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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 H_2O_2-induced brain endothelium breakdown, by employing human cerebral microvascular endothelial cells (hCMEC/D3). Our findings suggest that H_2O_2 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 H_2O_2 – 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

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Declaration of Competing Interest

None.

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 endonucleasel/redox factor-1 (APEl/Refl) 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. APEl/Refl exerts major inflammatory roles by regulating the DNA binding activity of NF-kB and hypoxia-inducible transcription factor lα. 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_2O_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 (86934S) 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^{TM}-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_2O_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_2O_2 suppresses P53 and upregulates APE1/Ref1 in hCMEC/D3

Human cerebral microvascular endothelial cells were treated with either vehicle (PBS) or H_2O_2 (10^{-3}, 10^{-4}, 10^{-5} M) for 24 h. Our results indicatethatH_2O_2 downregulated P53 expression levels in all three concentrations, as depicted in Figure (Fig.) 1A. On the other hand, H_2O_2 induced the expression of the inflammatory APE1/Ref1 in all treatments of H_2O_2 (10^{-3}, 10^{-4}, 10^{-5} M). The results appear in Fig. 1B. The means of the H_2O_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_2O_2 activates (phosphorylates) MLC2 in hCMEC/D3

To evaluate the effects of H_2O_2 in the phosphorylation (activation) of MLC2, hCMEC/D3 cells were subjected to treatment with either vehicle (PBS) or H_2O_2 (10^{-3}, 10^{-4}, 10^{-5} M) for 24 h. Our observations suggest that this reactive oxygen species inducer (H_2O_2) increased the phosphorylation of MLC2 in all cases of H_2O_2 treatments (Fig. 1C). The means of the H_2O_2 treatments (10^{-3}, 10^{-4}, 10^{-5} M) as related to pMLC2 expression were 1.858, 1.758, 2.479.

H_2O_2 activates (dephosphorylates) cofilin in the brain microvascular endothelium

The cerebral endothelial cells hCMEC/D3 were treated with either vehicle (PBS) or H_2O_2 (10^{-3}, 10^{-4}, 10^{-5} M) for 24 h. The results indicate that H_2O_2 suppressed the cofilin phosphorylation in those cells (Fig. 1D). The means of the H_2O_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_2O_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_2O_2 (10^{-4} M) for 8 h. The Hsp90 inhibitor 17-DMAG induced the expression of P53 in those cells (mean value: 1.88), while H_2O_2 suppressed this endothelium defender (P53) (mean value: 0.41). Furtermore, 17-DMAG counteracted the H_2O_2-induced suppression of P53 in hCMEC/D3 cells (mean value: 1.99). The results appear in Fig. 2A.

Hsp90 inhibition suppresses the H_2O_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_2O_2 (10^{-4} M) for 8 h. H_2O_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_2O_2-induced MDM2 augmentation (mean value: 0.63), as indicated in Fig. 2B.

Hsp90 inhibition counteracts the H_2O_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_2O_2 (10^{-4} M) for 8 h. H_2O_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_2O_2 exposure (mean value: 0.31) (Fig. 2C).

Hsp90 inhibition suppresses the H_2O_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_2O_2 (10^{-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_2O_2 increased ROS levels (mean value: 1.53). Moreover, 17-DMAG suppressed the H_2O_2 – triggered ROS induction (mean value: 0.96) (Fig. 3A).

Hsp90 inhibition protects against H_2O_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_2O_2 (700 μM) or vehicle (PBS). The transendothelial electrical resistance (TEER) measurements depicted in Fig. 3B revealed that H_2O_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_2O_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 trigerred 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_2O_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_2O_2 and have been associated with angiotensin II-induced vascular hypertrophy [33].

Exposure of hCMEC/D3 cells to H_2O_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_2O_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_2O_2 phosphorylates P53 and causes cellular death via apoptosis. On the other hand, exposure to the antioxidant agent (N-acetylcysteine) prevents H_2O_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_2O_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_2O_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_2O_2 is an important physiological second messenger and endogenous ROS which has been implicated in a variety of pathophysiologies including angiogenesis, hyperpermeability, and apoptosis [46]. Superoxide anions within the cells are frequently converted to H_2O_2 that can cross the cell membrane through aquaporin channels[44].

In the presence of transition metals, H_2O_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_2O_2 has been reported to result in excessive MDA generation. P53-inducible antioxidant enzymes such as glutathione peroxidase 1 (GPxl) and manganese superoxide dismutase (MnSOD) regulate mitochondrial ROS levels [17]. The seleniumdependent GPxl scavenges H_2O_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_2O_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_2O_2-induced generation of ROS in BBB endothelium (Fig. 3A). Exposing hCMEC/D3 cells to TNFα 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_2O_2-induced brain endothelial hyperpermeability, since AUY-922 protected the BBB cells against H_2O_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_2O_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_2O_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 α- and β-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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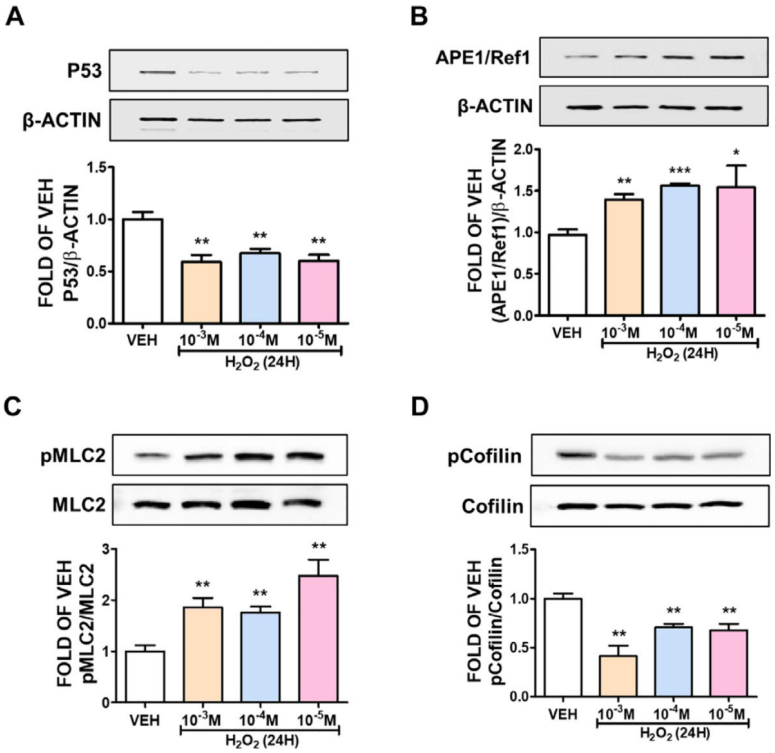

Fig. 1..

H_2O_2 induces brain endothelial barrier dysfunction.

Western Blot analysis of P53 and β-actin **(A)**, APEl/Refl 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 H_2O_2 (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.

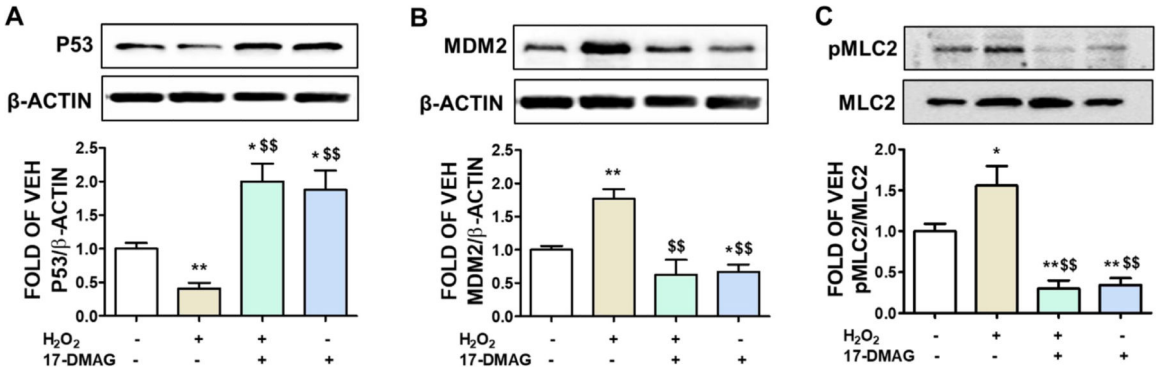

Fig. 2..

Hsp90 inhibition suppresses the H_2O_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_2O_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_2O_2. Means ± SEM.

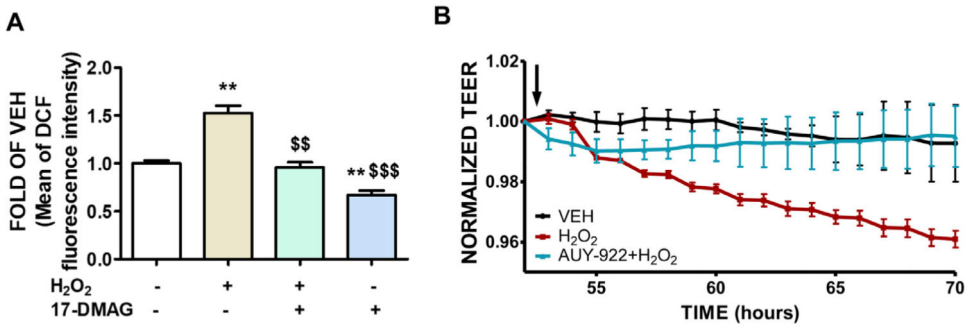

Fig. 3..

Hsp90 inhibitors protect against H_2O_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_2O_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_2O_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_2O_2 (black arrow). The cells that were treated with H_2O_2 shows a gradual decrease in their TEER values (increased permeability). However, the AUY-922-pretreated cells were protected against the H_2O_2-induced barrier breakdown. Means ± *S*.E, $n = 3$ per group.