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perceiving epigenetic marks that are specifically maintained when the endothelial cell is out of its previous hyperglycemic milieu.

**RESEARCH DESIGN AND METHODS**

**In vitro studies**

**Cell culture conditions and treatments.** Confluent bovine aortic endothelial cells were maintained in minimum essential medium (Gibco) containing 0.5% fetal bovine serum, nonessential amino acids (Gibco), and antibiotics Gentamicin (Pfizer). Cells were incubated with minimum essential medium with 5.5 mol/l low glucose or minimum essential medium with 30 mol/l high glucose or 30 mol/l mannitol for 16 h where stated.

**Set7 shRNA knockdown.** Human microvascular endothelial cells (HMECs) were infected with MISSION shRNA-expressing lentivectors targeted to Set7 coding regions according to the user instructions (Sigma). The sequence targeting Set7 corresponds to 5'-CCAGAATCTCATGAACTGAA-3' (TRCN0000076830). Cells transduced with MISSION Non-Target shRNA Control Vector were used as controls. HMECs were seeded at 5 x 10⁴ cells/dish in a 60-mm dish 20 h before infection, incubated with the lentivirus stock for 2 days, followed by selection in puromycin (1 μg/ml; Sigma) for 7 days. The cells were examined by protein blots using anti-Set7 rabbit antibody.

**Overexpression of Set7 in human endothelial cells.** HMECs expressing Set7 were created by retrovirus-mediated gene transfer as described previously (9). cDNAs encoding FLAG-tagged Set7 were inserted into the BamHI and NotI sites of the retroviral vector pCXneo (10) (a gift from Dr. T. Akagi, KAN Research Institute, Kobe, Japan) to create pJS14. pJS14 or pCXneo was co-transfected with retrovirus packaging vectors (Takara) into Akagi, KAN Research Institute, Kobe, Japan) to create pJS14. pJS14 or Set7 were created by retrovirus-mediated gene transfer as described previously (9).

**RNA isolation and first-strand cDNA synthesis.** Total RNA was extracted using Trizol reagent. Cells were subsequently treated with Turbo DNAse (Ambion). First-strand cDNA synthesis from the purified mRNA was performed using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions.

**RT-PCR.** PCR amplification was performed using ABI Prism 7500; 2 pmol of each forward and reverse primer was added to a total of 20 μl reaction containing 1× SYBR Green PCR Master Mix (Roche) and Rox Reference Dye (Invitrogen). Reactions were incubated for 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.

**In vivo studies**

**Animal model.** Male apolipoprotein knockout (apoE KO) mice (backcrossed 20 times to a C57BL/6 background; Animal Resource Centre, Canning Vale, WA, Australia) were housed at the Precinct Animal Centre, Baker IDI Heart and Diabetes Institute, and studied according to guidelines of the National Health and Medical Research Council of Australia. Mice were rendered diabetic via five daily intraperitoneal injections of 55 mg -kg⁻¹ -day⁻¹ streptozotocin (MP Biomedicals, Eschwege, Germany), resulting in a model of insulin deficiency (11). ApoE and apoE diabetic mice were monitored for 2 weeks. In over 90% of mice injected with these daily injections of streptozotocin, plasma glucose levels were >20 mmol/l within the first week of the study. In all injected mice, plasma glucose levels were measured every 2 weeks. This serial monitoring revealed that in a subgroup of mice (<20%), despite initially developing hyperglycemia, after ~10–12 weeks, these mice now had reduced plasma glucose levels (<15 mmol/l) despite no treatment, which remained decreased until the time of death at week 20. This group was termed “previously hyperglycemic” (HG—NG) and was compared to the control (NG—NG) and diabetic (HG—HG) mice. All these apoE KO mice received standard mouse food and water ad libitum. Mice were cycled by euthanasia using an intraperitoneal injection of Euthal (10 mg/kg) (Delvet Limited, Seven Hills, Australia) followed by exsanguination via cardiac puncture. The excised aortas were placed in 1% neutral buffered formalin and fixed for 2 days, followed by selection in Genetec (G418, 1 mg/ml; Invitrogen) for 7 days. The cells were then examined by protein blots using anti-FLAG M2 monoclonal antibody (Sigma) and anti-Set7 rabbit serum (a gift from Dr. P.L. Jones, University of Illinois at Urbana-Champaign) (7).

**Immunoprecipitation of FLAG-Set7.** Cell lysates were prepared from cells expressing FLAG-Set7 or from cells transduced with pCXneo as a control. The 1 x 10⁷ cells were washed twice with ice-cold PBS and extracted with 600 μl lysis buffer (50 mmol/l Tris HCl, pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, and proteinase inhibitor). Lysate was incubated with anti-FLAG M2 affinity gel (Sigma) for 2 h at 4°C. Immunoprecipitates were washed with Tris-buffered saline (50 mmol/l Tris HCl, pH 7.5, 150 mmol/l NaCl) three times and then eluted with 3 x 50 μl lysis buffer (Sigma) and analyzed by immunoblotting as well as measuring histone methyltransferase activity.

**Histone methyltransferase activity assay.** The histone methyltransferase activity assay was performed according to the manufacturer's (Upstate) instructions. One of the following substrates was used for methyltransferase reaction: 5 μg recombinant histone H3 (Upstate) or 0.4 nmol of a biotin-conjugated histone H3 peptide (amino acids 1–23), either K4 or R4 (Sigma). Reactions were determined by spotting on P-81 paper (Upstate) and scintillation counting.

**Chromatin immunoprecipitation.** Cells were treated with formaldehyde at a 1% concentration for 10 min and then treated with glycine at 0.15 mol/l for 10 min. Cell pellets were resuspended in SDS lysis containing 1% SDS, 10 mmol/l EDTA, 50 mmol/l Tris, pH 8.1 (Upstate), and a protease inhibitor cocktail (Roche). Cells were sonicated to shear chromatin to 700–300 bp in length, resuspended in chromatin immunoprecipitation dilution buffer containing 0.1% SDS, 1% Triton X-100, 1.2 mmol/l EDTA, 16.7 mmol/l Tris-HCl, pH 8.1, and 167 mmol/l NaCl, and 15 μl salmon sperm DNA-protein A agarose (Upstate) was added and precleared. To the soluble chromatin fraction, the following antibody of interest was added and incubated for a minimum of 8 h: H3K1m1 (Abcam ab8895), H3K4m2 (Upstate 07030), H3K4m3 (Upstate 07473), H3K4m4 (Abcam ab9045), H3K3m2 (Abcam ab7312–100), H3K3m3 (Abcam ab8888), Set7 (a gift from Dr. P. Jones, University of Illinois at Urbana-Champaign), LSD1 (Upstate 05539), and Suv39H1 clone MG14 (Upstate 05615). Immune complexes were collected with salmon sperm DNA-protein A agarose and subsequently washed and eluted with buffer (1% SDS, 0.1 mol/l HAcO). Protein-DNA cross-links were reversed overnight at 65°C and recovered by phenol-chloroform extraction and ethanol precipitated at ~−20°C. Analysis of chromatin immunoprecipitation DNA samples was performed by quantitative PCR (qPCR) of the nuclear factor (NF)-κB p65 promoter region (sequences available upon request). A 10 μl input sample was used as an internal control for variation in DNA between samples.

**RNA isolation and first-strand cDNA synthesis.** Total RNA was extracted using Trizol reagent. Cells were subsequently treated with Turbo DNAse (Ambion). First-strand cDNA synthesis from the purified mRNA was performed using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. The NFκB p65 gene was quantified using qRT-PCR.

**RT-PCR.** PCR amplification was performed using ABI Prism 7500; 2 pmol of each forward and reverse primer was added to a total of 20 μl reaction containing 1× SYBR Green PCR Master Mix (Roche) and Rox Reference Dye (Invitrogen). Reactions were incubated for 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.

**Statistical analysis.** Data are shown as means ± SE. One-way nonparametric ANOVA for repeated measures followed by Fisher’s post hoc test was used to compare treatment groups. Calculations were performed using GraphPad Prism (GraphPad Software).
RESULTS

Previous hyperglycemia is associated with persistent gene activity and H3K4m1. We recently demonstrated that transient hyperglycemia causes gene-activating H3K4m1 marks associated with NFκB-p65 mRNA expression (3). Despite these and other recent advances, understanding the complexity of H3K4 and H3K3 methylation remains an important challenge. We show that transient hyperglycemia induces long-lasting activation of the NFκB-p65 gene (Fig. 1A) and persistent changes in H3K4m1 (Fig. 1B), which were not seen for H3K4m2 (Fig. 1C) and H3K4m3 (Fig. 1D). The epigenetic changes persist for 6 days of subsequent normal glycemia. To differentiate the role of glucose in endothelial cells, we also measured the effect of transient hyperglycemia on the counteracting cytokine, interleukin (IL)-10. Expression of the IL-10 gene in bovine and human endothelial cells did not change after transient exposure to hyperglycemia (data not shown).

**Set7 mono-methylates H3K4 in human vascular cells.** Based on this specific change in H3K4m1, but not dimethylation or tri-methylation, we postulated that the Set7 enzyme, previously reported to act as a histone mono-methyltransferase, albeit in nonendothelial cells, might be involved (13). Indeed, because the crystal structure of Set7 (13) indicates specificity for this enzyme as a mono-methylase to its target K4 lysine of histone H3, we tested whether H3K4 is the unique methylation site of Set7 puriﬁed histone H34 and mutant H3R4 peptides. G: Aortic endothelial cells were exposed to glucose and soluble chromatin were immunopurified at the indicated times using H3K4m1 (LG) 16 h group, **P < 0.01 vs. LG 16 h group. H: The osmolyte mannitol does not increase NFκB-p65 mRNA levels. Samples were analyzed in triplicate, and data are presented as means ± SE. I: Mannitol does not increase Set7 association with the NFκB-p65 promoter. Samples were analyzed in triplicate, and data are presented as means ± SE.

**FIG. 1. Ambient and prior hyperglycemia sustains increased activating H3K4m1-associated Set7 enrichment on the NFκB-p65 gene in aortic endothelial cells.** A: Ambient and prior hyperglycemia sustains increased NFκB-p65 gene expression in bovine aortic endothelial cells. RNA was extracted, and NFκB-p65 mRNA levels were quantiﬁed by real-time RT-PCR (qRT-PCR) and normalized to the level of 18S. *P < 0.05 vs. low glucose (LG) 16 h group. Mono-methylation is the predominant H3K4 mark after transient hyperglycemia. Bovine aortic endothelial cells were exposed to glucose and soluble chromatin was immunopuriﬁed at the indicated times using H3K4m1 (B), H3K4m2 (C), and H3K4m3 (D) antibodies. qPCR was used to measure the level of enrichment on the NFκB-p65 promoter (~400 bp from the +1 transcription start site). Error bars represent SE. Samples were analyzed in triplicate, and data are presented as means ± SE. *P < 0.05 vs. LG 16 h group, **P < 0.01 vs. LG 16 h group. F: Lysine 4 of histone H3 (H3K4) is a major methylation site for the Set7 enzyme. Set7 was immunopuriﬁed by anti-FLAG antibody in overexpressed Set7 or control pCX4neo human endothelial cells. Substrates used to determine methyltransferase activity were synthesized histone H34 and mutant H3R4 peptides.
Set7 was assayed using H3K4 and H3R4 as substrate peptides (Fig. 1F). The results of these experiments indicate that Set7 has specific H3K4 methylation activity and mutation of the lysine residue to arginine (H3R4) eliminates the ability of the peptide to serve as a substrate for the Set7 histone methyltransferase.

**Glucose-induced changes in H3K4m1 are related to the recruitment of Set7.** Because Set7 appears to be the enzyme responsible for H3K4m1 in immortalized cancer cell types (14) and is now confirmed in endothelial cells (Fig. 1E and F), we subsequently tested the hypothesis that Set7 is mobilized to the NFκB-p65 promoter to maintain the active transcriptional state. Immunopurified chromatin from bovine aortic endothelial cells exposed to transient hyperglycemic conditions was associated with sustained enrichment for Set7 on the NFκB-p65 promoter ($P < 0.05$) in response to glucose (Fig. 1G). To exclude the possibility that the effects are a result of osmotic stress, cells were incubated in 30 mmol/L mannitol for 16 h and returned to normoglycemia for 2, 4, or 6 days. Analysis of NFκB-p65 gene expression showed no increase in the p65 transcript in response to mannitol stimulation (Fig. 1H). Chromatin immunoprecipitation analyses indicated no enrichment of the Set7 enzyme on the NFκB-p65 promoter consistent with persisting epigenetic marks being mediated by glucose (Fig. 1I).

**Set7 knockdown attenuates glucose-induced p65 gene activity.** Having established that Set7 has methyltransferase activity for H3K4 and specific recruitment to the NFκB-p65 gene, we next determined the functional consequence of knockdown of Set7 on epigenetic persistence of H3K4m1 marks and NFκB-p65 gene expression mediated by transient hyperglycemia. A protein blot of Set7 in wild-type and knockdown (Set7KD) cells indicated robust silencing by the shRNA to Set7 in endothelial cells (Fig. 2A). Transient hyperglycemia failed to induce NFκB-p65 gene expression in Set7KD cells (Fig. 2B). Parallel experiments using H3K4m2 antibody were also performed to illustrate the specificity of histone modification with no effect on H3K4m2 seen with Set7KD (Fig. 2D). We also assessed IL-10 gene expression in Set7KD and wild-type

**FIG. 2. Erasure of the activating H3K4m1 mark on the NFκB-p65 promoter in Set7 KD cells despite transient hyperglycemia.** A: Protein blot of Set7 knockdown in HMECs exposed to ambient and prior hyperglycemia for the indicated times. B: Ambient and prior hyperglycemia does not sustain increased NFκB-p65 gene expression in Set7 KD cells. RNA was extracted, and NFκB-p65 mRNA levels were quantified by real-time RT-PCR (qRT-PCR) and normalized to the level of 18s. C: Knockdown of the Set7 enzyme rescinds H3K4m1 mark on the NFκB-p65 promoter. Samples were analyzed in triplicate, and data are presented as means ± SE. *$P < 0.05$ vs. LG 16 h group. D: Set7 KD does not change the H3K4m2 mark on the NFκB-p65 promoter. HMECs were exposed to glucose, and soluble chromatin was immunopurified at the indicated times for SuV39h1 (E), H3K9m1 (F), H3K9m2 (G), H3K9m3 (H), and LSD1 (I) antibodies. qPCR was used to measure the level of enrichment on the NFκB-p65 promoter. Error bars represent SE. Samples were analyzed in triplicate, and data are presented as means ± SE. *$P < 0.05$ vs. LG 16 h group.
cells. The knockdown of Set7 did not change IL-10 mRNA levels in these cells (data not shown).

SuV39h1 has a conserved Set [Su(var)3–9, Enhancer-of-zeste, Trithorax] catalytic domain that belongs to the histone methyltransferase family and is capable of methylating lysine K9 of histone H3 (H3K9) to mediate transcriptional repression. We first determined whether SuV39h1 was inversely correlated with NFκB-p65 transcription and a component of hyperglycemic memory. Soluble chromatin fractions derived from cross-linked HMECs were immunoprecipitated against SuV39h1 antibody and enrichment was compared with baseline glucose controls by real-time PCR quantitation. Chromatin immunoprecipitation analysis indicates that the co-repressor SuV39h1 does not significantly change on the p65 promoter (Fig. 2E).

**Transient hyperglycemia causes a sustained reduction of H3K9 methylation on the NFκB-p65 promoter.** The degree of gene activation or inhibition is exquisitely regulated by multiple histone marks mediated by histone methyltransferase enzymes (15). Indeed, methylation of H3K4 and H3K9 can both inhibit each other and can be mutually antagonistic (14). Therefore, to further elucidate the underlying regulatory mechanism of NFκB-p65 gene expression as a result of transient and prior hyperglycemia in endothelial cells, we specifically investigated these repressive H3K9 methylation marks. Endothelial cells were incubated in high glucose for 16 h and then returned to normoglycemic conditions for 2, 4, or 6 days and analyzed for H3K9m1 (Fig. 2F). Chromatin immunoprecipitation measurements indicate that transient hyperglycemia causes a sustained reduction in both H3K9m2 (Fig. 2G) and H3K9m3 (Fig. 2H) on the NFκB-p65 promoter.

**Transient hyperglycemia induces recruitment of LSD1 H3 demethylase.** Although we have initially focused on various changes in histone methylation, it is increasingly appreciated that methyl groups can be removed or erased as a result of demethylation (16). Indeed, until recently, it was believed that methyl groups could not be removed and erased from histones. Recent experimental studies now show that demethylation is linked to both transcriptional repression and activation events (17,18). Lysine-specific demethylase 1 (LSD1) and Jumonji C (JmJC) have recently been characterized as H3 demethylases (19–22). LSD1 is a nuclear amine oxidase that uses oxygen as an electron acceptor to reduce methylated lysine to form lysine (20). LSD1 demethylates H3K4m1 and H3K4m2 and is consistent with its role in removal of the active methylation mark; LSD1 is found in co-repressor complexes and promotes suppression of gene expression (16). LSD1 is also associated with the activation of gene expression and demethylation of H3K9m1 and H3K9m2 (19,21,23). The JmJC protein family specifically demethylates H3K9 and is associated with the activation of gene expression (24,25). The preference for distinct H3K9 methylation erasure in the experiments we have performed (Fig. 2E and F) prompted us to test the hypothesis that demethylation at histone H3 lysine 9 might be associated with the cooperative recruitment of LSD1 at the NFκB-p65 gene. The role of the LSD1 enzyme in response to transient hyperglycemia is not well understood, and this is underscored by recent evidence suggesting the release of the demethylase on active genes is inversely linked with increased H3K4m2 (26). With these results in mind and our experimental evidence indicating that transient hyperglycemia could be associated with increased gene-activating H3K4m1 with no significant change in H3K4m2 or H3K4m3, we examined recruitment of LSD1 demethylase to the p65 promoter in response to hyperglycemia. Results shown in Fig. 2F indicate that transient hyperglycemia enriches the demethylase, LSD1, on the NFκB-p65 promoter and that this is inversely correlated with H3K9 methylation, which is consistent with its role as a co-activator of gene activity.

**In vivo studies**

**Metabolic parameters.** To explore the in vivo relevance of these changes in p65 expression, we examined changes in expression of NFκB-p65 not only in a well-validated model of diabetes-associated atherosclerosis, the diabetic apoE KO mice (27), but also have evaluated these changes in a group of mice where there has been a degree of β-cell recovery weeks after induction of diabetes, as has been previously reported with respect to streptozocin in rodents (28). This subgroup of mice has experienced a period of hyperglycemia followed by a significant period of near-normoglycemia (HG → NG), allowing us to determine if previous transient episodes of hyperglycemia can lead to sustained upregulation of genes implicated in diabetic vascular injury.

Glycated hemoglobin was increased significantly in the diabetic apoE KO group (17.6 ± 0.4%, n = 15) when compared with the control apoE KO group (4.7 ± 0.1%, n = 15). Both these parameters were decreased in the previously hyperglycemic group but were still elevated (6.4 ± 0.8%, n = 10), albeit modestly, when compared with the control apoE KO group (P < 0.05). Body weight was significantly decreased in the diabetic apoE KO group (20.9 ± 0.4 g) compared with control apoE KO mice (30.7 ± 0.3 g, P < 0.01). Mice in the previously hyperglycemic group had a modest decrease in body weight (27.0 ± 1.0 g) when compared with control apoE KO mice (P < 0.05) but not as low as seen in diabetic apoE KO mice (P < 0.01). Plasma lipids including total and LDL cholesterol were increased in diabetic apoE KO mice but were similar to control mice in the previously hyperglycemic animals (total cholesterol, control 14.5 ± 0.7 mmol/l [means ± SE], diabetic 33.0 ± 4.6 mmol/l [P < 0.01 vs. control], previously hyperglycemic 15.5 ± 1.3 mmol/l [P < 0.01 vs. diabetic]; LDL cholesterol, control 10.4 ± 0.6 mmol/l, diabetic 27.2 ± 4.4 mmol/l [P < 0.01 vs. control], previously hyperglycemic 10.5 ± 2.8 mmol/l [P < 0.01 vs. diabetic]).

**Plaque area remained increased and NFκB, VCAM, and MCP-1 mRNA levels remained elevated in previously hyperglycemic mice.** Plaque area was quantitated as a percentage area of aorta stained red with Sudan IV. Total plaque area was significantly increased in diabetic apoE mice (HG → HG) and was compared with control apoE mice (NG → NG) (Table 1). Importantly, the greater-than-threefold increase in plaque area was also seen in the previously hyperglycemic (HG → NG) mice. When plaque area was quantified in the three individual segments (arch, thoracic, and abdominal region), the same pattern was observed as for total plaque area with an increase in plaque area at all three sites in diabetic mice and a similar increase at these sites in the previously hyperglycemic mice (Fig. 3). Aortic mRNA levels for NFκB p65 were significantly upregulated in diabetic apoE KO mice when compared with the apoE KO controls (Table 2). NFκB p65 gene expression was also significantly upregulated in the aortas from the previously hyperglycemic mice. Gene expression of two important mediators of atherosclerosis,
particularly in the diabetic setting, that are known to be NFκB dependent—VCAM-1 (11) and MCP-1 (29)—were also significantly upregulated in the chronically diabetic mice. This increase in MCP-1 and VCAM-1 mRNA levels was also observed in the aortas from the previously hyperglycemic mice (Table 2).

**DISCUSSION**

The novel findings described in these in vitro studies represent a paradigm shift in understanding the relationship between epigenetic change and hyperglycemic memory. Using endothelial cell models of transient and prior hyperglycemia, we demonstrated that active NFκB-p65 gene expression is linked to persisting epigenetic marks that are maintained when the cell is removed from its hyperglycemic environment. The evidence for this came from cell culture experiments in which specified changes in epigenetic information are associated with transcriptional longevity. Three sets of experiments indicated that the underlying regulatory mechanism of NFκB-p65 gene activity induced by hyperglycemia involve specific epigenetic modifications. First, the persistence of NFκB-p65 gene activation mediated by transient hyperglycemia is associated with Set7 recruitment and H3K4 mono-methylation, which could be overcome by somatic knockdown of the Set7 methyl-lysine writer. Second, the consequent increase in activating epigenetic marks is directly correlated with distinct and persistent H3K9 demethylation events. Finally, chromatin immunopurification studies indicate hyperglycemia-induced recruitment of the methyl-lysine eraser, LSD1, occurs concomitantly with reduced H3K9 methylation and increased NFκB-p65 expression. Although none of the experiments alone constitutes evidence that any one epigenetic mark is solely important in long-lived gene activity, taken together, the evidence strongly suggests that changes in epigenetic information are associated and potentially could partly explain the phenomenon of “hyperglycemic memory.”

The importance of sustained effects of previous changes in glucose levels is increasingly being appreciated as a result of more recent findings from studies of vascular

**TABLE 1**

|                  | n | Total   | Arch     | Thoracic | Abdominal |
|------------------|---|---------|----------|----------|-----------|
| Control apoE KO (NG→NG) | 10 | 3.0 ± 0.5 | 8.0 ± 0.5 | 1.2 ± 0.3 | 1.1 ± 0.3 |
| Diabetic apoE KO (HG→HG)  | 10 | 13.0 ± 1.0* | 24.4 ± 1.0* | 8.7 ± 1.0* | 8.5 ± 0.7* |
| Previously hyperglycemic apoE KO (HG→NG) | 6  | 11.4 ± 1.9* | 21.3 ± 2.1* | 8.0 ± 2.7* | 8.2 ± 1.8* |

Data are means ± SE. *P < 0.05 compared with the control apoE KO group.

**TABLE 2**

|                  | n | NFκB-p65 | MCP-1  | VCAM-1  |
|------------------|---|----------|--------|---------|
| Control apoE KO (NG→NG) | 5  | 1.0 ± 0.2 | 1.1 ± 0.2 | 1.0 ± 0.2 |
| Diabetic apoE KO (HG→HG)  | 5  | 2.5 ± 0.9* | 3.2 ± 1.1* | 3.2 ± 0.5* |
| Previously hyperglycemic apoE KO (HG→NG) | 4  | 2.7 ± 1.1* | 3.7 ± 0.9* | 1.9 ± 0.06†† |

Data are means ± SE. *P < 0.05 compared with control apoE KO group; †P < 0.05 compared with diabetic apoE KO group.
they are linked to ongoing vascular changes in diabetes that are probably associated with the activity of methyl-reading and -erasing enzymes on the promoter. Interestingly, other evidence suggests that H3K4m2 is co-exist to regulate gene expression, and these methyl-marks are probably associated with the activity of methylwriting and -erasing enzymes on the promoter. Thus, it appears that periods of transient or prior hyperglycemia lead to various methyltransferase and demethylase changes associated with p65 gene expression, we used an extensively characterized model of diabetes-associated atherosclerosis (11,27). In this model, a subgroup of diabetic animals lost their severe hyperglycemia, as has recently postulated to be inversely correlated with LSD1 (26). Although these distinguishable results indicate a complicated and somewhat contradictory set of experimental data, we cannot exclude that these distinct differences indicate functional specialization of H3 enzymes in the various vascular cell populations.

To further explore the potential in vivo relevance of the changes associated with p65 gene expression, we used an extensively characterized model of diabetes-associated atherosclerosis (11,27). In this model, a subgroup of diabetic animals lost their severe hyperglycemia, as has previously been reported after injection of streptozotocin in various rodent models of diabetes (28,31,32). Despite a reduction in plasma glucose and lipid levels at death in these previously hyperglycemic mice, p65 gene upregulation persisted, as did the increase in gene expression of various NFkB-dependent proteins, MCP-1, and VCAM-1. Furthermore, the sustained increase in these pro-inflammatory molecules was associated with increased atherosclerosis in these mice with prior hyperglycemia. These changes are consistent with this model, exhibiting macrovascular disease as a result of “metabolic memory.” Thus, it appears that periods of transient or prior hyperglycemia lead to various methylation and demethylation events that, when integrated, have an impact on gene activity. These events lead to sustained activation of pro-inflammatory pathways, which are likely to participate in the progression of diabetic complications. Further understanding of the chromatin remodeling events and how they are linked to ongoing vascular changes in diabetes should lead to better strategies to reduce the burden of diabetes complications.

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