Diabetes increases oxidative stress in the retina and in its capillary cells, and increased oxidative stress is considered as one of the major metabolic abnormalities associated with the development of diabetic retinopathy.\textsuperscript{1-8} Our previous work has shown that in addition to increase in reactive oxygen species (ROS) in the retina, the antioxidant defense system is also compromised in diabetes.\textsuperscript{5-8} The levels of the intracellular antioxidant, glutathione (GSH) become subnormal, and the enzymes responsible for glutathione redox cycle (glutathione peroxidase and glutathione reductase) and biosynthesis/degradation (gamma-glutamyl transpeptidase) are compromised.\textsuperscript{5-9} To overcome oxidative stress, the cell is also equipped with a redox sensitive transcription factor, NF-E2-related factor 2 (Nrf2), and Nrf2 antioxidant response pathway is considered as one of the major cellular defenses against the cytotoxic effects of oxidative stress.\textsuperscript{10,11} Nrf2 is constitutively expressed in all tissues, and depending on the extent of detoxification the organ is required to do, its levels may vary.\textsuperscript{12} In the retina, Nrf2 is shown to act as a cytoprotective mechanism in response to ischemia-reperfusion injury.\textsuperscript{13} Nrf2 is retained in the cytosol by its binding to a cluster of proteins, including its cytosolic inhibitor, Kelch like-ECH-associated protein 1 (Keap1), but under oxidative stress, it dissociates from Keap1 and moves to the nucleus to bind with the antioxidant-response element (ARE) to regulate transcription of antioxidant genes.\textsuperscript{14,15} The role of Nrf2 in the development of diabetic retinopathy remains to be explored.

The Nrf2-signaling pathway activates the transcription of a number of genes important in protection against oxidative stress, including GSH biosynthesis,\textsuperscript{10} and glutamate cysteine ligase (GCL) is a rate-limiting enzyme in biosynthesis of GSH.\textsuperscript{16,17} The enzyme has catalytic (GCLC) and modifier (GCLM) subunits, and Nrf2 is considered a key transcription factor for the regulation of GCLC.\textsuperscript{18,19} We have shown that the gene transcripts of GCLC are decreased in the retina in diabetes.\textsuperscript{20} How diabetes affects Nrf2-mediated regulation of GCLC remains unclear.

The goal of this study is to understand the role of the Nrf2-Keap1-GCLC-GSH signaling pathway in the development of diabetic retinopathy. Using rat retina, we have investigated the effect of diabetes on Nrf2-Keap1-GCLC pathway, subcellular localization of Nrf2, and the binding of Nrf2 with Keap1 and at the GCLC enhancer. To further strengthen the significance of our results in the development of diabetic retinopathy, experiments were confirmed in isolated retinal endothelial cells, the cells that are the site of histopathology associated with diabetic retinopathy, exposed to high glucose, and also in the retina from human donors with diabetic retinopathy.
METHODS

Rats

Diabetes was induced in male Wistar rats by streptozotocin injection, and the rats were maintained diabetic for 6 to 8 months. At the end of the experiment, retina from one eye was crosslinked with 1% paraformaldehyde and the other retina was stored in liquid nitrogen for future analysis. The severity of hyperglycemia, as evidenced by glycated hemoglobin (GHB) and average body weight, was significantly different in diabetic rats (GHB ~11% and BW ~351 ± 47 g) compared with their age-matched normal control rats (GHB ~5.5% and BW ~502 ± 37 g). Treatment of animals conformed to the Association for Research in Vision and Ophthalmology’s Resolution on Treatment of Animals in Research, and was approved by the institutional guidelines.

Retinal Endothelial Cells

Bovine retinal endothelial cells (BRECs) were isolated from calf eyes, and cells from the fourth to seventh passage (~80% confluence) were incubated in normal (5 mM) or high (20 mM) glucose, as routinely performed in our laboratory. To further confirm the effect of Nrf2 on GCLC, cells were preincubated with a highly effective antioxidant, 15 μM tert-butylhydroquinone (tBHQ; Sigma-Aldrich, St. Louis, MO) for 24 hours before incubating with 5 mM or 20 mM glucose. The final concentration of dimethyl sulfoxide (DMSO), used to dissolve tBHQ, was <0.001% in the incubation medium. Parallel solvent (DMSO) and osmotic (20 mM mannitol) controls were included in each experiment.

To investigate the effect of silencing Keap1 on Nrf2-mediated signaling, the cells from the fourth and fifth passage were transfected with Keap1-siRNA using commercially available transfection reagent (sc-29528; Santa Cruz Biotechnology, Santa Cruz, CA) as previously reported by us, and confirmed by qPCR using gene and species specific primers. Controls included nontargeting scrambled RNA and transfection reagent, transfection controls.

Human Retina

Human postmortem eyes, enucleated within 6 to 8 hours after death, were obtained from the Midwest Eye Banks (Ann Arbor, MI). Donors with documented proliferative retinopathy had diabetes for 10 to 30 years (Table 1). Retina from age-matched nondiabetic donors was used as transfection controls.

Subcellular Fractionation

Nuclear fraction was prepared from retina or BRECs using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) as reported previously. Cytosolic fraction was prepared by gently homogenizing the retina or BRECs in 20 mM HEPES buffer (pH 7.5) containing 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 250 mM sucrose. The homogenate was centrifuged at 750g for 10 minutes at 4°C to remove nuclei and cell debris, and the supernatant was centrifuged at 100,000g for 90 minutes to obtain the cytosol. Protein was determined by the bicinchoninic acid assay (Sigma-Aldrich).

Gene Expression

Expression of genes was determined by SYBR green based quantitative PCR (q-PCR) using a commercial PCR system (7500 Real-Time PCR System; Applied Biosystems, Foster City, CA). Chromatin immunoprecipitated (ChIP) purified DNA or cDNA was amplified using the primers listed in Table 2. PCR conditions included denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 60 seconds. This was followed by 95°C for 15 seconds, 60°C for 60 seconds, 95°C for 15 seconds, and 60°C for 15 seconds for dissociation. The specific products were confirmed by SYBR green single melting curve and a single-correct-size product on a 1.2% agarose gel. Samples were measured in duplicate. Values in each cDNA were normalized to the Ct value from β-actin in the same sample, and values in each ChIP purified DNA were normalized to the Ct value from the input sample. Relative fold changes were calculated by setting the mean fraction of normal samples (normal rat retina or BRECs in 5 mM glucose) as one.

Protein Expression

Retina or BRECs were homogenized in 30 mM Tris-HCl buffer (pH 7.5) containing 2 mM EGTA, 1 mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM sodium fluorescein (NaF), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate (Na₃VO₄). The samples were centrifuged at 750g for 5 minutes to remove the cell debris. Protein (30–40 μg) was separated on a 4% to 20% gradient polyacrylamide gel, blotted onto membranes, blocked, and incubated with the primary antibody against the protein of interest. Loading controls included β-actin for total homogenate and histone H3 for nuclear samples. The band intensity was quantified using digitizing software (Un-Scan-It Gel; Silk Scientific, Orem, UT).

Nrf2-DNA Binding Activity

The activation of Nrf2 was investigated by quantifying the binding of Nrf2 to ARE using a binding kit (TransAM Nrf2 DNA; Active Motif), following the manufacturer’s protocol. In brief, nuclear protein (5–20 μg) was incubated for 1 hour in a 96-well

### Table 1. Human Donors

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Duration of Diabetes, y</th>
<th>Cause of Death</th>
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<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>Intracranial hemorrhage</td>
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<tr>
<td>2</td>
<td>70</td>
<td>Cerebrovascular accident</td>
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<td>3</td>
<td>72</td>
<td>Myocardial infarction</td>
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<td>4</td>
<td>55</td>
<td>Subarachnoid hemorrhage</td>
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<tr>
<td>5</td>
<td>77</td>
<td>Myocardial infarction</td>
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<tr>
<td>6</td>
<td>57</td>
<td>Myocardial infarction</td>
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<tr>
<td>7</td>
<td>70</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>Pulmonary edema</td>
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<tr>
<td>2</td>
<td>61</td>
<td>Acute myocardial Infarction</td>
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<tr>
<td>7</td>
<td>59</td>
<td>Renal failure</td>
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<tr>
<td>8</td>
<td>67</td>
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</table>
plate coated with ARE sequence oligonucleotide. The bound
Nrf2 was captured by anti-Nrf2 antibody and developed using
secondary antibody and the developing buffer. The resultant
color was measured spectrophotometrically at 450 nm/655
nm.

GSH levels were quantified by an enzymatic recycling
method using a GSH Assay Kit (catalog # 703002; Cayman
Chemical, Ann Arbor, MI). Protein (3–5 µg) was deproteinized
by phosphoric acid, and GSH was measured in the supernatant
after its pH was neutralized with triethanolamine.27,28

Binding of Nrf2 with Keap1 was determined by immuno-
precipitating Keap1 in the homogenate or Nrf2 in the cytosol
for 15 minutes, permeabilized with 0.2% Triton X-100 for 10
minutes, and blocked in 1% BSA for 1 hour. The cells were then
incubated with antirabbit-keap1 and antimouse-Nrf2 simulta-
nuously for 2 hours, and rinsed with 0.1% Tween 20 in PBS.
This was followed by incubation with antirabbit-DyLight 488
(green) and antimouse-Texas red (red; Vector Lab, Burlingame,
CA) for 1 hour, washing with PBS, and mounting with DAPI-
containing mounting media (blue). The slides were imaged
with commercial microscopy (Zeiss ApoTome; Carl Zeiss, Jena,
Germany) using ×40 magnification.21,22

Table 2. Primer Sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<td>GCLC; NM_012815.2</td>
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<td>Reverse: TCATCACCCTGGCAAACAGTC</td>
<td>1795–1776</td>
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<td>Forward: CTTCTTGAGACGCCCATGACT</td>
<td>549–570</td>
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<tr>
<td></td>
<td>Reverse: CTTGGCTGGAGACAGCTGGTAT</td>
<td>694–673</td>
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<td></td>
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<tr>
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<td>957–976</td>
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<tr>
<td></td>
<td>Reverse: CATCTGACTCTGTGTCTGTG</td>
<td>1171–1152</td>
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<tr>
<td>Bovine</td>
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<td></td>
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<td></td>
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<td></td>
<td>Reverse: CTTGGCTCTCAGATGCTGGTGG</td>
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<td>1008–1027</td>
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<td></td>
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<tr>
<td>Human</td>
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<td>Reverse: TCTCTTGGCTTGGGCCCTCTGCG</td>
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</table>

Statistical Analysis

The data are expressed as mean ± SD, and this allowed us to
show the variability within a group. Statistical analysis was
carried out using commercial statistical software (SigmaStat;
Systat Software, Inc., Chicago, IL). The Kolmogorov-Smirnov
test was used to test if the data were normally distributed.
For multiple group comparision, one-way ANOVA followed by
Student-Newman-Keuls test was used for data with normal
distribution, while Kruskal-Wallis one-way analysis followed
by Dunn’s test was performed for data that did not present normal
distribution. Keap1 mRNA in BRECs, and Nrf2 mRNA and Nrf2
at GCLC-ARE4 in rat were analyzed by Dunn’s test, while all
other data were analyzed by Student-Newman-Keuls test, and
P < 0.05 was considered as statistically significant.

Results

Retina From Diabetic Rats Have Impaired Nrf2-
Keap1-GCLC Signaling Pathway

The gene transcripts of Nrf2 and its total protein expression
were significantly increased in the retina from diabetic rats
compared with normal rats (Figs. 1a, 1b), but the levels of Nrf2...
in the nuclear fraction were decreased by 40% compared with
the values obtained from age-matched normal rats (Fig. 1c).
Consistent with a decrease in nuclear Nrf2, the DNA binding
activity of Nrf2 was also subnormal in diabetes ($P < 0.05$
versus normal; Fig. 1d).

To understand the mechanism responsible for decreased
nuclear Nrf2, Keap1 and its interactions with Nrf2 were
investigated. As shown in Figures 2a and 2b, gene and protein
expressions of Keap1 were significantly increased in the retina
from diabetic rats compared with those from normal control
rats. Although the binding of Keap1 with Nrf2 was similar in
the retinal homogenate from normal rats and diabetic rats (Fig.
2c), it was increased by over 2-fold in the cytosolic fraction of the
diabetic rat retina ($P < 0.05$ versus normal; Fig. 2d).

To investigate if this decreased activity has any effect on the
binding of Nrf2 at the enhancer of $GCLC$-$ARE4$, the region
considered to have a strong transcription activation function,\textsuperscript{18} Nrf2 was immunoprecipitated in the cross-linked DNA
complex and the DNA for $GCLC$-$ARE4$ was quantified by SYBR
green-based q-PCR. As shown in Figure 3a, the binding of Nrf2
at $ARE4$ was decreased by 90% in diabetes. Normal rabbit IgG, used as ChIP control, yielded DNA less than
10% compared with the values from Nrf2 antibody, which was
consistent with our previous studies.\textsuperscript{26,30,31} Decreased binding

of Nrf2 at $ARE4$ was accompanied by a significant decrease in
$GCLC$ expression (mRNA and protein; Figs. 3b, 3c).

**High Glucose Impairs Nrf2-Keap1-GLC Pathway
in Retinal Endothelial Cells**

To further confirm the role of Nrf2 in the development of
diabetic retinopathy, Nrf2-GLC pathway was quantified in
isolated retinal endothelial cells. Exposure of BRECs to high
glucose increased gene expression of Nrf2 by 1.5-fold, but its
nuclear expression and the DNA binding activity were
decreased by 50% to 65% compared with the cells exposed
to normal glucose (Figs. 4a–c). However, the cells incubated in
20 mM mannitol instead of 20 mM glucose did not present any
increase in Nrf2 mRNA nor any decrease in its binding activity,
suggesting that the effect of high glucose on Nrf2 was not due
to an increase in the osmolarity.

As with the retina from diabetic rats, high glucose increased
Keap1 gene expression by over 3-fold and its protein
expression by 30% compared with the values obtained from
cells in normal glucose (Figs. 5a, 5b). Immunofluorescence
results showed that Nrf2 was mainly in the nucleus, but under
high glucose conditions, its cytosolic expression was in-

\textsuperscript{18}P < 0.05 compared with the values obtained from normal rats.
creased, and this was accompanied by increased colocalization of Nrf2 and Keap1 in the cytosol (Fig. 5c).

Consistent with decreased DNA binding activity, Nrf2 binding at GCLC-ARE4 and the gene transcripts of GCLC were also decreased by over 60% in the cells exposed to high glucose (Figs. 6a, 6b). The decreased GCLC was accompanied by reduction in GSH levels by 20% compared with the cells incubated in normal glucose (Fig. 6c).

To confirm the direct regulation of Nrf2 on GCLC, the cells were treated with an Nrf2 activity inducer, tBHQ. Pretreatment with tBHQ prevented a high glucose–induced decrease in Nrf2 activity (Fig. 4c) and GCLC gene transcripts (Fig. 6b). However, preincubation of cells with DMSO, instead of tBHQ had no effect on a glucose-induced decrease in Nrf2 activity and GCLC gene transcripts.

To further confirm the role of Nrf2-Keap1 signaling in subnormal antioxidant defense system, the effect of Keap1-siRNA on GCLC was determined. As shown in Figure 4b, transfection of retinal endothelial cells with Keap1-siRNA prevented a glucose-induced decrease in Nrf2 accumulation in the nucleus, and this was accomplished by amelioration of decrease in GCLC expression (Fig. 6b). In contrast, transfection with scramble RNA did not help Nrf2 movement into the nucleus, and also failed to prevent decrease in GCLC expression induced by high glucose insult.

**Figure 3.** Binding of Nrf2 at GCLC-ARE4. (a) Nrf2 binding at GCLC-ARE4 was measured by ChIP technique in which the precipitated DNA was amplified for ARE4 region of the GCLC gene (~3701 to ~3898) by SYBR green-based q-PCR. GCLC (b) mRNA and (c) protein expressions were quantified by PCR array (PRAN-087) and by Western blot technique, respectively, using β-actin as internal/loading control. Data are represented as mean ± SD from four to six rats in each group, and values obtained from normal rat retina are considered as 1 for mRNA or 100% for protein expression. *P < 0.05 compared with the values obtained from normal rats.

**Figure 4.** High glucose and Nrf2 in retinal endothelial cells. BRECs were incubated in 5 mM or 20 mM glucose for 4 days with or without pretreatment with tBHQ for 24 hours. (a) Nrf2 gene transcript was measured by q-PCR using β-actin as a housekeeping gene. (b) Nrf2 expression was quantified in the nuclear fraction by Western blot technique, and histone H3 was used as a loading control. (c) Nrf2 activity was measured in the nuclear fraction using TransAM Nrf2 DNA binding kit. 5 and 20, cells incubated with 5 mM or 20 mM glucose for 4 days, respectively; si-K and SC, cells transfected with Keap1-siRNA or scramble RNA, respectively, followed by incubation in 20 mM glucose for 4 days; 20+tBHQ, cells pretreated with 15 μM tBHQ for 24 hours followed by incubation in 20 mM glucose for 4 days; mann, cells incubated in 20 mM mannitol instead of 20 mM glucose. Data are presented as mean ± SD from three to four preparations in each group with the values obtained from cells incubated in 5 mM glucose are adjusted to 1 for mRNA or 100% for protein expression and activity. *P < 0.05 and #P < 0.05 compared with the values obtained from the untransfected cells incubated in 5 mM glucose and 20 mM glucose, respectively.

**Human Donors With Diabetic Retinopathy Have Subnormal Retinal Nrf2-GCLC Singing**

As with the retina from diabetic rodents and retinal endothelial cells exposed to high glucose, Nrf2 transcript was increased by ~5-fold and its protein by 35% in the retina from human donors with diabetic retinopathy compared with the retina from nondiabetic donors (Figs. 7a, 7b). In the same diabetic retinopathy donor eyes, retinal gene and protein expressions of GCLC were significantly decreased (Figs. 7c, 7d). Unlike rat retina, GCLC in human retina showed as a doublet; the reason for this discrepancy is not clear, but could include the specificity of the antibody.

**DISCUSSION**

Diabetes increases ROS levels in the retina, compromises the antioxidant defense enzymes, and attenuates the levels of intracellular antioxidant, GSH, creating an environment with increased oxidative stress. In addition to GSH, the cell is equipped with another very efficient antioxidant defense system, Nrf2, and this redox-sensitive Nrf2 plays a key role in...
Nrf2 and Diabetic Retinopathy

Effect of high glucose on Keap1 levels and its interactions with Nrf2 in retinal endothelial cells. Keap1 (a) gene and (b) protein expressions were measured by q-PCR and Western blot, respectively, using β-actin as an internal control. (c) Colocalization of Nrf2 and Keap1 was detected by immunofluorescent microscopy using Texas red-conjugated Nrf2 antibody (red) and DyLight 488-conjugated Keap1 antibody (green). The coverslips were mounted using DAPI-containing mounting medium (blue). The images are representative of three or more different experiments. *P < 0.05 compared to the cells incubated in 5 mM glucose.

but despite increased production, it fails to reach the nucleus to augment the transcription machinery.

Nrf2 is anchored in the cytoplasm via its binding to Keap1, and to combat stress Nrf2 dissociates from Keap1 and translocates to the nucleus. Keap1 has 25 cysteine residues and here we show that in diabetes—although the overall expression of Nrf2 is increased in the retina—its DNA-binding activity and nuclear levels are decreased, possibly because of increased binding of cytosolic Nrf2 with Keap1. The binding of Nrf2 at the enhancer region of GCLC, which has high transcription activation function, is decreased, resulting in subnormal GSH levels. These results suggest an important role of Nrf2-GCLC-GSH signaling in the maintenance of retinal redox status in the development of diabetic retinopathy. The role of Nrf2-Keap1-GCLC-GSH in diabetic retinopathy is further supported by our data from isolated retinal endothelial cells showing similar glucose-induced abnormalities, which can be modulated by regulating Keap1; and also the retina from human donors with diabetic retinopathy presenting abnormalities in Nrf2-mediated signaling.

In normal conditions, Nrf2 is dormant within the cell and its transcriptional activities are quiescent, but when it is released under stress conditions, it moves from the cytosol to the nucleus to bind with ARE to promote the transcription of genes important in antioxidant defense. Here we show that in diabetes, despite increase in Nrf2 expression, its levels in the nucleus and the DNA binding activity are subnormal. This suggests that increased oxidative stress created by the diabetic environment signals the cell to produce more Nrf2, but despite increased production, it fails to reach the nucleus to augment the transcription machinery.

Nrf2 is anchored in the cytoplasm via its binding to Keap1, and to combat stress Nrf2 dissociates from Keap1 and translocates to the nucleus. Keap1 has 25 cysteine residues and the modification of Cys-151 aids the dissociation of Nrf2 from Keap1 preventing the degradation of Nrf2, and allowing it to translocate to the nucleus. Here we show that the expression of Keap1 in the retina is increased in diabetes, but the nuclear levels of Nrf2 are decreased. In addition, the binding of Nrf2 to Keap1 is also increased; however, the transcriptional activity of Nrf2 remains subnormal. The possible reason for this disparity could be that due to increased oxidative stress in diabetes, the redox-sensing capacity of Keap1 is altered hindering the dissociation of Nrf2 from the Keap1-Nrf2 complex. In support, despite age-associated increase in oxidative stress in the liver, decrease in nuclear Nrf2 levels are observed. The possibility that diabetes increases posttranslational modifications of Nrf2 and/or Keap1 altering the binding of Nrf2 with Keap1, however, cannot be ruled out. In agreement, diabetes is shown to favor nitrination, ribosylation and other posttranslational modifications of a number of retinal proteins. The other possibilities could include diabetic conditions epigenetically modifying Keap1 and/or Nrf2, resulting in altered expressions. Consistent with this, diabetes has been shown to epigenetically modify lens Keap1 and favor epigenetic modifications in many retinal proteins and transcriptional factors, including manganese superoxide dismutase, matrix metalloproteinase-9, and nuclear transcription factor B.
Nrf2 and Diabetic Retinopathy

The data from human donors with diabetic retinopathy showing increased retinal Nrf2 expression, but decreased GCLC levels compared with their age-matched nondiabetic counterparts further strengthen our hypothesis that Nrf2-GCLC signaling has a major role in the development of diabetic retinopathy.

Thus, we have provided strong evidence showing that Nrf2-Keap1-GCLC-GSH signaling has an important role in the development of diabetic retinopathy. These results raise the possibility that the regulation of Nrf2-Keap1 by pharmacological or molecular means could serve as a potential adjunct therapy to combat oxidative stress and protect diabetic patient from vision loss.

Acknowledgments

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Disclosure: Q. Zhong, None; M. Mishra, None; R.A. Kowluru, None

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