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Real-time measurements of nicotinamide adenine dinucleotide in live human trabecular meshwork cells: Effects of acute oxidative stress[☆]

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Abstract

The trabecular meshwork (TM) region of the eye is exposed to a constant low-level of oxidative insult. The cumulative damage may be the reason behind age-dependent risk for developing primary open angle glaucoma. Chronic and acute effects of hydrogen peroxide (H₂O₂) on TM endothelial cells include changes in viability, protein synthesis, and cellular adhesion. However, little if anything is known about the immediate effect of H₂O₂ on the biochemistry of the TM cells and the initial response to oxidative stress. In this report, we have used two-photon excitation autofluorescence (2PAF) to monitor changes to TM cell nicotinamide adenine dinucleotide (NADPH). 2PAF allows non-destructive, real-time analysis of concentration of intracellular NADPH. Coupled to reduced glutathione, NADPH, is a major component in the anti-oxidant defense of TM cells. Cultured human TM cells were monitored for over 30 min in control and H₂O₂-containing solutions. Peroxide caused both a dose- and time-dependent decrease in NADPH signal. NADPH fluorescence in control and in 4 mM H₂O₂ solutions showed little attenuation of NADPH signal (4% and 9% respectively). TM cell NADPH fluorescence showed a linear decrease with exposure to 20 mM H₂O₂ (–29%) and 100 mM H₂O₂ (37%) after a 30 min exposure. Exposure of TM cells to 500 mM H₂O₂ caused an exponential decrease in NADPH fluorescence to a final attenuation of 46% of starting intensity. Analysis of individual TM cells indicates that cells with higher initial NADPH fluorescence are more refractive to the apparent loss of viability caused by H₂O₂ than weakly fluorescing TM cells. We conclude that 2PAF of intracellular NADPH is a valuable tool for studying TM cell metabolism in response to oxidative insult.

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Keywords

glaucoma; trabecular meshwork; NADP/nicotinamide adenine dinucleotide phosphate; oxidative stress

1. Introduction

The trabecular meshwork (TM) region of the eye contains high levels of the anti-oxidant reduced glutathione (GSH) as well as glucose-6-phosphate dehydrogenase, the enzyme required for generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Kahn et al., 1983; Padgaonkar et al., 1994). Reduced GSH is able to neutralize reactive oxygen species via donation of reducing equivalents, and is itself regenerated through consumption of NADPH. Therefore, the oxidation state of TM cells depends on high levels of both reduced GSH and NADPH, and the large amounts of reduced GSH and NADPH suggest a great ability of TM cells to neutralize peroxides and superoxide radicals. Indeed, several *in situ* studies demonstrate that the ability of TM to neutralize reactive oxygen species may be important for normal aqueous outflow, although this appears to be a very complex problem. For example, exposure to H₂O₂ has no effect on outflow facility in untreated perfused eyes but reduces outflow facility by one-third in eyes depleted of reduced GSH (Kahn et al., 1983). However, in other perfusion studies combining GSH depletion with perfusion of other reducing equivalents demonstrate increased outflow facility (Epstein et al., 1990). These data suggests that there are multiple sites where reduced GSH/NADPH can regulate outflow.

In light of the above findings, real-time imaging of the oxidative state of TM cells may be a valuable tool in studying the pathophysiology of glaucoma. We describe here an approach to detect the fluorescent signal of NADPH in live TM cell cultures using a nondestructive microscopy technique, two-photon microscopy (2PM). The absorbance peak of NADPH lies in the near ultraviolet; however, prolonged excitation at this wavelength would be destructive to living cells. 2PM is based on non-linear optical absorption and fluorescence processes that involve two or more infra-red photons interacting simultaneously with a target molecule. We can therefore achieve NADPH excitation fluorescence using a high-intensity near infra-red laser with extremely short pulse duration (~100 fs), resulting in minimal thermal and photodamage to the TM cells.

2. Materials and methods

2.1. Cell culture

hTM42, a primary TM cell line, was a gift from Dr. Doug Rhee (Massachusetts Eye and Ear Infirmary, Boston MA). TM cells were grown to confluence at 37 °C and 5% CO₂ on gelatin-coated dishes in Dulbecco's modified Eagle's Media (DME; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 5 ng/mL human recombinant basic fibroblast growth factor (Invitrogen) plus penicillin and streptomycin (Invitrogen).

2.2. Solutions

Balanced salt solution (BSS; Alcon Laboratories, Inc., Fort Worth, TX) supplemented with glucose (BSS + glucose) contained the following: 1 g/L glucose ($C_6H_{12}O_6$), 6.5 g/L sodium chloride (NaCl), 0.75 g/L potassium chloride (KCl), 0.48 g/L calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 0.3 g/L magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 3.9 g/L sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$), 1.7 g/L sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), and sodium hydroxide and/or hydrochloric acid to adjust pH to approximately 7.5. BSS + glucose was used to make dilutions of hydrogen peroxide (30% H_2O_2 ; Fisher Scientific, Pittsburgh, PA).

2.3. Two-photon microscopy

2PM measurements in cell culture were performed using a Zeiss LSM 510 META confocal system on an inverted Axiovert 200M microscope platform (Carl Zeiss MicroImaging Inc., Göttingen, Germany) controlled by the Zen data acquisition software (Zeiss). The microscope stage was equipped with an incubator chamber (Solent Scientific) allowing experiments to be performed at 37 °C. The femtosecond excitation laser pulses were generated with a tunable mode-locked Ti:Sapphire two-photon laser (Cameleon™ ultra II, Coherent Inc., Santa Clara, CA) delivering ~120 fs pulses at 80 MHz repetition rate. The wavelength of the two-photon laser was tuned to 740 nm for maximum NADPH autofluorescent signal. The excitation pulses were scanned across the TM cells using the internal scanning Galvanometer mirrors of the confocal system and focused by an Olympus “UPlanSApo” 20×/0.75 NA objective. The average incident power on the sample was approximately 6 mW. The NADPH autofluorescent signal was collected by the objective in the epi-direction and subsequently separated by a 500DCXR (Chroma Technology Corp, Rockingham, VT) dichroic mirror, spectrally filtered with an HQ450/100m-2p (Chroma Technology Corp, Rockingham, VT) and finally focused to an external photomultiplier tube for data collection.

2.4. Cell treatment

Concentrations of H_2O_2 (500, 100, 20, and 4 mM) in BSS + glucose were prepared fresh immediately prior to the experiment and maintained at 37 °C. The microscope incubator was heated to 37 °C for several hours before the experiment to allow the system to reach thermal equilibrium to reduce artificial effects of temperature gradient throughout the experiment. Stacked time series images of TM cells maintained in BSS + glucose were recorded. The BSS + glucose solution was carefully removed from the dish, and pre-warmed H_2O_2 -containing solutions were subsequently added. There was a ~10 s lag time between the insertion of H_2O_2 solution and acquired data (time = 0).

2.5. Image analysis

The effect of H_2O_2 on the NADPH fluorescence within TM cells was quantified by collecting a stacked time series of 2PM images at ~2 min intervals for a total of 16 images (30 min of total measurement). At each time interval, 6 images (512×512 pixel size; 3.2 μ s pixel dwell time) were taken spaced at 2 μ m intervals (z-stack) in order to capture the NADPH signal throughout the entire TM cell, as well as to counter any drift of the

microscope stage or axial movements of the TM cells. All images were analyzed using commercial software Matlab (MathWorks, Natick, MA). The attenuation of the NADPH signal was quantified by taking the strength of signal energy by using Matlab's *trapz* function, which utilizes the trapezoidal method for finding the integral under the image, hence the energy of the signal. To analyze the standard deviation, each image was divided into four quadrants (except for the BSS data, where the mean and error was calculated through 4 separate measurement) and the energy of each quadrants were calculated using the *trapz* function. The mean and standard deviation was calculated using the *mean* and *std* function of Matlab.

3. Results

3.1. Detection of in vivo NADPH through 2-photon autofluorescence (2PAF)

Fig. 1 illustrates a time series of 2P images of TM cells maintained in BSS + glucose at 37 °C. Stable NADPH signal was observed over the entire time series images. The majority of NADPH signal was localized in 1–2 μm particles (most likely mitochondria), while a weaker uniformly diffuse NADPH signal was observed in the cytoplasm. During the image acquisition time interval, only small movements of cells were observed. Over the 30 min experiment, only a slight NADPH signal fluctuation (of 4%) was observed, which is most likely due to a combination of photobleaching of NADPH and small movements of TM cells. Nevertheless, this signal fluctuation is much smaller than the signal attenuation observed after the introduction of H_2O_2 (between 9 and 46% reduction).

3.2. The attenuation of NADPH autofluorescence signal of TM cells exposed to different H_2O_2 concentrations

Two-photon excitation autofluorescence (2PAF) of NADPH in living human TM cells was compared among treatments with BSS + glucose with a range of H_2O_2 concentrations. Fig. 2 illustrates the attenuation of NADPH autofluorescent signal through the oxidative stress introduced by H_2O_2 vs. time. Each point on the energy curve represents the integrated signal of the entire image as explained in the Section 2.4. All energy curves were normalized to the initial NADPH fluorescence at time zero (set to 1). As shown in Fig. 2, the attenuation of the NADPH signal is highly correlated to the H_2O_2 concentration. For the lowest concentration of H_2O_2 (4 mM), an immediate attenuation of signal is observed through the first 10 min, followed by a steady fluorescent energy signal. The maximum energy attenuation for this concentration is calculated to be about 9%. Continuous attenuation as a function of time of the NADPH fluorescent signal is observed with 20 mM and 100 mM H_2O_2 , which account for a final decrease in the NADPH signal of 29% and 37% respectively. For the 500 mM H_2O_2 concentration, a drastic change in the NADPH signal is observed, and the NADPH autofluorescence signal shows an exponential decrease of 46% of the time zero value. Fig. 3 shows the NADPH autofluorescence images of TM cells at different H_2O_2 concentrations at time zero and 30 min after the introduction of H_2O_2 . It is evident that the final NADPH autofluorescence signals are very different with 20, 100 and 500 mM H_2O_2 treatments, but very similar in control or 4 mM H_2O_2 treatments.

3.3. Differential responses of individual TM cells to H₂O₂

As mentioned in Section 3.2, we contribute the significant attenuation of the overall autofluorescence signal to introduction of oxidative stress through H₂O₂ solution. We contribute this attenuation qualitatively to the “death” of the TM cells due to the oxidative stress. In order to understand how a population of TM cells reacts differently to the effect of H₂O₂, we calculated the autofluorescence energy of a region of interest (ROI) enclosing individual TM cells and compared the NADPH autofluorescence attenuation rates among them over time. From this analysis, we attribute two different autofluorescence attenuation behaviors based on two different degrees of initial cell autofluorescence. These different cell responses to oxidation stress are highlighted in Fig. 4, which contrasts the NADPH fluorescence in BSS + glucose control cells (Panel A) to both 20 mM H₂O₂ (Panel B) and 500 mM H₂O₂ (Panel C). Individual cells with higher (red) or lower (yellow) initial NADPH are outlined in the image. For cells that have brighter initial fluorescence energy, we observed 1) a more gradual decrease of the NADPH autofluorescence signal and 2) different signal reduction rates which depend on the H₂O₂ concentration (as described in the Section 3.2). For cells that have dimmer initial fluorescence energy, a more sudden drop of the NADPH autofluorescence signal is observed where the TM cells seem to suddenly “vanish” from the image plane. This sudden apparent loss of viability is mostly observed within the first eight minutes of introduction of oxidative stress and when higher concentrations of H₂O₂ (100 mM and 500 mM) are added to the cell culture.

In BSS + glucose control TM cells, analysis of individual TM cell NADPH fluorescence shows that both strongly NADPH fluorescing cells (circled in red) and weakly fluorescing cell (yellow) have a stable intensity over the 30 min of imaging (Fig. 4A). This is not the case in H₂O₂ treated cells. With both 20 and 500 mM H₂O₂ treatment, analysis of individual TM cell NADPH autofluorescence indicates that strongly NADPH fluorescing cells (circled in red) show an decrease in fluorescence, while the weakly fluorescing cell (yellow) almost immediately lose NADPH fluorescence (Fig. 4B and C). Since NADPH is a critical cellular metabolite, we assume that the cells that vanish are non-viable. Animations of the three conditions (BSS + glucose control, 20 mM H₂O₂, and 500 mM H₂O₂) are available as supplemental videos (Videos 1–3).

Supplementary data related to this article can be found online at doi:10.1016/j.exer.2011.02.012.

4. Discussion

We have previously used 2PM to investigate the collagen structures of the TM region in their native unfixed state (Ammar et al., 2010). In this report, we used 2PM to analyze the real-time NADPH levels in human TM cells exposed to different concentrations of H₂O₂. 2PM has been used to image the NADPH within the rabbit cornea which allowed the monitoring of changes in oxidative metabolism of the basal epithelium in real-time (Piston et al., 1995) but, to our knowledge, never before in the TM region of the eye. We conclude that 1) H₂O₂ causes a dose-dependent decrease in the average NADPH fluorescence of TM cells and 2) that initial levels on intracellular NADPH levels correlate with apparent TM cell viability to H₂O₂ insult. The ability of TM cells to counter oxidative damage is critical to their survival,

since H₂O₂ in the human aqueous humor may be as high as 25 mM (Spector and Garner, 1981). The H₂O₂ in the aqueous humor is believed to be constantly generated through a light-dependent reaction with iris melanin and is enhanced by the high levels of ascorbate present in the aqueous humor (Pirie, 1965; Wielgus and Sarna, 2008).

Several lines of evidence suggest that a reduction in the antioxidant properties of the TM correlates to the progression of primary open angle glaucoma (POAG). The TM inducible glucocorticoid-response protein myocilin is induced by TM exposure to oxidative stress, and long-term exposure could lead to obstruction of aqueous outflow (Polansky et al., 1997). Short term treatment of cultured TM cells with sub-lethal doses of peroxide (1 mM) reduced TM cell adhesion to the growth matrix and repeated exposure could potentially lead to cell loss, compromised tissue integrity, and subsequent impaired outflow (Zhou et al., 1999). It has also been shown that the circulating plasma levels of both reduced GSH and total GSH were consistently lower in patients with POAG as compared to age matched controls (Gherghel et al., 2005). Long-term exposure of TM cultures to low doses (0.2 mM) H₂O₂ induced expression of inflammatory markers associated with TM from glaucoma donors (Li et al., 2007). Superoxide dismutase, a GSH-utilizing enzyme required for neutralizing superoxide radicals, shows an age-dependent decline in the TM (De La Paz and Epstein, 1996). Finally, two studies have found statistically higher levels of oxidative DNA damage in TM tissue taken from glaucoma vs. healthy human eyes (Izzotti et al., 2003; Sacca et al., 2005). Taken together, cumulative oxidative damage to TM cells by chronic exposure to peroxide may be one component for the age-dependent increase in incidence of POAG.

In conclusion, we report findings of 2PM imaging of *in vitro* human TM cells after exposure to H₂O₂. Our findings reveal a dose- and time-dependent decrease in intracellular NADPH fluorescence in the early stages after being exposed to oxidative stress. The use of 2PM for exploring the early changes that occur in TM cells after localized metabolic insults may help shed light on the pathophysiology of POAG as it relates to *in vivo* oxidative stress. While current ophthalmic imaging devices such as ultrasound and optical coherence tomography focus on structural imaging, 2PM has potential to provide both structural and functional data on imaged tissues. Further development of 2PM imaging devices that can access the aqueous drainage tissue of the eye *in vivo* may help with early detection of disease potentially leading to sight saving interventions in patients suffering from glaucoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Stable NADPH fluorescence from live trabecular meshwork (TM) cells in BSS + glucose. Human TM cells were incubated at 37 °C in BSS + glucose and imaged for NADPH fluorescence by two-photon autofluorescence (2PAF). Sample images at time zero, 8, 16, and 24 min are shown. The majority of NADPH signal was localized to 1–2μm particles (most likely mitochondria), with a weaker NADPH signal observed throughout the cytoplasm.

Fig. 2.

NADPH fluorescence is attenuated due to oxidative stress introduced by different concentration of H_2O_2 . H_2O_2 causes both a dose- and time-dependent decrease in NADPH signal in trabecular meshwork cells. Four different H_2O_2 concentrations are compared: 4 mM (pink), 20 mM (green), 100 mM (blue), and 500 mM (red).

Fig. 3.

A qualitative comparison of reduction in NADPH fluorescence in trabecular meshwork (TM) cells treated with increasing concentrations of H₂O₂. Two-photon autofluorescence (2PAF) images of the field of TM cells at time zero and 30 min (end of the experiment). While the average NADPH fluorescent is reduced in TM cells overall with H₂O₂ treatment, there is cell to cell variability in the response to H₂O₂.

Fig. 4.

Different NADPH fluorescent intensities highlight slow and fast NADPH attenuation in trabecular meshwork (TM) cells. Energy plots for individual cells with higher (red) or lower (yellow) than average NADPH intensity (normalized to the average energy of all cells in the image). After treated with a range of H_2O_2 , highly fluorescence cells (red) show greater apparent viability than cells that were weakly fluorescent (yellow). Panel A) BSS + glucose. Panel B) 20 mM H_2O_2 . Panel C) 500 mM H_2O_2 .