Heat Shock Protein 90 Inhibitors Protect and Restore Pulmonary Endothelial Barrier Function

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Heat shock protein 90 (hsp90) inhibitors inactivate and/or degrade various client proteins, including many involved in inflammation. Increased vascular permeability is a hallmark of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Thus, we tested the hypothesis that hsp90 inhibitors may prevent and/or restore endothelial cell (EC) permeability after injury. Exposure of confluent bovine pulmonary arterial endothelial cell (BPAEC) monolayer to TGF- β 1, thrombin, bacterial lipopolysaccharide (LPS), or vascular endothelial growth factor (VEGF) increased BPAEC permeability, as revealed by decreased transendothelial electrical resistance (TER). Treatment of injured endothelium with hsp90 inhibitors completely restored TER of BPAEC. Similarly, preincubation of BPAEC with hsp90 inhibitors prevented the decline in TER induced by the exposure to thrombin, LPS, VEGF, or TGF- β 1. In addition, hsp90 inhibitors restored the EC barrier function after PMA or nocodazole-induced hyperpermeability. These effects of the hsp90 inhibitors were associated with the restoration of TGF- β 1- or nocodazole-induced decrease in VE-cadherin and β -catenin expression at EC junctions. The protective effect of hsp90 inhibitors on TGF- β 1-induced hyperpermeability was critically dependent upon preservation of F-actin cytoskeleton and was associated with the inhibition of agonist-induced myosin light chain (MLC) and myosin phosphatase target subunit 1 (MYPT1) phosphorylation, F-actin stress fibers formation, microtubule disassembly, increase in hsp27 phosphorylation, and association of hsp90 with hsp27, but independent of p38MAPK activity. We conclude that hsp90 inhibitors exert barrier protective effects on BPAEC, at least in part, via inhibition of hsp27-mediated, agonist-induced cytoskeletal rearrangement, and therefore may have useful therapeutic value in ALI, ARDS, and other pulmonary inflammatory disease.

Keywords: endothelial permeability; TGF- β 1; hsp27; 17-AAG; MYPT1

Vascular endothelium forms a selective permeable barrier between blood and the interstitial space of all organs and participates in the regulation of macromolecule transport and blood cell trafficking through the vessel wall. It is generally accepted that inflammatory mediators, such as histamine, thrombin, bacterial lipopolysaccharide (LPS), and cytokines bind to their cognate receptors on the endothelial cell (EC) surface and activate intracellular signaling pathways, leading to the formation of paracellular gaps and increased transendothelial permeability for fluids and macromolecules (1–3). Endothelial barrier dys-

CLINICAL RELEVANCE

The finding that hsp90 inhibitors, including 17AAG (which is currently undergoing clinical trials related to cancer), prevent and restore endothelial cell permeability induced by several major inflammatory mediators may lead to a new intervention to control inflammation and endothelial barrier function in patients with acute lung injury.

function and increased endothelial monolayer permeability is an important early step in the development of inflammatory pulmonary conditions, such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and sepsis (4, 5), which are devastating lung disorders with mortality exceeding 30% (6).

Growing evidence indicates that inflammatory cytokines, such as TGF- β 1, increase EC permeability *in vitro* and play a critical role in the development of lung edema during lung injury *in vivo* (7–11). Our previous data indicate that TGF- β 1 induces a decrease in the transendothelial electrical resistance (TER). These studies as well as data from other laboratories (7, 12, 13) establish TGF- β 1 as a key mediator of increased pulmonary endothelial permeability in the development of pulmonary edema during acute lung injury.

Hsp90 is one of the most abundant cellular proteins, accounting for approximately 1 to 2% of total proteins under unstressed conditions (14). It functions as part of a multichaperone complex with a variety of co-chaperones and client proteins, many of which are crucial in inflammation. These complexes cycle between an “open” and a “closed” conformation, relative to the distance between the N-terminals of the hsp90 homodimer. Hsp90 inhibitors shortcut the cycle and lock the complex in the “open” state, resulting in client protein deactivation, destabilization, and proteosomal degradation (14–16). Although many hsp90 client proteins act as inflammatory mediators, little is known about the regulation of inflammatory responses by hsp90 inhibitors or about their effects on agonist-induced endothelial barrier dysfunction. We have previously reported that hsp90 inhibitors effectively protect from LPS-induced ALI and EC injury, *in vivo* and *in vitro* (17). The present study was thus conducted to investigate the protective and reparative effects of hsp90 inhibitors on receptor-mediated and non-receptor-mediated EC hyperpermeability and the mechanisms responsible for these effects. We employed three hsp90 inhibitors: radicicol (RA), the most effective hsp90 inhibitor, *in vitro* (16) and 17-AAG and 17-DMAG, which are currently undergoing phase I and II clinical trials as adjunct therapy for various neoplasms.

MATERIALS AND METHODS

Antibodies and Reagents

Primary antibodies were obtained as follows: MYPT1 and anti-phospho-MYPT1 (Thr850) were from Upstate Biotechnology (Lake Placid, NY);

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diphospho-MLC (Thr18/Ser19), phospho (Thr¹⁸⁰, Tyr¹⁸²)-p38MAPK, total p38MAPK, and anti-phospho (Ser⁸²)-hsp27 were from Cell Signaling (Beverly, MA); anti-VE-cadherin and anti- β -catenin antibodies were from Invitrogen (San Francisco, CA). Polyclonal anti-hsp27 antibody was from Stressgen (Ann Arbor, MI), and anti-hsp90 antibody was from BD Transduction Laboratories (Bedford, MA). Antibody to β -tubulin was from CRP (Covance Research Products, Denver, PA). Secondary antibodies conjugated with fluorescent dye Cy2 and Cy3 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Human TGF- β 1 was obtained from R&D Systems (Minneapolis, MN). 17-AAG and 17-DMAG were obtained from the National Cancer Institute (Bethesda, MD). Radicol was purchased from Sigma (St. Louis, MO). Protein A-agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). Unless specified, biochemical reagents were obtained from Sigma.

Cell Culture

In contrast to our previous studies of TGF- β -induced EC permeability, in which we used commercially available BPAEC, in this study we used the in-house harvested BPAEC, which we have previously extensively characterized for other permeability models (7, 18). Cultures were maintained in medium 199, supplemented with 10% fetal bovine serum, 5% iron-supplemented calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all Invitrogen, San Francisco, CA). In all experiments, confluent EC monolayers (Days 4–6 in culture) were used.

Endothelial Monolayer Permeability Assay

Changes in endothelial monolayer permeability were assessed by measuring electrical resistance across monolayers using the electrical cell impedance sensor technique (Applied Biophysics, Troy, NY), as our laboratory previously described (19, 20). Briefly, equivalent numbers of endothelial cells were plated on gelatin-coated gold electrode arrays (8W10E) and grown to confluence in the growth medium. Experiments were conducted after electrical resistance had achieved a steady state 1,000 to 1,200 Ω . Cells were then treated as described, and electrical resistance across monolayers was recorded over time.

Western Immunoblotting and Co-Immunoprecipitation

After treatments, BPAEC monolayers grown in 35-mm dishes were rinsed with ice-cold PBS, lysed with 2 $\times$ SDS sample buffer, and boiled for 5 minutes. Extracts were separated on SDS-PAGE, transferred to nitrocellulose or PVDF membranes, and reacted with an antibody of interest. Immunoreactive proteins were visualized with the enhanced chemiluminescent detection system (Amersham, Little Chalfont, UK). The relative intensities of the protein bands were quantified by scanning densitometry using National Institutes of Health ImageJ software (Bethesda, MD).

For immunoprecipitation, cells were lysed with ice-cold immunoprecipitation buffer (20 mM Tris HCl, pH 7.4; 137 mM NaCl; 10% glycerol; 1% Nonidet P-40; 2 mM EDTA; 1 mM Na₃VO₄; 20 mM Na₂MoO₄; 1 mM NaF; and protease inhibitor cocktail), sonicated three times for 15 seconds. The supernatant was immunoprecipitated with either control antibody (rabbit IgG) or anti-hsp27 (5 μ g/ml) overnight at 4°C followed by incubation with protein A-agarose beads for 4 hours at 4°C. Agarose beads were collected by centrifugation, washed three times with PBS, re-suspended in 60 μ l of 3 $\times$ SDS sample buffer, then boiled for 5 minutes. Protein was separated on 4 to 12% gradient SDS-PAGE. Resulted membranes were blotted with hsp90 and hsp27 antibodies and the amount of co-precipitated proteins was analyzed using ImageJ software.

Immunofluorescence Microscopy

Immunofluorescence microscopy studies were performed as we have previously described (7). After treatment, EC grown on gelatinized coverslips were rinsed with PBS, fixed in 3.7% paraformaldehyde for 10 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were then washed with PBS, blocked with PBS-Tween 20 containing 2% BSA for 30 minutes, and incubated with primary antibodies. Specimens were washed and exposed to secondary antibodies conjugated with either

Cy2 or Cy3. Coverslips were mounted on slides with ProLong antifade mounting medium (Molecular Probes, Eugene, OR) and analyzed under laser-scanning confocal microscope (LSM 510, Meta 3.2; Zeiss, Peabody, MA).

Silver Nitrate Staining

EC were cultured on plastic culture dishes for 10 to 12 days. After treatment, cells were rinsed with PBS, fixed in 3.7% paraformaldehyde for 30 minutes, and stained with 0.2% silver nitrate as described previously (21, 22).

Statistical Analysis

Values are reported as means $\pm$ SE. Comparisons between control and treated cells were performed using one-way ANOVA, unpaired *t* test, or Mann-Whitney test, as appropriate, using SigmaStat software (SPSS Inc., Chicago, IL). Differences of *P* $\leq$ 0.05 were considered significant.

RESULTS

Effect of hsp90 Inhibitors on the Loss of EC Barrier Integrity and on Cytoskeletal Changes Induced by TGF- β 1

We initially investigated the ability of RA to prevent the disruption of the endothelial monolayer and EC detachment induced by prolonged (18 h) exposure to a relatively high TGF- β 1 concentration (50 ng/ml), using phase contrast microscopy. Compared with control (Figure 1a), TGF- β 1 produced a dramatic change in EC monolayer morphology and significant EC detachment (Figure 1b, *arrows*). In the presence of RA (1 μ g/ml), TGF- β 1 did not cause EC detachment and maintained monolayer integrity (Figure 1d); morphologically, RA- and TGF- β 1-treated EC monolayers appear identical to controls (Figures 1a versus 1d). There was no evidence of cell death (necrosis or apoptosis) as reflected in lack of chromatin condensation in DAPI-stained cells (data not shown). RA alone did not induce visible changes in EC morphology (Figure 1c). Using silver nitrate staining as an indicator of monolayer integrity, we observed clear, continuous silver-stained lines between adjacent EC in control cells (Figure 1e); this staining completely disappeared after exposure of EC to 10 ng/ml TGF- β 1 for 6 hours (Figure 1f), without visible EC detachment or gap formation in the monolayer, as reflected by phase contrast microscopy (Figure 1h). In contrast, EC treated with TGF- β 1 in the presence of RA demonstrated strong silver staining of the borders between EC, which appeared even more intense (Figure 1g) than in untreated, control cultures. Furthermore, treatment with TGF- β 1 decreased VE-cadherin and β -catenin staining between adjacent EC (Figures 2c and 2g, respectively), compared with control (Figures 2a and 2e), indicating EC cytoskeletal rearrangement and barrier compromise. Stimulation of EC with TGF- β 1 in the presence of RA completely abolished the EC cytoskeletal rearrangement induced by TGF- β 1 (Figures 2d and 2h), whereas EC treated with RA alone (Figures 2e and 2f) demonstrated cytoskeletal organization identical to control EC. In addition, RA prevented TGF- β 1-induced increase in actin stress fibers (Figures 2j versus 2k) and depolymerization of peripheral microtubules (Figures 2n versus 2o).

Effect of hsp90 Inhibitors on TGF- β 1-Mediated BPAEC Hyperpermeability

To test the hypothesis that hsp90 inhibitors exert a protective effect on the EC barrier function, we examined changes in EC permeability by monitoring changes in TER, which inversely correlates with paracellular gap formation and reflects changes in the EC monolayer integrity (7, 13). In these experiments we used two approaches. To examine the ability of hsp90 inhibitors to prevent EC from injury induced by TGF- β 1, cells were first pre-

Figure 1. Effects of TGF- β 1 and radicicol on endothelial monolayer integrity. (a–d) bovine pulmonary arterial endothelial cells (BPAEC) were cultured in 35-mm dishes for 10 days to allow a confluent monolayer to form. Cells were then exposed for 18 hours to (a) vehicle (0.1% DMSO); (b) TGF- β 1 (50 ng/ml); (c) TGF- β 1 (50 ng/ml) and RA (1 μ g/ml), given at the same time; or (d) RA (1 μ g/ml) alone. Endothelial cell (EC) detachment induced by TGF- β 1 (b) is shown by arrows. Phase contrast: original magnification $\times 100$. (e–h) Under similar culture conditions, EC were treated with 10 ng/ml TGF- β 1 for 6 hours and stained with silver nitrate to reveal borders between adjacent EC; treatments: (e) vehicle (0.1% DMSO); (f) TGF- β 1 (10 ng/ml); (g) TGF- β 1 (10 ng/ml) and RA (1 μ g/ml), given at the same time; or (h) TGF- β 1 (10 ng/ml), alone (phase contrast). Original magnification $\times 320$.

incubated with hsp90 inhibitors for 3 hours and then stimulated with TGF- β 1. As we have previously reported with commercially available EC, in-house harvested BPAEC exposed to TGF- β 1 exhibited a time- and dose-dependent decrease in TER (Figure 3a). The maximum decline in TER was observed 2 to 2.5 hrs after the addition of TGF- β 1. Because nitric oxide has been implicated in EC hyperpermeability, we investigated whether NO participates in TGF- β 1-induced barrier dysfunction. Pretreatment with the nonselective NOS inhibitor L-NAME (100 μ M) had no effect on the TGF- β 1-induced decrease in TER; furthermore, by itself, L-NAME did not alter EC TER values over a 16-hour period (*see* Figure E1 in the online supplement). Next we examined the effect of hsp90 inhibitors on the TER of non-stimulated BPAEC. RA and 17-AAG caused moderate increases in TER, whereas 17-DMAG had no effect on BPAEC TER (Figure 3b). Pre-incubation with any of three hsp90 inhibitors (RA, 17-AAG, or 17-DMAG; all at 1 μ g/ml) for 3 hours abolished the TGF- β 1-induced decline in TER (Figure 3c). To address the question of whether barrier function of an already disintegrated EC monolayer can be restored by hsp90 inhibitors, we exposed BPAEC to TGF- β 1, as above, followed by hsp90 inhibitor treatment, 2 to 3 hrs after injury, at the nadir of the TER response. Post-treatment with hsp90 inhibitors completely restore the TER of BPAEC (Figure 3d).

Effect of hsp90 Inhibitors on TGF- β 1-Induced EC Contraction

We and others have shown that TGF- β 1-induced EC injury requires Rho activation followed by MLC and MYPT1 phosphorylation (7, 19, 20, 23–25). Thus, we investigated whether hsp90 inhibitors prevent MLC and MYPT1 phosphorylation. As previously shown (7), TGF- β 1 treatment increased MLC (Figure 4a) and MYPT1 (Figure 4b) phosphorylation in a time-dependent manner, as evidenced by increased immunoreactivity to di-phospho-MLC and phospho-MYPT1-specific antibodies, respectively. The increase in both MLC and MYPT1 phosphorylation induced by TGF- β 1 (Figures 4a and 4b) was inhibited by RA treatment, suggesting that inhibition of TGF- β 1-induced contraction may be involved in the barrier-protective effect of hsp90 inhibitors. RA alone had no effect on MLC and MYPT1 phosphorylation (Figures 4c and 4d).

Effect of hsp90 Inhibitors on TGF- β 1-Induced p38 MAPK Activation

The p38 MAPK plays a critical role in the TGF- β 1-induced increases of BPAEC permeability (11, 12). In agreement with previously published data, we observed that TGF- β 1 treatment activated p38 MAPK in BPAEC (Figures 5a). However, the increase in p38MAPK phosphorylation induced by TGF- β 1 was not significantly inhibited by RA (Figure 5a), suggesting that the p38MAPK activation is not the main mechanism leading to BPAEC protection from TGF- β 1-induced injury. Interestingly, radicicol itself increased phospho-p38 MAPK signal (Figure 5b).

Involvement of hsp27 in the Barrier-Protective Effect of hsp90 Inhibitors

Hsp27 is an hsp90 client protein involved in the regulation of endothelial barrier function via stabilization of actin microfilaments. Thus we examined the effect of RA and TGF- β 1 on hsp27 phosphorylation and association with hsp90. Treatment of BPAEC with TGF- β 1 for 6 hours significantly increased phospho-hsp27 content in BPAEC homogenates (Figure 6a). TGF- β 1-induced hsp27 phosphorylation was attenuated in cells stimulated with TGF- β 1 in the presence of radicicol. RA alone had no effect on phospho-hsp27 expression. Next, we addressed the question of whether TGF- β 1 and RA influence the amount of hsp90 associated with hsp27. Using co-immunoprecipitation techniques, we observed that incubation with TGF- β 1 increased the amount of hsp90 which co-immunoprecipitates with hsp27. RA significantly decreased the TGF- β 1-induced increase in hsp90–hsp27 association (Figure 6b). RA alone decreased association of hsp90 with hsp27 even below control levels.

Distinct Roles of Microtubules and Actin Microfilaments in the Barrier-Protective Effect of RA

Destabilization of actin microfilaments or disassembly of microtubules result in a hyperpermeable endothelial monolayer (1, 7, 26, 27). To test whether the barrier-protective effect of hsp90 inhibitors is mediated by stabilization of actin microfilaments or via targeting of the microtubule cytoskeleton, we examined the effect of RA on BPAEC hyperpermeability after actin microfil-

Figure 2. Effects of TGF- β 1 and radicicol on BPAEC cytoskeletal rearrangement. BPAEC grown on glass coverslips for 10 days were treated with (a, e, h, j) vehicle (0.1% DMSO), (b, f, i, m) RA (1 μ g/ml) for 6 hours, (c, g, j, n) TGF- β 1 (10 ng/ml, 6 h), or (d, h, k, o) TGF- β 1 plus RA (given at the same time, 6 h). Cells were then fixed and double-stained for VE-cadherin (a–d), β -catenin (e–h), F-actin (h–k), and β -tubulin (l–o) as described in MATERIALS AND METHODS. Bars = 20 μ m.

ament disruption by cytochalasin B or after microtubule disassembly by nocodazole. Treatment of BPAEC with either cytochalasin B (5 μ g/ml) or nocodazole (5 μ M) resulted in a rapid and

dramatic decrease in TER (Figures 7A and 7B). Pre-incubation of EC with RA for 3 hours or treatment with RA at the point when the decrease in TER reached its nadir had no effect on BPAEC

Figure 3. Effects of TGF- β 1 and hsp90 inhibitors on BPAEC transendothelial resistance (TER). BPAEC were plated on gold microelectrodes and TER was measured as described in MATERIALS AND METHODS. (a) EC were treated with either vehicle (0.1% DMSO) or TGF- β 1, at the time indicated by arrow, and TER was monitored for 18 hours. (b) EC were treated with vehicle or one of three hsp90 inhibitors at the time indicated by arrow. (c) Cells were pre-treated with vehicle or one of three hsp90 inhibitors for 3 hours (RA, 17-AAG, 17-DMAG; all 1 μ g/ml) followed by TGF- β 1 (10 ng/ml, time indicated by arrow) and TER was monitored for 18 hours. (d) Cells were exposed to TGF- β 1, as above (time indicated by

first arrow), followed by hsp90 inhibitor treatment, 2 to 3 hours after injury, at the nadir of the TER response (time indicated by the second arrow), and TER was monitored for 18 hours. Data are means $\pm$ SE ($n = 4$ for all groups).

Figure 4. Effects of TGF- β 1 and radicicol on MLC and MYPT1 phosphorylation. Confluent BPAEC monolayers were incubated with vehicle (0.1% DMSO), TGF- β 1 (10 ng/ml) alone or together with RA (1 μ g/ml) for the indicated periods of time. (a) MLC or (b) MYPT1 phosphorylation was monitored by Western blotting with di-phospho-MLC and phospho-MYPT-specific antibodies, respectively, as described in MATERIALS AND METHODS. Quantitative analysis of MLC and MYPT1 phosphorylation is expressed as the ratio of phosphorylated to total protein. (c, d) EC were treated with RA alone for 6 hours. Data are means $\pm$ SE ($n = 5$ for all groups). * $P \leq 0.05$ compared with vehicle; # $P \leq 0.05$ compared with corresponding TGF- β 1 alone treatment.

hyperpermeability induced by cytochalasin B (Figure 7A), suggesting that actin microfilament integrity is required for the barrier-protective actions of hsp90 inhibitors. Pretreatment of BPAEC with RA also had no protective effect on the first rapid stage of the decline in TER induced by nocodazole, but, in contrast to cytochalasin B, EC permeability was completely restored when RA was added at the nadir of the nocodazole hyperpermeability response (Figure 7A). In addition, the decreases in interendothelial junction VE-cadherin (Figure 7B, b) and β -catenin (Figure 7B, e) staining observed after nocodazole treatment (5 μ M, for 6 h) was prevented by RA (Figure 7B, c and f), indicating that EC maintained appropriate tight junctions and integrity.

The Barrier-Protective Effect of hsp90 Inhibitors Is Not Limited to TGF- β 1-Induced Hyperpermeability

We also tested the hypothesis that hsp90 inhibitors may protect EC barrier integrity from a diverse spectrum of injurious stimuli. Receptor-mediated agonists thrombin (100 nM), VEGF (50 ng/ml), and LPS (1,000 EU/ml) induced time-dependent decreases in TER, which reached plateau after 4 to 6 hours of treatment (Figures 8a–8c). Pre-incubation of BPAEC with RA significantly attenuated the increased EC permeability induced by all these agonists. The effects of 17-AAG and 17-DMAG were identical to that of RA (data not shown). Furthermore, post-treatment of injured cells with RA completely restored the TER of BPAEC after exposure to all tested agents. RA demonstrated strong protection and restoration of BPAEC barrier function even after nonreceptor, PMA (100 nM)-induced injury (Figure 8d).

DISCUSSION

Lung vascular endothelial cells form a dynamic barrier, which is critical for the regulation of vascular permeability. In the present study we demonstrated for the first time that the most advanced hsp90 inhibitors (RA, 17-AAG, 17-DMAG) prevented and, more importantly, restored EC permeability after stimulation with a crucial proinflammatory cytokine, TGF- β 1.

Previous studies, including our own, have described effects of TGF- β 1 on EC morphology and actin organization (7, 10–13). An important characteristic of intact, confluent endothelial or epithelial monolayers *in situ* and *in vitro* is the ability of borders between cells to stain with silver nitrate, a property that is lost when cells in the monolayer lose intercellular junctions or are not confluent (22).

In addition, VE-cadherin constitutes interendothelial adherens junctions through a homophilic binding of its extracellular domain and by the anchoring of its intracellular domain to actin cytoskeleton via catenins (28–32). VE-cadherin is the target of signaling pathways and agents such as VEGF, histamine, thrombin, and TGF- β 1 that increase vascular permeability (10, 33, 34). Indeed, a recent study demonstrated that the hsp90 inhibitor, geldanamycin, protected EC from VEGF-induced hyperpermeability via a mechanism associated with reduced VE-cadherin phosphorylation and actin stress fiber formation (35).

To directly monitor changes in EC permeability we used the electrical cell impedance sensor (ECIS) technique, which we have successfully used in our previous studies (19, 20). It is well

Figure 5. Effect of radicicol on TGF- β 1-induced p38 MAPK activation. BPAEC were treated with (a) vehicle (0.1% DMSO), TGF- β 1 (10 ng/ml), TGF- β 1 plus RA (1 μ g/ml), given together, or (b) RA alone for the indicated periods of time. Phosphorylated p38MAPK expression was detected by Western analysis using antibodies against phospho-p38MAPK as described in MATERIALS AND METHODS. Phosphorylation of proteins was quantified as the ratio of phospho-p38MAPK to total protein. Data are means $\pm$ SE ($n = 4$ for all groups). * $P \leq 0.05$ compared with vehicle.

documented that changes in TER inversely reflect changes in permeability of EC monolayers (7, 13). In this study we used two approaches: prevention of barrier dysfunction and restoration of barrier function. We demonstrated for the first time that hsp90 inhibitors not only protect EC from potential injury induced by TGF- β 1, but more importantly, that they also restore the barrier function of injured endothelium. We also demonstrated that the barrier-protective effect of hsp90 inhibitors is not selective to TGF- β 1 but, impressively general, as it protects EC from barrier dysfunction induced by other receptor-mediated edemagenic agents, such as thrombin, LPS, and VEGF as well as from barrier dysfunction induced by non-receptor-mediated stimulation with PMA or by the disassembly of microtubules with nocodazole. These findings imply that hsp90 inhibitors may be of therapeutic value in controlling lung edema. These findings agree with two

recent studies from our laboratory, where we demonstrated that 17-AAG and radicicol reduce LPS-induced inflammation, pulmonary dysfunction, and pulmonary edema in mice *in vivo*, as well as reduce LPS-induced endothelial hyperpermeability in EC in culture (17, 36). Furthermore, the protective effects of hsp90 inhibitors in culture, were associated with inhibition of pp60 src, VE-cadherin, and β -catenin phosphorylation, and actin stress fiber formation (36).

It is well known that the EC cytoskeleton has a critical role in EC barrier regulation (1). Destabilization of actin microfilaments or disassembly of microtubules results in a hyperpermeable endothelial monolayer (7, 20, 37). There is growing evidence that hsp90 actively participates in the organization of the cytoskeleton in different cell types. For example, hsp90 has F-actin-bundling activity and binds to F-actin and to Wiskott-Aldrich syndrome

Figure 6. Effects of TGF- β 1 and radicicol on hsp27 phosphorylation and hsp90/hsp27 complex formation. BPAEC were treated with vehicle (0.1% DMSO), TGF- β 1 (10 ng/ml), TGF- β 1 plus RA (1 μ g/ml), given together, or RA alone for 6 hours. (a) The level of phosphorylated hsp27 was detected by immunoblotting using antibody directed against phospho-hsp27, as described in MATERIALS AND METHODS. Hsp27 phosphorylation was quantified as the ratio of phospho-hsp27 to total hsp27. (b) Cell lysates were immunoprecipitated with antibody against hsp27 as described in MATERIALS AND METHODS. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies against

hsp90 or hsp27. The levels of hsp90 associated with hsp27 were quantified as ratio of hsp90 to hsp27. Data are means $\pm$ SE ($n = 4$ for all groups). * $P < 0.05$.

the second arrow. Data are means $\pm$ SE ($n = 4$ for all groups). (B) BPAEC grown on glass coverslips were treated (a, d): with vehicle (0.1% DMSO), (b, e): nocodazole (5 μ M), or (c, f): nocodazole plus RA (1 μ g/ml), given together, for 6 hours. Cells were then fixed and double-stained for VE-cadherin (a–c) and β -catenin (d–f) as described in MATERIALS AND METHODS. Bars = 20 μ m.

protein (N-WASP) (38, 39). Interaction of hsp90 with N-WASP protects N-WASP from proteasome-dependent degradation, and by promoting v-Src-dependent N-WASP phosphorylation, amplifies N-WASP-dependent actin polymerization (39). Hsp90 also stabilizes/activates LIM kinase, promoting actin polymerization by inactivation of the actin depolymerizing factor, cofilin (40). Protein Swol1, a fission yeast analog of hsp90, facilitates myosin II assembly (41). Conversely, inhibition of hsp90 attenuates or abolishes its effects on the actomyosin cytoskeleton (38–41). In the present study, TGF- β 1-induced hyperpermeability was associated with increased hsp27 phosphorylation and hsp90-hsp27 complex formation; both of these effects were prevented by radicicol. Hsp27 phosphorylation is mediated by MAPKAP kinase 2, which in turn is phosphorylated and activated by p38 MAPK (for review see Ref. 42). We did not observe a significant effect of hsp90 inhibitors on TGF- β -induced p38 MAPK activation. This suggests either direct inhibition of MAPKAP kinase 2 or dissociation of MAPKAP kinase 2 from hsp27, or both. Hsp27 is a well-known hsp90 client protein. It binds to actin and, when phosphorylated, it promotes actin-myosin association (43, 44). Hsp27 also mediates the RhoA-induced MYPT phosphorylation and cellular contraction. Hsp27 phosphorylation has been correlated with LPS-induced endothelial dysfunction in ALI (45).

Interestingly, all tested edemagenic agents (except PMA) induce EC barrier dysfunction, at least in part, via activation of

the contractile apparatus (10, 20, 46–49). A key EC contractile event in several models of agonist-induced barrier dysfunction is the phosphorylation of regulatory myosin light chain (MLC) catalyzed by Ca^{2+} /calmodulin-dependent MLC kinase and/or through the activity of the Rho/Rho kinase pathway (1). Rho kinase increases MLC phosphorylation by two potential mechanisms: directly via the phosphorylation of MLC at Ser19 and Thr18, and indirectly via the phosphorylation of the regulatory subunit of MLC phosphatase (MYPT1) at Thr 696 and 853, which suppresses MLC phosphatase activity (25, 50, 51). Our data demonstrate that treatment with hsp90 inhibitors abolished the increases in phospho-MLC and phospho-MYPT1 expression induced by TGF- β 1, which is correlated with the effect of hsp90 inhibitors on TGF- β 1-induced increase in TER, suggesting that the barrier-protective effect of hsp90 inhibitors on agonist-induced EC permeability may depend, at least in part, upon inhibition of EC contraction. Furthermore, radicicol failed to increase TER when added after cytochalasin B.

Taken together, these data strongly suggest that the barrier-protective effect of hsp90 inhibitors require a functional actin cytoskeleton and that hsp90 inhibitors prevent or repair endothelial barrier dysfunction, at least in part, by inducing or maintaining cortical actin formation via inhibition of activity and expression of key kinases, leading to suppression of MLC phosphorylation.

In summary, hsp90 inhibitors have emerged as an attractive therapeutic modality for various types of cancer, causing combi-

Figure 7. Role of microtubules and actin microfilaments on the barrier-protective effect of radicicol. (A) BPAEC were plated on gold microelectrodes and TER was measured as described in MATERIALS AND METHODS. (a) Cells were pretreated with RA (1 μ g/ml) for 3 hours (first arrow) followed by cytochalasin B (5 μ g/ml; blue line) indicated by the second arrow; or EC were first treated with cytochalasin B (indicated by the second arrow) and at the nadir of TER response they received RA (1 μ g/ml), indicated by the third arrow. Control groups were treated with vehicle (0.1% DMSO) or cytochalasin B alone. TER was monitored for 18 hours. (b) Cells were treated as above but instead of the actin microfilament disrupter, cytochalasin B, microtubules were disassembled by nocodazole (5 μ M), indicated by

Figure 8. Effects of radicicol on EC barrier disruption induced by receptor- and non-receptor-mediated agonists. BPAEC were plated on gold microelectrodes and TER was measured as described in MATERIALS AND METHODS. Cells were pretreated with RA (1 μ g/ml, indicated by the first arrow) for 3 hours and then exposed to (a) thrombin (100 nM), (b) VEGF (50 ng/ml), (c) LPS (1,000 EU/ml), or (d) PMA (100 nM), as indicated by the second arrow. To examine the effect of RA on restoration of injured EC, RA was added at the nadir of the TER response (indicated by the third arrow). Control groups were treated with vehicle (0.1% DMSO) or with agonists alone. TER was monitored for 18 hours. Data are means $\pm$ SE ($n = 4$).

natorial blockage of numerous growth-promoting and apoptosis-blocking pathways, but their involvement in the regulation of endothelial barrier function and lung inflammation has not been investigated. We conclude that hsp90 inhibitors protect from the EC barrier from dysfunction induced by several inflammatory mediators that are involved in pathogenesis of ALI, ARDS, and other pulmonary inflammatory diseases. These findings emphasize the potential therapeutic value of hsp90 inhibitors in lung inflammation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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