shown in Fig. 2B, MK2-AP was effective in reversing the effects of LeTx after 15 or 30 min (the point at which HSP27 phosphorylation was completely inhibited). MK2-AP had no effect on the level of total HSP27, supporting its role in MK2 activation rather than upregulation of HSP27. As expected, the drug was more effective if administered sooner after exposure to LeTx. To ascertain dose-response effect, different concentrations of MK2-AP were tested for their effects on LeTx inhibition of HSP27 phosphorylation. As shown in Fig. 2C, higher concentrations of MK2-AP were...
MK2-AP activates MK2 directly and induces HSP27 phosphorylation in vitro. Since we have only assayed MK2 activation by MK2-AP indirectly by looking at HSP27 phosphorylation in treated endothelial cells, we aimed to test whether the latter is mediated specifically by MK2 rather than other kinases. To accomplish this aim, we first knocked down the expression of MK2 in RPMEC using an siRNA against MK2 that we have employed previously (11). Next, these cells, along with cells treated with nonspecific control siRNA, were treated with MK2-AP. As shown in Fig. 3A, MK2 siRNA reduces the level of MK2 in transfected RPMEC. At the same time, knocking down MK2 blocked the activity of MK2-AP, which could no longer stimulate HSP27 phosphorylation, suggesting that it acts through MK2. Control siRNA did not block the MK2-AP effect on HSP27 phosphorylation. Knocking down MK2 had no significant effect on the phosphorylated or total level of p38 compared with scrambled siRNA control, suggesting that MK2 siRNA was specific (Fig. 3A). The apparent reduction in phospho-p38 compared with cells not treated with siRNA suggests a nonspecific effect, as it was also observed in cells treated with scrambled siRNA control. More importantly, this reduction does not correlate with the reduction in HSP27 phosphorylation, which did not occur in scrambled siRNA control-treated cells. These results suggest that the activation of HSP27 phosphorylation is dependent on MK2.

To further characterize the mechanism of action of MK2-AP and prove that it directly activates MK2, we immunoprecipitated MK2 from RPMEC. The immunoprecipitate was then split into two batches, one control and one treated with MK2-AP (50 µM). Next, purified recombinant unphosphorylated HSP27 was added in kinase assay buffer along with a magnesium-ATP cocktail. After 45 min the mixture was boiled and analyzed by SDS-PAGE and immunoblotting with antibodies against phospho-HSP27 and total HSP27. As shown in Fig. 3B, MK2-AP increased HSP27 phosphorylation by purified MK2 in vitro by 63%. The results described in Fig. 3 suggest that MK2-AP specifically targets and activates MK2, leading to HSP27 phosphorylation in vitro and in cells.

MK2 activation by MK2-AP is independent of p38 MAP kinase activation or MK2 phosphorylation. Since p38 MAP kinase is a stress-activated kinase, we wished to eliminate the possibility that MK2-AP is inducing some nonspecific stress in endothelial cells, leading to p38 MAP kinase activation, which could in turn lead to activation of its downstream substrate MK2 and phosphorylation of HSP27. Indeed, MK2-AP induced phosphorylation of p38, which is correlated with its activation (Fig. 4A). However, it has been reported previously that Kaposin B-derived peptides also increased p38 MAP kinase signaling, possibly as part of a positive feedback loop (13). Furthermore, we have shown previously that overexpression of phosphomimicking HSP27 increased the phosphorylation of p38, even after treatment with LeTx, which degraded its upstream kinase MKK3 (10).

To establish that HSP27 phosphorylation induced by MK2-AP is due to direct activation of MK2 rather than nonspecific activation of its upstream kinase p38, we knocked down the latter using specific siRNA against p38 MAP kinase. As shown in Fig. 4B, siRNA against p38 reduced the level of p38 significantly. However, it did not block the increased HSP27 phosphorylation induced by MK2-AP. These results suggest that the activation of p38 by MK2-AP is due to direct activation of MK2 independent of p38 MAP kinase activation or MK2 phosphorylation.

Just as MK2-AP increased p38 phosphorylation, it also increased MK2 phosphorylation in a dose- and time-dependent manner (Fig. 4C). Although it is possible that MK2-AP could induce MK2 phosphorylation directly by activating autophosphorylation, we speculated that MK2 phosphorylation was
MK2-AP blocks anthrax LeTx-induced vascular leak in rats. After demonstrating specificity of MK2-AP and its ability to block anthrax LeTx-induced intercellular gap formation, we evaluated its ability to block anthrax LeTx-induced vascular leak in vivo. Rats were catheterized via the jugular vein and administered saline or MK2-AP (20 mg/kg). After 15 min, rats were treated with anthrax LeTx (100 μg/kg) or saline. Rats received two more doses of MK2-AP or saline 30 and 60 min after the LeTx treatment. Ninety minutes after LeTx treatment, Evans Blue dye was injected through the catheter. Rats were then euthanized, and their lungs were removed. Upon gross examination, lungs treated with anthrax LeTx appeared edematous, whereas those treated with MK2-AP did not.

Vascular leak and edema can also be assessed by the accumulation of Evans Blue dye, which complexes with serum albumin when injected intravenously, in tissues where the permeability barrier is compromised. As shown in Fig. 5A, rats treated with the anthrax LeTx alone had bluer lungs with several intensely blue patches, consistent with vascular leak and edema. On the other hand, rats treated with MK2-AP and anthrax LeTx had much clearer lungs, which was consistent with the ability of MK2-AP to block vascular leak. To quantify vascular leak, we homogenized the lungs and extracted accumulated Evans blue with formamide and assayed its absorbance. Since there was day-to-day variability in the responses and assay conditions, we normalized vascular leak (Evans Blue accumulation) to the amount accumulated in LeTx-treated rat lungs studied on the same day.

As shown in Fig. 5B, MK2-AP significantly reduced anthrax LeTx-induced vascular leak. To further quantify permeability and vascular leak, we also compared lung weights among the different treatment groups, which when normalized to body weights is another measure of vascular leak and lung injury (16). As shown in Fig. 5C, anthrax LeTx increased the lung weight/body weight ratio significantly, whereas MK2-AP had no significant effect on that ratio. Furthermore, MK2-AP treatment blocked the anthrax LeTx-induced increase in lung weight, which is consistent with its effect on Evans Blue accumulation. Finally, to verify that MK2-AP was activating MK2 in rat lungs, we probed lung homogenates from rats treated with MK2-AP and/or anthrax LeTx. As shown in Fig. 5D, MK2-AP increased the level of phospho-HSP27 even in the presence of LeTx, which suggests that the MK2-AP is effective in activating MK2 in vivo. These results indicate that MK2-AP is active in vivo and can induce HSP27 phosphorylation while protecting lungs from the vascular leak induced by anthrax LeTx.

**DISCUSSION**

In this report, we describe the design and evaluation of a mechanism-based drug that causes kinase activation and reduces anthrax toxin-associated vascular leak. By activating MK2, a kinase that we have linked specifically to anthrax LeTx-induced increases in endothelial permeability (10), we were able to reverse LeTx’s effects on signaling and vascular permeability. Although progress in combating anthrax has been made on the vaccine and antibiotic front, situations arise when the patient is beyond help by these agents, especially if the level of anthrax toxin produced by the bacteria reaches a certain level.
The lung injury caused by anthrax, which is refractory to treatment with corticosteroids and diuretics (5, 15), is most probably due to its direct action on the endothelium, without the requirement for other immune modulators. For example, recent studies indicate that the action of LeTx on immune cells is important only for infection but not for the lethality of LeTx (3, 7). Anthrax LeTx activity resides in the lethal factor component, which is a metalloprotease that degrades MAP kinase kinases (MEKs, or MKKs), its only known substrates (10). We have shown that by degrading MKK3b in pulmonary endothelial cells, anthrax LeTx can perturb endothelial barrier permeability through disrupting p38/MK2/HSP27 signaling (10). Although other kinases such as Erk can also be affected by LeTx, our results indicate that only p38/MK2/HSP27 signaling is correlated with its barrier-altering effects (10).

Since our earlier studies indicated that overexpressing a phosphomimicking form of HSP27 could restore p38/MK2 signaling and protect the endothelial permeability barrier against LeTx in cultured cells (10), we explored approaches that activate HSP27 phosphorylation to treat anthrax LeTx-induced vascular leak. We focused on MK2, which despite having other substrates besides HSP27 remains the major immediate kinase that directly phosphorylates HSP27. In designing the MK2-activating drug, we built on a report that Kaposin B peptides can directly interact with and activate MK2 (13, 14). We picked a small sequence of the peptide to improve cell entry combined with a transduction domain to further enhance access to the cell. The experiments we describe above demonstrate that the drug MK2-AP is fairly specific and effective in activating HSP27 phosphorylation. Both dose response and time course experiments show that the effects are dose dependent, rapid, and reversible.

While the exact mechanism by which MK2-AP activates MK2 needs to be determined, it is reasonable to assume that, just as has been proposed with the Kaposin B peptide (13,
14), it might bind to certain regions of MK2 and block the autoinhibitory domain of MK2. However, the true nature of the molecular interaction between MK2-AP and MK2 needs to be characterized further by structural and mutation/deletion studies. Better understanding of the mechanism in future studies might lead to informed rational design of enzyme activators as drugs. Historically, drugs that inhibit enzymes have been easier to design because increased knowledge of enzyme structure and mechanism can guide the targeting of an active site or a substrate-binding site. Nevertheless, there are diseases where the problem is more likely related to insufficient protein phosphorylation, and the drug that is needed is a kinase activator and not an inhibitor. Although inhibiting a phosphatase might increase the phosphorylation of a kinase substrate, phosphatases tend to have a much broader spectrum of substrates than kinases, and their inhibition is likely to cause more nonspecific effects.

While our data support a role for MK2-AP in treating anthrax by targeting endothelial permeability barrier regulation, it might also be useful in other types of lung injury due to a variety of conditions, such as sepsis. This claim is supported by our previous studies in which we show that increased phosphorylation of HSP27 can protect the endothelial barrier against different permeability-inducing stimuli such as hypoxia and TGFβ (8, 9). One of the challenges in developing drugs against ALI/ARDS is that many of the rodent models used to evaluate therapeutics produce one or another but not all of the components of ALI/ARDS. The pathology of ALI/ARDS is complex and involves epithelial as well as inflammatory cell contribution beyond the vascular endothelium. However, the endothelial barrier is the major mediator of permeability and vascular leak that leads to lung flooding, which disrupts gas exchange and leads to different degrees of hypoxemia. The latter is what determines the severity of ARDS according to the most recent international consensus definition and classification of ARDS (Berlin Definition) (2). Indeed, a recent study demonstrates that extravascular lung water and pulmonary microvascular permeability in ARDS patients correlate with the severity of ARDS according to the Berlin Definition (6). Research in anthrax pathogenesis suggests that there is no anthrax pneumonia, even in inhalation anthrax (12, 18). Rather than infecting the lung epithelium, as happens in other bacterial or viral pneumonias, the anthrax bacteria migrates to the lymph nodes, where it propagates and releases the bacteria and anthrax toxin into the circulation. It is that toxin acting on the pulmonary vascular endothelium that causes the pleural effusions and pulmonary edema associated with anthrax infection. In that regard, anthrax-induced lung injury and ARDS are an example of an indirect cause of ARDS, as occurs in sepsis and other types of distal injuries. Inasmuch as anthrax LeTx causes endothelial cell barrier compromise in cultured rat cells and in vivo in rats injected intravenously with the toxin, the latter can be used as an animal model to evaluate drugs that target the endothelial permeability and vascular leak component of ARDS. In conclusion, we have developed a drug that specifically targets a mechanism by which anthrax might cause vascular leak, which could be an effective treatment against anthrax and acute lung injury due to a variety of causes.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

T.L. and R.R.W. performed experiments; T.L. and U.S.K. analyzed data; T.L., N.S.H., and U.S.K. interpreted results of experiments; T.L. and U.S.K. prepared figures; T.L., N.S.H., and U.S.K. edited and revised manuscript; T.L. and U.S.K. approved final version of manuscript; U.S.K. conception and design of research; U.S.K. drafted manuscript.

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