Free Radical Biology & Medicine 51 (2011) 216–224

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Nrf2 has a protective role against neuronal and capillary degeneration in retinal ischemia–reperfusion injury

Yanhong Wei [a], Junsong Gong [a], Takeshi Yoshida [a], Charles G. Eberhart [a,b], Zhenhua Xu [a], Ponvijay Kombairaju [c], Michael B. Sporn [d], James T. Handa [a], Elia J. Duh [a,*]

[a] Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA
[b] Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA
[c] Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21287, USA
[d] Department of Pharmacology and Toxicology, Dartmouth School of Medicine, Hanover, NH, USA

ARTICLE INFO

Article history:
Received 26 January 2011
Revised 11 April 2011
Accepted 13 April 2011
Available online 19 April 2011

Keywords:
Apoptosis
Capillary degeneration
Cytokines
Inflammation
Ischemia–reperfusion
Knockout mice
Nuclear factor erythroid-2 related factor 2
Reactive oxygen species
Retina
Triterpenoids

ABSTRACT

Retinal ischemia–reperfusion (I/R) involves an extensive increase in reactive oxygen species as well as proinflammatory changes that result in significant histopathologic damage, including neuronal and vascular degeneration. Nrf2 has a well-known cytoprotective role in many tissues, but its protective function in the retina is unclear. We investigated the possible role of Nrf2 as a protective mechanism in retinal ischemia–reperfusion injury using $Nrf2^{-/-}$ mice. I/R resulted in an increase in retinal levels of superoxide and proinflammatory mediators, as well as leukocyte infiltration of the retina and vitreous, in $Nrf2^{+/+}$ mice. These effects were greatly accentuated in $Nrf2^{-/-}$ mice. With regard to histopathologic damage, $Nrf2^{-/-}$ mice exhibited loss of cells in the ganglion cell layer and markedly accentuated retinal capillary degeneration, as compared to wild-type. Treatment with the Nrf2 activator CDDO-Me increased antioxidant gene expression and normalized I/R-induced superoxide in the retina in wild-type but not $Nrf2^{-/-}$ mice. CDDO-Me treatment abrogated retinal capillary degeneration induced by I/R in wild-type but not $Nrf2^{-/-}$ mice. These studies indicate that Nrf2 is an important cytoprotective mechanism in the retina in response to ischemia–reperfusion injury and suggest that pharmacologic induction of Nrf2 could be a new therapeutic strategy for retinal ischemia–reperfusion and other retinal diseases.

Introduction

Retinal ischemia is a major contributor to tissue damage in diseases including acute angle-closure glaucoma, retinal vascular occlusions, diabetic retinopathy, and retinopathy of prematurity [1]. An important aspect of this injury is the condition of ischemia–reperfusion (I/R), in which blood supply returns to a tissue bed after a period of ischemia. The absence of oxygen and nutrients during ischemia creates a condition in which restoration of circulation results in the generation of reactive oxygen species [2,3], leading to inflammation [4]. Animal models of retinal ischemia–reperfusion have been developed and have been widely used to study the effects of I/R on neuronal injury in the retina, especially ganglion cell loss [5,6]. More recently, it has been demonstrated that injury to the retinal microvasculature, including capillary degeneration, is another major sequela in this retinal ischemia/reperfusion model [7]. This is consistent with ischemia–reperfusion in systemic diseases, which is known to have a major impact on the vasculature [8].

Similar to other tissue beds, retinal I/R injury involves an extensive increase in oxidative stress as well as proinflammatory changes. The generation of excessive free radicals is known to be an important primary event in retinal I/R. Indeed, modulation of oxidative stress reduces histopathologic changes in retinal I/R injury, including leukocyte infiltration [9] and capillary degeneration [10]. Modulation of inflammation has also been found to be beneficial in retinal I/R [11]. Overall, the retinal I/R model serves as a useful approach for studying the regulation of oxidative stress and inflammation in the retina by various genes and molecules, including iNOS, manganese superoxide dismutase, and catalase [7,10–12]. This model is especially valuable in evaluating the importance of various molecules in protecting the retina against cellular damage, particularly ganglion cell loss and retinal capillary degeneration, which are critical endpoints in retinal diseases such as glaucoma and diabetic retinopathy, respectively.

Abbreviations: CDDO-Me, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid–methyl ester; COX-2, cyclooxygenase-2; DHE, dihydroethidium; GCLC, glutamate-cysteine ligase, catalytic subunit; GCLM, glutamate-cysteine ligase, modifier subunit; HO-1, heme oxygenase-1; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; IL-6, interleukin-6; ILM, internal limiting membrane; IOP, intraocular pressure; I/R, ischemia–reperfusion; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; NQO1, NAD(P)H dehydrogenase, quinone 1; Nrf2, NF-E2-related factor 2; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

* Corresponding author at: Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N. Broadway, Baltimore, MD 21287. Fax: +1 410 614 5471.
E-mail address: eduh@jhmi.edu (E.J. Duh).

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.04.026

Nrf2 (NF-E2-related factor 2) is a transcription factor that plays a significant role in protecting cells from endogenous and exogenous stresses [13]. In a wide range of tissues, Nrf2 acts as a master regulator of the antioxidant response, serving as one of the most important cellular pathways protecting against oxidative stress [14]. Nrf2 also plays an important role as a negative regulator of inflammation [15,16]. Nrf2 normally resides in the cytoplasm, bound by its cytosolic inhibitor, Keap1, which targets Nrf2 for proteosomal degradation. Various endogenous or exogenous inducing agents such as reactive oxygen species can disrupt the association of Nrf2 with Keap1, leading to nuclear translocation of Nrf2 and the transcriptional activation of an array of cytoprotective genes.

Although Nrf2 is known to play a critical role in many organs including the liver, lung, and brain [13], its role in the retina has been less investigated. In a model of oxygen-induced retinopathy, Nrf2 was found to have a beneficial effect on the retinal vasculature at postnatal day 9, although this effect was not seen at postnatal day 12 [17]. In a model of uveitis in which ocular inflammation is experimentally induced by administration of LPS, Nrf2 was found to modulate the inflammatory response and associated oxidative stress in the retina [18]. However, a retinal cytoprotective role for Nrf2 has not yet been established. In this study, we sought to determine whether Nrf2 prevents cellular injury in retinal ischemia–reperfusion, specifically neuronal and capillary degeneration. We found that Nrf2 is indeed a cytoprotective mechanism in the retina after I/R, and that pharmacologic activation of Nrf2 is a promising strategy for protecting the retina from damage in I/R injury.

Materials and methods

Mice

$Nrf2^{-/-}$ and wild-type ($Nrf2^{+/+}$) mice were generated as previously described [19] and backcrossed into C57BL/6 background. The animals were maintained on an AIN-76A diet and water ad libitum and housed at a temperature range of 20–23 °C under 12:12-h light–dark cycles. Mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine, and mouse studies adhered to the ARVO (Association of Research in Vision & Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.

Model of retinal ischemia–reperfusion

Retinal ischemia was induced as previously described [7]. Adult mice were anesthetized with a cocktail of 50 mg/kg ketamine, 10 mg/kg xylazine, and 2 mg/kg acepromazine. The anterior chamber of one eye was cannulated with a 30-gauge needle attached to a line infusing sterile saline. The intraocular pressure (IOP) was raised to 80–90 mm Hg by elevating the saline reservoir. The retina was monitored for blanching, indicating loss of blood flow. After 90 min of ischemia, the needle was withdrawn and the IOP was normalized to allow reperfusion. The other eye of the same mouse was set up as the control.

CDDO-Me treatment

Some mice were pretreated with three intraperitoneal injections of 1 μmol/kg CDDO-Me (Reata Pharmaceuticals, Irving, TX, USA; dissolved in 10% DMSO, 10% cremophor-EL, 80% PBS) or vehicle at 48, 24, and 0 h before being subjected to I/R, similar to a previous dosing scheme used for LPS-induced uveitis [18] and based on a previous study showing that the effect of CDDO-Me on Nrf2 activation lasts for at least 24 h [20]. After I/R, the mice were treated with 1 μmol/kg CDDO-Me or vehicle every 48 h. For gene expression analysis, the mice were treated with one intraperitoneal injection of 1 μmol/kg CDDO-Me or vehicle.

Superoxide measurement

Dihydroethidium (DHE; Invitrogen-Molecular Probes, Eugene, OR, USA) was used for histochemical evaluation of superoxide production. DHE is a cell-permeable compound that oxidized on reaction with superoxide to 2-hydroxyethidium, which binds to DNA in the nucleus and fluoresces red [21,22]. Serial cryosections (5 μm) were incubated with 2.5 μM DHE in DMSO at room temperature for 15 min. After fixation with 2% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 1 min, the cryosections were rinsed twice in PBS for 5 min, and visualized using a Zeiss Axiovert 200 M microscope (Zeiss Microimaging, Thornwood, NY, USA) under the conditions of absorption/emission: 518/605 nm.

Superoxide anion in retina was quantified by lucigenin assay as previously described [23] with minor modifications. Briefly, fresh retinas were put into 0.2 mL Krebs/HEPES buffer and incubated in the dark at 37 °C under 85% O_2/5% CO_2 for 45 min. Lucigenin (Sigma, St. Louis, MO) was added up to a final concentration of 0.5 mM and the plate was incubated for 10 min at room temperature. Photon emission was measured over 10 s by a luminometer (BMG Labtech, Inc., Durham, NC). Repeated measurements were made over a 30 min period for 15 measurements. After averaging the measurements, the values were then subtracted the background from blank and normalized by the protein concentration for each retina.

Histopathology and analysis of cells in retina

Following enucleation, eyes were immediately fixed with 10% formalin overnight at room temperature. The fixed eyes were then dehydrated with graded series of ethanol, embedded in paraffin, cross-sectioned (4 μm), and stained with hematoxylin and eosin (H&E). For ganglion cell layer counting, pictures were taken of three sections, randomly selected from each eye in the region of the optic nerve. The nuclei in the ganglion cell layer (excluding the nuclei in the vessels) were counted in each entire section. Counting was performed in a masked fashion.

For quantitation of inflammatory cells, H&E-stained sections of eyes were scored by an ophthalmic pathologist (CGE) who was masked with respect to animal genotype. Inflammatory cells in the vitreous and below the internal limiting membrane (ILM) were counted in 10 random 400× high-powered fields for each eye.

Apoptotic DNA cleavage ELISA

Apoptotic DNA cleavage was assayed using a Cell Death Detection ELISA Plus kit (Roche Applied Science, Indianapolis, IN) as previously described [12]. Briefly, fresh retina was immediately put into 200 μL chilled lysis buffer supplied in the kit and vortexed at the highest speed for 2 min. The tube with retina was incubated for 30 min at room temperature with gentle shaking. The supernatant was collected after centrifugation for 10 min at 10,000*g* at 4 °C. For each sample, 20 μL supernatant was subjected to the ELISA assay according to the manufacturer's instructions. Following the color reaction to detect captured DNA fragments, the samples were measured at 405 nm (with 490 nm as reference). The values were normalized by the weight of retina.

Quantitative PCR

Total RNA from retina was isolated using the RNeasy kit (Qiagen, MD), and then treated with DNase (Qiagen, Hilden, Germany). Single-stranded cDNA was synthesized from 0.5 μg total RNA using oligo $(dT)_{12–18}$ primer (Invitrogen, Carlsbad, CA), and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) in a final reaction volume of 25 μL. Real-time PCR was performed using SYBR Premix Ex Taq (Takara, Dalian, China) with the Stratagene Mx3005P qPCR system (Angilent Technologies, Santa Clara, CA). The primers were NQO-1