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ORIGINAL ARTICLE

Protective effects of GHRH antagonists against hydrogen peroxide-induced lung endothelial barrier disruption

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Abstract

Purpose Growth hormone-releasing hormone (GHRH) is a hypothalamic hormone, which regulates growth hormone release from the anterior pituitary gland. GHRH antagonists (GHRHAnt) are anticancer agents, which also exert robust anti-inflammatory activities in malignancies. GHRHAnt exhibit anti-oxidative and anti-inflammatory effects in vascular endothelial cells, indicating their potential use against disorders related to barrier dysfunction (e.g. sepsis). Herein, we aim to investigate the effects of GHRHAnt against lung endothelial hyperpermeability.

Methods The in vitro effects of GHRHAnt in H_2O_2-induced endothelial barrier dysfunction were investigated in bovine pulmonary artery endothelial cells (BPAEC). Electric cell-substrate impedance sensing (ECIS) was utilized to measure transendothelial resistance, an indicator of barrier function.

Results Our results demonstrate that GHRHAnt protect against H_2O_2-induced endothelial barrier disruption via P53 and cofilin modulation. Both proteins are crucial modulators of vascular integrity. Moreover, GHRHAnt prevent H_2O_2 – induced decrease in transendothelial resistance.

Conclusions GHRHAnt represent a promising therapeutic intervention towards diseases related to lung endothelial hyperpermeability, such as acute respiratory distress syndrome - related or not to COVID-19 - and sepsis. Targeted medicine for those potentially lethal disorders does not exist.

Keywords Vascular endothelium · Oxidative stress · Inflammation · Growth hormone-releasing hormone · P53

Introduction

Growth hormone-releasing hormone (GHRH) is a 44 amino-acid peptide [1], which binds to the GHRH receptors on the somatotrophs of the anterior pituitary gland, to regulate the synthesis and secretion of growth hormone (GH). The predominant source of GHRH production is the hypothalamus. However, it can be produced by different sites; such as lungs, kidney, liver, ovary, and testis [2]. GHRH is also secreted by carcinoid and pancreatic tumors, an event which contributed to its isolation, characterization, and sequencing [3, 4]. The full biological activity of GHRH is retained in the first 29 amino- acid sequence [4]. GHRH receptor (GHRHR) and its splice variants (SVs) have been identified in a variety of human cancers; including glioblastoma, pancreatic cancer, lymphoma, and small cell lung carcinoma (SCLC). That demonstrates their implication in cancers [1, 5].

GHRH antagonists (i.e. JV-1-36, JV-1-63, and MIA-602) were developed to counteract the growth-factor activities of GHRH in cancers [1, 6]. Those peptides exert anti-proliferative effects in various malignancies, such as ovarian [7], lung [8], breast, and prostate cancer [1, 9]. GHRH triggers the extracellular signal regulated kinase 1/2 (ERK1/2) [10] and Janus kinase 2/ signal transducer and activator of transcription 3 (JAK2/STAT3) [11]; while, GHRHAnt counteract those effects [12, 13]. Recent evidence suggests a possible role of GHRHAnt in endothelial barrier function [14, 15].

Endothelial cells (ECs) are polarized. Their apical side is exposed to the lumen, while their basolateral surface covers basement membrane [16]. Vascular endothelium is a single layer of endothelial cells; which constitutes the inner lining of arteries, veins, and capillaries. It functions as a semi-

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permeable barrier between the blood and the surrounding tissues [17]. Inter-endothelial junctions are composed of tight junctions (TJs), adherens junctions (AJs), and gap junctional complexes [18]. Tight and adherens junctions promote the adhesion of opposing endothelial cells to preserve endothelial barrier integrity; whereas gap junctions form channels between neighboring cells to communicate ions and signals [19]. In the quiescent vasculature, endothelium maintains blood fluidity and controls vessel permeability. That allows to the exchange of solutes, small molecules, and nutrients; while it restricts the extravasation of larger components [20]. In disease states, the integrity of endothelial barrier is compromised, causing leukocytes infiltration into the interstitium, lung edema, and respiratory failure [21, 22].

The partial reduction of oxygen molecules during normal cellular metabolism generates reactive oxygen species (ROS); which include superoxide anion (O^{2-}), hydroxyl radical (OH^{-}), and hydrogen peroxide (H_2O_2). Excessive accumulation of ROS in ECs modulates calcium homeostasis [23], induces cell adhesion molecules [24], and facilitates actin cytoskeleton remodeling. GHRHAnt reduce oxidative stress in the aging brain [25], and suppress cyclooxygenase 2 (COX-2) and cytochrome c oxidase IV (COX-IV) [26]. Recent studies demonstrate anti-oxidative effects of GHRHAnt in lung and brain endothelial cells [27, 28]. Herein we will elucidate the effects of GHRHAnt in H_2O_2-induced lung endothelial barrier disruption, as well as the molecular mechanisms involved in those effects.

Materials and methods

Reagents

GHRH (103663–156), RIPA buffer (AAJ63306-AP), anti-mouse IgG HRP-linked antibody (95017554), anti-rabbit IgG HRP-linked antibody (95017-556), and nitrocellulose membranes (10063-173) were purchased from VWR (Radnor, PA, USA). P53 (9282 S), phospho-Cofilin (3313 S), and cofilin (3318 S) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Hydrogen peroxide (H1009) and β-actin antibody (A5441) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GHRH antagonist JV-1-36 (031-23) was obtained from Phoenix Pharmaceuticals INC (Burlingame, CA, USA).

Cell culture

Bovine pulmonary artery endothelial cells (BPAEC) (PB30205) were purchased from Genlantis (San Diego, CA, USA). Those cells were cultured in DMEM (VWRL0101-0500) supplemented with 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2-95% air. All materials were purchased from VWR (Radnor, PA, USA).

Measurement of endothelial barrier function

The barrier function of endothelial cell monolayers was estimated by electric cell-substrate impedance sensing (ECIS), using ECIS model ZΘ (Applied Biophysics, Troy, NY). All experiments were conducted on confluent cells that had reached a steady-state resistance of at least 800 Ω.

Western blot analysis

Proteins were isolated using RIPA buffer. Equal amounts of proteins were loaded in each well, so to be separated by electrophoresis onto 10% sodium dodecyl sulfate (SDS-PAGE) Tris-HCl gels. Wet transfer was used to transfer the proteins onto nitrocellulose membranes, which were then incubated for 1 h at room temperature in 5% nonfat dry milk. The blots were incubated overnight with appropriate primary antibodies (1:1000) at 4 °C. The signals for immunoreactive proteins were developed using appropriate secondary antibodies (1:2000); and were visualized in ChemiDoc™ Touch Imaging System from Bio-Rad (Hercules, CA, USA).

Densitometry and statistical analysis

Image J software (National Institute of Health) was used to perform densitometry of immunoblots. The data are 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, and *n* represents the number of experimental repeats.

Results

H_2O_2 and GHRH reduce endothelial P53

Bovine lung endothelial cells were treated with vehicle (0.1% DMSO), or GHRHAnt (1 μM), or H_2O_2 (0.1 mM), or GHRH (1 μM) for 8 h. Our results demonstrate a significant induction of P53 expression in GHRHAnt-treated cells. In contrast, those cells treated with H_2O_2 or GHRH expressed less P53 (Fig. 1A).

GHRHAnt support barrier function via cofilin phosphorylation

Dephosphorylation of cofilin activates it, so to sever the actin filament in a pH-dependent manner [29]. To measure the phosphorylation of cofilin, BPAEC were exposed to

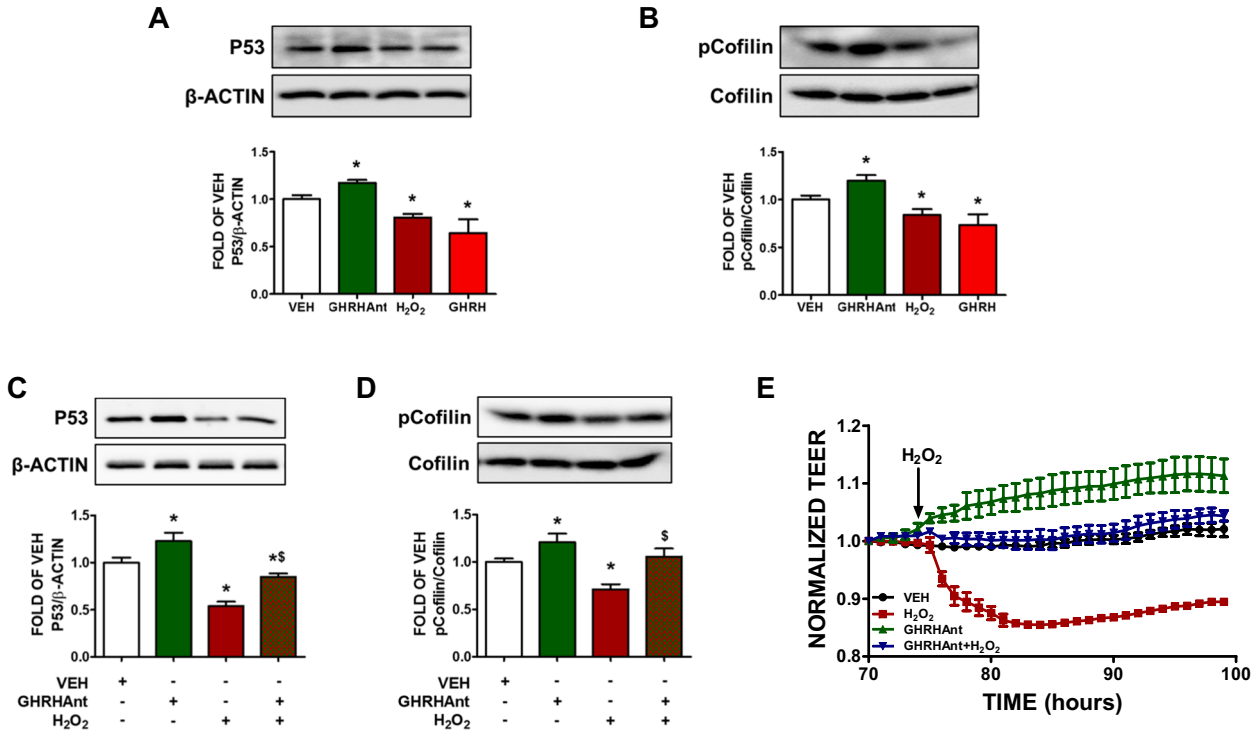

Fig. 1 GHRHAnt protect against H_2O_2-induced lung endothelial barrier dysfunction. Western blot analysis of P53 and β-actin (**A**), pCofilin and cofilin (**B**) after treatment of BPAEC with vehicle (0.1% DMSO), or GHRHAnt (1 μM), or H_2O_2 (0.1 mM), or GHRH (1 μM) (8 h). Western blot analysis of P53 and β-actin (**C**), pCofilin and cofilin (**D**) after treatment of BPAEC with vehicle (0.1% DMSO) or GHRHAnt (1 μM) for 8 h and post-treatment with vehicle (PBS), or H_2O_2 (0.1 mM) (8 h). The blots shown are representative of three independent experiments. The signal intensity of the bands was analyzed by densitometry. Protein levels of P53 and pCofilin were normalized to β-actin and cofilin, respectively. $^{*}P < 0.05$ vs. vehicle (VEH) and $^{\$}P < 0.05$ vs. H_2O_2. Means ± SEM. **E** Confluent monolayers of BPAEC were treated with vehicle (0.1% DMSO) or GHRHAnt (1 μM), and post-treated with vehicle (PBS) or H_2O_2 (0.1 mM). A gradual decrease in endothelial permeability (increased TEER) was observed in GHRHAnt-treated cells. H_2O_2 exposure decreased TEER values (increased permeability). GHRHAnt prevented the H_2O_2-induced endothelial barrier hyperpermeability. $n = 3$ per group. Means ± S.E. The black arrow indicates the addition of H_2O_2 in the confluent monolayers

vehicle (0.1% DMSO), or GHRHAnt (1 μM), or H_2O_2 (0.1 mM), or GHRH (1 μM) for 8 h. GHRHAnt significantly upregulated cofilin phosphorylation. In contrast, H_2O_2 and GHRH opposed those effects (Fig. 1B).

GHRHAnt counteract H_2O_2-induced suppression of P53 in BPAEC

BPAEC were treated with vehicle (0.1% DMSO), or GHRHAnt (1 μM) for 8 h and were consequently exposed to vehicle (PBS), or H_2O_2 (0.1 mM) (8 h). Our observations reveal that H_2O_2 suppressed P53 expression, and the expression levels of that protein were significantly increased in those groups pretreated with GHRHAnt (Fig. 1C).

Cofilin activation by H_2O_2 is inhibited due to GHRHAnt treatment

BPAEC were treated with vehicle (0.1% DMSO), or GHRHAnt (1 μM) for 8 h, before vehicle (PBS), or H_2O_2 (0.1 mM) exposure (8 h). Our results indicate that H_2O_2 potentiates the activation of cofilin in BPAEC. The GHRHAnt-pretreated cells showed significant protection against H_2O_2-induced cofilin activation (Fig. 1D).

GHRHAnt protect against H_2O_2-induced endothelial hyperpermeability in BPAEC

To investigate the effects GHRHAnt against H_2O_2-induced lung endothelial barrier disruption, BPAEC were seeded on gold-plated ECIS arrays and were left to reach steady transendothelial resistance values. Then, those cells were treated with vehicle (0.1% DMSO) or GHRHAnt (1 μM) prior to treatment with vehicle (PBS), or H_2O_2 (0.1 mM). Our observations suggest that treatment with H_2O_2 decreased TEER values (red line) (increased permeability), and GHRHAnt increased TEER values (green line) (decreased permeability). GHRHAnt pre-treatment prevented H_2O_2-triggered endothelial hyperpermeability (blue line) (Fig. 1E).

Discussion

P53 is a tumor suppressor protein which controls a plethora of biological processes; such as cell cycle arrest, DNA repair, senescence, and apoptosis to restrict abnormal cell growth [12]. The protective role of P53 is not limited to cancers. Indeed, this transcription factor also exerts anti-inflammatory activities in the vasculature, which in turn results to enhanced endothelial barrier function [30]. Nuclear factor-kappa B (NF-κB) and P53 are reciprocally connected [31]. In the vascular endothelium, NF-κB regulates the production of proinflammatory cytokines, chemotactic factors, and adhesion molecules; hence it promotes monocyte recruitment and disease progression [32]. On the other hand, P53 suppresses inflammation by inhibiting NF-κB [33] and has been reported to be involved in the glucocorticoids-mediated function by abrogating NF-κB [34].

Moreover, P53 protects the endothelium against inflammation by suppressing apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref1) [35], RhoA [36], and lipid peroxidation [37, 38]. Mice deficient in P53 were more susceptible to LPS than the wild type counteracts; in a model of lipopolysaccharide (LPS)-induced acute lung injury (ALI) [39]. It was also established that GHRHAnt induce the expression of P53 in endothelial cells to enhance barrier function [14, 40]. The mechanisms of P53 regulation by GHRHAnt have also been investigated; and unfolded protein response (UPR) has been suggested to act as a possible mediator [14, 41].

Accumulation of misfolded or unfolded proteins in the endoplasmic reticulum (ER) beyond a critical threshold levels activates UPR. This is a highly conserved molecular mechanism, comprised of three stress sensors: inositol requiring enzyme 1α (IRE1α), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6). UPR is involved in the regulation of endothelial integrity [42], since the UPR suppressor Kifunensine (KIF) induces endothelial hyperpermeability [43]. Recent evidence suggests that ATF6 protects against inflammation. Induction of ATF6 results in the suppression of paracellular and transcellular permeability due to LPS; whereas ATF6 downregulation potentiates LPS-induced endothelial hyperpermeability [44]. GHRHAnt potentiate UPR activation in endothelial cells and counteract KIF-induced decrease in transendothelial resistance [41], while GHRH agonist MR-409 exerts the opposite effects [41]. UPR positively regulates the expression of P53 in lung endothelium [45]. Hence, GHRHAnt utilize UPR and P53 to enhance the barrier functions of microvasculature [46–48].

Cofilin is an actin-binding protein. Upon activation, it depolymerizes filamentous actin (F-actin), and generates free filaments accessible to globular actin (G-actin). These events result to dynamic alterations in actin cytoskeleton necessary for activation of NF-κB and intercellular adhesion molecule-1 (ICAM-1) expression [49]. Moreover, cofilin reduces tight junction proteins and increases endothelial permeability [50]. P53 impairs the actin severing activity of cofilin by inducing the Rac1/pCofilin axis [51]. The present study provides evidence that GHRHAnt significantly suppress the activation of cofilin due to H_2O_2 treatment (Fig. 1D).

Adherens junctions are comprised of VE-cadherin complexes with catenin, and are dominant in most vascular beds [18]. AJs integrity is crucial for paracellular permeability. Breakdown of VE-cadherin adhesions destabilizes AJs and promotes different pathological processes (e.g. atherogenesis, inflammation, ALI) [52]. Phosphorylation of VE-cadherin provides the mechanism for AJs remodeling [19]. VE-cadherin phosphorylation at tyrosine residues modulates endothelial junctions during inflammation [53]. The receptor-type vascular endothelial protein tyrosine phosphatase (VE-PTP) interacts with VE-cadherin and decreases its phosphorylation [54]. VE-PTP null mice exhibit defects in angiogenesis which causes embryonic death, demonstrating a crucial role of VE-PTP in the maintenance and remodeling of vasculature [55].

In summary, our study substantiates previous observations on the anti-oxidative activity of GHRHAnt in human cells and supports that those peptides are promising therapeutic candidates for disorders related to increased oxidative stress and inflammation.

Author contributions M.S.A.: Investigation, data analysis and interpretation, draft preparation, K.-T.K.: Draft preparation, N.B.: Edited the manuscript, provided funds, and conceived the project.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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