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p53 protects against LPS-induced lung endothelial barrier dysfunction

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Barabutis N, Dimitropoulou C, Birmpas C, Joshi A, Thangjam G, Catravas JD. p53 protects against LPS-induced lung endothelial barrier dysfunction. Am J Physiol Lung Cell Mol Physiol 308: L776–L787, 2015. First published February 20, 2015; doi:10.1152/ajplung.00334.2014.—New therapies toward heart and blood vessel disorders may emerge from the development of Hsp90 inhibitors. Several independent studies suggest potent anti-inflammatory activities of those agents in human tissues. The molecular mechanisms responsible for their protective effects in the vasculature remain unclear. The present study demonstrates that the transcription factor p53, an Hsp90 client protein, is crucial for the maintenance of vascular integrity, protects against LPS-induced endothelial barrier dysfunction, and is involved in the mediation of the anti-inflammatory activity of Hsp90 inhibitors in lung tissues. p53 silencing by siRNA decreased transendothelial resistance (a measure of endothelial barrier function). A similar effect was induced by the p53 inhibitor pifithrin, which also potentiated the LPS-induced hyperpermeability in human lung microvascular endothelial cells (HLMVEC). On the other hand, p53 induction by nutlin suppressed the LPS-induced vascular barrier dysfunction. LPS decreased p53 expression in lung tissues and that effect was blocked by pretreatment with Hsp90 inhibitors both in vitro and in vivo. Furthermore, the Hsp90 inhibitor 17-allyl-aminodemethoxy-geldanamycin suppressed the LPS-induced overexpression of the p53 negative regulator MDMX as well as p53 and MDM2 (another p53 negative regulator) phosphorylation in HLMVEC. Both negative p53 regulators were downregulated by LPS in vivo. Chemically induced p53 overexpression resulted in the suppression of LPS-induced rhoa activation and mlc2 phosphorylation, whereas p53 suppression caused the opposite effects. These observations reveal new mechanisms for the anti-inflammatory actions of Hsp90 inhibitors, i.e., the induction of the transcription factor p53, which in turn can orchestrate robust vascular anti-inflammatory responses both in vivo and in vitro.

acute lung injury; hsp90; human pulmonary endothelium; lung barrier function; p53

LIPOLYSACCHARIDE (LPS) is the major component of the outer wall of gram-negative bacteria. It activates macrophages, neutrophils, dendritic, and other cells that induce inflammation, oxidative stress, and endothelial damage (6). Exposure to LPS leads to endothelial barrier dysfunction, increased endothelial permeability, acute lung injury, and acute respiratory distress syndrome (1). We have recently reported that heat shock protein 90 (Hsp90) is an important positive regulator of these effects of LPS and that inhibitors of Hsp90 prevent and repair the LPS-induced cellular damage, both in vitro and in vivo (3, 26, 59).

Hsp90 is a highly conserved cellular chaperone and one of the most abundant proteins in eukaryotic cells (27). It represents 1–2% of total cellular proteins and participates in the stabilization and activation of more than 200 “client” proteins (61). Hsp90 forms multichaperone complexes with a variety of cochaperones and proinflammatory client proteins. These complexes cycle between an open and a closed conformation of the dimeric Hsp90. Hsp90 inhibitors, such as 17-allyl-aminodemethoxy-geldanamycin (17-AAG), lock the complex in the open state, leading to client protein deactivation, destabilization, and proteasomal degradation (52).

p53 serves as an Hsp90 client protein able to regulate major metabolic pathways and cytokines, which are required for cellular growth and defense (34, 51). Hsp90 binds to a folded, native-like conformation of p53 in vitro with micromolar affinity. The DNA-binding domain of p53 and the middle and carboxy-terminal domains of Hsp90 are responsible for this interaction, which is essential to stabilize p53 at physiological temperatures and to prevent it from irreversible thermal inactivation (44). In fact, multiple DNA binding domains interact with multiple Hsp90 sites (21).

p53 was discovered 30 years ago as a cellular partner of simian virus 40 large T antigen, an oncprotein of this tumor virus. A decade later, it became clear that p53 is actually a tumor suppressor that is frequently mutated in most human cancers, is induced by stress, and promotes cell cycle arrest, apoptosis, and senescence (31). Additionally, p53 is involved in the induction of anti-inflammatory activities in various tissues (30, 35). Thus recent studies from several groups, including ours, have revealed a potent anti-inflammatory action of Hsp90 inhibitors in vascular tissues that involves modulation of Hsp90 activation (3, 16, 26). The molecular mechanisms that drive those effects remain unclear. We therefore tested the hypothesis that at least part of the anti-inflammatory effects of Hsp90 inhibitors may be mediated by the “guardian of the genome” and tumor suppressor protein, p53 (32).

MATERIALS AND METHODS

Reagents. 17-AAG was obtained from the National Cancer Institute (Bethesda, MD). Anti-Hsp90 antibody (624088) was purchased from BD Biosciences Transduction Laboratories (San Jose, CA). MDMX (sc-74468) antibody, p53 siRNA (sc-29435), and control siRNA
(sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). p53 (9282s), p\(^{\text{s15}}\)53 (9284p), p\(^{\text{s392}}\)53 (9281P), p21 Waf/Cip (2947s), pMDM2 (3521s), p-myosin light chain 2 (3674s), and RhoA (8789s) antibodies were obtained from Cell Signaling (Danvers, MA). β-Actin antibody (P8999), CellLyticM Lysis Reagent (C2978), nutlin-3a (SML0580), pifithrin a (P4359), and EZview Red Protein Affinity Gel beads (P6486) were purchased from Sigma-Aldrich (St. Louis, MO). Secondary mouse and rabbit antibodies were purchased from Licor (Lincoln, NE). Oligofectamine (12252011), Pierce BCA protein assay, and nitrocellulose membranes were obtained from Fisher Scientific (Pittsburgh, PA).

**Animals.** Seven to 8-wk-old male C57BL/6 mice from Jackson Laboratories were used in all experiments. Mice were maintained under pathogen-free conditions in a 12:12-h light-dark cycle. All animal care and experimental procedures were approved by the Old Dominion University IACUC and were in line with the principles of humane animal care adopted by the American Physiological Society.

**Cell culture.** In house harvested human lung microvascular endothelial cells (HLMVEC) were maintained in M199 media supplemented with 20% FBS and antibiotics/antimycotics, as described previously (5).

**Fig. 1. Effects of p53 expression on endothelial barrier dysfunction.** A: Western blot analysis of p53 expression after 4 h treatment with either vehicle (DMSO; VEH) or 25 μM or 50 μM pifithrin (PTH) in human lung microvascular endothelial cells (HLMVEC). Blot shown is representative of 3 independent experiments. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05 vs. vehicle, **P < 0.01 vs. vehicle. Means ± SE. B: vehicle (DMSO) or PTH (25 μM, 50 μM) was added to the media of confluent HLMVEC monolayers at 0 h. PTH (50 μM) caused a reduction in transendothelial resistance (TER). ****P < 0.0001 vs. corresponding untreated cell group; n = 4 per group. Means ± SE. C: cells were pretreated for 4 h with either vehicle (DMSO) or 50 μM PTH and were exposed to LPS (1 EU/ml, at arrow). A gradual increase in endothelial permeability (reduced TER) was observed in LPS-treated cells, which was more pronounced in PTH-treated cells. ****P < 0.0001 vs. vehicle, ####P < 0.0001 vs. 50 μM PTH alone, $$$P < 0.01 vs. VEH+LPS, & & & P < 0.01 vs. VEH; n = 4 per group. Means ± SE. D: HLMVEC pretreated with either vehicle (DMSO) or 10 μM nutlin for 4 h were exposed to LPS (1 EU/ml, at arrow). LPS did not induce a significant increase in endothelial permeability (reduced TER) in nutlin-treated cells. However, vehicle-pretreated cells exhibited reduced TER values after LPS exposure. ****P < 0.0001 vs. VEH, n = 3 per group. Means ± SE (left). Western blot analysis of p53 expression in HLMVEC after 4 h treatment with either vehicle (DMSO) or 10 μM nutlin. Blot shown is representative of 3 independent experiments. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05 vs. control siRNA. Means ± SE (left). Western blot analysis of p53 expression in HLMVEC 48 h after siCtr or sip53 transfection. Blot shown is representative of 3 independent experiments. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05 vs. control siRNA. Means ± SE (right).
**Measurement of endothelial barrier function.** The barrier function of endothelial cell monolayers grown on electrode arrays (8W10E+) was estimated by the electric cell-substrate impedance sensing (ECIS) method, as previously published (3), by using an ECIS model 1600R from Applied BioPhysics. Experiments were conducted on wells that achieved at least 800-Ω baseline, steady-state resistance.

**Immunoprecipitation and transfections.** Confluent cell culture dishes were placed on ice and washed three times with ice-cold PBS. PBS was removed and ice-cold lysis buffer was added. Adherent cells were scraped and the cell suspension was transferred to a cold microcentrifuge tube. Constant agitation was maintained for 30 min at 4°C and lysates were centrifuged for 30 min at 4°C. The protein concentration in supernatant was calculated by the BCA method according to manufacturer’s instructions. Protein-matched samples (40 μg per lane) were separated by electrophoresis through 12% SDS-PAGE Tris-HCl gels. Gel transfer was used to transfer the proteins onto nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in 5% nonfat dry milk in Tris-buffered saline-0.1% (vol/vol) Tween 20. The blots were then incubated at 4°C overnight with the appropriate antibody. The signal for the immunoreactive proteins was developed by using the appropriate secondary antibody and was visualized in a LICOR Odyssey CLx imaging system.

**In vivo experiments.** Stock solutions of Escherichia coli LPS were prepared in saline. Mice received either vehicle (saline) or LPS (3,000 unit/g body wt, intratracheally) 24 h before receiving vehicle (10% DMSO in saline) or the Hsp90 inhibitor AUY922 (AUY; 10 μg/g body wt dissolved in 10% DMSO), intraperitoneally. Mice were euthanized 48 h later (i.e., 72 h after LPS) by cervical dislocation, and the lungs were flushed with 5 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 precooled mortar, and stored at −80°C.

**RESULTS**

**p53 inhibition reduces endothelial barrier integrity.** HLMVEC were seeded on gold electrode arrays and were exposed to DMSO (vehicle) or 25 or 50 μM of the p53 expression inhibitor pifithrin. Figure 1A shows that pifithrin...
induced a concentration-dependent decrease in p53 expression. Furthermore, DMSO-treated cells maintained constant trans-endothelial resistance (TER) values, whereas cells exposed to 50 μM pifithrin exhibited a significant reduction in TER values (Fig. 1B).

Effects of p53 inhibition by pifithrin on LPS-induced endothelial barrier dysfunction. HLMVEC were seeded on gold electrode arrays and were exposed to either 10% DMSO (vehicle) or 50 μM pifithrin before a 4-h treatment with PBS (vehicle) or LPS (1 EU/ml). PBS-treated cells maintained constant TER values, in contrast to the pifithrin-treated cells, which exhibited reduced TER (Fig. 1C). LPS profoundly reduced TER in both vehicle and pifithrin-treated cells; however, the LPS-induced decrease in TER values was far greater in pifithrin-treated cells (Fig. 1C).

Effects of p53 induction by nutlin on endothelial barrier dysfunction. Figure 1D, top right, demonstrates that p53 expression increased significantly in cells treated for 4 h with 10 μM nutlin. In additional experiments, HLMVEC were seeded on gold electrode arrays and were exposed to DMSO (vehicle) or 10 μM nutlin for 4 h before PBS (vehicle) or LPS (1 EU/ml) treatment. As shown in Fig. 1D, cells treated with PBS maintained stable TER values (Fig. 1E). The addition of LPS significantly reduced TER in vehicle-treated cells, but not in cells pretreated with the p53 inducer nutlin.

Effect of p53 silencing on HLMVEC TER. Figure 1E, top right, demonstrates that p53 expression was significantly reduced by p53 silencing with a specific siRNA. In additional experiments, HLMVEC were seeded on gold electrode arrays and were exposed to control siRNA or p53 siRNA (t = 0). As shown in Fig. 1E, cells treated with control siRNA maintained stable TER values. On the other hand, cells subjected to p53 siRNA treatment exhibited significantly reduced TER values.

Suppression of p53 expression by LPS. HLMVEC were exposed to either vehicle (PBS) or LPS (1 EU/ml) for 2 h. Cells exposed to LPS exhibited decreased p53 expression compared with the vehicle-treated cells (Fig. 2A).

Induction of p53 expression by 17-AAG. HLMVEC were treated with either vehicle (DMSO) or the Hsp90 inhibitor 17-AAG (1 μM) for 4 or 16 h. As shown in Fig. 2B, 17-AAG treatment for either time period significantly increased p53 expression compared with vehicle-treated cells.

Fig. 3. Effects of LPS and 17-AAG on p53 and p21 expression in mouse lung. A: Western blot analysis of p53 levels in lungs retrieved from mice 24 h after treatment with LPS. Blot shown is representative of 3 experiments per group. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05 vs. VEH. Means ± SE. B: Western blot analysis of p53 levels in lungs retrieved from mice 72 h after treatment with LPS or vehicle and posttreated (24 h after LPS) with AUY or vehicle (10% DMSO). Blot shown is representative of 3 experiments per group. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05 vs. LPS. Means ± SE. C: Western blot analysis of p21 levels in lungs retrieved from mice 72 h after treatment with LPS or vehicle and posttreated (24 h after LPS) with AUY or vehicle (10% DMSO). Blot shown is representative of 3 experiments per group. Signal intensity of p21 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05, **P < 0.01 vs. VEH. Means ± SE. D: Western blot analysis of MDM2 expression in lungs retrieved from mice 72 h after treatment with LPS or vehicle and posttreated (24 h after LPS) with AUY or vehicle (10% DMSO). Blot shown is representative of 3 experiments per group. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05, **P < 0.01 vs. VEH. Means ± SE. E: Western blot analysis of MDMX levels in lungs retrieved from mice 72 h after treatment with LPS or vehicle and posttreated (24 h after LPS) with AUY or vehicle (10% DMSO). Blot shown is representative of 3 experiments per group. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05 vs. VEH. Means ± SE.
Suppression of p21 expression by LPS. HLMVEC were exposed to either vehicle (PBS) or LPS (1 EU/ml) for 2 h. Cells exposed to LPS demonstrated decreased expression of the p53 target, p21, compared with vehicle-treated cells (Fig. 2C).

Induction of p21 expression by 17-AAG. HLMVEC were treated with either vehicle (DMSO) or the Hsp90 inhibitor 17-AAG (1 μM) for 8 h. As shown in Fig. 2D, p21 expression was significantly induced in 17-AAG treated cells.

Effects of LPS and AUY on p53 and p21 expression in mouse lung. Mice received either vehicle or LPS (intratracheally) and were euthanized 24 h after LPS treatment. Lungs of LPS-treated mice exhibited reduced p53 expression compared with corresponding controls (Fig. 3A). In additional studies, mice received either vehicle or the Hsp90 inhibitor AUY intraperitoneally 24 h after vehicle or LPS (intratracheally) and tissues were analyzed 72 h after LPS. As shown in Fig. 3, lungs from LPS-treated mice exhibited significantly lower expression of p53, even after 72 h. AUY reversed the LPS-induced p53 downregulation (Fig. 3B) and induced p21 expression (Fig. 3C), in vivo.

Effects of LPS and AUY on MDM2 and MDMX expression in mouse lung. Mice received either vehicle or the Hsp90 inhibitor AUY intraperitoneally 24 h after vehicle or LPS (intratracheally) and tissues were analyzed 72 h after LPS. As shown in Fig. 3, lungs from LPS-treated mice exhibited significantly higher expression of MDM2 and MDMX. AUY reversed the LPS-induced MDM2 (Fig. 3D) and MDMX (Fig. 3E) upregulation in vivo.

LPS reduces and 17-AAG increases the abundance of Hsp90/p53 complexes in HLMVEC. HLMVEC were exposed to either vehicle, LPS (1 EU/ml) for 2 h, or 17-AAG for 8 h. LPS reduced and 17-AAG increased p53 expression (Fig. 4, top). Immunoblotting of samples that were immunoprecipitated with p53 antibody showed that LPS-treated cells contained less of the Hsp90/p53 complex than untreated controls. On the other hand, 17-AAG induced the abundance of that complex (Fig. 4, bottom left). However, when expressed as the ratio of p53 to Hsp90, to reflect the binding affinity of the components in the complex, neither LPS nor 17-AAG exhibited any effect.

### Fig. 4. 17-AAG increases and LPS reduces the abundance of Hsp90/p53 complexes in HLMVEC. Top: Western blot analysis of p53 in HLMVEC treated with either vehicle (10% DMSO) or LPS for 2 h or 17-AAG for 8 h. Signal intensity of p53 was analyzed by densitometry. Protein levels were normalized to β-actin. *P < 0.05, **P < 0.01, vs. vehicle. Means ± SE. Bottom: Western blot analysis of samples at top, after immunoprecipitation (IP) with antibody against p53 and immunoblotting (IB) for p53 and Hsp90. Each blot is representative of 3 independent experiments. *P < 0.05, ****P < 0.0001, vs. vehicle. Means ± SE.
LPS increases the phosphorylation of MDM2 at Ser166. HLMVEC were treated with vehicle or 1 EU/ml LPS for 0.25 to 4 h. LPS induced Ser166 phosphorylation of the negative regulator of p53, MDM2, at each time point. Densitometric analysis of immunoblots revealed that maximal increase in pMDM2 occurred after 1 h exposure to LPS (Fig. 5A, top).

17-AAG reduces the LPS-induced MDM2 phosphorylation. HLMVEC were treated for 4 or 8 h with either vehicle (DMSO) or 17-AAG (1 µM) before LPS or vehicle treatment (2, 3, or 4 h). The results shown in Fig. 5B, demonstrate that LPS induced the phosphorylation of MDM2 and that 17-AAG was able to suppress that effect.

LPS induces and 17-AAG blocks MDMX expression. HLMVEC were treated with either vehicle (DMSO) or 17-AAG (1 µM) for 4 h before LPS (1 EU/ml) or vehicle treatment (2 h). Results shown in Fig. 5C demonstrate that LPS induced and 17-AAG blocked the expression of another p53 negative regulator, MDMX (Fig. 5C).

LPS induces and 17-AAG suppresses p53 phosphorylation. p53 phosphorylation increases its targeting to MDM2 and its degradation. HLMVEC were treated with vehicle, LPS (0.25–4 h), or 17-AAG (1 µM; 4 or 16 h). As shown in Fig. 6A, LPS induced p53 phosphorylation at S15 (Fig. 6, B and C) and S392 (Fig. 6, D and E), whereas 17-AAG decreased p53 phosphorylation (Fig. 6, A–D). The maximal effect of LPS on p53 phosphorylation occurred at 0.25 h (Fig. 6, B and D) or 2 h (Fig. 6, C and E). As shown before, p53 expression levels were reduced by LPS and induced by 17-AAG (Fig. 6, A and F).

17-AAG counteracts the LPS-induced p53 phosphorylation. HLMVEC were pretreated with either vehicle or 17-AAG (1 µM) for 4 h before LPS (1 EU/ml, for 0.25 h) or vehicle treatment. LPS induced p(Ser15)p53 (Fig. 7B) and p(Ser392)p53 (Fig. 7D) expression. 17-AAG suppressed both (Fig. 7, B–E). Additionally, 17-AAG significantly suppressed the baseline phosphorylation at both Ser15 and Ser392.

p53 negatively regulates RhoA activation. RhoA activation is a major pathway leading to endothelial barrier dysfunction. We investigated whether the beneficial effects of p53 on endothelial barrier function involve suppression of RhoA activation. HLMVEC were treated for 4 h with vehicle, 50 µM pifithrin, or 10 µM nutlin prior to vehicle or LPS treatment (2 h). LPS induced RhoA activation. Nutlin reduced baseline RhoA activity and completely blocked LPS-induced RhoA activation. Conversely, pifithrin significantly increased the LPS-induced RhoA activation (Fig. 8A).

p53 induction blocks the LPS-induced MLC2 phosphorylation. HLMVEC were pretreated with either vehicle or nutlin for 4 h before LPS treatment. LPS induced MLC2 phosphorylation and nutlin pretreatment suppressed that induction (Fig. 8B).

p53 suppression potentiates the LPS-induced MLC2 phosphorylation. HLMVEC were pretreated with either vehicle or pifithrin for 4 h before LPS or vehicle treatment. LPS induced MLC2 phosphorylation, which was further induced by pifithrin pretreatment (Fig. 8C).

DISCUSSION

Hsp90 is a major regulator of important physiological processes, through the stabilization and activation of various enzymes and transcription factors (8). Hsp90 inhibitors were
initially developed to fight cancer, but recent reports suggest that they may also have a beneficial role in other diseases, since they possess strong anti-inflammatory properties (2, 3, 6).

p53 is an Hsp90 client protein that functions as a node in numerous intracellular pathways and regulates important biological activities from fertility and development to maintaining genomic stability and cell death (45). It has been recently suggested that it is also involved in the mediation of anti-inflammatory responses, since p53 can drive inflammatory cell apoptosis in vivo (25) and there is a reciprocal negative regulation between p53 and NF-κB (10, 18). Increased NF-κB activation results in decreased p53 activity and vice versa. Proinflammatory NF-κB-induced cytokines such as IL-6 and macrophage migration inhibitory factor reduce p53 transcriptional activity (23, 46, 68) and agents that downregulate NF-κB cause p53 activation (67). p53 is also known to suppress the cyclooxygenase 2 gene (58) and to antagonize pp60src-induced cell migration and proliferation in atherosclerosis (43). Additionally, neutrophils and macrophages from p53 knockout mice exhibit elevated responses to LPS stimulation, stronger induction of proinflammatory cytokines, and enhanced NF-κB DNA binding activity; the p53 knockouts are more susceptible to LPS-induced acute lung injury, compared with wild type (35). Autoimmune diseases, including collagen-induced arthritis (67) and experimental autoimmune encephalitis (48), are more severe in p53-deficient mice. Inflammatory cell infiltration into the lung and subsequent disruption of alveolar architecture caused by chronic exposure to bleomycin are markedly increased in p53-null mice and in transgenic mice expressing the mutant p53 in the lung, compared with wild-type mice (9, 15).
Ionizing radiation induces faster and stronger invasion of inflammatory cells and fibroblasts into damaged tissues in p53-null mice than in wild type (29). Mice with a p53 P72R mutation have a markedly enhanced response to inflammatory challenges (11).

The present study reports for the first time that p53 expression levels are crucial for the maintenance of lung vascular integrity. Overexpression of p53 by nutlin, an inhibitor of the MDM2-p53 interaction (42), protected against LPS-induced lung barrier dysfunction. On the other hand, inhibition of p53 transcriptional activity and stability by pifithrin (28) resulted in the potentiation of LPS-induced barrier dysfunction. Pifithrin has been shown to also induce NF-κB activity, via the reciprocal regulation of NF-κB with p53 (49). Additionally, inhibition of the p53 gene expression by siRNA confirmed the importance of p53 on barrier integrity, since treated cells exhibited increased permeability (Fig. 1E). Furthermore, LPS was able to reduce the expression of p53 both in vitro and in vivo and that effect was blocked by Hsp90 inhibitors. We have employed two different Hsp90 inhibitors that represent two different generations of these compounds. 17-AAG is a geldanamycin derivative and it is the first Hsp90 inhibitor selected by for clinical use; AUY is a isoxazole derivative that is now being developed and is undergoing clinical trials in cancer patients (24). The key role of p53 in cellular defense against inflammatory stimuli was further demonstrated in a study showing that the activation of lung p53 by nutlin prevents and reverses pulmonary hypertension and that hypoxia-exposed p21-null mice exhibit exacerbated pulmonary hypertension compared with wild type (42).

We also investigated the effects of LPS and 17-AAG on p21 expression, since p21, the direct downstream target of p53, is involved in the vascular defense against pathogens. p21-null mice demonstrate an overactive inflammatory phenotype that is similar to that of p53-null mice and display increased susceptibility to endotoxic shock and increased serum levels of cytokines. Elevated NF-κB activity and secretion of cytokines was detected in LPS-stimulated p21-deficient mice and in human macrophages compared with similarly treated wild-type cells (55, 60). Our study shows that LPS reduces p21 expres-
sion in vitro, an effect that probably contributes to the LPS-induced endothelial barrier dysfunction. That effect was opposed by Hsp90 inhibitors (Figs. 2 and 3).

Furthermore, we show for the first time that Hsp90 forms a complex with p53 in HLMVEC and that the abundance, but not the affinity, of that complex in the cellular environment is influenced by both by LPS and 17-AAG (Fig. 4). When HLMVEC were treated with LPS the amount of the Hsp90-p53 complex was reduced, and this may be the cause of the lower p53 expression, as seen in Fig. 2A. On the other hand, 17-AAG induced the formation of that complex, which has been previously shown to partially stabilize p53 (21, 64, 65).

To further elucidate the mechanisms which regulate the p53 expression by LPS, we treated HLMVEC with LPS for various time points and evaluated the phosphorylation of the ubiquitin ligase MDM2, which plays a key role in the regulation of p53 stability by serving as its major negative regulator (31). LPS, an Akt activator (41), can significantly increase MDM2 phosphorylation, a posttranslational modification that is crucial for p53 degradation (40). Akt-mediated phosphorylation of MDM2 at Ser166 and Ser186 increases its interaction with p300,
allowing MDM2-mediated ubiquitination and degradation of p53 (22, 39, 69). Phosphorylation of MDM2 also blocks its binding to p19ARF, thus increasing the degradation of p53 (17, 69).

To investigate the mechanisms by which 17-AAG induces p53 expression, we pretreated HLMVEC with 17-AAG prior to LPS treatment. 17-AAG suppressed the LPS-induced MDM2 phosphorylation. LPS also induced the expression of the p53 negative regulator MDMX (56), and this effect was opposed by 17-AAG pretreatment. p53 phosphorylation has been shown to regulate its activity (38). In the present work we evaluated the phosphorylation of p53 by LPS, since that posttranslational modification has been shown to accelerate the degradation of p53 through the polyubiquitination-proteasome pathway (19). LPS induced p53 phosphorylation at Ser15 and Ser392. On the other hand, 17-AAG suppressed both the basal p53 phosphorylation levels and the LPS-induced p53 phosphorylation at both sites. We thus conclude that 17-AAG inhibits Akt leading to reduced MDM2 phosphorylation, reduced p53 ubiquitination, degradation, and thus increased p53 survival.

The Rho family of GTPases regulates a wide variety of cellular processes, including apoptosis, cell cycle progression and migration. Although this family of protein is comprised of some 22 members, the best characterized are RhoA, RhoB, RhoC, Rac1, and Cdc42 and most of them are involved in the regulation of endothelial barrier function (7). RhoA is involved in the regulation of actomyosin contractility, cytokinesis, focal adhesion and cell polarity and has been shown to be activated by LPS (54, 57). In the present study we demonstrate that p53 negatively regulates the LPS-induced RhoA activation. Our results are in line with others’ observations that p53 induction by nutlin reduces RhoA activity and that RhoA silencing increases p53 expression (37). Although pitfhrin potentiated the LPS-induced RhoA activation, by itself it did not affect RhoA activity, even though it suppressed TET; this suggests that pitfhrin may also affect endothelial barrier function through additional pathways. Loss of p53 has been also associated to the disruption of the RhoA signaling (12). Myosin light chain phosphorylation is a target of Rho kinase. Nutlin suppressed the LPS-induced MLC2 activation, and p53 inhibition potentiated this phosphorylation. MLC2 phosphorylation is crucial in the LPS-mediated endothelial barrier dysfunction (54).

Previous reports have suggested that p53 negatively modulates Rho GTPases (13, 14, 20) and restricts the Ras stimulation is crucial in the LPS-mediated endothelial barrier dysfunction potentiated this phosphorylation. MLC2 phosphorylation is a target of Rho kinase. Nutlin through additional pathways. Loss of p53 has been also associated with acute lung injury and acute respiratory distress syndrome. The 17-AAG-mediated p53 induction suppresses the pp60src activation (43) and induces p21 expression, which protects against LPS-induced responses, perhaps through negatively controlling NF-kB activation (60). Furthermore, our data suggest that p53 can negatively regulate LPS-induced RhoA activation. This working hypothesis is now depicted schematically in Fig. 9. In conclusion, our work reveals novel intracellular mechanisms responsible for the mediation of the anti-inflammatory role of Hsp90 in endothelial barrier dysfunction associated with acute lung injury and acute respiratory distress syndrome.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

N.B. and J.D.C. conception and design of research; N.B., C.D., C.B., A.J., and G.T. performed experiments; N.B. and C.D. analyzed data; N.B., C.D., and J.D.C. interpreted results of experiments; N.B. prepared figures; N.B. drafted manuscript; N.B., C.D., C.B., A.J., G.T., and J.D.C. approved final version of manuscript; C.B., A.J., and J.D.C. edited and revised manuscript.
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ENDOTHELIAL BARRIER PROTECTION BY p53


