Epigenetic Modification of *Sod2* in the Development of Diabetic Retinopathy and in the Metabolic Memory: Role of Histone Methylation

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PURPOSE. Mitochondrial superoxide levels are elevated in the retina in diabetes, and their scavenging enzyme, MnSOD, becomes subnormal. The objective of this study is to investigate the role of histone methylation of *Sod2*, the gene that encodes MnSOD, in the development of diabetic retinopathy and in the metabolic memory phenomenon associated with its continued progression after termination of hyperglycemia.

METHODS. Effect of high glucose on monomethyl H3K4 (H3K4me1), dimethyl H3K4 (H3K4me2), and lysine-specific demethylase-1 (LSD1) was quantified at *Sod2* by chromatin immunoprecipitation in isolated retinal endothelial cells. The role of histone methylation in the metabolic memory phenomenon was investigated in the retina of rats maintained in poor glycemic control (PC, approximately 12% glycated hemoglobin [GHb]) for 3 months followed by in good glycemic control (GC, approximately 6% GHb) for 3 months.

RESULTS. Hyperglycemia reduced H3K4me1 and -me2, and increased the binding of LSD1 and Sp1 at *Sod2*. Regulation of LSD1 by LSD1-siRNA ameliorated glucose-induced decrease in H3K4 methylation at *Sod2*, and prevented decrease in *Sod2* gene expression. In rats, re-institution of GC failed to reverse decrease in H3K4me1 and -me2 at *Sod2*, and LSD1 remained active with increased binding of LSD1 and Sp1 at *Sod2*. Retina from human donors with diabetic retinopathy also had decreased H3K4me2 and increased LSD1 at *Sod2*.

CONCLUSIONS. Histone methylation of retinal *Sod2* has an important role in the development of diabetic retinopathy and in the metabolic memory phenomenon associated with its continued progression. Targeting enzymes important for histone methylation may serve as a potential therapy to halt the development of diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2013;54:244–250) DOI:10.1167/iops.12-10854

Diabetes increases oxidative stress, and increased oxidative stress is considered as an important factor in the development of its microvascular complications, including retinopathy.^{1–4} Our studies have shown that retinal mito-

chondrial dysfunction has a crucial role in the apoptosis of capillary cells, a phenomenon that precedes the development of retinal histopathology characteristic of diabetic retinopathy.^{5–7} The enzyme responsible for scavenging mitochondrial superoxide, MnSOD, is inhibited,^{3,6} and overexpression of MnSOD protects retinal mitochondria dysfunction, capillary cell apoptosis, and the development of diabetic retinopathy in mice.^{8,9}

Regulation of gene transcription is associated with chromatin structure and remodeling.^{10–12} Expression of *Sod2*, the gene that encodes MnSOD, is coordinated by upstream promoter and intron-2 enhancer,^{13,14} and post-translational modifications of histone tails are associated with regulation of *Sod2* in chronic diseases, for example diabetes and cancer.^{15,16} We have shown that in the development of diabetic retinopathy, methylation of lysine 20 of histone H4 (H4K20), and acetylation of lysine 9 of histone H3 (H3K9) at the promoter and enhancer regions of retinal *Sod2* are increased, and SUV4-20h2, the enzyme important for trimethylation of H4K20, is elevated.¹⁶ Histone methylation is a complex process, and the lysine residues can be mono-, di-, and trimethylated. The position of the lysine residue on the histones and the degree of methylation of lysine exert different biologic outcomes; for example, methylation of lysine 4 of histone 3 (H3K4) is involved in the transcriptional activation, while H3K9 and H4K20 methylation is involved with gene repression.^{17–19} Methylation process is regulated by a balance between histone methylating and demethylating enzymes, and the removal of methyl groups from methylated H3K4 by a lysine-specific histone demethylase-1 (LSD1) is shown to induce transcriptional repression, while that from methylated H3K9 in activation.²⁰ The effect of diabetes on the methylation status of H3K4 in retinal *Sod2* remains unclear.

Epidemiologic and prospective studies have shown that diabetic retinopathy continues beyond the point when good glycemic control has been achieved, and have suggested a “metabolic memory” phenomenon.^{21,22} Recent studies have suggested an important role of epigenetic modifications in the metabolic memory phenomenon associated with the continued progression of diabetic complications after the removal of hyperglycemic insult.^{16,23–26} We have shown that retinal MnSOD continues to be inactive and H4K20me3 at *Sod2* elevated even when good glycemic control is instituted in diabetic rats.^{16,27,28}

The aim of our study was to investigate the role of H3K4 methylation in the regulation of retinal *Sod2* in the development of diabetic retinopathy, and also in the metabolic memory associated with its failure to halt after re-establishment of normal glycemic control. The specific role of LSD1 in histone methylation was investigated in retinal endothelial cells in which LSD1 was regulated by its specific si-RNA. Histone

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TABLE 1. Severity of Hyperglycemia in Rats

	Duration, mo	Body Weight, g	GHb, %
Normal (12)	6	431 ± 37	5.5 ± 0.5
PC (14)	6	335 ± 54*	12.2 ± 2.3*
PC (11) → GC	3 → 3	300 ± 35* → 459 ± 29	11.1 ± 0.9* → 5.6 ± 1.2
GC (13)	6	445 ± 14	6.6 ± 0.9

The values are means ± SD, and the numbers in parentheses represent number of rats in each group.

methylation status of *Sod2* also was confirmed in the retina from human donors with diabetic retinopathy.

METHODS

Endothelial Cells

Bovine retinal endothelial cells (BRECs) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal calf serum (heat inactivated), 5% replacement serum (Nu-serum; BD Bioscience, San Jose, CA), heparin (50 µg/mL), endothelial growth supplement (50 µg/mL; BD Bioscience), and antibiotic/antimycotic in an environment of 95% O₂ and 5% CO₂.^{6,7,16,29} The cells from the fourth to fifth passage were transfected with LSD1-siRNA using transfection reagent sc-29528 from Santa Cruz Biotechnology (Santa Cruz, CA) as performed routinely in our laboratory.^{16,29} Briefly, the transfection complex containing LSD1-siRNA, transfection reagent, and transfection medium (sc-36868; Santa Cruz Biotechnology) was pre-incubated for 45 minutes at room temperature, followed by the incubation of cells with this transfection complex for 8 hours at 37°C. Parallel incubations were done using nontargeting scrambled RNA as control. After transfection, the cells were rinsed with PBS, and incubated in 5 or 20 mM glucose medium for 4 days. Cells incubated in 20 mM mannitol, instead of 20 mM glucose, served as osmotic control.

Rats

Rats (male, Wistar), soon after the induction of diabetes with streptozotocin, were divided into three groups. Rats in group 1 were maintained in poor glycemic control (PC, glycated hemoglobin [GHb] approximately 12%) for 6 months, while in group 2 the rats were maintained in PC for 3 months followed by good glycemic control (GC,

GHb approximately 6%) for another 3 months (Rev group), and in group 3 the rats remained in GC for the entire 6 months (Table 1). GC was maintained by administering insulin two times per day (total of 5–7 international units [IU]/day). These methods were in compliance with the Declaration of Helsinki and are used routinely in our laboratory.^{16,25,27,28} The animal procedures conformed to the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the Wayne State University's Institutional Animal Care and Use Committee.

Human Retina

Retina was isolated from eyes of human donors with clinically documented diabetic retinopathy (obtained from Midwest Eye Banks, Ann Arbor, MI), and the eye globes were enucleated within 8 hours of death. These methods are routinely used in our laboratory.^{30,31} As shown in Table 2, average age of the diabetic donor was 63.1 ± 9.8 years, with a range of 10 to 30 years for diabetes, and majority of the donors were treated with laser therapy. Age-matched nondiabetic donors (63.6 ± 11.8 years) served as controls.

Chromatin Immunoprecipitation (ChIP)

Protein-DNA complex (100–120 µg) was immunoprecipitated with the antibody against H3K4me1, H3K4me2, LSD1, Sp1, or rabbit normal IgG (Abcam, Cambridge, MA). DNA fragments were recovered by phenol-chloroform-isoamyl alcohol extraction, followed by ethanol precipitation, and then resuspended in 14 µL water for PCR. Promoter and enhancer regions of *Sod2* were quantified by Sybr green-based real-time quantitative PCR (q-PCR) using the 7500 real-time PCR System (Applied Biosystem, Foster City, CA). Normal rabbit IgG was used as negative antibody control and DNA from the input (20–40 µg protein-

TABLE 2. Human Donors

	Age, y	Duration of Diabetes, y	Cause of Death	Laser Therapy
Nondiabetic donors				
1	44	–	Intracranial hemorrhage	
2	70	–	Cerebrovascular accident	
3	72	–	Myocardial infarction	
4	55	–	Subarachnoid hemorrhage	
5	77	–	Myocardial infarction	
6	57	–	Myocardial infarction	
7	70	–	Myocardial infarction	
Donors with diabetic retinopathy				
1	75	25	Pulmonary edema	+
2	61	10	Acute myocardial infarction	+
3	47	27	Acute myocardial infarction	+
4	59	25	Pneumonia	+
5	68	10	Congestive heart failure	+
6	66	30	Acute myocardial infarction	+
7	54	10	Unknown	
8	75	30	Acute myocardial infarction	+

Diabetic donors had clinically documented retinopathy.

TABLE 3. PCR Primers

	Primer Sequence
Rat	
<i>Sod2</i> promoter	Forward CCAGGAATGGAAAAGGAGTG Reverse CTTGTAACAGAGCGGCACAC
<i>Sod2</i> enhancer	Forward CTGGGAAACGGGTTGAGTAA Reverse TACCACAGCCTTCCCAAATC
<i>Sod2</i> off target	Forward CAGGCCCGTGGTGGGTGTTT Reverse TCCCCACAGGCCCAACACA
<i>LSD1</i>	Forward ACGCAAGCGGGCGAAGGTAG Reverse ACGGCTCCTCGGGCTCACTT
β -actin	Forward CCTCTATGCCAACACAGTGC Reverse CATCGTACTCCTGCTTGCTG
Bovine	
<i>Sod2</i> promoter	Forward GAGCCGTACCATCTCTTTTCG Reverse CTTCCTCACTCCGGGGAAAT
<i>Sod2</i> enhancer	Forward AGCCTGTGTATGCAACCATCT Reverse CCCCCTAATTATGTGTCCAG
β -actin	Forward CGCCATGGATGATGATATTGC Reverse AAGCCGGCCTTGACAT
Human	
<i>Sod2</i> promoter	Forward ATACGGGTTGGAAGGGCGCTG Reverse TGAGTTTTGGTTGCGCTGCCG
<i>Sod2</i> enhancer	Forward CACCTCTGCTAGGTCCTTCATCAGT Reverse GGCTGAAGAGCTATCTGGGCTGT
<i>Sod2</i>	Forward CCTGCTCCCCGCGCTTTCTT Reverse CGGGGAGGCTGTGCTTCTGC
<i>LSD1</i>	Forward GCAGCCACCAGCCGTCAGT Reverse TCACCCCTGCTGGCAGTCGT
β -actin	Forward AGCCTCGCCTTTGCCGATCCG Reverse TCTCTTGCTCTGGGCCTCGTCG

DNA complex) as an internal control. Rat *Sod2* off target region (+5511–5790 base pairs [bp]) served as a negative target control for promoter and enhancer. Each ChIP measurement was made with 5 to 6 samples per group, as used routinely in our laboratory.^{16,29}

Gene Expression

Gene expressions were confirmed by q-PCR. Amplification was performed in 1 μ L of ChIP purified DNA or cDNA using the primers provided in Table 3. PCR conditions included denaturation at 95°C for 10 minutes, followed by 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. The specific products were confirmed by Sybr green single melting curve and a single correct-size product when running in 2% agarose gel. Values in each immunoprecipitate were normalized to the Ct value from the input sample, and those in cDNA were normalized to the Ct value from β -actin in the same sample using the ddCt method. Relative fold changes were calculated by setting the mean fraction of normal or 5 mM glucose as one. Each sample was measured in duplicate.^{16,25,27,29} Transcripts of *Sod2* and *LSD1* in BRECs were measured by q-PCR using Taqman primers and *18s rRNA* as a housekeeping gene.

To confirm the specificity of ChIP assay, semiquantitative PCR was run for rat *Sod2* promoter occupied by H3K4me2. ChIP with normal rabbit IgG served as a negative antibody control, and off target region as target control. The PCR was run by denaturing for 2 minutes at 95°C, followed by 30 cycles of denaturation for 45 seconds at 92°C, annealing for 1 minute at 60°C, and extension for 45 seconds at 72°C. PCR

FIGURE 1. Effect of high glucose exposure of retinal endothelial cells on the gene expression of *Sod2*, and H3K4me1 and H3K4me2 at *Sod2*. Retinal endothelial cells, untransfected, or transfected with LSD1-siRNA or scramble RNA, were incubated in 5 or 20 mM glucose for 4 days. (a) Gene expression of *Sod2* was measured by real time-PCR using Taqman primers and *18s rRNA* as a housekeeping gene. (b) H3K4me1 and (c) H3K4me2 at *Sod2* promoter and enhancer were quantified by ChIP technique. IgG was used as a negative antibody control for ChIP and is labeled as Δ . The qPCR value in each immunoprecipitate was normalized to the input samples using the ddCt method. 5 and 20 indicate cells incubated in 5 or 20 mM glucose, respectively. 20+si and 20+SC indicate cells incubated in 20 mM glucose after being transfected with LSD1-siRNA or scramble RNA, respectively. 5+si indicates LSD1-siRNA transfected cells incubated in 5 mM glucose. Data are represented as mean $\pm$ SD from 4 or more experiments, each experiment performed in duplicate. * $P < 0.05$ and # $P < 0.05$ compared to the values obtained from untransfected cells incubated in 5 or 20 mM glucose, respectively.

products were visualized on a 1.2% agarose gel¹⁶ and imaged by Benchtop 3UV Transilluminator (UVP, Inc., Upland, CA).

Western Blotting

Proteins were separated on a 4% to 20% gradient polyacrylamide gel. The band intensity was quantified using Un-Scan-It Gel digitizing software (Silk Scientific, Orem, UT).

LSD1 Activity

LSD1 activity was quantified in the nuclear fraction prepared from rat retina using a chemiluminescent assay kit (BPS Bioscience, San Diego, CA). De-methylated H3 was captured using specific antibody, and developed by HRP-labeled secondary antibody.

Statistical Analysis

Results are presented as mean $\pm$ SD, and were analyzed using SigmaStat. One way ANOVA followed by Bonferroni's test was used for

FIGURE 2. Effect of high glucose on LSD1 in retinal endothelial cells. (a) *LSD1* gene expression was quantified by using its Taqman primers and *18s rRNA* as a housekeeping gene. (b) LSD1 protein expression was measured by Western blot technique. 5 and 20 indicate cells incubated in 5 or 20 mM glucose respectively. 20+si and 20+SC indicate cells transfected with LSD1-siRNA or scramble RNA, respectively, and incubated in 20 mM glucose. 5+si indicates LSD1-siRNA transfected cells incubated in 5 mM glucose. Mann indicates cells incubated in 20 mM mannitol. Values are mean $\pm$ SD from 4 to 6 measurements in each group. * $P < 0.05$ and # $P < 0.05$ compared to the values from untransfected cells incubated in 5 or 20 mM glucose, respectively.

data with normal distribution, while Kruskal-Wallis one way analysis followed by Dunn's were used for data that did not present normal distribution. $P < 0.05$ was considered statistically significant.

RESULTS

Retinal Endothelial Cells

High glucose, as expected, decreased the gene transcripts of *Sod2* by over 50% (Fig. 1a). This was accompanied by 40% decrease in H3K4me1, and 60% to 80% decrease in H3K4me2 at the promoter and the enhancer of *Sod2* compared to the values obtained from the cells incubated in normal glucose (Figs. 1b, 1c). IgG (ChIP control) yielded less than 10% of the ChIP antibody (Figs. 1b, 1c) and this is consistent with our previous results.¹⁶ Due to limitation of the sample, IgG-ChIP control for H3K4me2, however, was performed only at the enhancer of *Sod2*. The validity of the ChIP assay was confirmed further by using a positive control, transcription factor IIB (TFIIB) at β -actin promoter and, as reported previously,¹⁶ the occupancy of TFIIB at β -actin promoter was similar in the cells incubated in normal or high glucose.

To investigate the role of lysine-specific demethylation enzyme in hypomethylation of methylated H3K4 in *Sod2* gene regulation, LSD1 was regulated by its specific siRNA. Transfection of cells with LSD1-siRNA ameliorated glucose-induced decrease in *Sod2* gene transcripts, and this was accompanied by

FIGURE 3. Effect of diabetes on the occupancy of H3K4me1, H3K4me2, LSD1, and Sp1 at retinal *Sod2*. Protein-DNA complex was immunoprecipitated with the antibody against (a) H3K4me1, (b) H3K4me2, (c) LSD1, and (d) Sp1 or normal IgG (marked as*), and the promoter and the enhancer regions of *Sod2* were amplified and quantified by Sybr green-based q-PCR. (e) The specificity of ChIP was verified using semiquantitative PCR. Crosslinked retina was immunoprecipitated with anti-H3K4me2 or normal rabbit IgG, and the purified precipitated DNA was amplified for *Sod2* promoter (within -500+1 bp) and off target region (exon 5, +5511+5790bp). Each ChIP measurement was made with 5 to 6 samples/group. N, normal; PC, rats in poor glycemic control for 6 months; Rev, rats in poor control for 3 months followed by good glycemic control for 3 months; GC, rats in good control for 6 months. Data are represented as mean $\pm$ SD. from 5 to 6 rats in each group, * $P < 0.05$ compared to normal rats. Input-*Sod2* promoter represents the *Sod2* promoter region abundance in the DNA before it was subjected to ChIP.

amelioration of decrease in H3K4me1 at *Sod2* promoter and enhancer, and in H3K4me2 at the promoter only (Figs. 1a-c). The values obtained from the untransfected cells incubated in 20 mM glucose were significantly higher compared to the cells transfected with LSD1-siRNA. However, transfection of cells with scramble RNA did not prevent the damaging effects of high glucose, and the values obtained were significantly different from those obtained from cells transfected with LSD1-siRNA. Figure 2 is included to show that the transfection of cells with LSD1-siRNA prevents glucose-induced increase in its mRNA and protein levels; the values obtained from cells transfected with scramble RNA were significantly different from those transfect-

FIGURE 4. Effect of diabetes on retinal LSD1. (a) Rat retina nuclear fraction (5 to 10 μ g), prepared by Nuclear Extract kit (Active Motif, Carlsbad, CA), was used to measure LSD1 activity by LSD1 Chemiluminescent assay kit. The values obtained from normal rat retina are considered as 100%. (b) Gene and (c) protein expressions of LSD1 were measured by q-PCR (Sybr green) and by Western blot technique, respectively, using β -actin as internal control. Data are represented as mean $\pm$ SD from 5 to 6 rats in each group, * P < 0.05 compared to the values obtained from normal control rats.

ed with LSD1-siRNA. Incubation of cells in 20 mM mannitol, instead of 20 mM glucose, had no effect on LSD1 expression.

Diabetic Rats

In the retina of rats diabetic for 3 months, H3K4me1 was decreased by 40% at *Sod2* enhancer, but it did not change at its promoter (Fig. 3a). In contrast, H3K4me2 was decreased significantly at *Sod2* promoter, but was unchanged at its enhancer (Fig. 3b). Consistent with the binding of p65 of NF- κ B,¹⁶ the binding of LSD1 and Sp1 at *Sod2* promoter also was increased significantly compared to the values obtained from age-matched normal rats (Figs. 3c, 3d). Normal rabbit IgG, used as ChIP control, resulted in less than 10% of the precipitated DNA by ChIP antibodies (Fig. 3). To verify the specificity of the ChIP assay further, the DNA from ChIP with anti H3K4me2 was amplified for an off target region (located at exon 5, +5511–+5790 bp). As shown in Figure 3c, diabetes decreased H3K4me2 at *Sod2* promoter, and the occupancy of H3K4me2 at the off target was negligible compared to its enrichment at the promoter, but input DNA, the DNA before ChIP, had almost equal abundance of the *Sod2* promoter in the retina from normal and diabetic rats. Furthermore, products produced by IgG were almost negligible in the retina from normal and diabetic rats.

In the same diabetic rats, LSD1 enzyme activity was increased by 50% and its expression (gene and protein) was elevated by over 2-fold compared to the values obtained from age-matched normal rats (Figs. 4a–c).

Metabolic Memory Phenomenon

Our recent work has shown that epigenetic modifications of *Sod2* have an important role in the metabolic memory phenomenon associated with the continued progression of

FIGURE 5. *Sod2* and *LSD1* gene expressions and H3K4me2 at *Sod2* in the retina from patients with diabetic retinopathy. (a) *Sod2* gene expression by q-PCR, (b) H3K4me2 at *Sod2* promoter and enhancer by ChIP technique, and (c) *LSD1* gene expression by q-PCR were analyzed in the retina from donors with diabetes for over 10 years with clinically documented diabetic retinopathy, and from age-matched nondiabetic donors. Nor, nondiabetic donors; Dia, donors with diabetic retinopathy. Values are represented as mean $\pm$ SD from 4 to 6 donors in each group. * P < 0.05 compared to the values from nondiabetic donors.

diabetic retinopathy after reversal of hyperglycemia.¹⁶ Results presented in Figures 3a and 3b show that the re-institution of normal glycemia for 3 months after 3 months of poor glycemia did not provide any benefit to the decreased levels of H3K4me1 and H3K4me2 at *Sod2*. Similarly, the binding of LSD1 and Sp1 at *Sod2* remained elevated (Figs. 3c, 3d). This was accompanied by continued activation of LSD1 in the retina of rats in the Rev group (Fig. 4). Although the values obtained from the rats in the Rev group were significantly different from those in normal group, they were not different from those in the PC group. However, when GC was initiated soon after the induction of diabetes, H3K4me1 and H3K4me2 at *Sod2* were not significantly different from normal, and the activity and expression of LSD1 also were similar to those observed in normal rats (Figs. 3, 4).

Retina from Human Donors

Consistent with the results from rat retina and endothelial cells, gene expression of retinal *Sod2* was decreased by 40% in the donors with diabetic retinopathy compared to age-matched nondiabetic donors (Fig. 5a). In addition, in the same retina samples, promoter and enhancer regions of *Sod2* had decreased H3K4me2 (Fig. 5b), and this was accompanied by increased *LSD1* mRNA levels (Fig. 5c).

DISCUSSION

In the development of diabetic retinopathy, superoxide levels are elevated in the retina, antioxidant defense system is compromised, MnSOD is inhibited, and mitochondria are swollen and dysfunctional.^{7,8,16,28,30} Overexpression of MnSOD protects diabetes-induced mitochondrial damage and the development of retinopathy.^{6,8,9} Our recent work has shown that *Sod2* is modified epigenetically in diabetes, and methyl H4K20 and acetyl H3K9 are increased at the promoter and enhancer regions of *Sod2*.¹⁶ Since multiple lysine residues at histones can be modified, and these modifications can produce divergent effects, we showed here that the methylation of

H3K4 at *Sod2* is decreased in diabetes, and the possible mechanism for such decrease is the activation of the LSD1.

Lysine methylation is associated with distinct transcriptional states depending on which lysine residue is modified. Lysine on histones can be mono-, di-, or trimethylated, and the position of the lysine residue and the degree of methylation could have different biologic associations. For example, methylation of H3K9 represses chromatin conformation, but that of H3K4 opens up chromatin structure where transcription becomes active.^{17,32,33} We showed that the exposure of retinal capillary cells to high glucose decreases H3K4 methylation at *Sod2* promoter and enhancer regions, suggesting the role of H3K4 methylation in *Sod2* repression. In support, we have shown decreased retinal *Sod2* in diabetes,¹⁶ and others have shown dynamic modulation of histone methylase and demethylases in aortic endothelial cells.³³

Histone H3K4 methylation and LSD1 are considered as essential epigenetic targets in cancer cells,³⁴ and LSD1-mediated histone demethylation is associated with hypertension and enhanced vascular contraction.³⁵ LSD1 specifically demethylates mono- or dimethylated H3K4 and H3K9, and removal of methyl groups from H3K4 is linked with transcriptional repression.²⁰ High glucose exposure of human aortic microvascular endothelial cells, in addition to increasing methylation of H3K4 and hypomethylation of H3K9, also increases the recruitment of LSD1 at the promoter of p65 subunit of NF- κ B, and this is considered as one of the mechanisms associated with the activation of NF- κ B.³³ We showed that demethylation of retinal H3K4 at *Sod2* in hyperglycemia is accompanied by increased recruitment of LSD1 at *Sod2* promoter. These data strongly suggested that the demethylation of H3K4 by LSD1 has an important role in the downregulation of *Sod2*. Consistent with this, we showed that LSD1-siRNA protects demethylation of H3K4 at *Sod2*. In support, we have shown that the activity and gene expression of retinal MnSOD are decreased at 2 to 4 months of diabetes in rats, and these abnormalities continue for 10 to 12 months of diabetes.^{3,16,27} However, contrary to our results and those of Brasacchio et al.³³ showing increase in LSD1 recruitment at the promoters in hyperglycemic environment, others have shown reduced LSD1 occupancy at the promoters of the inflammatory genes in vascular smooth muscle cells from db/db mice.³⁶ The reason for such discrepancy is not clear, but could include variant response of different cell types and genes to the hyperglycemic insult, or differences in type I and type II diabetes models.

The promoter region of *Sod2* is considered essential for constitutive transcription, while the enhancer is to magnify promoter activity.^{14,37,38} The promoter region of *Sod2* gene is rich in GC and contains multiple binding sites for the transcription factors Sp-1 and AP-2, and binding of these factors directly to the CpG islands differently regulates promoter activity.³⁸ The intronic enhancer region of *Sod2* has an NF- κ B binding site, and is important in the induction of *Sod2*.¹⁴ Sp1 at the promoter and NF- κ B at the enhancer crosstalk by a connection protein, nucleophosmin, which binds to an 11-G single strand loop structure in the promoter, and this allows the promoter and the enhancer to regulate *Sod2* synergistically.³⁸ The data presented here demonstrate clearly that, despite increase in Sp1 binding at the promoter of *Sod2*, mRNA of *Sod2* remains subnormal in diabetes.¹⁶ One of the possible reasons could be that because of demethylation of methylated H3K4, the crosstalk between Sp1 at the promoter and NF- κ B at the enhancer of *Sod2* is affected. In addition, the repression of H4K20me3 and p50 of NF- κ B, and demethylation of H3K4me2 could alter the physical interactions and abolish the activation effects of Sp1. Consistent with this, our previous work has shown increased p65 at the promoter and enhancer of *Sod2* in diabetes, but only p50 at the enhancer of *Sod2*,¹⁶ and others

have shown that the activation of p50 inhibits the activation effects of p65.³⁹ Furthermore, data presented here also showed that high glucose decreases H3K4me1 and H3K4me2 at promoter and enhancer of *Sod2* in retinal endothelial cells, but in the retina, while H3K4me1 is decreased at the enhancer, it remains unchanged at the promoter. However, in contrast, H3K4me2 is decreased only at the promoter, but not at the enhancer. The reason for this discrepancy in the results from endothelial cells and the retina could be due to the species differences or the complexity of the retina structure where the methylation could be greater in capillaries compared to other cell type. In support of our results, different protein levels of H3K4me3, H3K4me2 are reported in oral squamous cell carcinoma, while H3K4me2 is increased, but H3K4me3 is decreased and H3K4me1 remains unchanged.⁴⁰ The possibility that decreased H3K4me1 at *Sod2* enhancer and H3K4me2 at its promoter in diabetes might be contributing synergistically to the compact chromatin structure by blocking the general transcription machinery cannot be ruled out.

Our in vitro and in vivo results showing hypomethylation of methyl H3K4 at retinal *Sod2* in hyperglycemic conditions are supported further by decreased H3K4me2 at *Sod2*, and also by decreased *Sod2* gene expression in the retina from human donors with diabetic retinopathy. These results strengthen further the role of H3K4 methylation in the regulation of *Sod2* in the development of diabetic retinopathy.

Epigenetic modifications have been implicated in the metabolic memory phenomenon associated with diabetic complications. Continued active transcriptional state of NF- κ B after removal of high glucose in aortic cells is linked with persisting methylation of H3K4 and hypomethylation of H3K9, possibly due to alterations in LSD1 and methyltransferases.³³ We have shown that re-institution of GC in rats after a period of PC does not benefit decrease in retinal *Sod2* and increase in mitochondrial superoxide levels, and *Sod2* continues to be modified epigenetically with increased H4K20me3, acetyl H3K9, and p65.¹⁶ Furthermore, the alterations in histone acetylating enzymes in the retina also persist after normal glycemia is re-instituted in diabetic rats.²⁵ We demonstrated that three months of GC after three months of PC does not benefit the methylation status of H3K4 at *Sod2*, and LSD1 continues to be active. These results suggest clearly that diabetes modifies multiple lysine residues at various histones at retinal *Sod2*, and these epigenetic modifications do not benefit from the reversal of hyperglycemic insult, thus confirming further the role of these epigenetic modifications in the failure of diabetic retinopathy to reverse. Consistent with this, we have shown that the re-institution of 6 months of GC that follows 6 months of PC in rats fails to provide any benefit to the histopathology associated with diabetic retinopathy, but the rats that are maintained in GC for the entire duration (GC group) showed little or no increase in capillary cell apoptosis and number of acellular capillaries compared to the age-matched normal control rats.^{27,41}

Our study provided a strong evidence that histone methylation, especially that of H3K4 at *Sod2*, has an important role in the development of diabetic retinopathy and in the metabolic memory phenomenon associated with its continued progression, and the methylation status of H3K4 is regulated by LSD1. Thus, this raises the possibility to use therapeutic modalities targeted toward regulation of methylation status of histones to prevent inhibition of MnSOD and protect mitochondrial damage.

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