Epigenetic Modifications of Keap1 Regulate Its Interaction With the Protective Factor Nrf2 in the Development of Diabetic Retinopathy

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PURPOSE. Diabetes induces oxidative imbalance in the retina and impairs Nrf2-mediated antioxidant response, and elevates Keap1, the cytoplasmic repressor of Nrf2. The goal of this study was to understand the role of epigenetic modifications at Keap1 promoter in regulation of Nrf2 function.

METHODS. The effect of high glucose on the binding of transcriptional factor Sp1 at Keap1 promoter and histone methylation status of the promoter was investigated in retinal endothelial cells. Role of histone methylation was confirmed in cells transfected with siRNA of methyltransferase enzyme Set7/9 (SetD7). In vitro results were confirmed in the retina from streptozotocin-induced diabetic rats. The role of epigenetic modifications of Keap1 promoter in the metabolic memory was examined in rats maintained in poor control for 3 months followed by good control for 3 months.

RESULTS. Hyperglycemia increased the binding of Sp1 at Keap1 promoter, and enriched H3K4me1 and activated Set7D. SetD7-siRNA prevented increase in Sp1 binding at Keap1 promoter and Keap1 expression, and ameliorated decrease in Nrf2-regulated antioxidant genes. Cessation of hyperglycemia failed to attenuate increased binding of Sp1 at Keap1, and the promoter continued to be methylated with increased expression of Keap1 and decreased expression of Nrf2-regulated genes.

CONCLUSIONS. Epigenetic modifications at Keap1 promoter by SetD7 facilitate its binding with Sp1, increasing its expression. Keap1 restrains Nrf2 in the cytosol, impairing its transcriptional activity. Reversal of hyperglycemia fails to provide any benefit to epigenetic modifications of Keap1 promoter, suggesting their role in both the development of diabetic retinopathy and the metabolic memory phenomenon.

Keywords: diabetic retinopathy, epigenetic modifications, Keap1, Nrf2, metabolic memory
epigenetic modifications play any role in regulating retinal Keap1 expression remains elusive.

Clinical and experimental studies have demonstrated that the progression of diabetic retinopathy does not halt after termination of hyperglycemic insult, and the disease continues to progress beyond the point when good control is achieved, suggesting a “metabolic memory” phenomenon. Recent studies have shown the role of epigenetic modifications in the metabolic memory associated with microvascular complications of diabetes, including retinopathy and nephropathy. Histones at retinal Gele-ARE/I continue to be modified and Nrf2 transcriptional activity compromised even after normal glycemia is maintained for 3 months after a period of 3 months of hyperglycemia. The role of epigenetic modifications of Keap1 promoter in the continued inhibition of Nrf2 activity remains to be determined.

The goal of this study was to understand the role of epigenetic modifications in the regulation of Nrf2 in diabetic retinopathy. Using retinal endothelial cells, the effect of high glucose on the regulation of Keap1 transcription was investigated by evaluating the binding of Sp1 at its promoter, and histone methylation status of the promoter. To understand the mechanism responsible for altered histone methylation in Keap1 transcription, the effect of genetic regulation of the histone methyltransferase enzyme Set7/9 (SetD7) on Sp1 binding and methylation status of lysine 4 of histone 3 (H3K4) at Keap1 promoter was investigated. To transition in vitro results to an in vivo setting, modifications at Keap1 promoter were also quantified in the retina from diabetic rats, and effect of reversal of glycemic insult on Sp1 binding and histone methylation was evaluated, and the results were confirmed in the retina from human donors with documented diabetic retinopathy.

**METHODS**

Retinal endothelial cells, prepared from bovine retina (BRECs), were incubated in normal (5 mM) or high (20 mM) glucose for 4 days or in high glucose for 4 days, followed by normal glucose for 4 additional days (20-5). For transfection, the transfection complex containing SetD7-siRNA, transfection reagent, and transfection medium (Santa Cruz Biotechnology, Paso Robles, CA, USA) were used. After transfection, the cells were rinsed twice with PBS, and incubated in normal or high glucose media for 4 days. The transfection efficiency, as determined by quantifying the protein and gene expressions of SetD7, was approximately 50% (Figs. 1a, 1b). Cells incubated with scramble RNA served as transfection control, and incubated in 20 mM mannitol as osmotic control. These procedures are routinely performed in our laboratory.

Male Wistar rats, obtained from Harlan Laboratories (South Easton, MA, USA), were made diabetic by streptozotocin, and then allow establishment of diabetes (blood glucose > 250 mg/dL, approximately 3 days after induction of diabetes) were either maintained in poor glycemic control (PC, glycated hemoglobin GHb approximately 12%) for 6 months, or maintained in good glycemic control (GC, GHb approximately 6%) for 6 months, or maintained in control for 3 months followed by control for good control for 3 additional months (Rev group), as reported by us. These procedures are in compliance with the Declaration of Helsinki, and conformed to the ARVO Resolution on Treatment of Animals.

Human retina was isolated from the eye globes enucleated within 6 to 8 hours after death (obtained from Midwest Eye Banks, Ann Arbor, MI, USA) from donors (45–75 years of age) with diabetes for 10 to 20 years and established retinopathy. For controls, nondiabetic donors (45–75 years of age) were used. Retina from these donors has been used in our laboratory.

Chromatin immunoprecipitation (ChIP) was performed in the protein-DNA complex isolated from cross-linked retina or cells by immunoprecipititating Sp1 (sc-56774; Santa Cruz Biotechnology) or H3K4me1 or Nrf2 using their specific antibodies (ab8895 or ab89443 respectively; Abcam, Cambridge, MA, USA). DNA fragments recovered by phenol-chloroform-isoomyl alcohol extraction were ethanol precipitated, and resuspended in nuclease free water.

The Sp1 binding region of the Keap1 promoter was quantified by SYBR green-based real-time quantitative PCR (qPCR) using the 7500 PCR System (Applied Biosystems, Foster City, CA, USA). In human retina, semiquantitative PCR was performed to quantify the binding of Sp1 or level of H3K4me1 at Keap1 promoter. Controls included rabbit anti-IgG (ab64940; Abcam) and DNA from the input.

Gene expression was quantified by SYBR green-based qPCR and the specific products were confirmed by SYBR green single melting curve using species-specific primers (Table). Relative fold changes were calculated by setting the mean values from normal rat retina or cells incubated in 5 mM glucose as one.

Protein expression was determined by Western blot technique using protein-specific antibodies (SetD7, sc-56774 and Keap1, sc-365626; Santa Cruz Biotechnology), and β-actin (A5441; Sigma-Aldrich Corp., St. Louis, MO, USA) was used as a loading control.

Activity of SetD7 methyltransferase, which methylates H3K4, was quantified using the SetD7 Fluorescent Assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Nuclear protein (15-25 μg) was incubated with the assay mixture for 20 minutes, and H2O2 production was measured at 540 nm excitation and 590 nm emission wavelengths.

Statistical analysis was performed using SigmaStat (San Jose, CA, USA), and the results are presented as mean ± SD. The data presenting normal distribution were analyzed by ANOVA followed by Bonferroni’s test and, for the data that did not present normal distribution, Kruskal-Wallis one-way analysis followed by Dunn’s was performed; P < 0.05 was considered statistically significant.
RESULTS

Retinal Endothelial Cells

Hyperglycemia decreases the transcriptional activity of Nrf2 and increases Keap1 expression in the retina and its endothelial cells.\textsuperscript{7} Keap1 transcription is considered to be regulated by the transcriptional factor Sp1.\textsuperscript{14} To examine the role of Sp1 in increased Keap1 expression in diabetes, the binding of Sp1 at Keap1 promoter was evaluated. Figure 2a shows that high glucose increased the binding of Sp1 at Keap1 by more than approximately 2-fold compared with the cells incubated in normal glucose; however, normal rabbit IgG control had less than 2% binding. In the same cell preparations, as expected, Keap1 gene expression was also increased by more than 2-fold (Fig. 2b).

Because methylation of H3K4 favors binding of the transcription factor,\textsuperscript{31} to understand the mechanism responsible for increased binding of Sp1 at Keap1, histone methylation at Sp1 binding region of the Keap1 promoter was investigated. The Keap1 promoter showed increased H3K4me1 by high glucose compared with cells in normal glucose. But, in contrast, 20 mM mannitol did not alter H3K4me1 levels, and the values obtained from cells in normal glucose were not different from those obtained in cells in mannitol (Fig. 3a). As a control, normal rabbit IgG yielded values that were less than 1% compared with those obtained from H3K4me1 antibody (Fig. 3b).

Because SetD7 mediates monomethylation of H3K4,\textsuperscript{32} we quantified the effect of glucose on SetD7 in endothelial cells. Gene transcripts and methyltransferase activity of SetD7 were elevated by approximately 2-fold in high glucose compared with normal glucose (Figs. 4a, 4b).

Regulation of SetD7

To specifically investigate the role of histone methylation in Keap1 transcription, SetD7-siRNA transfected cells were used. Regulation of SetD7 prevented glucose-induced increase in Sp1 binding at Keap1, but transfection with scramble RNA had no beneficial effect on Sp1 binding (Fig. 2a). In the same cell

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Figure 2. High glucose increases the binding of Sp1 at Keap1 promoter and elevates its expression. Untransfected cells, or cells transfected with SetD7-siRNA or scramble RNA, were incubated in 20 mM glucose for 4 days, or untransfected cells incubated in 20 mM glucose for 4 days followed by 5 mM glucose for 4 additional days (20-5) were analyzed for (a) Sp1 binding at Keap1 by ChIP technique. The q-PCR value in each immunoprecipitate was normalized to the Ct value from the input sample by using the delta-delta-Ct (ddCt) method. Rabbit IgG (\textsuperscript{*}) was used as a negative antibody control. (b) Keap1 mRNA was quantified by q-PCR using β-actin as a housekeeping gene. Set-si and SC, cells transfected with SetD7-siRNA or scramble RNA, respectively, and incubated in 20 mM glucose for 4 days. Data are represented as mean ± SD from four to six preparations in each group. *P < 0.05 compared with 5 mM glucose and #P < 0.05 versus high glucose.
Figure 3. Enrichment of H3K4me1 at Keap1 promoter impairs Nrf2 signaling. (a) H3K4me1 at Keap1 was quantified by ChIP technique using rabbit IgG as negative antibody control (^), and (b) the products were confirmed by agarose gel electrophoresis. (c) Nrf2 binding at Gclc promoter was determined by ChIP technique, and mRNA levels of (d) Gclc and (e) HO1 were quantified by q-PCR. Data are mean ± SD from three to four preparations in each group. *P < 0.05 and #P < 0.05 compared with 5 mM glucose and 20 mM glucose, respectively.
and promoter and decreased expressions of Gclc mRNA also was significantly different from each other (Figs. 3c–e). The values from the cells transfected with scramble RNA (Figs. 3a, 3b). This was accompanied by amelioration of glucose-induced decreased binding of Nrf2 at Gclc promoter and decreased expressions of Nrf2-regulated Gclc and heme oxygenase1 (HO1). The values obtained from the cells transfected with SetD7 or untransfected cells, incubated in high glucose medium, were significantly different from each other (Figs. 3c–e).

**Rat Retina**

Consistent with the results from retinal endothelial cells, Sp1 binding at retinal Keap1 promoter was increased by more than 2-fold in diabetic rat retina compared with normal rats (Fig. 5a). In the same samples, mRNA and protein expressions of Keap1 were also increased by more than 1.5-fold (Figs. 5b, 5c). Similarly, Keap1 promoter had increased H3K4me1 levels, and the ChIP antibody negative control (normal rabbit IgG) yielded values that were less than 1% compared with those obtained from Keap1 antibody (Figs. 6a, 6b). In the same samples, SetD7 expression and its methyltransferase activity were also significantly elevated in diabetes (Figs. 7a, 7b).

**Human Retina**

To validate these findings in diabetic patients with retinopathy, Sp1 binding at KEAP1 promoter was determined in the retina from human donors. Figure 8a shows increased SP1 binding at KEAP1 promoter in the diabetic donors compared with their age-matched nondiabetic donors, and, as expected, there was no change in the input samples. This was accompanied by increased expressions of KEAP1 and SETD7 in diabetic retinopathy donors (Figs. 8b, 8c).

**Metabolic Memory**

Retina continues to experience oxidative stress even after hyperglycemic insult is terminated. To understand the role of epigenetic modifications of Keap1 promoter in the metabolic memory phenomenon, Sp1 binding and H3K4me1 at the Keap1 promoter were quantified in the retina of rats maintained in poor glycemic control for 3 months followed by good glycemic control for 3 additional months. Figures 5a–c show that even after termination of hyperglycemic insult, the binding of Sp1 at Keap1 promoter remained high with increased mRNA and protein expressions of Keap1, and this was accompanied by continued increased H3K4me1 at Keap1 promoter (Fig. 6). In the same rats, reversal of hyperglycemia also failed to ameliorate diabetes-induced increase in SetD7 expression and methyltransferase activity (Fig. 7). The values obtained from rats in the PC-Rev group were not different from the rats in the PC group, but were significantly higher than those obtained from ones that remained normal throughout the experiment (P < 0.05). Maintenance of good glycemia, soon after induction of diabetes in rats (GC group), prevented diabetes-induced increase in Keap1 expression and H3K4me1 at its promoter (Figs. 5b, 6). The values obtained from the GC group were significantly different from those in PC or PC-Rev groups (P < 0.05), but were not different from the normal group (P > 0.05).

Consistent with the results from rat retina, glucose-induced increase in Sp1 binding and H3K4me1 at Keap1 remained elevated with increased Keap1 and SetD7 in the endothelial cells exposed to normal glucose for 4 days, which had followed 4 days of high glucose (20-5 group). The binding of Nrf2 at Gclc promoter remained compromised with decreased levels of the cytoprotective genes that are under the control of Nrf2, Gclc, and HO1 (Figs. 2–4). The values from the cells in continuous high glucose were not different from the cells in the 20-5 group.

**DISCUSSION**

The transcriptional activity of the protective factor Nrf2 and its binding at the ARE4 are decreased in the retina in diabetes, and the expression of its downstream target gene, Gclc, becomes subnormal. Here, we show that the binding of the transcriptional factor Sp1 at Keap1 promoter is significantly increased in diabetes, and the reason for this appears to be the methylation of H3K4 due to activation of SetD7. However, regulation of increase in SetD7 by its siRNA prevents increase in Sp1 binding at Keap1 promoter and Keap1 expression, and ameliorates decrease in the Nrf2-regulated genes, Gclc and
In addition to the epigenetic modifications of the Keap1 promoter in the development of diabetic retinopathy, the results also show the role of these modifications in the metabolic memory phenomenon. Cessation of high glucose insult fails to attenuate increased binding of Sp1 at Keap1, and the promoter continues to be methylated with increased expression of Keap1 and decreased expression of Nrf2-regulated defense genes. The results clearly suggest the role of epigenetic modification in the regulation of retinal antioxidant status in the development of retinopathy, and in the metabolic memory phenomenon associated with its progression.

Keap1, an intracellular inhibitor of Nrf2, is one of the primary regulators of Nrf2, and its cysteine-rich Kelch/DGR domain maintains its interaction with Nrf2. Under stress conditions, critical cysteine residues within Keap1, especially Cys151, is covalently modified and loses its ability to ubiquitinate Nrf2, and Nrf2 becomes free to move into the nucleus. Sp1, a ubiquitously expressed transcription factor, acts as an essential transcriptional activator of Keap1 by binding at its promoter. We have shown that the expression of Keap1 is increased in diabetes, and here we show that the possible mechanism of increased Keap1 expression could be the increased binding of Sp1 at its promoter. In addition, we also present similar increase in the retina of human donors with diabetic retinopathy, further strengthening the role of increased Keap1-Nrf2 in the regulation of retinal Nrf2 activity.

**Figure 5.** Diabetes increases the binding of Sp1 at Keap1 promoter. Retina from rats in poor glycemic control for 3 months followed by good glycemic control for 3 months (PC-Rev), or continuous PC or GC for 6 months was analyzed for (a) Sp1 binding at Keap1 using ChIP assay. Keap1 (b) mRNA was quantified by q-PCR using β-actin as a housekeeping gene, and (c) its protein expression by Western blotting technique and β-actin was used as a loading control. Results are represented as mean ± SD from five to six rats in each group, each analysis performed in duplicate: *P < 0.05 versus normal and #P < 0.05 versus PC rats.
Because the binding of the transcription factor at the promoter of a gene is regulated by epigenetic modifications, including histones methylation/acetylation and DNA methylation, and diabetes epigenetically modifies the transcriptional binding sites of a number of retinal genes, the role of histone methylation in the increased binding of Sp1 at Keap1 promoter was evaluated. H3K4me1 is significantly enriched at the Keap1 promoter in the retina. In support, H3K4me1 enrichment at the promoter regions is generally associated with transcriptional activation, and decreased H3K4me1 levels at retinal Sod2 are implicated with the suppression of its expression in diabetes.

Lysine 4 of histone 3 can be mono-, di-, or trimethylated, and depending on the methylation status, it can act as a transcriptional activator or repressor. Our recent study has shown that although H3K4me2 is increased at Gclc-ARE4 in the retina in diabetes, H3K4me3 and H3K4me1 are decreased, resulting in impaired binding of Nrf2 at Gclc-ARE4. Here we show that the activity of SetD7 is increased in diabetes, and regulation of SetD7 by its specific siRNA, in addition to ameliorating increase in H3K4me1 at Keap1 promoter, also prevents increased binding of Sp1 at the Keap1 promoter and Keap1 expression. Histone methylation is carried out by methyltransferases, and SetD7, a monomethyltransferase, methylates H3K4 and other proteins. Consistent with our results showing increased SetD7, this enzyme has been shown to regulate NF-κB-dependent inflammatory genes in diabetic nephropathy, and NF-κB is activated in the retina in diabetes. As Nrf2 binding with Keap1 regulates the movement of Nrf2 to the nucleus and controls the transcriptional activity of Nrf2, to further validate the role of histone methylation in Nrf2 signaling, we show that SetD7-siRNA also regulates the expressions of Gclc and HO1, the genes that are under the control of transcriptional activity of Nrf2. These results clearly suggest that epigenetic modifications at Keap1 promoter play important role in the regulating Nrf2-mediated ARE genes.

Intensive glycemic control, after a period of hyperglycemia, does not halt the progression of diabetic retinopathy. Metabolic memory phenomenon is also observed in animal models and in vitro models of diabetic retinopathy, and persistent hyperglycemia-driven changes in many genes associated with diabetic complications, including retinopathy, have been identified. As epigenetic modifications can persist in a system, recent comprehensive epigenomic profiling on the cells from subsets of The Diabetes Control and Complications Trial and Follow-up Epidemiology of Diabetes Interventions and Complications Study participants with varied complications have shown that epigenetics could be playing an important role in the further progression of complications during EDIC. In animal models, good control, after a period of poor control, fails to provide any benefit to the histopathology associated with diabetic retinopathy.
does not remove epigenetic modifications in the retinal genes. H3K4me2 continues to be increased and H3K4me3 and H3K4me1 decreased at Gclc-ARE4, and Nrf2 activity compromised after hyperglycemic insult is terminated. Here, we show that H3K4me1 enrichment and Sp1 binding remain increased, and Keap1 continues to be elevated. Furthermore, SetD7 continues to be active even after hyperglycemia is replaced by normal glycemia, confirming the role of epigenetic modifications in continuing increased oxidative stress that the retina experience after hyperglycemia is terminated. In support, transient glucose-induced SetD7-mediated H3K4me1 enrichment is associated with the persistent transcriptional activation inflammatory genes in human and bovine vascular endothelial cells. However, if good glycemic control is initiated soon after induction of diabetes, consistent with the retina escaping epigenetic modifications of Sod2, and accelerated apoptosis and histopathology, the binding of Sp1 and H3K4me1 enrichment at Keap1 promoter also do not increase.

In conclusion, we have presented results showing that epigenetic modifications at Keap1 promoter by SetD7 facilitate Sp1 binding with Keap1, increasing its expression, and increased Keap1 restrains Nrf2 in the cytosol, impairing the transcriptional activity of Nrf2. Reversal of hyperglycemia provides no benefit to Keap1 and its histone modifications, suggesting their role in both the development of diabetic retinopathy and in the metabolic memory phenomenon.

Figure 8. Retinal KEAP1 promoter is epigenetically modified in the human donors with diabetic retinopathy. (a) SP1 binding at KEAP1 was measured by ChIP technique by amplifying for the SP1 binding region at KEAP1 promoter in protein-DNA complex using normal rabbit IgG (^) as antibody control. (b) KEAP1 and (c) SETD7 mRNA levels were quantified by qPCR using β-actin as a housekeeping gene. The values obtained from nondiabetic donors were considered as 1. Diab, donors with documented diabetic retinopathy; Norm, age-matched nondiabetic donors. Data are presented as mean ± SD from four donors in each group. *P < 0.05 versus nondiabetic donors.
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References


