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Hsp90 inhibitors suppress P53 phosphorylation in LPS - induced endothelial inflammation

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ABSTRACT

P53 has been recently involved in the defense against inflammation. The “guardian of the genome” appears to orchestrate cellular responses against bacterial toxins, by regulating crucial pathways that orchestrate the vascular barrier functions. Indeed, an emerging body of evidence suggests that this tumor suppressor is involved in the mediation of the beneficial effects of Hsp90 inhibition in the inflamed endothelium. Interestingly, those compounds augment the abundance of P53 in the intracellular niche, while LPS dramatically reduces it. The current study focuses on the outcome of LPS and Hsp90 inhibition on P53 phosphorylation, since this modification negatively affects P53 stability. In an in *“vitro”* model of LPS – induced vascular leak in bovine pulmonary arterial endothelial cells, LPS induced P53 phosphorylation in four distinct residues, namely Ser. 6, Ser. 15, Ser. 33 and Ser. 392. Furthermore, LPS triggered the activation of the myosin light chain 2, which produces endothelial barrier dysfunction by cellular retraction and intercellular gap formation. Indeed, mice exposed to the toxin demonstrated elevated levels of the pro - inflammatory cytokines IL-2 and IL-10 in the bronchoalveolar lavage fluid. In bold contrast, the HSP90 inhibitor 17-DMAG, counteracted the LPS - induced effects both *in vivo* and in *vitro*. Specifically, this hsp90 inhibitor reduced phosphorylated P53 levels and lessened the activation of myosin light chain 2 (phosphorylation) in the bovine endothelium. Moreover, 17 - DMAG suppressed inflammation in mouse lungs, as reflected in reduced IL-2 and IL-10 BALF levels. In summary, the present results support previous observations on the protective role of P53 against inflammation and clarify mechanisms that govern vascular barrier function.

1. Introduction

The “guardian of the genome” P53 dictates cellular fate by regulating cellular responses to a plethora of environmental and intracellular stresses. It regulates the progression of cell cycle and possesses the capacity to induce cellular senescence, as well as apoptosis [1]. Although this transcription factor was initially considered to be involved exclusively in cellular defense against cancers, a battery of recent evidence are now suggesting otherwise [2].

Recent studies in the field of vascular biology, reveal that P53 exerts a prominent anti -inflammatory role on the endothelium [3]. Hence, it mediates the protective effects of Hsp90 inhibitors against LPS - triggered vascular barrier hyperpermeability [4]. Hsp90 inhibitors consist of a class of anti - cancer agents, which vigorously target the progression of severe and lethal malignancies [5]. Remarkable, cancer and inflammation are tightly associated conditions that frequently co-exist "Barabutis N, et al, P53, GHRH, inflammation and cancer, EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.10.034 and [6]".

Vascular barrier dysfunction, is considered both a cause and a consequence of inflammation. The endothelium consists of metabolically active cells that form a semipermeable barrier separating blood from parenchyma. The pulmonary endothelium appears to form a monolayer that determines the composition of blood moving downstream. A volatile dysfunction of this barrier is established in those suffering from with Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) [7]. Both clinical conditions affect thousands of people worldwide, with frequently lethal outcomes [8].

Our recent discoveries suggest that P53 organizes the first line of defense against inflammation, by devising meticulous responses that repair impaired endothelium monolayers. Our findings suggest that P53

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strengthens the barrier integrity by disrupting the formation of actin stress fibers. Further, it burdens the actin severing activity of cofilin by mediating Rac1 signaling following Hsp90 inhibition [9]. The activity of P53 is regulated by post-translational modifications, such as acetylation, phosphorylation and ubiquitination [10].

Thus, here we investigated the effects of LPS and Hsp90 inhibition on P53 phosphorylation both *in vivo* and *in vitro* in an experimental model of Acute Lung Injury. Now, we report for the first time, that the Hsp90 inhibitor 17-DMAG suppresses the LPS - induced P53 phosphorylation in bovine pulmonary arterial endothelial cells and disrupts the LPS-induced MLC2 activation.

In order to investigate these anti -inflammatory outcomes *in "vivo"*, we employed the Hsp90 inhibitor, AUY-922, in a mouse model of ALI. AUY-922 successfully suppressed the LPS - induced elevation in the inflammatory cytokines IL-2 and IL-10 [10,11]. Our results are in line with our previous findings on the protective role of P53 on the function of pulmonary endothelium, and further substantiate the anti - inflammatory actions of Hsp90 inhibitors in the vasculature.

The present manuscript reveals new information on the signaling cascades that defines the regulation of endothelial permeability. Hence, it contributes to the mapping of the etiologies that lead to ALI/ARDS. Most importantly, the dephosphorylion of P53 now appears to consist a post - translational modification, which may serve as the target for the development of new therapies against manifestations of cardiovascular dysfunctions, including ALI/ARDS.

2. Materials and methods

Reagents: The Hsp90 inhibitor 17- DMAG (cat. no. 102513-662), RIPA buffer (cat. no. AAJ63306-AP), anti - mouse Ig G HRP linked whole antibody from sheep (cat. no. 95017-554), anti - rabbit Ig G HRP linked whole antibody from donkey (cat. no. 95017-556) and nitrocellulose membranes (cat. no. 10063-173) were obtained from VWR (Radnor, PA). AUY – 922 was purchased from Selleckchem (Houston, TX, USA). P53 (cat. no. 9282S), p-Myosin Light Chain 2 (cat. no. 3674S), Myosin Light Chain 2 (cat. no. 3672S), phospho-P53 (Ser6) (cat. no. 9285S), phospho-P53 (Ser15) (cat. no. 9284S), phospho-P53 (Ser33) (cat. no. 2526S), and phospho-P53 (Ser392) (cat. no. 9281S) antibodies were obtained from Cell Signaling Technology (Danvers, MA). The β-actin antibody (cat. no. A5441) was purchased from Sigma-Aldrich (St Louis, MO).

Animals: 7–8 weeks old male C57BL/6 mice were used in all experiments. The animals 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.

***In vivo* treatments:** Stock solutions of *E. coli* LPS (0111:B4) were prepared in saline. Mice received vehicle (saline) or LPS (1.5 mg/Kg) via intra-tracheal instillation. 24 h after LPS administration, mice received AUY-922 (10 mg/kg each) via an intra-peritoneal injection. The animals were examined 72 h after LPS treatment.

Isolation of bronchoalveolar lavage fluid: Bronchoalveolar lavage fluid (BALF) was obtained by instilling and withdrawing 1 ml 1 × PBS via a tracheal cannula. The cells in the BALF were pelleted at 2500 g for 10 min, and the supernatant was removed for cytokine analysis. The cell pellet was re-suspended in water for 15 s to lyse the red blood cells. The remaining leukocytes were re-suspending in 1 ml of 1 × PBS, and the total cell count was determined using a haemocytometer.

BALF cytokine measurement: BALF samples were sent to Quansys Biosciences and evaluated using Q-Plex assay kit for mouse cytokines and chemokines.

Cell Culture: In-house harvested bovine pulmonary arterial endothelial cells (BPAEC) were subcultured from primary cultures and used at an early passage. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 - 95% air in DMEM (cat. no. VWRL0101-0500) medium supplemented with 10% fetal bovine serum (cat. no. 89510-186), and 1 × penicillin/streptomycin (cat. no. 97063-708). All the reagents for cell cultures were purchased from VWR (Radnor, PA).

Protein isolation and Western Blot Analysis: Proteins were isolated from cells or tissues using RIPA buffer. Protein-matched samples were separated by electrophoresis through 12% sodium dodecyl sulfate (SDS–PAGE) Tris-HCl gels. Wet transfer was used to transfer the proteins onto nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in 5% non-fat dry milk in Tris-buffered saline (TBS) – 0.1% (v/v) Tween 20. The blots were then incubated at 4 °C overnight with the appropriate primary antibody (1:1000). The signal for the immunoreactive proteins was developed by using the corresponding secondary antibody (1:2000) and was visualized in a ChemiDoc™ Touch Imaging System from Bio-Rad (Hercules, CA). β-Actin antibody (1:5000) was used as a loading control.

Densitometry and Statistical Analysis: Image J software (National Institute of Health) was used to perform densitometry of immunoblots. All data are expressed as mean values ± SEM (standard error of mean). A value of $P < 0.05$ was considered significant. GraphPad Prism 5 (version 5.01, Graph Pad Software) was used for data analysis. The letter n represents the number of experimental repeats.

3. Results

LPS increases the phosphorylation of P53 at Ser6. BPAEC were treated with either vehicle, 1 μg/ml LPS, or 10 μg/ml LPS for 0.5, 1 or 2 h. LPS significantly induced p53 phosphorylation after one hour of LPS treatment at both concentrations. At the higher concentration, LPS was able to trigger P53 phosphorylation after only a 30 min exposure. The densitometry analysis of immunoblots revealed that the maximum increase in P53 phosphorylation occurred after an hour of exposure to 10 μg/ml LPS (Fig. 1A).

LPS increases the phosphorylation of P53 at Ser15. BPAEC were treated with either vehicle, or 1 μg/ml LPS, or 10 μg/ml LPS for 0.5, 1 and 2 h. LPS significantly increased the phosphorylation of P53 at Ser 15 at both concentrations. The lower concentration of LPS significantly increased P53 phosphorylation after 2 h of treatment, whereas. 10 μg/ml LPS significantly induced P53 phosphorylation after both 0.5 and 1 h treatments (Fig. 1B).

LPS increases the phosphorylation of P53 at Ser33. Cells were treated as above. LPS significantly induced P53 phosphorylation at both concentrations. The lower concentration of LPS triggered a significant rise in P53 phosphorylation after 2 h of treatment, while the higher concentration upregulated P53 phosphorylation at all three time points (Fig. 1C).

LPS increases the phosphorylation of P53 at Ser392. BPAECs were treated as above. The phosphorylation of P53 at Ser 392 occurred at both concentrations, but only after a 2 h treatment (Fig. 1D).

17-DMAG prevents the LPS - induced p53 phosphorylation at Ser6. BPAEC were treated with vehicle (PBS) or the Hsp90 inhibitor, 17-DMAG (5μM) for 16 h, prior to an 1 h treatment with vehicle or LPS (10 μg/ml). As shown in Fig. 2A, LPS induced the phosphorylation of P53, in line with the results of Fig. 1A. The Hsp90 inhibitor completely prevented the LPS- induced P53 phosphorylation, to a level even lower than baseline (Fig. 2A).

17-DMAG prevents the LPS - induced p53 phosphorylation at Ser15. BPAEC were treated as above. In agreement with our earlier data, LPS induced p53 phosphorylation, while 17-DMAG suppressed that effect (Fig. 2B).

17-DMAG prevents the LPS - induced p53 phosphorylation at Ser33. The cells were exposed to conditions similar to those previously described. 17-DMAG pre- treatment (5 μM for 16 h) completely prevented the LPS - triggered P53 phosphorylation (Fig. 2C).

17-DMAG prevents the LPS – induced p53 phosphorylation at

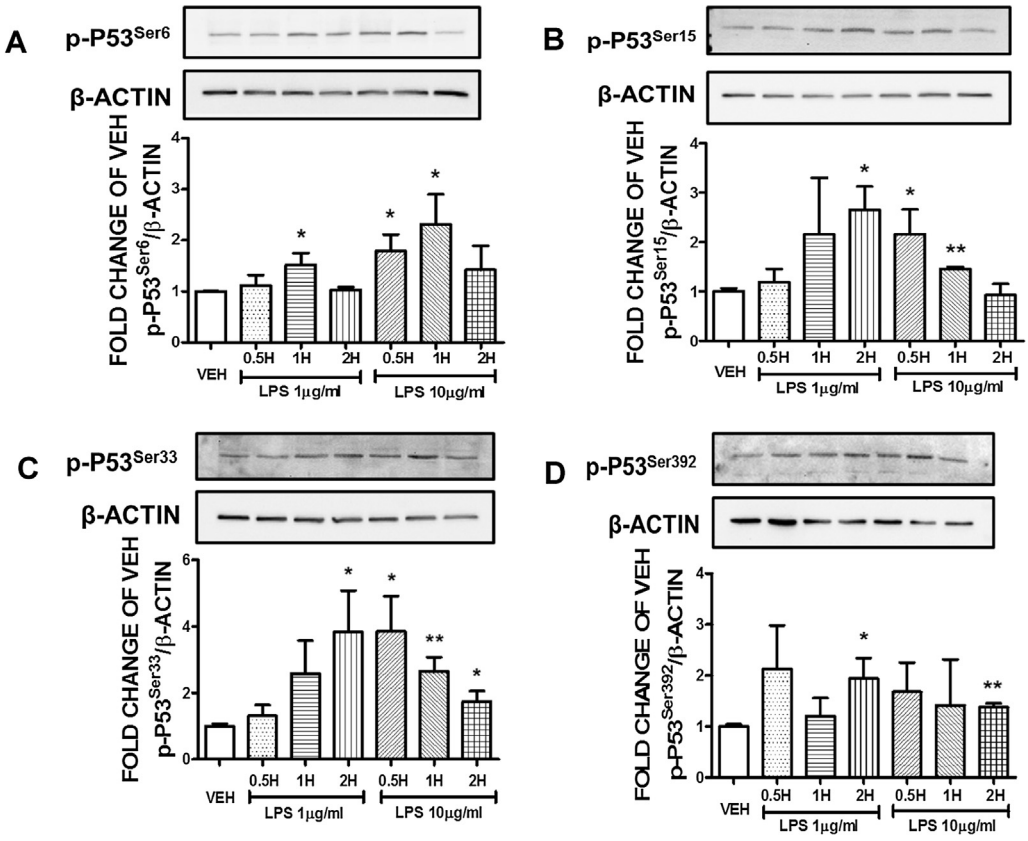

Fig. 1. LPS induces p53 phosphorylation. Western Blot analysis of (A) p-p53^{Ser6}, (B) p-p53^{Ser15}, (C) p-p53^{Ser33}, (D) p-p53^{Ser392} and β actin levels after treatment of BPAEC with vehicle (PBS), 1 μg/ml or 10 μg/ml LPS. The blots shown are representative of 3 independent experiments. The signal intensity of the p-p53^{Ser6}, p-p53^{Ser15}, p-p53^{Ser33}, and p-p53^{Ser392} bands were analyzed by densitometry. Protein levels were normalized to β actin. *P < 0.05, **P < 0.01 vs vehicle. Means ± SEM.

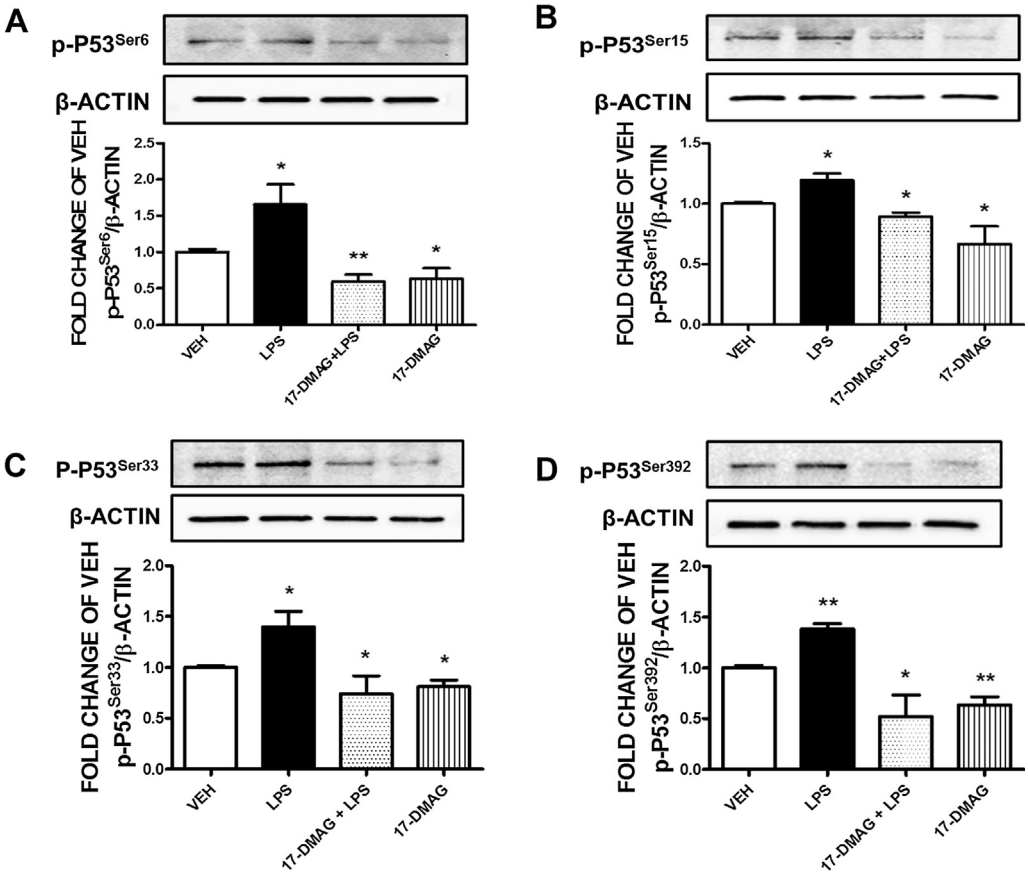

Fig. 2. 17-DMAG suppresses the LPS-induced p53 phosphorylation. Western Blot analysis of (A) p-p53^{Ser6}, (B) p-p53^{Ser15}, (C) p-p53^{Ser33}, (D) p-p53^{Ser392} and β actin levels after treatment of BPAEC with 17-DMAG or vehicle (10% DMSO) and post - treated with LPS or vehicle (PBS). The blots shown are representative of 3 independent experiments. The signal intensity of the p-p53^{Ser6}, p-p53^{Ser15}, p-p53^{Ser33}, and p-p53^{Ser392} bands were analyzed by densitometry. Protein levels were normalized to β actin. *P < 0.05, **P < 0.01 vs vehicle. Means ± SEM.

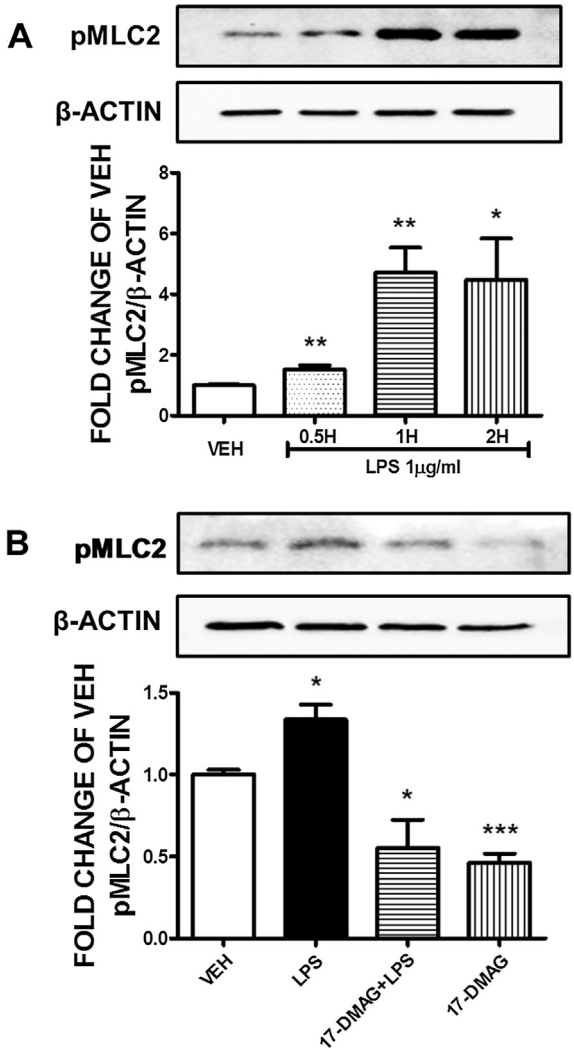

Fig. 3. Effects of LPS and 17-DMAG on MLC2 phosphorylation. (A) Western blot analysis of phosphorylated MLC2 (pMLC2) levels in BPAEC treated with vehicle (PBS) or LPS (1 μg/ml) for (0.5, 1 or 2 h). The blots shown are representative of 3 independent experiments. Signal intensity of pMLC2 was analyzed by densitometry. Protein levels were normalized to β actin. *P < 0.05, **P < 0.01 vs vehicle. Means ± SEM. (B) Western blot analysis of ppMLC2 levels in BPAEC treated with 17-DMAG or vehicle (10% DMSO) and post - treated with LPS (0.5 h) or vehicle (PBS). The blot shown is representative of 3 independent experiments. Signal intensity of pMLC2 was analyzed by densitometry. Protein levels were normalized to β actin. *P < 0.05, ***P < 0.001 vs vehicle. Means ± SEM.

Ser392. BPAEC were treated as above. Hsp90 inhibition completely prevented the LPS-induced p53 phosphorylation at Ser392 (Fig. 2D).

LPS increases the phosphorylation of MLC2. BPAEC were treated with vehicle (PBS) or LPS (1 μg/ml) for 0.5, 1 or 2 h. LPS induced the phosphorylation of MLC2 after one and two hours of treatment. The maximum effect was observed after one hour of exposure to LPS (Fig. 3A).

17-DMAG suppresses the LPS - induced MLC2 phosphorylation. Cells were exposed to 17-DMAG (5 μM) or vehicle (DMSO) for 16 h prior to LPS (10 μg/ml) exposure. The results shown in Fig. 3B, demonstrate the suppression of pMLC2 phosphorylation by 17-DMAG in both vehicle-treated as well as LPS - treated cells.

Hsp90 inhibition by AUY-922 suppresses the LPS – induced elevation of IL-2 and IL-10 in an *in vivo* model of LPS - induced inflammation: Male C57Bl/6 mice received vehicle (saline) or LPS (1.5 mg/Kg) via intra-tracheal instillation. 24 h after LPS administration

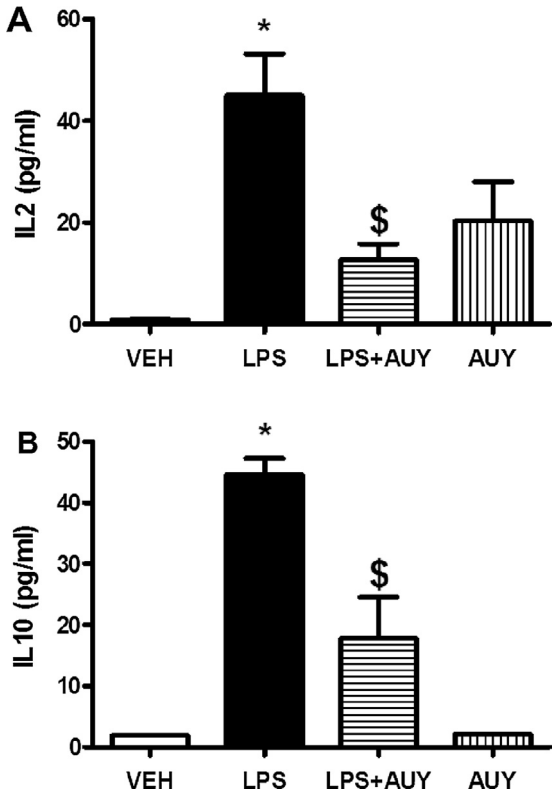

Fig. 4. Effects of LPS and AUY-922 on the IL 2 and IL 10 expression levels in an *in vivo* model of Acute Lung Injury. Male C57Bl/6 mice received vehicle (saline) or LPS (1.5 mg/Kg). 24 h after LPS administration the mice received AUY-922, and BALF was taken and examined 72 h after LPS treatment. (A) IL-2 and (B) IL-10 concentrations in BALF. n = 5 animals per group. Means ± SEM. $^{\$}P < 0.01$ vs LPS - treated mice. Means ± SE.

the mice received AUY-922 (10 mg/kg each) via an intra-peritoneal injection. The animals were examined 72 h after LPS treatment. BALF concentrations of IL-2 (Fig. 4A) and IL-10 (Fig. 4B) were dramatically elevated in mice treated with LPS. However, AUY-922 treatment strongly suppressed the LPS - induced elevation in both interleukins.

4. Discussion

P53 is considered to be a very attractive target for the development of novel therapeutic approaches towards a wide spectrum of human disease [12]. Although it was first identified as an oncogene, it is now considered the frontier of the cellular defense against toxic environmental factors and harmful genomic alterations [13]. In our previous studies, we have introduced the protective role of P53 on LPS - induced vascular barrier hyperpermeability, and we have established the synergistic action of Hsp90 inhibitors and P53 induction towards the enhancement of pulmonary vascular barrier structure. The increased abundance of intracellular P53 due to Hsp90 inhibition, results in robust protective responses against LPS [4].In human lung microvascular endothelial cells, LPS suppresses P53 levels via the induction of the negative P53 inhibitors MDM2 and MDM4, and the reduction of cytoplasmic P53/Hsp90 complexes, which protect P53 against degradation [4,14].

The current study focuses on the effects of Hsp90 inhibition on the LPS - induced P53 phosphorylation in Bovine Pulmonary Endothelial Cells. That P53 modification has been previously associated with P53 reduction [4]. Moreover, the study supports the importance of Hsp90 inhibition against inflammation by assessing the outcome of 17-DMAG on MLC2 phosphorylation, as well as the effect of AUY-922 on the expression of IL 2 and IL 10 in an *in vivo* model of ALI/ARDS.

The regulation of P53 occurs by a variety of mechanisms, including phosphorylation. The serines at positions 4, 6, and 9, relative to the N terminus of murine p53, are phosphorylated both *in vivo* and *in vitro* by casein kinase 1 (CK1) in response to DNA damage - inducing agents such as ionizing radiation, adriamycin and UV light [15]. The Ser6 epitope of P53 appears to be one of the most strongly phosphorylated sites in response to DNA damage; and it is now appearing to be a target of LPS exposure to BPAEC. Phosphorylation of Ser6 and Ser 9 by CK1 mediates interaction of p53 with Smad proteins and is important for the contribution of p53 to transforming growth factor beta signaling (TGF-beta) [16]. More importantly, P53 phosphorylation on six key serine residues (Ser^{6}, Ser^{15}, Ser^{20}, Ser^{37}, Ser^{46}, and Ser^{392}) has been associated with P53 instability [17].

Ser-15 is an evolutionarily conserved residue, corresponding to Ser-18 in murine p53. It can be phosphorylated in vitro by several protein kinases belonging to the ataxia-telangiectasia mutated (ATM) family, such as DNA-PK, ATR, and ATM itself [18]. The phosphorylation of P53 at ser 15 has been shown to regulate both p53 activation and stabilization. Stabilization of p53 after ionizing radiation results from the inhibition of the P53 - MDM2 binding due to DNA damage – induced p53 phosphorylation at the site Ser15 [18].

Phosphorylation of mouse p53 at Ser-18 is essential for an extended p53 - mediated response induced by exposure to either IR or UV light, thus this post translational modification is essential for the cellular response to DNA damage. In cancer, the inhibition of p53 phosphorylation at serine 15 leads to an increase of the mutant p53 stability. That phosphorylation is mediated by JNK kinase and is blocked by the JNK kinase inhibitor SP600125 [19].

The residue Ser392 (Ser389 in mice) is a major phosphorylation site in p53 which is a target of CK2, p38 MAPK, PKR, and CDK9 kinases. Phosphorylation in this site occurs at low levels under normal, unstressed conditions. However, upon appropriate stimuli, Ser392 phosphorylation may be induced by up to 5-fold [20]. Phosphorylation on Ser^{392} of p53 plays an important role in its stabilization and tetramer formation, and may serve as a switch that activates p53 as a transcription factor in response to DNA alterations [21]. All the aforementioned studies, support the crucial role of P53 phosphorylation on its stability and function.

LPS induces P53 phosphorylation in multiple sites in bovine pulmonary arterial endothelial cells (Fig. 1), and this effect is associated with the phosphorylation of myosin light - chain (MLC2) (Fig. 3). MLC kinases (MLCKs) are a family of Ca^{2}/calmodulin (CaM) - dependent protein kinases that phosphorylate the regulatory MLC2. MLC phosphorylation triggers contraction, resulting in endothelial cell membrane retraction, intercellular gap formation, and vascular barrier disruption [7]. Hence, P53 phosphorylation “propels” the barrier dysfunction by increasing the vascular permeability of pulmonary endothelium.

Hsp90 inhibitors are anti - inflammatory compounds, which have been previously shown to prolong survival, attenuate inflammation and reduce lung injury in murine sepsis [22]. In addition, those compounds may increase the transendothelial resistance of endothelial monolayers by targeting key inflammatory mediators [23]. Remarkably, those compounds can not only protect but also repair and restore pulmonary endothelial barrier function after its exposure to toxins [24]. This protection, is due to their ability to inactivate kinases, Hsp90 clients, that “drive” inflammatory responses and phosphorylate P53, such as ERK1/2 and CK1 [25]. The ability of Hsp90 inhibitors to suppress a wide variety of kinases may be explained by the fact that Hsp90 recognizes a common surface on client kinases [26].

The Hsp90 inhibitor, 17-DMAG is a water soluble analog of 17-AAG and geldanamycin. It binds to the ATP binding site of Hsp90 and inhibits its chaperone activity. Further, it displays more potent activity than 17-AAG, thus it was chosen to further our previous studies on the exploration of the anti -inflammatory role of P53 in experimental models of ALI [4]. Figs. 1 and 2 report for the first time that 17-DMAG suppresses the phosphorylation of P53 by LPS in four different sites. These observations explain our previous findings on the induction of P53 by Hsp90 inhibitors [4], since elevation of P53 phosphorylation levels have been shown to regulate P53 ubiquitination and subsequent degradation. Treatment of mouse and human fibroblasts with the PKC inhibitors H7 or bisindolylmaleimide caused P53 accumulation and increased its half - life [27,28].

Moreover, Fig. 3 demonstrates that 17-DMAG prevents the LPS - induced MLC2 activation in BPAEC. ROCK inhibitors and P53 overexpression have reported to exert similar effects [4,29] to Human Lung Microvascular Endothelial Cells, suggesting that the ROCK-MLC2 pathway is of crucial importance on the development of new anti - inflammatory agents. Interestingly, both P53 and MLC2 are phosphorylated by common kinases, that serve as Hsp90 client proteins and are negatively affected and targeted by Hsp90 inhibitors [30].

In order to further substantiate our findings on the anti - inflammatory role of Hsp90 inhibition in the vasculature, we employed an *in vivo* experimental model of acute lung Injury. We assessed the expression of two major inflammatory markers, IL 2 and IL 10 [31,32]. The results depicted in Fig. 4, indicate that Hsp90 inhibition strongly suppressed the LPS triggered inflammation in mouse lungs, providing the link between p53 phosphorylation levels and pulmonary inflammation. Namely, LPS is associated with P53 phosphorylation and MLC2 activation, whereas Hsp90 inhibition counteracts the LPS -induced effects.

Future studies may focus on genetically modified P53 mutants, which will be treated with toxins to evaluate the potential importance of pulmonary P53 expression levels on LPS – induced vascular dysfunction.

5. Conclusions

The current study supports our previous endeavors on the mechanisms that regulate pulmonary endothelial barrier function, by identifying the role of P53 on the maintenance of endothelial permeability. It introduces the inhibitory role of 17-DMAG on the LPS - induced myosin light chain 2 and P53 phosphorylation, and suggests that P53 is an appealing target in the investigation of new theurapeutical avenues towards toxin - induced inflammatory disorders.

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