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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 (H_2O_2) on TM endothelial cells include changes in viability, protein synthesis, and cellular adhesion. However, little if anything is known about the immediate effect of H_2O_2 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 H_2O_2-containing solutions. Peroxide caused both a dose- and time-dependent decrease in NADPH signal. NADPH fluorescence in control and in 4 mM H_2O_2 solutions showed little attenuation of NADPH signal (4% and 9% respectively). TM cell NADPH fluorescence showed a linear decrease with exposure to 20 mM H_2O_2 (−29%) and 100 mM H_2O_2 (37%) after a 30 min exposure. Exposure of TM cells to 500 mM H_2O_2 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 H_2O_2 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.

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 H_2O_2 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 non-destructive microscopy technique, two-photon microscopy (2PM). The absorbance peak of NADPH lies in the near ultraviolet;

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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 ($\sim$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% CO_2 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 ($C_2H_{12}O_6$), 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, Pittsburg, 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 $\sim$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 $\sim$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 $\sim$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 μs pixel dwell time) were taken spaced at 2 μm 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, were 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 st 37 °C. Stable NADPH signal was observed

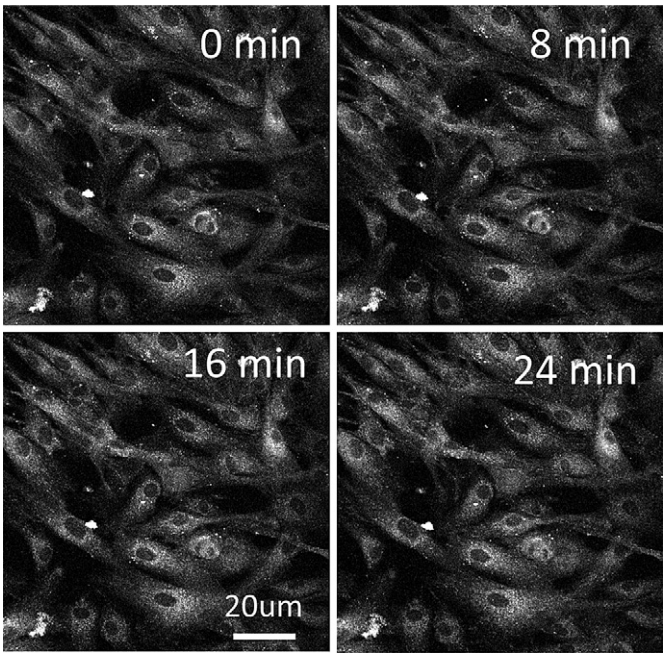

Fig. 1. Stable NADPH fluorescence from live trabecular meshwork (TM) cells in BSS + glucose. Human TM cells were incubated at 37 °C in BSS + glucose and imaged for NADPH fluorescence by two-photon autofluorescence (2PAF). Sample images at time zero, 8, 16, and 24 min are shown. The majority of NADPH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