Hsp90 inhibition protects the brain microvascular endothelium against oxidative stress

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Abstract

The brain endothelium is an integral element of the blood-brain barrier (BBB). Dysfunction of this formation due to increased generation of reactive oxygen species (ROS) progresses the establishment of neurological disorders including stroke and traumatic brain injury. Heat shock protein 90 inhibitors are anti-inflammatory agents, and their activities are mediated, at least in part, by P53. This is a tumor suppressor protein which regulates the opposing activities of Rac1 and RhoA in the cellular cytoskeleton. In the present study we investigated the role of Hsp90 inhibitors in the H2O2-induced brain endothelium breakdown, by employing human cerebral microvascular endothelial cells (hCMEC/D3). Our findings suggest that H2O2 downregulates P53 by enhancing the P53 suppressor mouse double minute 2 homolog (MDM2), as well as by increasing the apyrimidinic endonuclease 1/redox factor 1 (APE1/Ref1). The H2O2 – triggered violation of the brain endothelium barrier was reflected in measurements of transendothelial resistance, and the increased expression of the key cytoskeletal modulators cofilin and myosin light chain 2 (MLC2). Treatment of the hCMEC/D3 cells with Hsp90 inhibitors counteracted those events, and reduced the generation of the hydrogen peroxide – induced reactive oxygen species. Hence, our study suggests that Hsp90 inhibition supports the BBB integrity, and may represent a promising therapeutic approach for disorders associated with brain endothelium breakdown; including COVID-19.

Keywords

Inflammation; Reactive oxygen species; Oxidative damage; P53; Heat shock protein 90 inhibitors

Introduction

The blood-brain barrier (BBB) is a structural and functional barrier that restricts the movement of soluble mediators and leukocytes from the blood to the central nervous system (CNS). It maintains the exchange of multiple chemical substances required for synaptic and...
neuronal functioning. If the BBB has been compromised, cellular infiltration will occur, allowing neurotoxic blood components and microbial pathogens into the CNS. Dysfunction of the BBB will also affect the transport mechanism which removes waste from the CNS [1].

Impaired BBB function is a hallmark of many neurological diseases, including cerebral ischemia/reperfusion (I/R) and traumatic brain injury (TBI). The pathophysiology of chronic neurodegenerative disorders such as Huntington’s disease, multiple sclerosis, Alzheimer’s, and Parkinson’s disease are also a result of BBB dysregulation [1, 2]. BBB endothelial cells interact with the surrounding cells of the CNS cells, including the neurons and astrocytes. Those cells are important for maintaining brain homeostasis, and for preventing the entry of toxic substances.

P53 is a tumor suppressor molecule that restricts aberrant cell growth by repressing the cell cycle and inducing DNA repair. In case of irreversible damages, it will trigger apoptosis [3]. Recent observations reveal that P53 regulates endothelial barrier function by balancing the opposing activities of key-cytoskeletal proteins. This “endothelium defender” protects the lung microvascular endothelium by disrupting the inflammatory RhoA/MLC2 pathway via p190RhoGAP (p190) induction [4]. P190 is a multidomain protein that negatively regulates Rho and causes inactivation of Rho-GTP via hydrolysis to RhoGDP [5].

P53 opposes the severing of actin filaments by deactivating cofilin, thus supporting endothelial barrier function [4]. Induction of P53 in lung cells is associated with the activation of Racl/p21-activated kinase/LIM Kinase, the upstream effector of cofilin [4]. The suppression of the redox signaling factor apurinic/apyrimidinic endonuclease/redox factor-1 (APE1/Ref1) in the pulmonary endothelium and the reduction of the reactive oxygen species (ROS) generation in both lung and brain endothelium was also associated with P53 induction. APE1/Ref1 exerts major inflammatory roles by regulating the DNA binding activity of NF-kB and hypoxia-inducible transcription factor 1α. This multifunctional enzyme modulates the production of ROS and reactive nitrogen species (RNS) by regulating thioredoxin, catalase, and superoxide dismutase. Hence, APE1/Ref1 is considered to be a potential therapeutic target for neurodegenerative pathologies (e.g. Alzheimer’s and Parkinson’s disease) [6].

Heat shock protein 90 (Hsp90) is an ATP-dependent chaperone protein that is conserved from bacteria to humans, and serves to ensure the proper folding and maturation of its client proteins. Moreover, Hsp90 is involved in fundamental cellular processes including DNA repair, development, and immune responses [7]. Aberrant activation of Hsp90 has been linked to the development of multiple pathological conditions including cancer, inflammation, and viral infection [8]. Cancers employ Hsp90 to promote their growth and metastasis [9]. On the other hand Hsp90 inhibition is associated with the degradation of oncogenic client proteins including cell-cycle regulatory proteins, tyrosine kinases, and transcription factors [10].

In addition to their protective effects on malignancies and vascular damage, Hsp90 inhibitors reduce inflammation and oxidative stress by suppressing NF-kB, and signal transducer and activator of transcription (STAT) signaling pathways [11]. Those protective effects have been
associated with the induction of the P53 and downregulation of its negative regulators mouse double minute 2 (MDM2), MDMX [12], and NF-kB [13]. Recent works emphasize on the fact that P53 mediates the protective effects of Hsp90 inhibitors in the LPS-induced acute lung injury (ALI) [12, 14, 15], and that Growth Hormone Releasing Hormone (GHRH) antagonists support BBB function [16]. In the present study we demonstrate that Hsp90 inhibitors enhance brain endothelial function and protect against H$_2$O$_2$-induced breakdown.

**Materials and methods**

**Reagents**

The Hsp90 inhibitor AUY-922 (101756–820) and 17-DMAG (102513662), 2,7-Dichlorodihydrofluorescein diacetate (10180–506), antimouse IgG HRP linked whole antibody from sheep (95017–554), antirabbit IgG HRP linked whole antibody from donkey (95017–556), nitrocellulose membranes (10063–173), and RIPA buffer (AAJ63306-AP) were obtained from VWR (Radnor, PA). APEl/Refl (4128S), Phosphocofilin (pCofilin) (3313S), Cofilin (3318S), p-Myosin Light Chain 2 (pMLC2) (3674S), Myosin Light Chain 2 (MLC2) (3672S), MDM2 (8693S) and P53 (9282S) antibodies were purchased from Cell Signaling (Danvers, MA). Hydrogen peroxide (H1009) and β-actin antibody (A5441) were purchased from Sigma-Aldrich (St Louis, MO).

**Cell culture**

The hCMEC/D3 cells (SCC066) were purchased from Millipore Sigma (Temecula, CA). This BBB cell line was derived from human temporal lobe micro-vessels, which was enriched incerebral endothelial cells, and consequently immortalized by lentiviral transduction. These cells were maintained at 37 °C in a humidified atmosphere of 5% CO$_2$-95% air, in EndoGRO™-MV Complete Media Kit (SCME004) supplemented with 1 ng/mL FGF-2 (GF003), as we have previously done [16, 17].

**Western blot analysis**

Proteins were isolated from the cells using RIPA buffer. An equal amount of proteins were separated according to their molecular weight by electrophoresis onto sodium dodecyl sulfate (SDS-PAGE) Tris-HCl gels. A wet transfer technique was used to transfer the proteins onto the nitrocellulose membranes. The membranes were incubated 1 hat room temperature in a solution of 5% non-fat dry milk. The blots were then exposed to appropriate primary antibodies (1:1000) at 4 °C overnight. The following day, the membranes were incubated with the corresponding secondary antibodies (1:2000) and exposed to SuperSignal™ West Pico PLUS chemiluminescent substrate (PI34578). The signal for the protein bands were detected in a ChemiDoc™ Touch Imaging System from BioRad (Hercules, CA). The β-actin was the loading control unless otherwise stated in the graph of densitometry. All reagents were obtained from VWR (Radnor, PA).

**ROS measurement**

The hCMEC/D3 cells were treated with either vehicle (0.01% DMSO) or 17-DMAG (1 μM) for 24 h prior to the exposure to either vehicle (PBS) or H$_2$O$_2$ (10$^{-4}$ M) for 8 h. Cells were then incubated with 2,7-Dichlorodihydrofluorescein diacetate (20 μM) for 30 min. The
fluorescence intensity was measured in a Synergy HI Hybrid Multi-Mode Reader from Biotek (Winooski, VT).

**Measurement of endothelial barrier function**

The permeability of the endothelial cell monolayers was estimated by electric cell-substrate impedance sensing (ECIS), utilizing the ECIS model ZΘ (Applied Biophysics, Troy, NY, USA) as previously described [18, 19]. All experiments were conducted on confluent cells which had reached a steady-state resistance of at least 800 Ω [20].

**Densitometry and statistical analysis**

Image J software (National Institute of Health) was used to perform densitometry of immunoblots. All data is expressed as Means ± SEM (standard error of the mean). Student's t-test was used to determine statistically significant differences among the groups. A value of $P<0.05$ was considered significant. GraphPad Prism (version 5.01) was used to analyze the data. The letter n represents the number of experimental repeats.

**Results**

**H$_2$O$_2$ suppresses P53 and upregulates APE1/Ref1 in hCMEC/D3**

Human cerebral microvascular endothelial cells were treated with either vehicle (PBS) or H$_2$O$_2$ ($10^{-3}$, $10^{-4}$, $10^{-5}$ M) for 24 h. Our results indicate that H$_2$O$_2$ downregulated P53 expression levels in all three concentrations, as depicted in Figure (Fig.) 1A. On the other hand, H$_2$O$_2$ induced the expression of the inflammatory APE1/Ref1 in all treatments of H$_2$O$_2$ ($10^{-3}$, $10^{-4}$, $10^{-5}$ M). The results appear in Fig. 1B. The means of the H$_2$O$_2$ treatments ($10^{-3}$, $10^{-4}$, $10^{-5}$ M) as related to P53 expression were 0.58, 0.67 and 0.60, respectively. In the case of APE1/Ref1 expression, the corresponding means were 1.40, 1.56 and 1.54.

**H$_2$O$_2$ activates (phosphorylates) MLC2 in hCMEC/D3**

To evaluate the effects of H$_2$O$_2$ in the phosphorylation (activation) of MLC2, hCMEC/D3 cells were subjected to treatment with either vehicle (PBS) or H$_2$O$_2$ ($10^{-3}$, $10^{-4}$, $10^{-5}$ M) for 24 h. Our observations suggest that this reactive oxygen species inducer (H$_2$O$_2$) increased the phosphorylation of MLC2 in all cases of H$_2$O$_2$ treatments (Fig. 1C). The means of the H$_2$O$_2$ treatments ($10^{-3}$, $10^{-4}$, $10^{-5}$ M) as related to pMLC2 expression were 1.858, 1.758, 2.479.

**H$_2$O$_2$ activates (dephosphorylates) cofilin in the brain microvascular endothelium**

The cerebral endothelial cells hCMEC/D3 were treated with either vehicle (PBS) or H$_2$O$_2$ ($10^{-3}$, $10^{-4}$, $10^{-5}$ M) for 24 h. The results indicate that H$_2$O$_2$ suppressed the cofilin phosphorylation in those cells (Fig. 1D). The means of the H$_2$O$_2$ treatments ($10^{-3}$, $10^{-4}$, $10^{-5}$ M) as related to cofilin expression were 0.41, 0.71 and 0.68.
**Hsp90 inhibition opposes the H\textsubscript{2}O\textsubscript{2}-induced P53 suppression**

hCMEC/D3 cells were treated with either vehicle (0.01% DMSO) or 17-DMAG (1 μM) for 24 h prior to treatment with either vehicle (PBS) or H\textsubscript{2}O\textsubscript{2} (10\textsuperscript{-4} M) for 8 h. The Hsp90 inhibitor 17-DMAG induced the expression of P53 in those cells (mean value: 1.88), while H\textsubscript{2}O\textsubscript{2} suppressed this endothelium defender (P53) (mean value: 0.41). Furthermore, 17-DMAG counteracted the H\textsubscript{2}O\textsubscript{2}-induced suppression of P53 in hCMEC/D3 cells (mean value: 1.99). The results appear in Fig. 2A.

**Hsp90 inhibition suppresses the H\textsubscript{2}O\textsubscript{2}-induced increase of MDM2 in the hCMEC/D3 cells**

The cells were pre-treated with either vehicle (0.01% DMSO) or 17-DMAG (1 μM) for 24 h and post-treated with either vehicle (PBS) or H\textsubscript{2}O\textsubscript{2} (10\textsuperscript{-4} M) for 8 h. H\textsubscript{2}O\textsubscript{2} induced the levels of the P53 suppressor MDM2 (mean value: 1.77), and 17-DMAG exerted the opposite results (mean: 0.67). This Hsp90 inhibitor (17-DMAG) prevented the H\textsubscript{2}O\textsubscript{2}-induced MDM2 augmentation (mean value: 0.63), as indicated in Fig. 2B.

**Hsp90 inhibition counteracts the H\textsubscript{2}O\textsubscript{2}-induced activation of MLC2**

The hCMEC/D3 cells were exposed to either vehicle (0.01% DMSO) or 17-DMAG for 24 h before treatment with either vehicle (PBS) or H\textsubscript{2}O\textsubscript{2} (10\textsuperscript{-4} M) for 8 h. H\textsubscript{2}O\textsubscript{2} increased the expression levels of pMLC2 (mean value: 1.56), and 17-DMAG suppressed those levels (mean: 0.35). This Hsp90 inhibitor downregulated the phosphorylation of MLC2 (pMLC2) due to H\textsubscript{2}O\textsubscript{2} exposure (mean value: 0.31) (Fig. 2C).

**Hsp90 inhibition suppresses the H\textsubscript{2}O\textsubscript{2}-induced ROS generation in hCMEC/D3**

The human cerebral microvascular endothelial cells were treated with either vehicle (0.01% DMSO) or 17-DMAG (1 μM) for 24 h prior to an 8 h exposure to PBS or H\textsubscript{2}O\textsubscript{2} (10\textsuperscript{-4} M). Our observations reveal that 17-DMAG suppressed the ROS generation in hCMEC/D3 cells (mean value: 0.67). On the other hand, H\textsubscript{2}O\textsubscript{2} increased ROS levels (mean value: 1.53). Moreover, 17-DMAG suppressed the H\textsubscript{2}O\textsubscript{2}–triggered ROS induction (mean value: 0.96) (Fig. 3A).

**Hsp90 inhibition protects against H\textsubscript{2}O\textsubscript{2}-induced cerebral endothelial breakdown**

Confluent hCMEC/D3 cells seeded onto ECIS arrays were treated with either vehicle (0.01% DMSO) or 5μM of Hsp90 inhibitor AUY-922 for 20 h prior to their exposure to H\textsubscript{2}O\textsubscript{2} (700 μM) or vehicle (PBS). The transendothelial electrical resistance (TEER) measurements depicted in Fig. 3B revealed that H\textsubscript{2}O\textsubscript{2} increases the endothelial permeability (reduces TEER) of hCMEC/D3 (red line). Remarkably, those cells pre-treated with the Hsp90 inhibitor AUY-922 were protected against the H\textsubscript{2}O\textsubscript{2}-induced endothelial barrier disruption. Those protective effects appear in blue line.

**Discussion**

An excess of 10 million individuals are diagnosed with neurodegenerative diseases related to the BBB dysfunction [21]. Recent evidence indicate that COVID-19 impairs the central nervous system, endothelium, and the permeability of BBB [22–26]. The SARS-CoV-2
spike protein alters barrier function in 2D static and 3D microfluidic in vitro models of the human blood–brain barrier [27]. Neurological complications associated with BBB damage were reported due to the inflammatory response triggered by the SARS-CoV-2 [28, 29]. High-titer anti-SARS-CoV-2 antibodies were detected in the cerebral spinal fluid of comatose or encephalopathic patients demonstrating intrathecal IgG synthesis or BBB disruption [30]. Another study suggested that patients with COVID-19 developed endothelial activation and BBB dysfunction [31]. In our study we employed commercially available hCMEC/D3 cells from human temporal lobe microvessels to investigate the protective role of Hsp90 inhibition towards the H$_2$O$_2$-induced endothelial barrier impairment.

Hydrogen peroxide accumulated from its progenitor molecule superoxide anion (O$_2$.−) serves as a second messenger and exerts prolonged signaling effects [32]. This is a major contributor to oxidative stress in cells and tissues and activates p90RSK, which is a downstream effector of extracellular signal-regulated kinases 1/2 (ERK1/2). The activation of those kinases upregulates the vascular endothelial growth factor (VEGF), hence it contributes to endothelial growth, angiogenesis, and atheroma. Hypoxia-induced activation of ERK1/2 and P38 MAPK is also mediated by H$_2$O$_2$ and have been associated with angiotensin II-induced vascular hypertrophy [33].

Exposure of hCMEC/D3 cells to H$_2$O$_2$ resulted to reduced P53 expression, disrupted endothelial function, and increased levels of APE1/Ref1. Pre-treatment of those cells with the Hsp90 inhibitor 17-DMAG counteracted the deteriorating effects of H$_2$O$_2$ towards BBB integrity, and induced P53. This is a protein which regulates a wide variety of cellular responses against cellular threats. An emerging body of studies suggest the robust anti-inflammatory activities of P53 in a diverse variety of human tissues in the context of the unfolded protein response (UPR) element [34–37]. UPR is involved in diverse aspects of cardiovascular [36] and pulmonary conditions [23, 34, 38]. Interestingly, the activation of UPR increases P53 expression, while UPR suppression exerts the opposite effects [39].

Hsp90 inhibitors exert their anti-inflammatory properties at least in part via P53 [12], and have been associated with the activation of UPR both in vivo and in vitro [37, 40]. Those compounds exhibit a higher affinity towards activated Hsp90, thus moderate doses of those compounds in non-inflamed cells do not induce lethal effects [9, 41]. The UPR suppressor Kifunensine induced downregulation of P53, causing endothelial barrier dysfunction [19] counteracted by the Hsp90 inhibitor AUY-922 [42]. Induction of P53 by tunicamycin, which is an endoplasmic reticulum (ER) stress inducer, suppressed the redox sensor APE1/Ref1 in vascular endothelium [20]. These observations suggest an intense crosstalk between P53 and UPR in the intracellular niche. However, the exact interrelations of that connection remain largely unknown [34].

In granulosa cells, H$_2$O$_2$ phosphorylates P53 and causes cellular death via apoptosis. On the other hand, exposure to the antioxidant agent (N-acetylcysteine) prevents H$_2$O$_2$-induced apoptosis and phosphorylation of P53 [43]. Our previous observations have revealed that the phosphorylation of P53 is associated with the activities of barrier-disrupting agents, and that Hsp90 suppresses those effects [14]. Herein we demonstrate that H$_2$O$_2$ suppresses the
expression level of P53 (Figs. 1A and 2A) and that the Hsp90 inhibitor 17-DMAG opposes those effects in hCMEC/D3 cells (Fig. 2A) and prevents the H$_2$O$_2$-induced upregulation of MDM2 (Fig. 2B).

Ischemia/reperfusion and traumatic brain injury results in excessive ROS generation, which is the central event of various inflammatory diseases [44, 45]. Oxidative stress due to ROS damages nucleic acids, proteins and lipids. This damage leads to increased permeability to the vasculature in the BBB that contributes to inflammation and breakdown of this barrier. H$_2$O$_2$ is an important physiological second messenger and endogenous ROS which has been implicated in a variety of pathophysologies including angiogenesis, hyperpermeability, and apoptosis [46]. Superoxide anions within the cells are frequently converted to H$_2$O$_2$ that can cross the cell membrane through aquaporin channels [44].

In the presence of transition metals, H$_2$O$_2$ can produce a highly reactive hydroxyl radical which can oxidize proteins and cause lipid peroxidation. Brain tissue is rich in oxygen supply, polyunsaturated fatty acids, and iron, making it highly susceptible to lipid peroxidation [47]. Oxidative stress associated with protein oxidation and lipid peroxidation causes neuronal cytotoxicity, altered cell membrane fluidity, increased membrane permeability, and membrane disruption. All of which may lead to cellular injury. MDA production is an indicator of oxidative stress and lipid peroxidation [17, 48].

Treatment of brain endothelial cells with H$_2$O$_2$ has been reported to result in excessive MDA generation. P53-inducible antioxidant enzymes such as glutathione peroxidase 1 (GPx1) and manganese superoxide dismutase (MnSOD) regulate mitochondrial ROS levels [17]. The selenium-dependent GPx1 scavenges H$_2$O$_2$ [49]. MnSOD, an essential mitochondrial survival enzyme, decomposes superoxide [50]. Knockdown of P53 in retinal ganglion cells increased basal ROS levels and susceptibility to oxidation to H$_2$O$_2$-mediated cell death [51]. Here in we report that the upregulation of P53 by the Hsp90 inhibitor 17-DMAG reduces the basal ROS levels, and suppresses the H$_2$O$_2$-induced generation of ROS in BBB endothelium (Fig. 3A). Exposing hCMEC/D3 cells to TNF-α or IFN-γ increased their intracellular permeability [52], via occludin and claudin-5 suppression [52]. In this study, we report the protective role of Hsp90 inhibition against the H$_2$O$_2$-induced brain endothelial hyperpermeability, since AUY-922 protected the BBB cells against H$_2$O$_2$-triggered breakdown (Fig. 3B).

Alterations in the actin fiber formation regulate endothelial barrier function and permeability. Activation of MLC2 increases vascular permeability via the actomyosin contraction leading to reduced endothelial integrity [53]. We investigated the effects of H$_2$O$_2$ in the phosphorylation of MLC2 (Fig. 1C). It was previously shown in the lungs that P53 suppresses the MLC2 activation [4, 12, 42]. We are now reporting in hCMEC/D3 cells that H$_2$O$_2$ increases the phosphorylated MLC2 (pMLC2), and Hsp90 inhibition due to 17-DMAG treatment strongly opposes that effect (Fig. 2C).

Brain endothelial tight junctions (TJ) exhibit high transendothelial electrical resistance (TEER) and restrict passive diffusion [54]. The major TJ proteins include occludins and claudins, which form a continuous blood vessel to seal the inter endothelial cleft [55]. The
junctional integrity of the endothelial cells also depends on the endothelial cytoskeleton, which is mainly composed of microtubules, actin microfilaments, and intermediate filaments. Microtubules composed of $\alpha$- and $\beta$-tubulin create a lattice network from the nucleus to the periphery to ensure cellular rigidity. These microtubules interact with microfilaments and participate in the process of actin filament assembly. Actin depolymerizing factor (ADF)/cofilin family proteins are the actin-binding proteins that regulate the assembly and disassembly of actin filaments. The crosslinked F-actin and myosin motor proteins interact to form actomyosin contractile bundles that link the cell-cell matrix adhesion [56].

Rac1 mediates cortical actin and junctional complex stabilization as well as stress fiber destabilization by regulating cofilin. Abnormal cofilin dynamics may cause BBB endothelial dysfunction during inflammation, ischemia, and other stress conditions. Cofilin binding to actin is prevented by its phosphorylation at ser3 residue, thus phosphorylation deactivates this protein [57].

In our experiments $H_2O_2$ dephosphorylated cofilin in hCMEC/D3 cells (Fig. 1D), causing endothelial hyperpermeability (Fig. 3B). Previous studies reported that both Hsp90 inhibitors and GHRH antagonists support endothelial barrier function by deactivating cofilin [4,58]. Moreover, GHRH antagonists activate UPR and protect against the kifunensine (UPR suppressor)-induced barrier disruption [59].

Conclusions and future perspectives

Brain endothelial dysfunction contributes in the development and establishment of serious neurodegenerative diseases and cerebral disorders. Herein we report the capacity of Hsp90 inhibitors (P53 inducers) to support the BBB function under severe oxidative conditions. Remark-able, Hsp90 inhibitors were reported to cross the BBB [60–62]. Thus, we suggest that these anti-inflammatory agents may deliver new possibilities for the treatment of diseases related to BBB dysfunction, including COVID-19, cerebral ischemia/reperfusion (I/R) (stroke), and traumatic brain injury (TBI). Future in vivo and in vitro studies will further delineate the highly interrelated Hsp90/P53/UPR universe to deliver targeted therapeutical interventions towards the BBB-related pathologies [63,64]

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References

[1]. Sweeney MD, Sagare AP, Zlokovic BV, Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders, Nat. Rev. Neurol 14 (3) (2018) 133–150. [PubMed: 29377008]
[2]. Stephenson J, et al., Inflammation in CNS neurodegenerative diseases, Immunology 154 (2) (2018) 204–219. [PubMed: 29513402]
[3]. Barabutis N, Schally AV, Siejka A, P53, GHRH, inflammation and cancer, EBioMedicine 37 (2018) 557–562. [PubMed: 30344124]
[4]. Barabutis N, et al., Wild-type p53 enhances endothelial barrier function by mediating RAC1 signalling and RhoA inhibition, J. Cell. Mol. Med. 22 (3) (2018) 1792–1804. [PubMed: 29363851]
[5]. Kubra KT, et al., P53 versus inflammation: an update, Cell Cycle 19 (2) (2020) 160–162. [PubMed: 31880200]
[6]. Thakur S, et al., APE1/Ref-1 as an emerging therapeutic target for various human diseases: phytochemical modulation of its functions, Exp. Mol. Med. 46 (2014) e106. [PubMed: 25033834]
[7]. Schopf FH, Biebl MM, Buchner J, The HSP90 chaperone machinery, Nat. Rev. Mol. Cell Biol. 18 (6) (2017) 345–360. [PubMed: 28429788]
[8]. Hoter A, El-Sabban ME, Naim HY, The HSP90 family: structure, regulation, function, and implications in health and disease, Int. J. Mol. Sci 19 (9) (2018) 2560.
[9]. Barabutis N, Heat shock protein 90 inhibition in the inflamed lungs, Cell Stress Chaperones 25 (2) (2020) 195–197. [PubMed: 31950341]
[10]. Garcia-Carbonero R, Carnero A, Paz-Ares L, Inhibition of HSP90 molecular chaperones: moving into the clinic, Lancet Oncol. 14 (9) (2013) e358–e369. [PubMed: 23896275]
[11]. Lazaro I, et al., Targeting HSP90 ameliorates nephropathy and atherosclerosis through suppression of NF-kappaB and STAT signaling pathways in diabetic mice, Diabetes 64 (10) (2015) 3600–3613. [PubMed: 26116697]
[12]. Thangjam GS, et al., Novel mechanism of attenuation of LPS-induced NF-kappaB activation by the heat shock protein 90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin, in human lung microvascular endothelial cells, Am. J. Respir. Cell Mol. Biol. 50 (5) (2014) 942–952. [PubMed: 24303801]
[13]. Barabutis N, Uddin MA, Catravas JD, Hsp90 inhibitors suppress P53 phosphorylation in LPS-induced endothelial inflammation, Cytokine 113 (2019) 427–432. [PubMed: 30420201]
[14]. Uddin MA, et al., P53 deficiency potentiates LPS-Induced acute lung injury in vivo, Curr. Res. Physiol 3 (2020) 30–33. [PubMed: 32724900]
[15]. Barabutis N, et al., GHRH Antagonists protect against hydrogen peroxide-induced breakdown of brain microvascular endothelium integrity, Horm. Metab. Res. 52 (5) (2020) 336–339. [PubMed: 32403147]
[16]. Akhter MS, et al., P53-induced reduction of lipid peroxidation supports brain microvascular endothelium integrity, J. Pharmacol. Sci 141 (1) (2019) 83–85. [PubMed: 31607444]
[17]. Barabutis N, et al., LPS induces pp60c-src-mediated tyrosine phosphorylation of Hsp90 in lung vascular endothelial cells and mouse lung, Am. J. Physiol. Lung Cell. Mol. Physiol 304 (12) (2013) L883–L893. [PubMed: 23585225]
[18]. Akhter MS, et al., Kifunensine compromises lung endothelial barrier function, Microvasc. Res 132 (2020) 104051. [PubMed: 32730762]
[19]. Uddin MA, et al., P53 supports endothelial barrier function via APE1/Ref1 suppression, Immunobiology 224 (4) (2019) 532–538. [PubMed: 31023490]
[20]. Gaugler JE, et al., Reconsidering frameworks of Alzheimer’s dementia when assessing psychosocial outcomes, Alzheimers Dement. 5 (2019) 388–397.
[21]. Pellegrini L, et al., SARS-CoV-2 infects the brain choroid plexus and disrupts the blood-CSF barrier in human brain organoids, Cell Stem Cell 27 (6) (2020) 951–961.e5, doi:10.1016/j.stem.2020.10.001. [PubMed: 33113348]
[22]. Barabutis N, Unfolded protein response in the COVID-19 context, Aging Health Res 1 (1) (2021) 100001, doi:10.1016/j.aehr.2020.100001. [PubMed: 33330852]
[23]. Kaur S, Tripathi DM, Yadav A, The Enigma of Endothelium in COVID-19, Front. Physiol 11 (2020) 989. [PubMed: 32848893]
[25]. Marshall M, How COVID-19 can damage the brain, Nature 585 (7825) (2020) 342–343. [PubMed: 32934351]

[26]. Najjar S, et al., Central nervous system complications associated with SARS-CoV-2 infection: integrative concepts of pathophysiology and case reports, J. Neuroinflammation 17 (1) (2020) 231. [PubMed: 32758257]

[27]. Buzhdyan TP, et al., The SARS-CoV-2 spike protein alters barrier function in 2D static and 3D microfluidic in-vitro models of the human blood-brain barrier, Neurobiol. Dis 146 (2020) 105131. [PubMed: 33053430]

[28]. Alquisiras-Burgos I, et al., Neurological complications associated with blood-brain barrier damage induced by the inflammatory response during SARS-CoV-2 infection, Mol. Neurobiol. (2020) 1–16, doi:10.1007/s12055-020-02134-7.

[29]. Achar A, Ghosh C, COVID-19-associated neurological disorders: the potential route of CNS Invasion and blood-brain relevance, Cells 9 (11) (2020) 2360.

[30]. Alexopoulos H, et al., Anti-SARS-CoV-2 antibodies in the CSF, blood-brain barrier dysfunction, and neurological outcome: studies in 8 stuporous and comatose patients, Clin Immunol. 221 (2020) 108619. [PubMed: 33127563]

[31]. Perrin P, et al., Cytokine release syndrome-associated encephalopathy in patients with COVID-19, Eur. J. Neurol. (2020), doi:10.1111/ejn.14491.

[32]. Cai H, Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences, Cardiovasc. Res. 68 (1) (2005) 26–36. [PubMed: 16009356]

[33]. Javadov S, Jang S, Agostini B, Crosstalk between mitogen-activated protein kinases and mitochondria in cardiac diseases: therapeutic perspectives, Pharmacol. Ther. 144 (2) (2014) 202–225. [PubMed: 24924700]

[34]. Barabutis N, Unfolded protein response in lung health and disease, Front. Med 7 (2020) 344.

[35]. Barabutis N, P53 in acute respiratory distress syndrome, Cell. Mol. Life Sci. 77 (22) (2020) 4725–4727. [PubMed: 32886127]

[36]. Kubra KT, et al., Unfolded protein response in cardiovascular disease, Cell Signal. 73 (2020) 109699. [PubMed: 32592779]

[37]. Uddin MA, Kubra KT, Sonju JJ, Akhter MS, Seetharama J, Barabutis N, Effects of heat shock protein 90 inhibition in the lungs, Med Drug Discov 6 (2020) 100046 Epub 2020 May 17. doi:10.1016/j.medidd.2020.100046. [PubMed: 32728665]

[38]. Akhter MUMA, Kubra KTN; Barabutis, Autophagy, unfolded protein response and lung disease, Curr. Res. Cell Biol 1 (2020) 100003, doi:10.1016/j.crccbio.2020.100003. [PubMed: 33163960]

[39]. Akhter MS, Uddin MA, Barabutis N, Unfolded protein response regulates P53 expression in the pulmonary endothelium, J. Biochem. Mol. Toxicol. 33 (10) (2019) e22380. [PubMed: 31339623]

[40]. Kubra KT, et al., Hsp90 inhibitors induce the unfolded protein response in bovine and mice lung cells, Cell Signal. 67 (2020) 109500. [PubMed: 31837463]

[41]. Butler LM, et al., Maximizing the Therapeutic Potential of HSP90 Inhibitors, Mol. Cancer Res. 13 (11) (2015) 1445–1451. [PubMed: 26219697]

[42]. Kubra KT, et al., Luminespib counteracts the Kifunensine-induced lung endothelial barrier dysfunction, Curr. Res. Toxicol 1 (2020) 111–115, doi:10.1016/j.crtox.2020.09.003. [PubMed: 33094291]

[43]. Yang H, et al., Oxidative stress-induced apoptosis in granulosa cells involves JNK, p53 and Puma, Oncotarget 8 (15) (2017) 25310–25322. [PubMed: 28445976]

[44]. Angeloni C, et al., Traumatic brain injury and NADPH oxidase: a deep relationship, Oxid. Med. Cell. Longev. 2015 (2015) 370312. [PubMed: 25918580]

[45]. Barabutis N, Schally AV, Antioxidant activity of growth hormone-releasing hormone antagonists in LNCaP human prostate cancer line, Proc. Natl. Acad. Sci. USA 105 (51) (2008) 20470–20475. [PubMed: 19075233]

[46]. Anasooya Shaji C, et al., The tri-phasic role of hydrogen peroxide in blood-brain barrier endothelial cells, Sci. Rep 9 (1) (2019) 133. [PubMed: 30644421]

[47]. Collin F, Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases, Int. J. Mol. Sci 20 (10) (2019) 2407.
[48]. Ayala A, Munoz MF, Arguelles S, Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal, Oxid. Med. Cell. Longev 2014 (2014) 360438. [PubMed: 24999379]

[49]. Ekoue DN, et al., Manganese superoxide dismutase and glutathione peroxidase-1 contribute to the rise and fall of mitochondrial reactive oxygen species which drive oncogenesis, Biochim. Biophys. Acta Bioenergy 1858 (8) (2017) 628–632.

[50]. Kegler A, et al., Involvement of MnSOD Ala16Val polymorphism in epilepsy: a relationship with seizure type, inflammation, and metabolic syndrome, Gene 711 (2019) 143924. [PubMed: 31212050]

[51]. O’Connor JC, et al., A novel antioxidant function for the tumour-suppressor gene p53 in the retinal ganglion cell, Investig. Ophthalmol. Vis. Sci 49 (10) (2008) 4237–4244. [PubMed: 18487368]

[52]. Lopez-Ramirez MA, et al., Role of caspases in cytokine-induced barrier breakdown in human brain endothelial cells, J. Immunol. 189 (6) (2012) 3130–3139. [PubMed: 22896632]

[53]. Schnoor M, et al., Actin dynamics in the regulation of endothelial barrier functions and neutrophil recruitment during endotoxemia and sepsis, Cell. Mol. Life Sci. 74 (11) (2017) 1985–1997. [PubMed: 28154894]

[54]. Butt AM, Jones HC, Abbott NJ, Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study, J. Physiol. 429 (1990) 47–62. [PubMed: 2277354]

[55]. Stamatovic SM, Keep RF, Andjelkovic AV, Brain endothelial cell-cell junctions: how to “open” the blood brain barrier, Curr. Neuropharmacol 6 (3) (2008) 179–192. [PubMed: 19506719]

[56]. Prasain N, Stevens T, The actin cytoskeleton in endothelial cell phenotypes, Microvasc. Res 77 (1) (2009) 53–63. [PubMed: 19028505]

[57]. Nakano K, et al., Cofilin phosphorylation and actin polymerization by NRK/NESSK, a member of the germinable center kinase family, Exp. Cell Res. 287 (2) (2003) 219–227. [PubMed: 12837278]

[58]. Uddin MA, et al., GHRH antagonists support lung endothelial barrier function, Tissue Barriers 7 (4) (2019) 1669989. [PubMed: 31578921]

[59]. Akhter MS, Uddin MA, Schally AV, Kubra KT, Barabutis N, Involvement of unfolded protein response in the protective effects of growth hormone releasing hormone antagonists in the lungs, J. Cell Commun. Signal. 13 (2020) 1–5, doi:10.1007/s12079-020-00593-0.

[60]. Chen H, et al., Abrain-penetrating Hsp90 inhibitor NXD30001 inhibits glioblastoma as a monotherapy or in combination with radiation, Front. Pharmacol 11 (2020) 974. [PubMed: 32695001]

[61]. Putcha P, et al., Brain-permeable small-molecule inhibitors of Hsp90 prevent al-pha-synuclein oligomer formation and rescue alpha-synuclein-induced toxicity, J. Pharmacol. Exp. Ther. 332 (3) (2010) 849–857. [PubMed: 19934398]

[62]. Fogliatto G, et al., NMS-E973, a novel synthetic inhibitor of Hsp90 with activity against multiple models of drug resistance to targeted agents, including intracranial metastases, Clin. Cancer Res. 19 (13) (2013) 3520–3532. [PubMed: 23674492]

[63]. Campisi M, et al., 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, pericytes and astrocytes, Biomaterials 180 (2018) 117–129. [PubMed: 30032046]

[64]. Sivandzade F, Cucullo L, In-vitro blood-brain barrier modeling: a review of modern and fast-advancing technologies, J. Cereb. Blood Flow Metab. 38 (10) (2018) 1667–1681. [PubMed: 30058456]
Fig. 1.
H₂O₂ induces brain endothelial barrier dysfunction.
Western Blot analysis of P53 and β-actin (A), APE1/Ref1 and β-actin (B), phosphorylated MLC2 (pMLC2) and MLC2 (C), phosphorylated Cofilin (pCofilin) and Cofilin (D) expression levels after treatment of hCMEC/D3 cells with either vehicle (PBS) or H₂O₂ (10⁻³, 10⁻⁴, 10⁻⁵ M) for 24 h. The blots shown are representative of 3 independent experiments. The signal intensity of the protein bands was analyzed by densitometry. Protein levels were normalized to β-actin, unless otherwise indicated. *P<0.05, **P<0.01, ***P<0.001 vs vehicle. Means ± SEM.
Fig. 2.

Hsp90 inhibition suppresses the H\(_2\)O\(_2\)-induced hCMEC/D3 dysfunction
The hCMEC/D3 cells were pre-treated with either vehicle (0.01% DMSO) or Hsp90 inhibitor 17-DMAG (1 μM) for 24 h and post-treated with either vehicle (PBS) or H\(_2\)O\(_2\) (10\(^{-4}\) M) for 8 h. Western Blot analysis of (A) P53 and β-actin, (B) MDM2 and β-actin, (C) phosphorylated MLC2 (pMLC2) and MLC2. The blots shown are representative of 3 independent experiments. The signal intensity of the protein bands was analyzed by densitometry. Protein levels were normalized to β-actin, unless otherwise indicated.

*P<0.05, **P<0.01 vs vehicle, $P<0.01$ vs H\(_2\)O\(_2\). Means ± SEM.
Hsp90 inhibitors protect against H$_2$O$_2$-induced breakdown of hCMEC/D3.

(A) The hCMEC/D3 cells were pre-treated with either vehicle (0.01% DMSO) or Hsp90 inhibitor 17-DMAG (1 μM) for 24 h and post-treated with either vehicle (PBS) or H$_2$O$_2$ ($10^{-4}$ M) for 8 h. ROS was measured in the cells using 2,7-Dichlorodihydrofluorescein diacetate. **$P<0.01$ vs vehicle, $$$P<0.01$, $\$\$\$P<0.001$ vs H$_2$O$_2$. Means ± SEM. (B) The hCMEC/D3 cells were seeded onto gold plated arrays and treated with either vehicle (0.01% DMSO) or AUY-922 (5μM) for 20 h. Then the cells were exposed to either vehicle (PBS) or 700 μM H$_2$O$_2$ (black arrow). The cells that were treated with H$_2$O$_2$ shows a gradual decrease in their TEER values (increased permeability). However, the AUY-922-pretreated cells were protected against the H$_2$O$_2$-induced barrier breakdown. Means ± S.E, $n = 3$ per group.