HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2022 December 10.

Published in final edited form as:

Biochem Biophys Res Commun. 2021 December 10; 582: 105–110. doi:10.1016/j.bbrc.2021.10.032.

Adropin decreases endothelial monolayer permeability after cell-free hemoglobin exposure and reduces MCP-1-induced macrophage transmigration

William S. Dodd[a], **Devan Patel**[b], **Brandon Lucke-Wold**[a], **Koji Hosaka**[a], **Nohra Chalouhi**[a], **Brian L. Hoh**[a,*]

[a]Department of Neurosurgery, College of Medicine, University of Florida, Gainesville, FL, USA

[b]College of Medicine, Florida State University, Tallahassee, FL, USA

Abstract

Background: Cell-free heme-containing proteins mediate endothelial injury in a variety of disease states including subarachnoid hemorrhage and sepsis by increasing endothelial permeability. Inflammatory cells are also attracted to sites of vascular injury by monocyte chemotactic protein 1 (MCP-1) and other chemokines. We have identified a novel peptide hormone, adropin, that protects against hemoglobin-induced endothelial permeability and MCP-1-induced macrophage migration.

Methods: Human microvascular endothelial cells were exposed to cell-free hemoglobin (CFH) with and without adropin treatment before measuring monolayer permeability using a FITC-dextran tracer assay. mRNA and culture media were collected for molecular studies. We also assessed the effect of adropin on macrophage movement across the endothelial monolayer using an MCP-1-induced migration assay.

Results: CFH exposure decreases adropin expression and increases paracellular permeability of human endothelial cells. Treating cells with synthetic adropin protects against the increased permeability observed during the natural injury progression. Cell viability was similar in all groups and *Hmox1* expression was not affected by adropin treatment. MCP-1 potently induced macrophage migration across the endothelial monolayer and adropin treatment effectively reduced this phenomenon.

Conclusions: Endothelial injury is a hallmark of many disease states. Our results suggest that adropin treatment could be a valuable strategy in preventing heme-mediated endothelial injury

*Corresponding author. 1149 Newell Drive, L2-100, Gainesville, FL, 32610, USA. brian.hoh@neurosurgery.ufl.edu (B.L. Hoh).

Author contributions

WSD conceived and designed experiments, collected data, performed data analysis, interpreted results, and drafted the manuscript. DP collected data, performed data analysis, and revised the manuscript. KH conceived and designed experiments, performed data analysis, interpreted results, and revised the manuscript. BL, NC & BLH conceived and designed experiments, interpreted results, and revised the manuscript. All authors provided critical feedback, helped shape the research, and approved of the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Brian L. Hoh reports financial support was provided by National Institutes of Health. William S. Dodd & Brian L. Hoh reports financial support was provided by The Brain Aneurysm Foundation.

and macrophage infiltration. Further investigation of adropin therapy in animal models and human tissue specimens is needed.

Keywords

Hemoglobin; Endothelium; Adropin; Hemorrhage; Sepsis

1. Introduction

Heme is a cytotoxic molecule due to its ready participation in reactive oxygen species (ROS)-generating reactions. Under homeostatic conditions, heme is contained within hemoglobin, myoglobin, and other hemoproteins; however, certain disease states are characterized by the pathologic exposure of cell-free hemoglobin (CFH) to healthy tissues. Sepsis, which is responsible for millions of deaths annually, and subarachnoid hemorrhage (SAH), which portends exceptionally high morbidity and mortality [1], are two such diseases in which heme is central to the disease pathophysiology [2-4]. In septic states, free hemoglobin is produced through the hemolytic properties of the infecting microbes as well as through membrane disruption that occurs as a result of disseminated intravascular coagulation or capillary thrombosis [2,5,6]. Mouse models with reduced heme-catabolizing capacity (*Hmox1*$^{-/-}$ mice) have much greater mortality than wild-type mice suggesting free hemoglobin is crucial to sepsis pathology [6]. After SAH, the extravasated RBCs lyse and CFH begins to permeate throughout the subarachnoid space. Haptoglobin proteins function to aid in heme clearance by binding free heme molecules and an individual's haptoglobin genotype can predict complications after SAH [7], indicating that pathologic heme exposure is a major mediator of SAH pathology. CFH-induced permeability of cerebral microvasculature is also responsible for the development of vasogenic edema, an independent risk factor for clinical outcome [8]. Disruption of the endothelial monolayer, both in sepsis and SAH, also contributes to immune cell infiltration. Immune cells, especially monocytes/macrophages, respond to heme binding CD163 as well as the pro-inflammatory cytokines produced by damaged vasculature [9-12]. Expression of monocyte chemotactic protein 1 (MCP-1) is induced during sepsis and SAH [13,14], increasing the transmigration of pro-inflammatory monocytes/macrophages through the affected endothelium. Investigation into strategies for minimizing the toxic effects of CFH will improve the outcomes for patients with sepsis, SAH, and a range of other heme-mediated disease states.

Adropin is a conserved peptide hormone highly expressed in the brain and liver [15,16]. While the full range of its function and regulation are still being intensely investigated, there is compelling evidence to suggest adropin acts directly on endothelial cells to confer cytoprotective and vasculoprotective effects in part through stimulating nitric oxide (NO) production [17]. Evaluation of adropin and the adropin signaling pathway as a potential therapeutic has accelerated since these landmark studies. The goal of ongoing studies is to demonstrate the molecular and cellular mechanisms underlying adropin's function. Yang et al. found that adropin protects rat brain endothelial cells exposed to simulated ischemia [18]. In a mouse model of intracerebral hemorrhage, adropin treatment preserved the blood-brain barrier and improved outcome scores [19]. Further, Sato et al. found that adropin treatment

caused reduced monocyte/macrophage attachment to an endothelial monolayer [20]. These studies support the premise that adropin promotes endothelial homeostasis; however, there remains a gap in knowledge pertaining to the effect of adropin on endothelial cells after cell-free hemoglobin exposure. We hypothesize that adropin decreases endothelial permeability in response to CFH exposure and prevents macrophage transmigration in response to inflammatory stimuli.

2. Materials and methods

2.1. Cell culture methods

Cultured cells were grown in 10 cm culture dishes (Corning, Corning, NY) in a humidified, 5% CO_2 incubator (New Brunswick Scientific, Edison, NJ) at 37 °C. Cells were passaged at 80% confluence and only passages 3-5 were used for experiments. Human coronary artery endothelial cells (HCAECs) were obtained from Lonza Bioscience (Cat. #: CC-2585, Basel, Switzerland) and J774A macrophage cells were obtained from ATCC (Cat. #: TIB-67, Manassas, VA). Endothelial cell growth media (Cat. #: LL-003, Lifeline Cell Technology, Carlsbad, CA) was supplemented with 5 ng/mL rhFGF, 50 μg/mL ascorbic acid, 1 mg/mL hydrocortisone hemisuccinate, 10 mM L-glutamine, 15 ng/mL rhIGF-1, 5 ng/mL rhEGF, 0.75 U/mL heparin sulfate, and 5% FBS (all from Lifeline Cell Technology). The macrophage growth media consisted of Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (Cat. #: 30-2002 & 30-2020, ATCC); media was replaced every 48 h unless otherwise noted.

Cell-free hemoglobin (Cat. #: P5228, Abnova, Taipei, Taiwan) was diluted to 10 mg/mL in complete HCAEC growth media to make the stock solution. CFH stock was added to cultured cells to a final concentration of 1 mg/mL for all experiments. Synthetic adropin$^{34\text{-}76}$ peptide was manufactured Bachem (Bubendorf, Switzerland) and diluted in 0.1% BSA in normal saline as a stock solution. Adropin was added to cultured cells to a final concentration of 100 ng/mL for all experiments.

2.2. mRNA collection and qPCR

mRNA was harvested from endothelial cells after 18 h of CFH exposure using a binding column-based kit following manufacturer recommended protocol (Cat. #: 74104, Qiagen, Hilden, Germany) and reverse transcription was performed using kit obtained from New England Biolabs (Cat. #: E6560, Ipswich, MA). cDNA was amplified using a master mix kit (Cat. #: 1725271, BioRad, Hercules, CA) and the primers specified in Table 1. Relative cDNA abundance was calculated using the ΔΔCT method.

2.3. Adropin peptide ELISA

Immediately before mRNA harvesting, 1 mL of cell culture media was removed and stored at −20 °C. Adropin peptide concentration in the media was then measured using a commercially-available ELISA kit (Cat. #: NBP2-66433, Novus Biologicals, Centennial, CO) following manufacturer recommended protocols. Briefly, 100 μL of samples, standards, and controls were added to each well of the 96-well plates and incubated at 37 °C for 90 min. Next, wells were emptied and 100 μL of the biotinylated detection antibody was added

to each well and incubated for 1 h at 37 °C. Next, the plate was washed thrice, 100 mL of HRP-conjugate was added to each well, and the plate was incubated again at 37 °C. After 30 min incubation, the plate was washed 5 more times and 90 μL of the HRP substrate reagent was added to each well before the plate was returned to the 37 °C incubator for 15 min. Finally, 50 μL of the stop solution was added to each well and the optical density (OD) at 450 nm was measured using a microplate reader. The standard curve and sample concentrations were determined following manufacturer guidelines.

2.4. FITC-dextran permeability assay

HCAECs were grown in 24-well plates (Corning, Corning, NY) with transwell inserts (Cat. #: 662641, Greiner Bio-One, Monroe, NC) with a pore size of 0.4 μm until 100% confluent. CFH (or vehicle) and adropin (or vehicle) were introduced to both the luminal and abluminal compartments. After 18 h incubation at 37 °C in 5% CO_2 incubator, the luminal and abluminal compartments were washed with pre-warmed Earle's balanced salt solution (EBSS) and filled with 200 μL and 800 μL of EBSS, respectively. FITC-conjugated dextrans of molecular weight 70 kDa (MilliporeSigma, Burlington, MA) were added to a final concentration of 1 mg/mL in the luminal compartment of each transwell insert. After 15 min incubation, the transwell inserts were removed and the concentration of FITC-dextran in the lower (abluminal) compartment was measured with a multimodal microplate reader (BioTek, Winooski, VT) using Ex/Em wavelengths of 485 nm and 528 nm, respectively and then compared to a standard curve of known FITC-dextran concentrations (1—1000 ng/mL). The apparent permeability coefficient (P_{app}) was calculated as previously described [18,21].

2.5. Cell viability assay

Immediately before mRNA harvesting, approximately 10^4 cells were aliquoted into 1.5 mL microcentrifuge tubes and washed with PBS. Cell viability was measured by trypan blue exclusion as determined by a blinded observer using a hemocytometer and phase-contrast microscope.

2.6. Macrophage migration assay

The macrophage migration assay was performed as previous described [22]. Briefly, HCAECs were seeded in 24-well plate with transwell inserts at an initial density of 5×10^4 cells per well and were grown until 100% confluent. Separately, macrophages were cultured until 80% confluent in 10 cm culture dishes. The luminal and abluminal compartments of the transwell inserts were then washed and replaced with serum-free HCAEC media. MCP-1 (or vehicle) was then added to the abluminal compartment only with a final concentration of 10 ng/mL. Macrophages were harvested, washed, and reconstituted in serum-free HCAEC media before being added (2×10^4 cells each well) along with 100 ng/mL adropin (or vehicle). The 24-well plates with transwell inserts were then returned to humidified, 5% CO_2 incubator. After incubation for 48 h at 37 °C, the transwell inserts were removed from the 24-well plates. The luminal (top) side of the insert was thoroughly brushed with a sterile cotton-tipped applicator to remove the remaining macrophages and the entire insert was then submerged in 4% paraformaldehyde (PFA) in PBS.

The fixed inserts were then removed from the structural support frame and placed on a glass microscope slide (Leica Biosystems Inc., Buffalo Grove, IL). The slides were stained with an anti-F4/80 antibody (Cat. #: MCA497, BioRad, Hercules, CA) and Alex-Fluor594-conjugated secondary antibody to visualize migrated macrophages. The slides were counterstained with DAPI (Vector Labs, Burlingame, CA), cover slipped, and imaged using a widefield fluorescence microscope (Olympus America, Center Valley, PA). A blinded observer captured five images at random points on the insert using the 40x objective lens and a second blinded observer counted the number of $F4/80^+$ cells per image.

2.7. Statistical analysis

All values are expressed as mean ± SEM unless otherwise noted. Comparison of means between two groups was performed using an unpaired Student's t-test. Comparison of means between four groups was performed using the non-parametric Kruskal-Wallis with Dunn's post-hoc multiple comparisons test. P values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Hemoglobin exposure decreases adropin expression in cultured endothelial cells

Adropin is normally expressed in the arterial and venous endothelial cells of multiple tissues; however, there is still little known about the effect of disease states on adropin expression. We hypothesized exposure to cell-free hemoglobin would decrease adropin expression in human coronary artery endothelial cells (HCAECs). At the transcriptional level, we found that exposure to 1 mg/mL CFH for 18 h resulted in decreased *ENHO* expression (0.64 ± 0.08 -fold expression compared to vehicle control, $p < 0.01$) (Fig. 1A). We also measured secreted adropin in the cell media and found a consistent decrease in adropin peptide levels (30.43 ± 2.70 pg/mL in vehicle-treated cells vs. 13.50 ± 1.04 pg/mL in CFH-treated cells, $p < 0.01$) (Fig. 1B).

3.2. Adropin reduces hemoglobin-induced endothelial monolayer permeability

Increased endothelial monolayer permeability is a hallmark of hemorrhagic and hemolytic diseases, a phenomenon demonstrated *in vitro* to be mediated by direct effect of CFH on the endothelium. We hypothesized that adropin treatment would reduce the CFH-induced permeability as measured by the passive diffusion of large FITC-conjugated dextrans across the endothelial monolayer. We observed that adropin treatment alone had no effect on endothelial permeability (1.01 ± 0.04 relative permeability coefficient compared to control), whereas 18-h exposure to 1 mg/mL CFH caused an increase in permeability (1.45 ± 0.11 relative permeability coefficient compared to control, $p = 0.02$) (Fig. 2A). Adropin treatment blunted this effect of CFH exposure and prevented a significant increase in permeability (1.17 ± 0.05 relative permeability coefficient compared to control, $p > 0.05$) (Fig. 2A).

To ensure the changes in permeability were not due to cell death, we chose a CFH concentration previously demonstrated to be non-lethal [23] and also conducted a trypan blue exclusion assay to confirm cell viability after CFH exposure. All experimental conditions tested had >98% cell viability and none were significantly different from vehicle

control ($p > 0.99$ for all groups) (Fig. 2B). The other possible confounding variable for our experiment was heme metabolism and detoxification. We wanted to rule out the possibility that adropin was simply interfering with CFH-endothelial cell interactions and blunting the realized CFH exposure effect. We found that adropin treatment had no effect of *HMOX1* expression (15.00 ± 0.63 fold increase in CFH + vehicle group & 13.67 ± 0.17 fold increase in CFH + adropin group, $p > 0.05$) (Fig. 2C), indicating that endothelial cells have similar *HMOX1* response magnitudes after CFH exposure.

3.3. Adropin decreases MCP-1-induced macrophage transendothelial migration

The deleterious consequences of increased endothelial permeability are not limited to fluid/ion balance or protein leakage; immune cell extravasation also contributes to pathologic inflammation after hemoglobin exposure. We sought to determine if adropin could decrease macrophage trafficking across the endothelial cell monolayer in response to pro-inflammatory stimuli. As described in Fig. 3A, we introduced macrophage cells into the luminal compartment of a transwell culture dish containing confluent endothelial cells and simultaneously added MCP-1 to a final concentration of 10 ng/mL in the abluminal compartment. Macrophage cells readily migrated across the endothelial monolayer in response to MCP-1 (39.67 ± 5.23 $F4/80^+$ cells/HPF in the MCP-1 + vehicle group vs. 0.40 ± 0.25 $F4/80^+$ cells in the vehicle + vehicle group, $p < 0.01$). Adropin treatment decreased the number of migratory macrophage cells (5.33 ± 2.80 $F4/80^+$ cells/HPF, $p > 0.05$ vs vehicle + vehicle group) (Fig. 3B and C).

4. Discussion

The release of free hemoglobin induces endothelial injury, which then potentiates further tissue damage through increased permeability and eventually immune cell infiltration. The aim of this study was to define the potential of adropin treatment to decrease endothelial permeability and immune cell infiltration after simulated injury. First, we found adropin expression is greatly decreased in endothelial adropin expression after free hemoglobin exposure *in vitro* (Fig. 1). We observed this decrease to be present at both the transcriptional (Fig. 1A) and protein (Fig. 1B) levels. These findings suggest hemoglobin exposure alone is sufficient to decrease adropin expression in endothelial cells.

Next, we observed treatment with synthetic adropin prevents increased endothelial permeability after CFH exposure (Fig. 2A). Our data are consistent with previous studies demonstrating adropin promotes endothelial homeostasis in response to a wide variety of stressors and injury mechanisms [17-20]. Importantly, we have also demonstrated that cell viability and *HMOX1* expression are unaffected by CFH and/or adropin exposure (Fig. 2B&C). These data suggest the permeability changes observed are not due to cell death or rapid detoxification of CFH. We speculate the responsible mechanism could be modulation of cytoskeleton dynamics, as was demonstrated by Yang et al. after simulated hypoxia [18]. Future *in vivo* investigation of vascular permeability could also determine the effect of endothelial dysfunction on vascular smooth muscle cells and other cell types.

After hemoglobin-induced endothelial injury, circulating immune cells migrate through the endothelial monolayer into the local tissue. Monocyte chemoattractant protein (MCP)-1 is

upregulated in a variety of diseases, including heme-mediated diseases such as SAH and sepsis, and acts as a chemotactic cytokine for monocytes/macrophages, making it an ideal candidate to simulate immune cell migration induced by vascular injury. We found that adropin reduces monocyte/macrophage transmigration across endothelial cells in response to MCP-1 (Fig. 3). These data are consistent with recent literature suggesting adropin inhibits TNFα-induced ICAM-1 & VCAM-1 expression in human umbilical vein endothelial cells (HUVECs) [20]; however, we cannot rule out a direct effect of adropin on monocyte/macrophage cells that modulated their migratory activity.

Heme-mediated diseases induce permeability changes in affected vasculature and cause infiltration of immune cells. We have shown adropin can reduce these pathologic mechanisms *in vitro* and is a potentially valuable therapeutic to combat hemeinduced vascular injury. An *in vivo* study of SAH and/or sepsis models in addition to analysis of human patient tissue samples would be extremely valuable in validating the potential of adropin as a therapeutic agent. Additionally, our study was conducted using human coronary artery endothelial cells (HCAECs) due to their utility as a microvascular cell model. It remains possible that other endothelial subpopulations could be differentially affected by adropin treatment, yet current literature with brain *in vivo* injury models supports shared mechanisms. In summary, we have demonstrated adropin reduces CFH-induced endothelial permeability and MCP-1-induced macrophage transmigration. We also call for more investigation in adropin as a therapeutic for heme-mediated diseases.

Sources of funding

This work was supported with funding from the following grants and organizations: National Institutes of Health (R01-NS110710) and the Brain Aneurysm Foundation.

References

[1]. Hop JW, Rinkel GJE, Algra A, Van Gijn J, Case-fatality rates and functional outcome after subarachnoid hemorrhage: a systematic review, Stroke 28 (3) (1997) 660–664, 10.1161/01.STR.28.3.660. [PubMed: 9056628]

[2]. Effenberger-Neidnicht K, Hartmann M, Mechanisms of hemolysis during sepsis, Inflammation 41 (5) (Oct. 2018) 1569–1581, 10.1007/S10753-018-0810-Y. [PubMed: 29956069]

[3]. Meegan JE, et al. , Cell-free hemoglobin increases inflammation, lung apoptosis, and microvascular permeability in murine polymicrobial sepsis, PLoS One 15 (2) (Feb. 2020), 10.1371/journal.pone.0228727.

[4]. Dodd WS, et al. , Pathophysiology of delayed cerebral ischemia after subarachnoid hemorrhage: a review, J. Am. Heart Assoc 10 (15) (Aug. 2021) 21845, 10.1161/JAHA.121.021845.

[5]. Bateman RM, Sharpe MD, Jagger JE, Ellis CG, Sepsis impairs microvascular autoregulation and delays capillary response within hypoxic capillaries, Crit. Care 19 (1) (Nov. 2015), 10.1186/S13054-015-1102-7.

[6]. Larsen R, et al. , A central role for free heme in the pathogenesis of severe sepsis, Sci. Transl. Med 2 (51) (Sep. 2010), 10.1126/SCITRANSLMED.3001118.

[7]. Blackburn SL, et al. , Unique contribution of haptoglobin and haptoglobin genotype in aneurysmal subarachnoid hemorrhage, Front. Physiol 9 (May 2018), 10.3389/fphys.2018.00592.

[8]. Claassen J, Carhuapoma JR, Kreiter KT, Du EY, Connolly ES, Mayer SA, Global cerebral edema after subarachnoid hemorrhage: frequency, predictors, and impact on outcome, Stroke 33 (5) (May 2002) 1225–1232. Accessed: Jun. 03, 2019. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/11988595. [PubMed: 11988595]

[9]. Atangana E, et al. , Intravascular inflammation triggers intracerebral activated microglia and contributes to secondary brain injury after experimental subarachnoid hemorrhage (eSAH), Transl. Stroke Res 8 (2) (Apr. 2017) 144–156, 10.1007/s12975-016-0485-3. [PubMed: 27477569]

[10]. Provencio JJ, Altay T, Smithason S, Moore SK, Ransohoff RM, Depletion of Ly6G/C+ cells ameliorates delayed cerebral vasospasm in subarachnoid hemorrhage, J. Neuroimmunol 232 (1–2) (Mar. 2011) 94–100, 10.1016/j.jneuroim.2010.10.016. [PubMed: 21059474]

[11]. Gris T, et al. , Innate immunity activation in the early brain injury period following subarachnoid hemorrhage, J. Neuroinflammation 16 (1) (Dec. 2019), 10.1186/s12974-019-1629-7.

[12]. Kubota T, Handa Y, Tsuchida A, Kaneko M, Kobayashi H, Kubota T, The kinetics of lymphocyte subsets and macrophages in subarachnoid space after subarachnoid hemorrhage in rats, Stroke 24 (12) (1993) 1993–2000, 10.1161/01.STR.24.12.1993. [PubMed: 8248982]

[13]. Niwa A, Osuka K, Nakura T, Matsuo N, Watabe T, Takayasu M, Interleukin-6, MCP-1, IP-10, and MIG are sequentially expressed in cerebrospinal fluid after subarachnoid hemorrhage, J. Neuroinflammation 13 (1) (Aug. 2016), 10.1186/S12974-016-0675-7.

[14]. Chao J, et al. , Detection of early cytokine storm in patients with septic shock after abdominal surgery, J. Transl. Intern. Med 8 (2) (Jun. 2020) 91–98, 10.2478/JTIM-2020-0014.

[15]. Kumar KG, et al. , Identification of adropin as a secreted factor linking dietary macronutrient intake with energy homeostasis and lipid metabolism, Cell Metab. 8 (6) (Dec. 2008) 468–481, 10.1016/j.cmet.2008.10.011. [PubMed: 19041763]

[16]. Yang C, DeMars KM, Candelario-Jalil E, Age-dependent decrease in adropin is associated with reduced levels of endothelial nitric oxide synthase and increased oxidative stress in the rat brain, Aging Dis. 9 (2) (Apr. 2018) 322, 10.14336/AD.2017.0523. [PubMed: 29896421]

[17]. Lovren F, et al. , Adropin is a novel regulator of endothelial function, Circulation 122 (11_suppl_1) (Sep. 2010) S185–S192, 10.1161/CIRCULATIONAHA.109.931782. [PubMed: 20837912]

[18]. Yang C, DeMars KM, Hawkins KE, Candelario-Jalil E, Adropin reduces paracellular permeability of rat brain endothelial cells exposed to ischemia-like conditions, Peptides 81 (Jul. 2016) 29–37, 10.1016/J.PEPTIDES.2016.03.009. [PubMed: 27020249]

[19]. Yu L, et al. , Adropin preserves the blood-brain barrier through a Notch1/Hes1 pathway after intracerebral hemorrhage in mice, J. Neurochem 143 (6) (Dec. 2017) 750–760, 10.1111/jnc.14238. [PubMed: 29030969]

[20]. Sato K, et al. , Adropin contributes to anti-atherosclerosis by suppressing monocyte-endothelial cell adhesion and smooth muscle cell proliferation, Int. J. Mol. Sci 19 (5) (Apr. 2018) 1293, 10.3390/ijms19051293.

[21]. Liu J, Jin X, Liu KJ, Liu W, Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage, J. Neurosci 32 (9) (Feb. 2012) 3044–3057, 10.1523/JNEUROSCI.6409-11.2012. [PubMed: 22378877]

[22]. Hoh BL, et al. , Estrogen deficiency promotes cerebral Aneurysm rupture by upregulation of Th17 cells and interleukin-17A which downregulates E-Cadherin, J. Am. Heart Assoc 7 (8) (Apr. 2018), e008863, 10.1161/JAHA.118.008863. [PubMed: 29654199]

[23]. Kuck JL, et al. , Ascorbic acid attenuates endothelial permeability triggered by cell-free hemoglobin, Biochem. Biophys. Res. Commun 495 (1) (Jan. 2018) 433–437, 10.1016/j.bbrc.2017.11.058. [PubMed: 29129689]

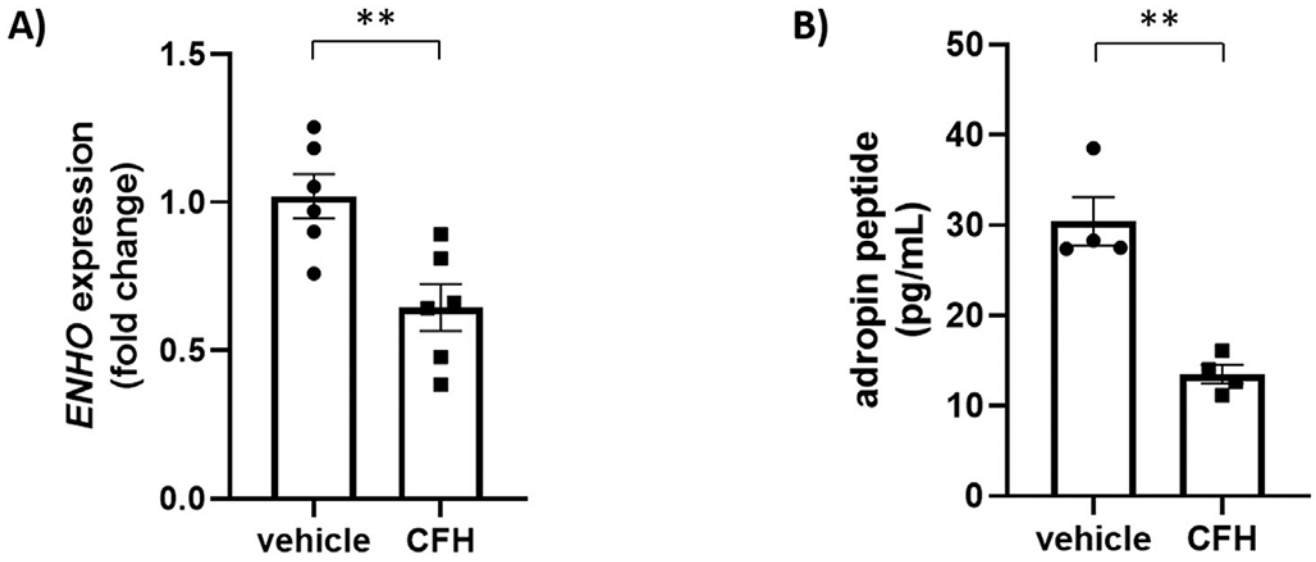

Fig. 1. Hemoglobin exposure decreases adropin expression in cultured endothelial cells.

A) Transcriptional expression of the adropin-encoding gene, *ENHO*, in HCAECs exposed to cell-free hemoglobin 18 h, ** indicates $p < 0.01$ by unpaired Student's *t*-test. **B)** Adropin peptide concentration in cell culture media after 18 h of cell-free hemoglobin exposure, ** indicates $p < 0.01$.

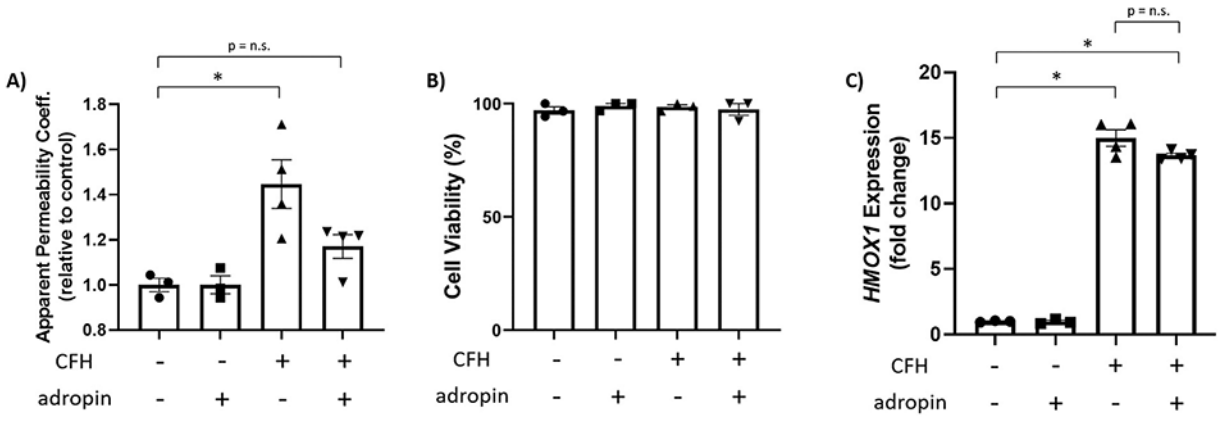

Fig. 2. Adropin reduces hemoglobin-induced endothelial monolayer permeability.

A) HCAEC monolayer permeability to 70 kDa FITC-labeled dextrans after 18 h of CFH exposure. Data presented as relative permeability compared to control, * indicates $p < 0.05$. **B)** Cell viability after CFH exposure as measured by trypan blue exclusion, $p > 0.99$ between all groups. **C)** Heme oxygenase 1 *(HMOX1)* gene expression after CFH exposure, * indicates $p < 0.05$.

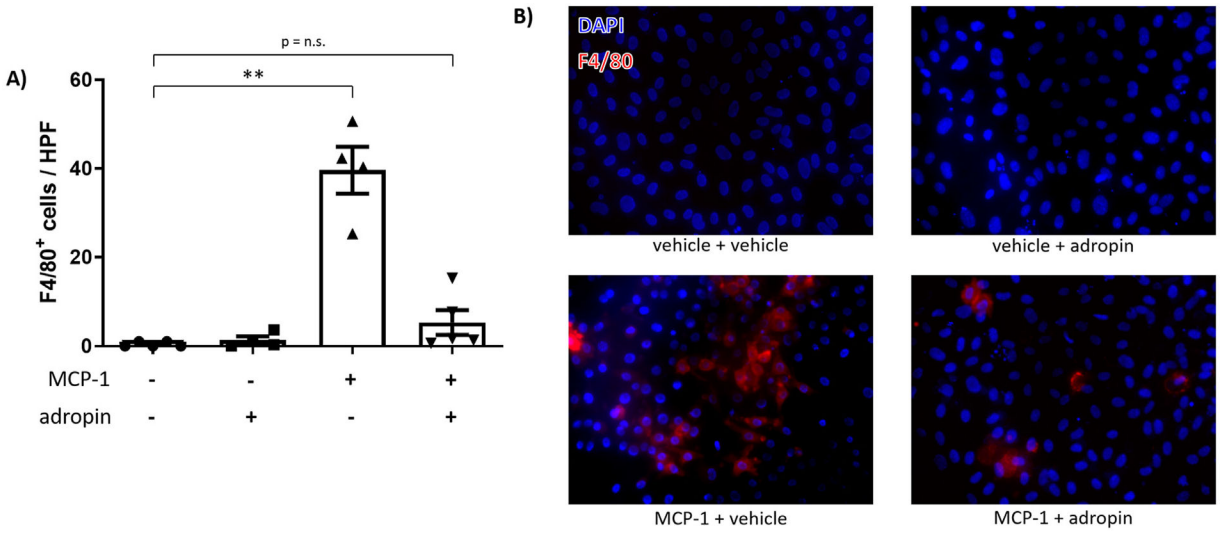

Fig. 3. Adropin decreases MCP-1-induced macrophage transendothelial migration.

A) Quantitative image analysis of F4/80$^+$ cells which migrated across HCAEC monolayer, ** indicates $p < 0.01$. **B)** Representative images of the abluminal surface of the well inserts using 40x objective lens. F4/80$^+$ cells are shown in red with DAPI nuclear counterstain (blue).

Table 1

Primers used for quantitative PCR.

Primer	Forward primer sequence	Reverse primer sequence
GAPDH	5′ – GACAGTCAGCCGCATCTTCT – 3′	5′ – TTAAAAGCAGCCCTGGTGAC – 3′
ENHO	5′ – GGGTGGGGGCTTATGAGTTG – 3′	5′ – CTAGGGAAAGAGTGGACCCG – 3′
HMOX1	5′ – CTCAAACCTCCAAAAGCC – 3′	5′ – TCAAAAACCACCCCAACCC – 3′