Nrf2 Activation Is a Potential Therapeutic Approach to Attenuate Diabetic Retinopathy


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PURPOSE. Oxidative stress is a causal factor in the development of diabetic retinopathy; however, clinically relevant strategies to treat the disease by augmenting antioxidant defense mechanisms have not been fully explored. We hypothesized that boosting nuclear factor erythroid-2-related factor 2 (Nrf2) antioxidant capacity with the novel Nrf2 activator dh404, would protect the retina in diabetes including vision-threatening breakdown of the blood-retinal barrier (BRB) and associated damage to macroglial Müller cells.

METHODS. Sprague-Dawley rats were randomized to become diabetic or nondiabetic and administered dh404 by gavage for 10 weeks. Complementary in vitro studies were performed in cultured Müller cells exposed to hyperglycemia.

RESULTS. In diabetes, dh404 prevented vascular leakage into the retina and vitreous cavity as well as upregulation of the vascular permeability and angiogenic factors, VEGF, and angiopoietin-2, and inflammatory mediators, including TNF-α and IL-6. Müller cells, which maintain BRB integrity and become gliotic in diabetes with increased immunolabeling for glial fibrillary acidic protein, were protected by dh404. In diabetes, dh404 bolstered the antioxidant capacity of the retina with an increase in hemeoxygenase-1, nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) quinine oxidoreductase-1, and Nrf2. Further, dh404 attenuated the diabetes-induced increase in oxidative stress as measured by dihydroethidium and 8-oxo-2'-deoxyguanosine (8-OHdG) immunolabeling as well as NADPH oxidase isoform expression. Studies in Müller cells supported these findings with dh404 attenuating the hyperglycemia-induced increase in vascular permeability, angiogenic and inflammatory mediators, and oxidative stress.

CONCLUSIONS. Our data demonstrate the ability of dh404 to protect the retina against diabetes-induced damage and potentially prevent vision loss.

Keywords: diabetic retinopathy, Müller cells, Nrf2, oxidative stress

Diabetic retinopathy (DR) is a leading cause of vision impairment and blindness across the globe. Preventative treatments for DR are limited and most approaches target vision-threatening end-stage disease that is characterized by damage to the microvasculature, vascular leakage, and macular edema. These approaches are invasive and include laser photocoagulation to remove damaged blood vessels. Newer treatments that inhibit the potent vascular permeability and angiogenic mediator, VEGF, have provided a significant advance in the treatment of DR. However, repeated intraocular injections are required and a substantial number of eyes are resistant to anti-VEGF treatments. Therefore, there is a necessity for further investigation into more effective treatment strategies that prevent the underlying causal factors involved in the development of DR.

Retinal Müller cells are of interest due to the key role these macroglia have in the vascular dysfunction and inflammation that occurs in DR. The processes of Müller cells are in close contact with the retinal microvasculature to maintain the integrity of the inner retinal blood-retinal barrier (BRB), and damage to retinal Müller cells can result in the amplified production of VEGF, vascular leakage, and the increased production of proinflammatory mediators. Furthermore, oxidative stress is a major causal factor in the development of vascular and glial cell pathology in DR and arises when there is an imbalance between the excess production of reactive oxygen species (ROS) and compromised antioxidant defense mechanisms. Considerable attention has been given to reducing excess ROS levels in retinal disease, including DR. However, less explored is the therapeutic use of targeting key transcription factors involved in bolstering antioxidant defenses to prevent vascular and glial cell damage in DR.

There is increasing interest in the ability of the transcription factor nuclear factor erythroid-2 related factor2 (Nrf2) to improve tissue pathology by boosting the transcription of various antioxidant genes, including hemeoxygenase-1 (HO-1) and nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) quinine oxidoreductase 1 (NQO1). Nrf2 is a redox sensitive transcription factor that is kept in a latent state by its interaction with Kelch ECH associated protein 1 (Keap1). In response to increased free...
radicals, Keap1 is oxidized or covalently modified resulting in the release of Nrf2. Subsequently, Nrf2 enters the cell nucleus and binds to the antioxidant response element, which initiates the transcription of antioxidant genes.14 There has been considerable interest in developing Nrf2 activators for the treatment of diabetic complications that feature an imbalance in antioxidants and pro-oxidants.15–18 Synthetic small molecule activators of the Nrf2/Keap1 pathway include bardoxolone methyl, which belongs to the antioxidant inflammation modulator class of drugs. Although preclinical studies showed a reduction in kidney disease with bardoxolone methyl,19,20 this treatment was associated with heart failure in at risk patients.21 Subsequently, there has been interest in an analogue of bardoxolone methyl, dh404 (CDDO-9,11-dihydro-trifluoro-ethyl amide [CDDO-dhTFEA]), which exhibits similar biological properties to bardoxolone methyl but has been designed to improve efficacy and minimize toxicity.20,22 However, whether dh404 exerts protective effects in DR is unknown.

To determine if dh404 prevented breakdown of the BRB and Müller cell injury in DR we administered dh404 to rats with streptozotocin-induced diabetes. Further, we performed complementary in vitro studies in Müller cells exposed to hyperglycemia to elucidate the potential mechanisms responsible for retinal protection. Indeed, we now demonstrated that dh404 prevents damage to retinal Müller cells and the upregulation of angiogenic and inflammatory factors as well as oxidative stress by increasing antioxidant capacity, via Nrf2.

Materials and Methods

Animals
All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Alfred Medical Research and Education Precinct (AMREP) animal ethics committee (E1598/2015/M). Sprague Dawley rats were obtained from AMREP Animal Services (Melbourne, VIC, Australia) and received normal rat Chow (Certified Rodent Diet #5002; LabDiet, St. Louis, MO, USA) and drinking water ad libitum, and were housed at 22 ± 1°C with a 12-hour light/dark cycle. Following an overnight fast, 6-week-old rats (180–200 g) were randomized to become diabetic or nondiabetic. Diabetes was induced by a single tail vein injection of 55 mg/kg streptozotocin (Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in vehicle (0.1 M citrate buffer, pH 4.5). Nondiabetic rats received a single tail vein injection of vehicle only. Rats then were randomized to receive the Nrf2 activator, dh404 (3 mg/kg; Reata Pharmaceuticals, Irving, TX, USA) dissolved in 200 μL sesame oil (Sigma-Aldrich Corp.) or 200 μL sesame oil alone each day by oral gavage. Treatments were administered 5 days after administration of streptozotocin or citrate buffer and continued for 10 weeks. As reported previously, this dose of dh404 is optimal in reducing diabetic atherosclerosis,15 nephropathy,15 and endothelial cell dysfunction.16 Each week, rats were weighed and their blood glucose levels measured (AccuCheck Advantage II Blood Glucose Monitor; Roche Diagnostics, Indianapolis, IN, USA). Only rats with blood glucose levels >15 mmol/L were considered diabetic and studied. Insulin was administered three times per week to diabetic rats to reduce mortality and promote weight gain (2–4 units by intraperitoneal injection, Humulin NPH; Eli Lilly and Co., Indianapolis, IN, USA). At the end of the studies, rats received an anesthetic overdose of pentobarbital sodium (Lethabarb, 60 mg/kg; Virbac, NSW, Australia).

Primary Cultures of Macrogial Müller Cells
Primary cultures of Müller cells were established from the retinas of neonatal Sprague Dawley rats as described previously.23 Separate dishes containing Müller cells were exposed to normoglycemia (5.5 mmol/L D-glucose and 20 mmol/L mannitol), hyperglycemia (25 mM D-glucose), and treated with dh404 (0.25 μM) or vehicle (0.0001% dimethylsulfoxide) for 72 hours. Supernatants were harvested and cells were lysed using radioimmunoprecipitation assay (RIPA) buffer containing 1:100 Halt protease inhibitor cocktail (Thermo Fisher Scientific, VIC, Australia). The dose of dh404 was based on previous studies.23 Experiments were repeated three times with three replicates in each experiment.

Vascular Leakage

As described previously,23,24 albumin levels were measured in single retinas using a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) and normalized to dry retinal weight. The vitreous humour was collected from enucleated eyes by a 26-gauge needle attached to a 1 mL syringe, which was inserted behind the limbus at 45° downward into the vitreal space. The vitreous humour (~40 μL) was collected immediately in tubes containing 100 μL of 0.1 M PBS, pH 7.4, and the Halt protease inhibitor cocktail (1:100; Thermo Fisher Scientific) and then mixed vigorously. The samples were centrifuged at 12,879g for 10 minutes to separate the liquid components from the residual gel and cells. Vitreal samples then were diluted 1:2 in PBS to measure albumin levels by ELISA. Five to seven rats per experimental group were evaluated.

Quantitative Real-Time PCR (qPCR)

qPCR was performed as described previously.23,24 The primer sequences for VEGF, angiopoietin-2, the NADPH oxidase (Nox) family of enzymes, HO-1, NQO1, TNF-α, IL-6, and intercellular adhesion molecule-1 (ICAM-1) have been reported previously.23,25,26 Total RNA was isolated from single retina using the RNAeasy mini kit (Qiagen, VIC, Australia). One μg of RNA from each retina was subjected to DNase treatment (DNA-free kit; Ambion, Carlsbad, CA, USA) and reverse transcription (First Strand cDNA synthesis kit; Roche, Basel, Switzerland). Seven to eight rats per experimental group were evaluated. For cultured Müller cells, RNA was extracted using the RNAeasy mini kit (Qiagen) and 500 ng RNA was used. mRNA expression was normalized to 18S rRNA endogenous control and the relative fold difference in expression was calculated using the comparative 2–ΔΔCt method.

Müller Cell Gliosis

Using an established method,23 5 μm paraffin sections of retina were incubated overnight at 4°C with anti-glial fibrillary acidic protein (GFAP, 1:500; DakoCytomation, Glostrup, Denmark). A negative control (PBS instead of anti-GFAP) and isotype IgG control were included in each experiment. GFAP immunolabeling was visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Life Technologies, Carlsbad, CA, USA). Nonspecific binding was blocked with 3% normal goat serum (NGS) for 20 minutes. Immunolabeling was visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Life Technologies, Carlsbad, CA, USA). ImageJ software (v3.1, National Institutes of Health [NIH], Bethesda, MD, USA) was used to set a threshold for immunolabeling, which was applied to all fields. Data are
presented as the percentage of immunolabeling per field of retina. Five to six rats per group were evaluated.

Oxidative Stress in Retina

ROS levels were measured using an established method.²⁴ Eyes were embedded in OCT and immersed in liquid nitrogen and 10 μm cryosections stained with dihydroethidium (DHE; 5 μM in PBS; Sigma-Aldrich Corp.) for 30 minutes at room temperature. Four randomly selected sections per eye were selected randomly and labeling intensity was measured in the entire retina using ImageJ software. In addition, immunohistochemistry was performed for 8-hydroxy-2-deoxyguanosine (8-OHdG), an oxidized derivative of deoxyguanosine. Three μm paraffin sections of eyes were incubated with anti-8-OHdG (1:1000, #12501; QED Bioscience, San Diego, CA, USA) overnight at 4°C. The sections then were washed with PBS and incubated for 45 minutes with biotinylated goat anti-mouse IgG (1:500, #E43301; Dako, Carpinteria, CA, USA), washed with PBS, and then incubated with the avidin-biotin complex ( Vectastain ABC kit, #PK6100; Vector Laboratories, Burlingame, CA, USA). Labeling was developed with the 3,3′-diaminobenzidine substrate chromagen system (DakoCytomation) and the sections were coverslipped. Immunolabeling for 8-OHdG was quantitated as described above for GFAP. Five rats per group were evaluated.

Western Blotting

Snap frozen retina and pellets from cultured Müller cells were homogenized in RIPA buffer (Sigma-Aldrich Corp.) containing 1:100 Halt protease inhibitor cocktail (Thermo Fisher Scientific) using a sonicator. Total retinal protein was quantitated using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Fifty μg of retinal or Müller cell lysates were subjected to electrophoresis and immunoblotting. For nuclear Nrf2 measurements, retinas were extracted using the NE-PER protein extraction kit (Thermo Fisher Scientific) as published.²³ Membranes were blocked with 1:100 Halt protease inhibitor cocktail (Thermo Fisher Scientific) overnight at 4°C. The membranes were washed and reprobed with a mouse anti-β-actin (1:2000, #sc-47778; Santa Cruz Biotechnologies, Dallas, TX, USA) or H3 antibody (1:1000, #3638; Cell Signaling Biotechnologies) or H3 antibody (1:1000, #ab5621; Santa Cruz Biotechnologies, Dallas, TX, USA). Following an overnight incubation at 4°C, membranes were washed and incubated with a goat anti-rabbit IgG conjugated with HRP (1:5000; Cell Signaling Technology, Danvers, MA, USA). Membranes were developed with the Pierce ECL Western blotting substrate (Thermo Fisher Scientific) according to the manufacturer’s protocol. Immunolabeling was quantitated using the ImageJ software (v.2, Bio-Rad Laboratories) and the signal was expressed as the ratio between corresponding protein and β-actin or H3. Five rats per experimental group were evaluated.

Enzyme-Linked Immunosorbent Assay (ELISA)

Snap frozen retina and cultured retinal Müller cells were homogenized in 0.1 M sodium phosphate buffer (pH 9.5) containing 1:100 Halt protease inhibitor cocktail (Thermo Fisher Scientific). Total protein was quantitated using the Bradford assay (Bio-Rad Laboratories). Rat VEGF (#DY594, R&D Systems, Inc., Minneapolis, MN, USA), MCP-1 (#555130, BD Biosciences, San Jose, CA, USA) and IL-6 (#555019, BD Biosciences) protein levels were measured. Müller cell supernatants for TNF-α levels were concentrated ×11 using 10K MW cut-off centrifugal filters (¾UFCA0124; Millipore, Cork, Ireland) and measured using a rat TNF-α ELISA kit (#88-7340-86; Life Technologies, Rockford, IL, USA). Five to eight rats per experimental group were evaluated.

Statistics

Data were analyzed using the GraphPad Prism Software (v.5, GraphPad Software, San Diego, CA, USA). Data were assessed first for normality by Kolmogorov-Smirnov, D’Agostinos, and Pearson omnibus, as well as Shapiro-Wilk normality tests. Analysis was then performed by 1-way ANOVA followed by Bonferroni post-test analysis (for data that passed normality tests) or by nonparametric Kruskal-Wallis tests followed by Dunns post-test (for data that did not pass normality tests). For comparison between two groups, either Student’s t-test or Mann-Whitney U tests were used, on data that passed and failed normality tests, respectively. P < 0.05 was considered significant. Investigators were masked to the experimental groups.

Results

Body Weight and Blood Glucose

Diabetic rats did not gain as much body weight as age-matched nondiabetic rats, and dh404 did not affect body weight (Table). Blood glucose levels were increased in diabetic rats compared to nondiabetic rats and dh404 had no effect on blood glucose levels (Table).

Dh404 Prevented Retinal Vascular Leakage and the Increase in Angiogenic Factors in Diabetics

Breakdown of the BRB and subsequent vascular leakage is a major cause of vision loss in diabetes.²⁷ We first examined if dh404 influenced vascular leakage by measuring the levels of albumin in the retina and vitreous cavity. In diabetic rats, albumin levels were increased by 1.5-fold in retina and 3.5-fold in vitreous compared to nondiabetic controls (Figs. 1A, 1B). Treatment with dh404 did not influence vascular leakage in nondiabetic rats, but in diabetic rats dh404 prevented the rise in albumin levels (Figs. 1A, 1B). As VEGF is a main causal factor in the vascular permeability that can develop in DR,²⁸ we measured retinal VEGF levels. VEGF mRNA and protein levels were increased by 2.4- and 2-fold, respectively, in retinas of diabetic compared to nondiabetic rats (Figs. 1C, 1D). Dh404 did not influence retinal VEGF levels in nondiabetic rats, but prevented the diabetes-induced increase in retinal VEGF mRNA and reduced retinal VEGF protein to the level of nondiabetic control (Figs. 1C, 1D). Angiopoietin-2 has potent vasoactive properties in the retina²⁹ and we found that angiopoietin-2 expression was increased in the retinas of diabetic rats compared to nondiabetic controls (Fig. 1E). Dh404 had no effect on retinal angiopoietin-2 mRNA levels in nondiabetic rats, and in diabetic rats reduced the expression of this angiogenic factor to control levels (Fig. 1E).

| Table. Body Weight and Blood Glucose Levels in Sprague Dawley Rats |
|-----------------|-----------------|-----------------|
| Groups          | Body Weight, g  | Blood Glucose, mmol/L |
| Nondiabetic     | 275.17 ± 7.70   | 9.39 ± 0.58      |
| Nondiabetic + dh404 | 257.36 ± 10.92  | 10.06 ± 0.89    |
| Diabetic        | 238.17 ± 5.69*  | 30.77 ± 1.32*    |
| Diabetic + dh404 | 248.07 ± 4.87*  | 29.79 ± 1.89*    |

*P < 0.001 to NDiab. n = 12 to 24 rats per group. Values are Mean ± SEM.
Dh404 Prevented Müller Cell Gliosis in Diabetes

Müller cells when injured in DR contribute to breakdown of the BRB.\textsuperscript{7} When Müller cells become damaged they exhibit increased expression of the intermediate filament protein, GFAP.\textsuperscript{7} In nondiabetic controls, GFAP immunolabeling was sparse and restricted to the retinal surface (Figs. 1F, 1G). In diabetic rats, GFAP immunolabeling was increased throughout the processes of Müller cells. Dh404 did not influence GFAP immunolabeling in retinas from nondiabetic rats, but reduced the diabetes-induced increase in GFAP in Müller cell processes (Figs. 1F, 1G).

Dh404 Prevented the Increased Expression of Inflammatory Mediators in Diabetic Retina

Inflammation contributes to the pathogenesis of DR.\textsuperscript{30} Thus, the expression of key inflammatory mediators in retinas from diabetic rats was evaluated. Indeed, dh404 prevented the diabetes-induced increase in TNF-α, IL-6, ICAM-1, and MCP-1 (Figs. 2A–E).
Increased Nrf2-Responsive Antioxidant Genes in Diabetic Retina

In diabetic retinas, HO-1 mRNA and protein levels were increased moderately compared to nondiabetic rat groups, and further increased with dh404 treatment (Figs. 3A, 3B). In diabetic retinas, NQO1 mRNA levels were similar to nondiabetic control groups, while NQO1 protein levels were reduced compared to controls. Dh404 increased the mRNA levels of NQO1 and restored the protein levels of NQO1 to control levels (Figs. 3C, 3D). Nrf2 levels in the nuclear fraction of diabetic retinas were reduced compared to nondiabetic groups and increased to the level of nondiabetic controls with dh404 (Fig. 3E).

Dh404 Prevented Oxidative Stress in Diabetic Retina

ROS levels, as measured by DHE labeling, were increased in diabetic retinas compared to nondiabetic control groups, and further increased with dh404 treatment (Figs. 3A, 3B). In diabetic retinas, NQO1 mRNA levels were similar to nondiabetic control groups, while NQO1 protein levels were reduced compared to controls. Dh404 increased the mRNA levels of NQO1 and restored the protein levels of NQO1 to control levels (Figs. 3C, 3D). Nrf2 levels in the nuclear fraction of diabetic retinas were reduced compared to nondiabetic groups and increased to the level of nondiabetic controls with dh404 (Fig. 3E).

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In Cultured Müller Cells, dh404 Reduced the Hyperglycemia-Induced Increase in Angiogenic and Inflammatory Factors

To gain further insight into the protection afforded by dh404 we studied primary cultures of rat Müller cells. The protein levels of VEGF, MCP-1, IL-6, and TNF-α in cell supernatant all were markedly increased by hyperglycemia (6-, 3-, and 3-fold,
respectively; Figs. 5A–D). Dh404 had no effect on the protein levels of these factors in Müller cells grown under normoglycemic conditions, but in hyperglycemia dh404 reduced all of the studied factors (Figs. 5A–D). In Müller cells exposed to hyperglycemia, the mRNA levels of IL-6 and TNF-α were increased, and dh404 reduced their expression to control levels (Figs. 5E, 5F).

In Cultured Müller Cells, dh404 Increased Nrf2-Responsive Antioxidant Genes

We next evaluated if dh404 boosted antioxidant defense mechanisms in Müller cells. The mRNA and protein levels of HO-1 were increased moderately by hyperglycemia, and dh404 markedly increased HO-1 expression in cells exposed to normoglycemia or hyperglycemia (Figs. 6A, 6C). Although the mRNA and protein levels of NQO1 mRNA were unchanged by hyperglycemia, dh404 increased NQO1 expression in both normoglycemia and hyperglycemia conditions (Figs. 6B, 6D). Of the 3 Nox isoforms measured, Nox1 and Nox4 but not Nox2 mRNA levels were increased by hyperglycemia, and only Nox1 and Nox4 mRNA levels were reduced with dh404 (Figs. 6E–G). p22phox was increased by hyperglycemia and reduced with dh404 (Fig. 6H).

**DISCUSSION**

The main findings of this study are that a treatment approach to augment the transcription factor Nrf2, prevented vision-threatening events in DR, including vascular leakage and associated damage to macroglial Müller cells. Specifically, dh404 administered shortly after onset of streptozotocin-induced diabetes abrogated vascular leakage into the retina and vitreous cavity. Further, dh404 prevented the diabetes-induced increase in key events linked to retinal vascular leakage, including elevated angiogenic and inflammatory factors as well as the gliosis of Müller cells. The mechanisms by which dh404 prevented retinal pathology in diabetes involved an increase in the Nrf2-responsive antioxidants, HO-1 and NQO1, as well as a decrease in oxidative stress elements, such as ROS and Nox isoform expression in retina and Müller cells. Our data together with previous reports that Nrf2 influenced retinal injury,31,32 highlight the potential of Nrf2 activators, such as dh404, as a treatment approach to prevent damage to the retinal neurovascular unit in diabetes.

Current treatments for DR are focused on the microvascular lesions that develop as a consequence of hyperglycemia and tissue ischemia. It is increasingly appreciated that DR is a disease of the retinal neurovascular unit, which is comprised of neurons and glial cells that are connected anatomically to the vasculature.2 This physical arrangement is critical to maintain retinal health including local blood flow, ion and fluid transport and the integrity of the BRB, and hence prevent vision-threatening vascular leakage.2 In this context, macroglial Müller cells have an important role, and in DR damage to this cell population results in gliosis, which negatively impacts on the integrity of the BRB.6,7 Further, in DR, Müller cells produce increased amounts of VEGF and inflammatory factors that promote damage to the retinal microvasculature, including vascular leakage.7 Consistent with the retinal vascular leakage that occurs in patients with proliferative DR, we found in diabetic rats, increased levels of albumin in the neural retina and also the vitreous cavity.7 The ability of dh404 to prevent diabetes-induced vascular leakage as well as Müller cell gliosis and the increase in retinal VEGF levels in retina and cultured
FIGURE 4. Oxidative stress in the retina of diabetic rats is reduced by treatment with dh404. (A) Representative images of 3 μm sections of retina showing that in diabetic rats, DHE labeling is increased in the inner retina (from retinal surface to INL) as well as in the ONL where photoreceptor nuclei reside. In diabetic rats, dh404 reduced DHE labeling. Scale bar: 40 μm. (B) Quantitation of DHE labeling in the inner retina (retinal surface to INL). n = 5 rats per group. (C) Representative images of 3 μm sections of retina showing increased 8-OHdG immunolabeling in the GCL and INL as well as Müller cells processes (arrows) in diabetes, which is reduced with dh404 treatment. (D) Quantitation of 8-OHdG immunolabeling in the inner retina. Scale bar: 40 μm. n = 5 to 6 rats per group. mRNA levels of (E) Nox1, (F) Nox2, and (G) Nox4 as well as (H) p22phox protein levels in retina. n = 5 to 8 rats per group. *P < 0.05, **P < 0.01, ***P < 0.001 to NDiab. *P < 0.05, **P < 0.05 to Diab. Values are mean ± SEM.
Müller cells exposed to hyperglycemia indicates the potential of this pharmacologic approach to prevent vision-threatening events in DR. Moreover, dh404 reduced the expression of angiopoietin-2, a growth factor that enhances the permeability of retinal endothelial cells, has angiogenic properties and potentiates the actions of VEGF on the vasculature. Indeed, angiopoietin-2 is a potential new target for the treatment of DR and diabetic macula edema. Overall, these findings are consistent with previous studies in other retinal diseases showing that Nrf2 influences the integrity of the BRB.

Increasing evidence indicates that DR is a pro-inflammatory condition, and IL-6 and TNF-α as well as the chemokine MCP-1 and the leukocyte adhesion molecule, ICAM-1 are implicated in the pathogenesis of DR. Müller cells are recognized as an important mediator and source of the increased levels of these pro-inflammatory factors in DR. The ability of dh404 to reduce the increased production of IL-6, TNF-α, MCP-1, and ICAM-1 in the retina of diabetic rats as well as Müller cells exposed to hyperglycemia highlights the importance of Müller cells in DR and the powerful anti-inflammatory capacity of Nrf2 activation.

Dh404 exerts its actions by promoting the translocation of Nrf2 into the nucleus and increasing the production of Nrf2-responsive genes by preventing Keap1 from binding to Nrf2. Although these mechanisms were not fully explored in our study, we demonstrated that dh404 increased Nrf2 translocation into the nucleus in the diabetic retina, and also increased Nrf2-responsive genes that provide key antioxidant and cytoprotective defense mechanisms in damaged tissues including the retina. HO-1 is of particular importance as it serves as an adaptive mechanism to protect cells in tissues including the retina from stress-induced oxidative damage. Consistent with previous studies, the expression of HO-1 was increased in the retinas of diabetic rats, and HO-1 expression was amplified further by dh404 treatment. NQO1 is a highly inducible enzyme that is regulated by Nrf2 and confers efficient antioxidant properties. We demonstrated that dh404's retinoprotective effects in diabetes were associated with increased NQO1, data that are in agreement with studies on ischemic retinopathy and human aortic endothelial cells exposed to a diabetic environment.

Dh404 not only promoted antioxidant defense but also reduced oxidative stress in the retina of diabetic rats. The Nox family of enzymes are a major source of ROS, and Nox1, Nox2 and Nox4 are implicated in the development of retinal diseases including DR. Our finding that dh404 reduced the levels of p22phox, a critical component of Nox enzyme activity as well as the expression of all three studied Nox isoforms in the diabetic retina indicated that dh404 has the potential to reduce inflammation and oxidative stress in the retina of diabetic rats.

**Figure 5.** In primary cultures of rat Müller cells, angiogenic and inflammatory factors are reduced with dh404 treatment. NG, normoglycemia; HG, hyperglycemia; The hyperglycemia-induced increase in (A) VEGF protein, (B) MCP-1 protein, (C) IL-6 protein, (D) TNF-α protein, (E) IL-6 mRNA, and (F) TNF-α mRNA was prevented by dh404 treatment. n = 3 independent experiments with 3 samples in each experiment. *P < 0.05, **P < 0.01 to NG. #P < 0.05 to HG. Values are mean ± SEM.
FIGURE 6. In primary cultures of rat Müller cells, Nrf2-responsive antioxidant genes are increased with dh404 treatment. Dh404 increased (A) HO-1 mRNA, (B) NQO1 mRNA, (C) HO-1 protein, and (D) NQO1 protein levels. (E) Nox1 mRNA, (F) Nox2 mRNA, (G) Nox4 mRNA, and (H) p22phox protein levels are reduced with dh404 treatment. β-actin is the loading control for HO-1, NQO1 and p22phox. *n = 3 independent experiments with 3 samples in each experiment. *P < 0.05, **P < 0.01 to NG. *#P < 0.05, **#P < 0.01, ###P < 0.001 to HG. Values are mean ± SEM.
ability to correct the imbalance in oxidative stress in DR. Supporting these findings, treatment with dh404 reduced the diabetes-induced increase in DHE and 8-OHdG labeling in Müller cells and also ganglion cells and photoreceptors. Importantly, although dh404 reduced excess ROS levels it did not completely abolish ROS in the retina of diabetic animals, which suggested that this treatment approach is likely to be retinoprotective yet allow sufficient ROS to be available to support normal cellular functions. Further, we acknowledge that we did not directly determine if dh404’s reduction of ROS was entirely due to the reduction of ROS derived from Nox. It should be noted that other retinal cell types, such as ganglion cells and photoreceptors, which may contribute to oxidative stress in DR and be responsive to dh404, were not studied, but rather we focused our attention on Müller cells due to their key role in the retinal pathology that occurs in DR.

Our in vitro findings strongly indicated that dh404 boosts antioxidant capacity and reduces oxidative stress in rats with DR. Using primary cultures we demonstrated that a major target for dh404’s correction of oxidative stress imbalance in hyperglycemia is the Müller cell. Indeed, in vitro, dh404 amplified the antioxidants HO-1 and NQO1, which are highly expressed in this cell type where they have a protective role. With respect to oxidative stress, previous studies have indicated that Müller cells increase their expression of Nox1 and Nox4, but not Nox2, in response to hypoxia. We demonstrated the same pattern of Nox expression in Müller cells exposed to hyperglycemia. Importantly, dh404 effectively reduced the hyperglycemia-induced amplified expression of p22phox as well as Nox1 and Nox4 in Müller cells, indicating that in diabetic animals dh404’s reduction in Nox2 is likely to be attributed to other retinal cell populations.

In conclusion, our study demonstrated that strategies to effectively boost the activation of Nrf2, such as dh404, reduces oxidative stress and repletes antioxidant pathways to result in retinoprotective effects in DR that include the prevention of damage to the neurovascular unit and particularly the injury to macroglial Müller cells.

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References


