Heat shock protein 90 inhibitors attenuate LPS-induced endothelial hyperpermeability

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Chatterjee A, Snead C, Yetik-Anacak G, Antonova G, Zeng J, Catravas JD. Heat shock protein 90 inhibitors attenuate LPS-induced endothelial hyperpermeability. Am J Physiol Lung Cell Mol Physiol 294: L755–L763, 2008. First published February 1, 2008; doi:10.1152/ajplung.00350.2007.—Endothelial hyperpermeability leading to vascular leak is an important consequence of sepsis and sepsis-induced lung injury. We previously reported that heat shock protein (hsp) 90 inhibitor pretreatment improved pulmonary barrier dysfunction in a murine model of sepsis-induced lung injury. We now examine the effects of hsp90 inhibitors on LPS-mediated endothelial hyperpermeability, as reflected in changes in transendothelial electrical resistance (TER) of bovine pulmonary arterial endothelial cells (BPAEC). Vehicle-pretreated cells exposed to endotoxin exhibited a concentration-dependent decrease in TER, activation of pp60src, phosphorylation of the focal adhesion protein paxillin, and reduced expression of the adherens junction proteins, vascular endothelial (VE)-cadherin and β-catenin. Pretreatment with the hsp90 inhibitor, radicicol, prevented the decrease in TER, maintained VE-cadherin and β-catenin expression, and inhibited activation of pp60src and phosphorylation of paxillin. Similarly, when BPAEC hyperpermeability was induced by endotoxin-activated neutrophils, pretreatment of neutrophils and/or endothelial cells with radicicol protected against the activated neutrophil-induced decrease in TER. Increased paxillin phosphorylation and decreased expression of β-catenin and VE-cadherin were also observed in mouse lungs 12 h after intraperitoneal endotoxin and attenuated in mice pretreated with radicicol. These results suggest that hsp90 plays an important role in sepsis-associated endothelial barrier dysfunction.

endothelial permeability; radicicol; endotoxin; acute respiratory distress syndrome

Gram-negative bacterial sepsis affects more than 750,000 people annually in the United States alone (31); it is often associated with endothelial hyperpermeability leading to vascular leak syndromes, including acute respiratory distress syndrome (ARDS; Refs. 32, 44). Endotoxin or LPS, the bacterial cell envelope component responsible for most of the septic response, activates macrophages, neutrophils, and other cells to produce inflammatory mediators and free radicals that cause endothelial damage (25). Direct activation of endothelial cells by LPS is one of the earliest causes of endothelial dysfunction in sepsis (3, 19).

Heat shock protein 90 (hsp90), an abundant molecular chaperone, is involved with folding, maturation, and stabilization of numerous client proteins ranging from tyrosine kinases to several different types of transcription factors and proteins controlling growth and survival (49). Hsp90 inhibitors, such as radicicol, interact with the NH2-terminal ATP binding site of hsp90 and result in destabilization and degradation of client proteins (47). Hsp90 inhibitors have emerged as an attractive therapeutic modality for various types of cancer, causing combinatorial blockade of numerous growth-promoting and apoptosis-blocking pathways (45, 55).

Regulation of endothelial permeability is a complex process involving signaling components that are associated with cytoskeletal and membrane-bound proteins that maintain cell-cell or cell-matrix contacts. LPS phosphorylates paxillin, a constituent of the focal adhesion complex (FAC; 3, 4), by mechanisms that include pp60src, a client protein of hsp90 (8, 51). Furthermore, pp60src phosphorylates adherens junction components, such as p120 catenin (p120catenin) and β-catenin, resulting in loss of vascular endothelial (VE)-cadherin and disruption of cell-cell adhesion (43).

Neutrophils are a major contributing factor to the pulmonary pathophysiology associated with sepsis (1, 35). Activated neutrophils produce reactive nitrogen species, as a result of increased expression of the inducible nitric oxide synthase (iNOS; Ref. 48), and are responsible for endothelial monolayer barrier disruption through protein nitration of key elements regulating cell-cell barrier function such as β-catenin (26) and tight junction proteins (33). A critical role of hsp90 in iNOS-mediated cytotoxic action was suggested when hsp90 was demonstrated to allosterically enhance iNOS, dose-dependently increasing its activity and NO output in vitro (68).

Recent evidence indicates an anti-inflammatory effect of hsp90 inhibitors (10, 24, 30, 37, 64, 67). We (12) recently reported that hsp90 inhibitors significantly improve survival, reduce lung injury, and attenuate systemic and pulmonary inflammation in a murine model of LPS-induced sepsis. The goal of the present study was to determine the direct effect of hsp90 inhibition on LPS-induced endothelial permeability. We hypothesized that hsp90 inhibition would reduce pp60src activation and neutrophil-mediated nitric oxide production resulting in attenuation of LPS- and activated neutrophil-mediated endothelial barrier dysfunction. Our results demonstrate that hsp90 inhibitors attenuate LPS-induced activation of endothelial pp60src and paxillin phosphorylation and confer a significant protection of the endothelial barrier.

METHODS

Reagents and animals. Anti-paxillin antibody was from Santa Cruz Biotechnology (cat. no. sc-5574), and anti-phospho (Tyr118) paxillin antibody was from BD Biosciences (cat. no. 611724). Anti-pp60src antibody was obtained from BioSource (cat. no. AHO 1152), as was anti-active pp60src antibody (cat. no. AHO 0051) that recognized only the dephosphorylated Tyr529 pp60src. Anti-VE-cadherin antibody was from Santa Cruz Biotechnology (cat. no. sc-28644). Anti-
active β-catenin antibody (recognizing the nonphosphorylated Ser37 and Thr41 residues) was from Upstate Biotechnology (cat. no. 05-655). Anti-rabbit IgG conjugated with fluorescent dye Cy3 (cat. no. 111-165-003, Jackson ImmunoResearch) was used as the secondary antibody for VE-cadherin immunostaining, and anti-mouse IgG conjugated with fluorescent dye Cy2 (cat. no. 111-225-003, Jackson ImmunoResearch) was used as the secondary antibody for active pp60src immunostaining. All other reagents were purchased from Sigma. Male C57BL/6 mice (7–8 wk old, Harlan Laboratories) were used in all experiments. Mice were maintained in pathogen-free conditions with 12-h light-dark cycle.

**Cell culture.** In-house harvested bovine pulmonary arterial endothelial cells (BPAEC) were subcultured from primary cultures and used in experiments during passage 3. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2-95% air in medium 199 supplemented with 10% fetal bovine serum, 5% iron-supplemented calf serum, 20 mg/ml l-glutamine, 1 × MEM amino acid and vitamin solutions, 0.6 mg/ml thymidine, 500 IU/ml penicillin, and 500 mg/ml streptomycin. The endothelial cells grew to contact-inhibited monolayers exhibiting typical cobblestone morphology and uptake of acetylated LDL (Biomedical Technologies).

**Estimation of endothelial barrier function from measurements of transendothelial electrical resistance.** The method is based on an established process to monitor cell behavior by electric cell-substrate impedance sensing or ECIS (58) using an ECIS Model 1600R and electrode arrays (8W1E) obtained from Applied BioPhysics. Each well of these 8-well arrays has a small gold film surface active electrode (250 mm diameter) and a large counter electrode. A uniform confluent endothelial monolayer reduces the amount of current flowing across the gold electrode to the counter electrode and thus increases the resistance. The total electrical resistance measured dynamically across the monolayer reflects the combined resistance between the ventral surface of the cell and the electrode, reflective of focal adhesion as well as the resistance between cells. Intercellular gaps increase current flow and reduce the resistance. Thus a change in transendothelial electrical resistance (TER) represents a change in cell-cell adhesion and/or cell-matrix adhesion. Experiments were conducted on wells that achieved >800 Ω of baseline, steady-state resistance.

**Experiments with mouse neutrophils.** Adult male mice (8 wk old) were injected intraperitoneally with 3% Difco thioglycolate medium (cat. no. 225640, BD Biosciences). Four hours later, the animals were killed and peritoneal lavage was performed six times (5 ml of PBS each time). With this procedure, neutrophils are the predominant cell type harvested (~95%; Ref. 62). The combined lavage suspensions were spun at 600 g for 15 min, and neutrophils were resuspended in 2 ml of PBS. This method allows the harvesting a substantial number of neutrophils from a small group of animals (5). Before each experiment, neutrophils were tested for activation by measuring basal and PMA-stimulated release of superoxide radicals by the luminol derivative L-012 (cat. no. 120-04891, Wako Chemicals; Ref. 41). All isolations produced nonactivated neutrophils. Neutrophils were incubated with LPS [1,000 endotoxin units (EU)/ml; n = 4, all groups]. A significant attenuation in the LPS-induced decrease in TER was observed in all RA-treated groups from as early as 30 min post-LPS. Error bars represent SE; where not shown, error bars are within the symbol. All RA-pretreated groups are significantly different (*P < 0.01) from the DMSO + LPS group.

Fig. 1. LPS reduces transendothelial electrical resistance (TER). PBS (vehicle) or LPS was added to the media of confluent bovine pulmonary arterial endothelial cell (BPAEC) monolayers at 0 h. Resistance was recorded over 4 h and normalized to the 0-h value. A gradual increase in endothelial permeability (reduced TER) was observed with increasing concentrations of LPS. *P < 0.05 from PBS group; n = 4 per group. Error bars represent SE; where not shown, error bars are within the symbol.

Fig. 2. The heat shock protein 90 (hsp90) inhibitor, radicicol (RA), attenuates the LPS-induced endothelial hyperpermeability. BPAEC were preincubated for 2.5 h with RA (0.1–1 µg/ml) or vehicle (10% DMSO) followed by LPS [1,000 endotoxin units (EU)/ml; n = 4, all groups]. A significant attenuation in the LPS-induced decrease in TER was observed in all RA-treated groups from as early as 30 min post-LPS. Error bars represent SE; where not shown, error bars are within the symbol. All RA-pretreated groups are significantly different (*P < 0.01) from the DMSO + LPS group.

Fig. 3. RA attenuates LPS-induced paxillin phosphorylation in BPAEC. Compared with vehicle (VEH), LPS produced a 2-fold increase in the ratio of phosphorylated paxillin to pan-paxillin; this effect was prevented in BPAEC pretreated with RA. n = 6 in all groups. *P < 0.05 from Vehicle; #P < 0.05 from Vehicle + LPS. Error bars represent SE.
from the BPAEC monolayers 1 h before the start of the experiment and replaced with serum-free culture media. Neutrophils in PBS were added to each well, and TER was monitored over the specified period of time.

**Immunofluorescence studies.** BPAEC were grown to confluence on 13-mm coverslips. Cells were first exposed to vehicle or radicicol for 2.5 h and then to PBS or LPS for 3 h (a time corresponding to the maximum effect of LPS on TER), washed with PBS, fixed with 4% paraformaldehyde, permeabilized with Triton X-100 for 15 min, and blocked with 1% BSA for 1 h at room temperature. BPAEC were then treated with appropriate primary and secondary antibodies, washed three times in PBS (0.1% BSA), mounted onto glass slides with mounting medium (ProLong antifade kit), and viewed under a Zeiss Axiohot 2 epifluorescence microscope equipped with SPOT camera (Diagnostic Instruments).

**In vivo experiments.** All animal experiments were approved by the Committee on Animal Use in Research and Education of the Medical College of Georgia and adhered to the principles of humane animal care adopted by the American Physiological Society. Stock solutions of *Escherichia coli* LPS (0111:B4) were prepared in saline. Mice received vehicle (10% DMSO in saline) or hsp90 inhibitor (dissolved in 10% DMSO) intraperitoneally (5 μg/g body wt) 24, 12, 6, and 0 h before LPS (6.75 × 10^4 EU/g body wt). Mice were killed at 12 h after LPS injection by cervical dislocation, and the lungs were flushed with 1 ml of ice-cold PBS (5 mM EDTA), excised, dipped in saline, blotted dry, quickly snap-frozen in liquid nitrogen, crushed to powder in a prechilled mortar, and stored at −80°C.

**Western blotting and immunoprecipitation experiments.** Portions of stored lung samples were Dounce-homogenized in 1 ml of ice-cold RIPA buffer with protease inhibitors, sodium orthovanadate and sodium molybdate. Additionally, at the end of the experiment, BPAEC grown in 100-mm dishes were washed three times with ice-cold PBS (with sodium orthovanadate) and scraped with modified RIPA lysis buffer. After end-over-end rotation for 2 h at 4°C, samples were centrifuged at 21,000 g for 15 min, and the supernatants were loaded on a 7.5% polyacrylamide SDS gel for electrophoresis. Proteins were transferred on polyvinylidene difluoride membranes (Amersham), reacted with primary antibodies of interest, incubated with appropriate horse radish peroxidase-conjugated secondary antibodies, and detected by enhanced chemiluminescence (Amersham).

**Statistical analysis.** Data are presented as means ± SE. Statistical analyses between or among groups were performed using one-way ANOVA or Student’s t-tests as appropriate. Differences at *P < 0.05* were considered significant.

**RESULTS**

**LPS dose-dependently reduces TER.** BPAEC were grown on gold electrode arrays and, after 4 days, were exposed to PBS (vehicle) or LPS. PBS maintained constant TER values (shown in Fig. 1 normalized to the 0-h value), suggesting a confluent endothelial monolayer and minimal experimental variability over the 4 h of the study. LPS profoundly reduced TER at a

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**Fig. 4. RA attenuates LPS-induced pp60Src activation in BPAEC.** *A and B:* compared with vehicle, LPS caused a 1.8-fold increase from vehicle in the ratio of active pp60Src to pp60Src, as indicated by Western blotting; this effect was prevented in LPS-treated BPAEC that were pretreated with RA. *n = 6* in all groups except for the Vehicle-LPS group where *n = 5.* *P < 0.05* from Vehicle; *#P < 0.05* from Vehicle + LPS. Error bars represent SE. *C and D:* confluent BPAEC were pretreated with RA or vehicle for 2.5 h followed by a 3-h exposure to PBS or LPS. Fluorescence of Cy2-tagged secondary antibody recognizing anti-active pp60Src primary antibody is shown. *Insets:* magnified view of an endothelial cell. Note the enhanced fluorescence of Vehicle-LPS-exposed cells compared with the other groups. Bar graph (*D*) represents quantification performed using Zeiss LSM 510 META 3.2 software. *n = 3* per group; *#P < 0.05* from Vehicle; *#P < 0.05* from Vehicle + LPS-treated group. Error bars represent SE. IF, immunofluorescence.

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concentration as low as 100 EU/ml and produced incrementally greater time- and concentration-dependent suppressions of TER.

The hsp90 inhibitor, radicicol, protects against LPS-induced endothelial barrier dysfunction. Confluent BPAEC monolayers were incubated with different concentrations of radicicol (0.1, 0.5, and 1 μg/ml) or vehicle (10% DMSO) for 2.5 h followed by 1,000 EU/ml LPS. A steep decrease in TER was observed in the vehicle-LPS group, whereas all radicicol-treated groups exhibited a significant attenuation in the fall in TER starting as early as 30 min after the addition of LPS and lasting until the end of the experiment (Fig. 2). All radicicol-treated groups recovered to their original starting resistance value within 7 h from LPS treatment and then exhibited a continuing increase in TER, suggesting supranormal strengthening of BPAEC barrier function by the hsp90 inhibitor. Conversely, TER of vehicle/LPS-treated wells continued to drop for the duration of the experiment, bringing the resistance to ~60% of starting values.

Radicicol reduces LPS-induced paxillin phosphorylation and pp60Src activation. BPAEC grown to confluence in 60-mm dishes were treated with vehicle or radicicol (1 μg/ml) for 2.5 h followed by PBS or LPS (1,000 EU/ml) for 3 h. Cells were then lysed, subjected to PAGE, and probed for phosphorylated paxillin and pan-paxillin as well as activated and total pp60Src. Since activation of pp60Src requires dephosphorylation of the COOH-terminal tyrosine, Y-529 (50), we used a monoclonal antibody that recognized that site. Compared with vehicle, LPS produced ~2-fold increase in immunoreactive phosphorylated paxillin (Fig. 3), which was reduced significantly to control levels in radicicol-pretreated and LPS-treated cells. LPS also produced a ~85% increase in the ratio of activated to total pp60Src from vehicle, which was reduced to control levels in radicicol-pretreated and LPS-treated cells (Fig. 4). These findings were confirmed in additional experiments of immunofluorescent analyses of activated pp60Src (Fig. 4), which exhibited enhanced active pp60Src fluorescence in LPS-treated BPAEC compared with vehicle-treated or with radicicol-pretreated and LPS-treated cells.

Radicicol prevents LPS-induced loss of VE-cadherin. Indirect immunofluorescence studies revealed that vehicle-treated cells exhibit strong expression of VE-cadherin, an endothelium-specific transmembrane glycoprotein that plays a major role in the organization of adherens junctions; VE-cadherin expression was concentrated in interendothelial junctions (Fig. 5A). Vehicle-
pretreated, LPS-treated cells exhibited a profound loss of VE-cadherin, which was significantly prevented by pretreatment of BPAEC with radicicol. These findings were confirmed in experiments where VE-cadherin expression was detected by Western blotting (Fig. 5B). Taken together, the data shown in Figs. 4 and 5 suggest that pp60Src may mediate the LPS-induced loss in VE-cadherin expression. To confirm this, BPAEC were pretreated with either vehicle or the pp60Src inhibitor PP2 (10 μM). As shown in Fig. 6, PP2 pretreatment prevented the LPS-induced loss in VE-cadherin expression.

**Antioxidant treatment fails to prevent the LPS-induced hyperpermeability of cultured BPAEC.** LPS increases oxidative stress (21), and hsp90 inhibitors have been associated with increased cellular levels of antioxidants (34, 46). We therefore examined whether pretreatment of BPAEC with either N-acetylcysteine (NAC) or ascorbic acid (AA) might mimic the protective effects of hsp90 inhibitors and prevent LPS-induced hyperpermeability. As shown in Fig. 7, neither NAC nor AA was able to prevent the decrease in TER induced by LPS.

**Radicicol attenuates the activated neutrophil-induced increase in endothelial permeability.** Since neutrophils are important mediators of sepsis-induced lung hyperpermeability, we studied whether hsp90 inhibitors would ameliorate neutrophil-induced endothelial monolayer barrier dysfunction. LPS-activated neutrophils produced a concentration-dependent profound decrease in TER (Fig. 8A) within 2–4 h, reaching a plateau after ~5 h. At the highest concentration (500,000 per well), nonactivated neutrophils did not reduce TER and exhibited a profile similar to vehicle. To test the effects of hsp90 inhibitors, neutrophils (Fig. 8B), BPAEC (Fig. 8C), or both (Fig. 8D) were preincubated with radicicol for 2 h before neutrophils were activated with LPS, washed, and placed on top of the endothelial monolayer. As observed before, vehicle preincubation produced a profound drop in TER in response to LPS-activated neutrophils. However, pretreatment of either neutrophils or both neutrophils and endothelial cells completely prevented the decrease in TER (Fig. 8, B and D); even radicicol pretreatment of endothelial cells alone resulted in a dramatic protection of barrier dysfunction (Fig. 8C).

**Radicicol prevents sepsis-induced phosphorylation of lung paxillin and maintains expression of lung VE-cadherin and β-catenin.** In mice pretreated with vehicle before injecting LPS, a profound decrease in VE-cadherin (Fig. 9A) and active β-catenin (Fig. 9B) expression in lung homogenates was observed 12 h post-LPS compared with controls. However, in mice pretreated with radicicol (3 mg/kg), VE-cadherin and β-catenin expression remained at normal levels. Furthermore, in mice pretreated with vehicle before LPS, lung paxillin phosphorylation was dramatically increased 12 h post-LPS (Fig. 10) but remained at normal levels in radicicol-pretreated mice.

**DISCUSSION**

We (12) recently reported that in a murine model of LPS-induced severe sepsis, hsp90 inhibitors, such as radicicol, effectively prolong survival and prevent the development of acute lung injury, including significant inhibition of lung leak. These findings suggest the possibility that hsp90 inhibitors protect endothelial barrier function either by directly acting on the endothelium or indirectly. The present study provides evidence in support of the hypothesis that hsp90 inhibitors protect from LPS-induced endothelial barrier dysfunction by acting both directly on the endothelium and indirectly by inhibiting neutrophil-induced toxicity. These data further suggest that hsp90 inhibitors act, at least in part, by preventing pp60Src activation and paxillin phosphorylation and by maintaining optimal expression of VE-cadherin and β-catenin.
Endothelial cells lack the membrane-bound CD14 receptor recognized by LPS-LBP complexes, which is expressed in neutrophils, resulting in their activation. LPS-mediated signaling events in endothelial cells are unclear, but it is hypothesized that LBP-bound LPS interacts with soluble CD14 and that this complex plays an important role in LPS-mediated endothelial cell responses (3). LPS can also initiate these responses independently of LBP and soluble CD14 but at higher LPS concentrations. Accordingly, in our experiments, a much higher LPS concentration was required to stimulate endothelial cells than neutrophils (as reflected in decreases in TER).

FAC, consisting of a highly specialized array of integrins and other proteins, provide the primary means of anchoring the endothelial cell monolayer to the extracellular matrix (11). Many FAC proteins require tyrosine phosphorylation for their activation and translocation out of the complex (54). The tyrosine kinase pp60Src is essential for FAC protein phosphorylation and is believed to regulate focal adhesion kinase activity (28). Accordingly, LPS-treated BPAEC exhibited a twofold increase in active pp60Src that was associated with an increase in permeability, whereas the hsp90 inhibitor, radicicol, blocked both pp60Src activation and BPAEC hyperpermeability. Total levels of pp60Src did not change significantly, suggesting that radicicol might have interfered with pp60Src Y-509 dephosphorylation without altering its expression. This is in agreement with a recent report that in a human bladder cell line, 2 h after treatment with the hsp90 inhibitor, geldanamycin, activity of pp60Src decreased without any changes in total pp60 Src protein levels (27). Our findings are the first to show LPS-mediated pp60Src activation in endothelial cells but agree with studies of LPS-induced pp60Src activation in other cell types (15, 29). Src kinases are potential proinflammatory mediators in sepsis and acute lung injury (42). pp60Src regulates endothelial cell contraction through tyrosine phosphorylation of myosin light chain kinase and cortactin, thus signaling increased endothelial permeability (9, 18). Inhibition of pp60Src...
blocks the TNF-α augmentation of thrombin-induced increase in endothelial permeability by inhibiting Ca^{2+} entry (59). pp60Src also increases endothelial cell permeability in response to superoxide anions (53) and VEGF (65). pp60Src is one of the first recognized hsp90 client proteins, and hsp90 inhibitors reduce hsp90-p60Src complex formation (66). Therefore, pp60Src is a potentially relevant therapeutic tool for modulating inflammatory signaling.

Endothelial cells are connected to each other by a complex set of junctional proteins that comprise tight junctions, adherens junctions, and gap junctions. Adherens junctions are of fundamental importance in regulating endothelial barrier function (6). Endothelial adherens junction contain VE-cadherin as the major structural protein that mediates homophilic binding and adhesion of adjacent cells (38). Catenins form an important class of proteins that associate with actin filaments and VE-cadherins and are involved in the proper assembly and function of the adherens junction complex (40). Our in vivo studies are the first to show a remarkable decrease in the expression of functional VE-cadherin and β-catenin in lungs from septic mice. This occurs at a time of significant pulmonary hyperpermeability, as we (12) reported recently. VE-cadherin is associated with p120cat through a cytoplasmic juxtamembrane domain. The relative levels of VE-cadherin expressed on the junctional surface are dictated by p120cat levels. Disruption of this interaction has been shown to trigger cadherin internalization and endosomal degradation (61). Members of the Src family kinases phosphorylate VE-cadherin and p120cat (2) and result in profound disorganization of cadherin-dependent cell-cell contacts (7, 56, 63), suggesting an additional pathway through which Src kinases may regulate endothelial permeability. Indeed, inhibition of Src kinases have conferred protection from LPS-induced lung injury in mice (52). It is possible that in the present model, Src kinases played a role in VE-cadherin downregulation and that, by blocking pp60Src activation, radi-cicol attenuated VE-cadherin degradation and affected a higher VE-cadherin expression in lung tissue. A near-complete loss of functional β-catenin was observed in sepsis 12 h post-LPS. β-Catenins serve as linker proteins between VE-cadherins and α-catenins. The interaction between β-catenin and VE-cadherin is vulnerable to dissociation through pp60Src-mediated tyrosine phosphorylation of β-catenin (22, 36). Phosphorylation of this membrane-bound β-catenin results in its translocation to the cytosolic compartment where it is phosphorylated by other kinases leading to proteasomal degradation. We have probed lung lysates with an antibody that recognizes the nonphosphor-ylated, membrane-bound catenin (referred to as active β-catenin) and show that its expression is reduced in sepsis. This suggests a potential involvement of pp60Src toward the regulation of functional β-catenin expression as previously shown for neutrophil-induced hyperpermeability of endothelial cells.
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(57). Additionally, we observed that the pp60Src inhibitor, PP1, ameliorated the LPS loss in VE-cadherin expression.

LPS activates neutrophils to adhere to the endothelium (13), secrete destructive elastases and lipid mediators (14, 16), release reactive oxygen radicals (20), and produce reactive nitrogen radicals such as peroxynitrite (17). Peroxynitrite increases endothelial permeability by nitration of key cell-junction proteins (23, 26, 39). It is therefore possible that at least part of the BPAEC hyperpermeability produced by activated neutrophils is mediated by reactive nitrogen species and that the protective effect of hsp90 inhibitors is due to reduced reactive nitrogen species. Additionally, LPS activates neutrophil NF-κB, which mediates increased synthesis and production of many proinflammatory mediators that also regulate endothelial permeability (1). Since hsp90 inhibitors also attenuate NF-κB activity (60), it is reasonable to assume that this might also act as a protective mechanism in neutrophil-induced endothelial permeability.

Our data also show that even pretreatment of only endothelial cells with hsp90 inhibitors protects them from activated neutrophil-induced endothelial monolayer barrier dysfunction. A recent study has shown that pretreatment of cells with the hsp90 inhibitor, 17-allylamino-demethoxygeldanamycin (17AAG), resulted in an increase in glutathione (GSH) levels (34). GSH is a powerful antioxidant, beneficial to endothelial cells in the presence of activated neutrophils. It was thus possible that the observed protective effect of radicicol in the present study might have been GSH-mediated. However, pretreatment of endothelial cells with either of two potent anti-oxidants (NAC or AA) failed to inhibit the LPS-induced hyperpermeability.

In conclusion, the present findings demonstrate that hsp90 inhibitors exert barrier-enhancing effects and suggest that they may prove beneficial in improving endothelial barrier function not only in sepsis, but also under other inflammatory conditions.

REFERENCES
