# Elevated hydrostatic pressure increases the sensitivity of optic nerve head astrocytes to an oxidative challenge

Vidhya R. Rao,[1,2,3] Alexandra D. Hegel,[1,2,3] Jonathan Lautz,[3,4] Jamie C. Floss,[2] Vicki Husak,[3] Evan B. Stubbs Jr.,[1,3] Simon Kaja[1,2,3]

[1] Department of Ophthalmology, [2] Department of Molecular Pharmacology &Therapeutics, [4] Graduate Program in Neuroscience, Loyola University Chicago, Maywood, IL, U.S.A.
[3] Research Service, Edward Hines Jr. Veterans Administration Hospital, Hines, IL, U.S.A.

*Corresponding author: Simon Kaja, Ph.D. (skaja@luc.edu)*

Visual Neurobiology and Signal Transduction Laboratory
Loyola University Chicago

## Abstract

Primary open angle glaucoma (POAG) is often associated with elevated intraocular pressure (IOP), manifesting in a pathological triad of optic nerve head remodeling, damage to the optic nerve, and retinal ganglion cell loss.

Optic nerve head astrocytes (ONHAs), the primary cell type in the optic nerve head, undergo significant pathological changes in POAG.

Increased levels of oxidative stress, secondary to elevated IOP, are strongly implicated in the pathophysiology of POAG.

Here, the cellular and molecular consequences of elevated hydrostatic pressure on cultured ONHAs responses to an exogenous oxidative challenge were investigated.

## Materials and Methods

**Cell Culture:** Primary adult rat optic nerve head astrocyte (ONHA) culture was prepared and maintained as described by us previously (Kaja et al., Exp. Neurol. 2015, 265: 59-680 and Kaja et al., Exp. Eye Res. 2015; 138: 159-166).

**Exposure to elevated hydrostatic pressure:** ONHA culture was exposed to control ambient pressure (AP) or elevated hydrostatic pressure (EHP; 25-30 mm Hg above ambient pressure) using a custom-built cell culture pressure chamber.

**Exposure to oxidative stress:** ONHAs were subsequently challenged with chemically-induced oxidative stress using *tert*-butylhydroperoxide (tBHP; 0-500 µM for 5h). For proof-of-concept experiments, some ONHAs cultures were pre-treated with the prototypic antioxidant Trolox (100 µM) dissolved in ethanol.

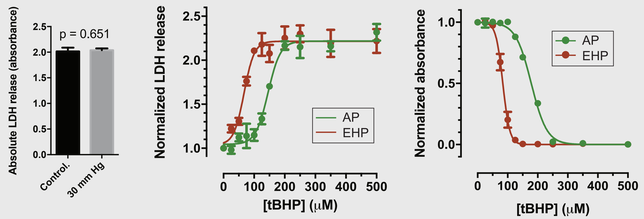
**Cell viability assays and detection of Reactive Oxygen Species (ROS):** Cell viability was measured using MTT and LDH assays; levels of oxidative stress were quantified using the fluorescent indicator dye, CellROX®, or by using the cell-permeable 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), which is converted to the fluorescent 2',7'-dichlorofluorescein (DCF) by endogenous esterases and oxidation (Kaja et al., Exp. Eye Res. 2015; 138: 159-166).

**Quantitative PCR and immunoblotting:** qPCR was performed using standard Taqman® chemistry in an AriaMX Realtime PCR System machine (Agilent Genomics). Immunoblotting for NOS2 was performed using a validated rabbit polyclonal anti-NOS2 antibody (sc-650; 1:500 dilution) using standard protocols and Luminata Forte HRP substrate (Millipore).

**Data analysis:** Data was graphed and analzed in Prism 6.0 (GraphPad Software, Inc.) and is presented as mean ± s.e.m. or S.D., as indicated.

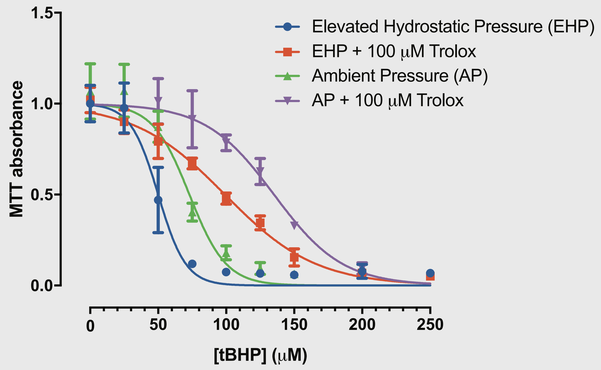
## Results

### 1. Exposure to elevated hydrostatic pressure sensitizes primary ONHAs to subsequent oxidative insults




*Exposure to elevated hydrostatic pressure (30 mm Hg for 16 hr) did not alter cell viability. Absolute LDH release was unaltered (n = 6; P = 0.651). However, subsequent exposure to chemically-induced oxidative stress revealed significantly increased LDH release and reduced conversion of MTT, indicative of reduced cell viability.*

### 2. The prototypic antioxidant, Trolox, can prevent hydrostatic pressure-induced sensitization to subsequent oxidative stress insult

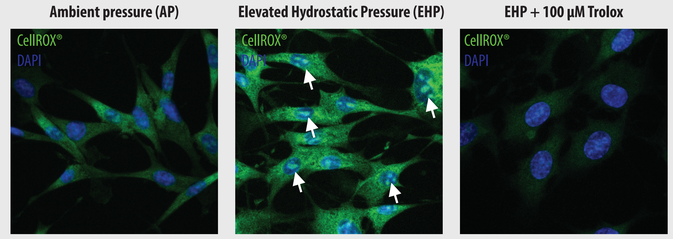



*In a subsequent series of experiments, we exposed cells to the prototypic antioxidant, Trolox (100 µM in 100% ethanol), or vehicle (100% ethanol), for 1 hr prior to and during the exposure to oxidative stress. Trolox shifted the $EC_{50}$ equally in ambient and elevated hydrostatic pressure-treated groups (see Table 1).*

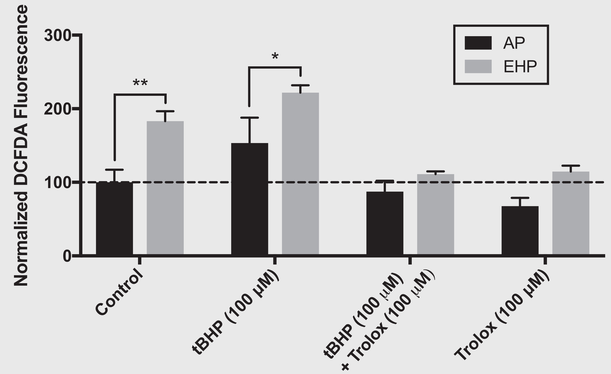
| | *EHP + Vehicle* | *EHP + Trolox* | *AP + Vehicle* | *AP + Trolox* |
|---|---|---|---|---|
| *$EC_{50}$* | *50.0 ± 1.9* | *99.1 ± 2.2* | *73.0 ± 2.4* | *135.2 ± 2.6* |
| *$R^2$* | 0.946 | 0.980 | 0.954 | 0.975 |
| *n (plates)* | *3* | *3* | *3* | *3* |

**Table 1:** *Parameters from non-linear fit of MTT assays. Data is presented as mean ± s.e.m. and was generated from three separate experiments (n=3), with eight technical replicates each.*

### 3. Exposure to hydrostatic pressure generates elevated levels of oxidative stress in ONHAs

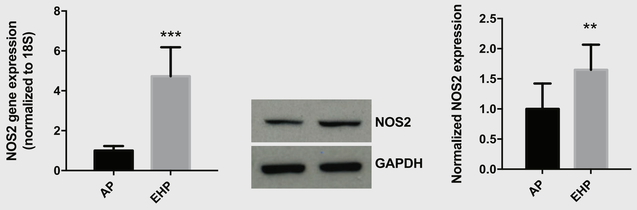



*CellROX® staining showed strong nuclear fluorescence (white arrows), indicative of elevated ROS levels, after hydrostatic pressure challenge for 16 hrs. Trolox (100 µM) completely prevented generation of hydrostatic pressure-induced ROS.*




*In a separate experiment, we exposed ONHAs to elevted hydrostatic pressure for 2 hours in cells pre-treated with sublethal conditions of tBHP (100 µM) and/or Trolox (100 µM) and quantified ROS using DCFDA fluorescence. Elevated hydrostatic pressure challenge significantly increased ROS to levels comparable of those of sublethal tBHP exposure. Trolox completely prevented generation of hydrostatic pressure-and tBHP-induced ROS. Data is mean ± S.D.*
** P < 0.05, ** P < 0.01.*

### 4. Hydrostatic pressure increases nitric oxide synthase 2 expression in ONHAs




*Exposure of ONHAs to elevated hydrostatic pressure caused an approximately 5-fold up-regulation of NOS2 gene expression and a 2-fold increase of NOS2 protein.*
**** P < 0.001, ** P < 0.01.*

## Summary

- Short-term exposure of cultured ONHA to elevated hydrostatic pressure does not cause cell viability, but sensitizes to subsequent oxidative challenge.
- Elevated hydrostatic pressure leads to a statistically significant increase in Reactive Oxygen Species, as determined by quantificaiton of CellROX® and DCFDA fluoresnce.
- Elevated hydrostatic pressure increases NOS2 expression, as described previously.
- Trolox protects ONHAs against elevated hydrostatic pressure- and tBHP-induced ROS.

## Conclusions

- Our data suggest that even modest exposure to elevated IOP in POAG may significantly alter the oxidation response of ONHAs.
- Our experimental system provides a standardized *in vitro* model to study the intracellular pathways leading to the generation of hydrostatic pressure-induced increases in ROS and NO.
- Our *in vitro* model is amenable to high-throughput screening approaches for the testing of novel glioprotectants for POAG.

## References

- Kaja S, Payne AJ, Patel KR, Naumchuk N, Koulen P. Differential subcellular $Ca^{2+}$ signaling in a highly specialized subpopulation of astrocytes. Experimental Neurology 2015, 265: 59-68
- Kaja S, Payne AJ, Naumchuk Y, Levy D, Zaidi DH, Altman AM, Nawazish S, Ghuman JK, Gerdes BC, Moore MA, Koulen P. Plate reader-based cell viability assays for glioprotection using primary rat optic nerve head astrocytes. Experimental Eye Research 2015; 138: 159-166.

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## Disclosures

Disclosures: VRR - none, ADH - none, JL - none, JCF - none, VH - none, EBS - none, SK - K&P Scientific LLC (I), Experimentica Ltd. (I, C, R, S).