Hyperglycemia induces a Dynamic Cooperativity of Histone Methylase and Demethylase Enzymes associated with Gene-Activating Epigenetic Marks that co-exist on the Lysine Tail

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Submitted 2 December 2008 and accepted 3 February 2009.
ABSTRACT

Objective. Results from The Diabetes Control Complications Trial (DCCT) and the subsequent Epidemiology of Diabetes Interventions and Complications (EDIC) study and more recently from the United Kingdom Prospective Diabetes Study (UKPD) have revealed that the deleterious end-organ effects that occurred in both conventional and more aggressively treated subjects continued to operate more than 5 years after the patients had returned to usual glycemic control and is interpreted as memory or a legacy of past glycemia known as “hyperglycemic memory”. We have hypothesized that transient hyperglycemia mediates persistent gene-activating events attributed to changes in epigenetic information.

Research Design and Methods. Models of transient hyperglycemia were used to link NFκB-p65 gene expression with H3K4 and H3K9 modifications mediated by the histone methyltransferases (Set7 and SuV39h1) and the lysine specific demethylase (LSD1) by the immunopurification of soluble NFκB-p65 chromatin.

Results. The sustained upregulation of NFκB-p65 gene as a results of ambient or prior hyperglycemia, was associated with increased H3K4m1 but not H3K4m2 or H3K4m3. Furthermore, glucose was shown to have other epigenetic effects including the suppression of H3K9m2 and H3K9m3 methylation on the p65 promoter. Finally, there was increased recruitment of the recently identified histone demethylase LSD1 to the p65 promoter as a result of prior hyperglycemia.

Conclusions. These studies indicate that the active transcriptional state of the NFκB-p65 gene is linked with persisting epigenetic marks such as enhanced H3K4 and reduced H3K9 methylation, which appear to occur as a result of effects of the methyl-writing and methyl-erasing histone enzymes.

Abbreviations:
DCCT, The Diabetes Control Complications Trial
EDIC, Epidemiology of Diabetes Interventions and Complications
BAECs, bovine aortic endothelial cells
HMECs, human microvascular endothelial cells
ChIP, Chromatin immunopurification
H3K4m1, histone H3 lysine K4 monomethylation
H3K4m2, histone H3 lysine K4 dimethylation
H3K4m3, histone H3 lysine K4 trimethylation
H3K9m1, histone H3 lysine K9 monomethylation
H3K9m2, histone H3 lysine K9 dimethylation
H3K9m3, histone H3 lysine K9 trimethylation
LSD1, lysine specific demethylase
Vascular complications are the major source of morbidity and mortality in diabetes and are considered, based on both epidemiological data and from more mechanistic studies to occur primarily as a result of the long term deleterious effects of hyperglycemia. Interestingly, these vascular complications often persist and may progress despite improved glucose control, possibly as a result of prior episodes of hyperglycemia. Results in both type 1 and type 2 diabetes as observed in the DCCT/EDIC study and in the recent follow up of the UKPDS trial have revealed that end-organ effects that occurred in both conventional and intensified glycemic control groups continued to operate more than 5 years after the patients had returned to their usual level of glycemic control (1; 2). These studies suggest that the injurious effects of exposure to high glucose levels persist for many years after these episodes of altered metabolic control and this is typically referred to as either “hyperglycemic memory” (3) or the legacy effect (4). Recently, several studies including the ADVANCE (5) and ACCORD studies (6), failed to demonstrate that intensified glycemic control for 3 to 5 years markedly reduced macrovascular complications, emphasising the lack of rapid reversibility of glucose related vascular changes by improved glycemic control. Indeed, such studies are consistent with the view that previous episodes of transient hyperglycaemia may induce longstanding deleterious changes in the vasculature.

Until now, our view of susceptibility to hyperglycemia-induced vascular complications focused predominantly on genetic polymorphisms, but recent studies exploring epigenetic mechanisms such as chromatin remodelling, histone modifications, and DNA methylation are increasingly appreciated to be critical to the way we view changes in gene activity. This gene-environment interaction involving epigenetic changes may be particularly relevant to the pathogenesis of diabetic complications (7) with glucose inducing chromatic remodelling and conferring epigenetic marks by specifically modulating histone methylation (8). In this study, we have extended these findings to characterise in more detail the nature of histone methylation of the promoter region of the p65-NFκB gene, which is upregulated in a sustained manner in response to prior transient hyperglycaemia (3). These results highlight the importance of histone modifications that control the active transcriptional expression of gene activity, which is linked with persisting epigenetic marks that are specifically maintained when the endothelial cell is out of its previous hyperglycemic milieu.

MATERIALS AND METHODS
In vitro studies
Cell culture conditions and treatments. Confluent bovine aortic endothelial cells (BAEC) were maintained in minimum essential medium (MEM, Gibco) containing 0.5% foetal bovine serum, non-essential amino acids (Gibco) and antibiotics Gentomicin (Roche). Cells were incubated with MEM 5.5mM low glucose (LG) or MEM with 30mM high glucose (HG) or 30mM Mannitol for 16hrs where stated.

Set7 shRNA knockdown. Human microvascular endothelial cells (HMECs) were infected with MISSION shRNA-expressing lentiviral vectors targeted to Set7 coding regions according to the user instructions (Sigma). The sequence targeting Set7 corresponds to 5’-CCAGATCCTTATGAATCAGAA-3’ (TRCN0000078630). The cells transduced MISSION Non-Target shRNA Control Vector were used as controls. HMECs were seeded at 5 × 10^5 cells/dish in a 60-mm dish 20 hours before infection, incubated with the lentivirus 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 was inserted between the BamHI and NotI sites of the retroviral vector pCX4neo (10) (a kind gift from Dr T. Akagi, KAN Research Institute, Kobe, Japan) to create pJS14. pJS14 or pCX4neo were co-transfected with retrovirus packaging vectors (Takara) into 293T cells (10). Two days after transfection, culture supernatants were collected and used as viral stocks. HMECs were seeded at $5 \times 10^5$ cells/dish in a 60-mm dish 20 hours before infection, incubated with the virus stock for 2 days, followed by selection in Geneticin (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 kind gift from Dr P.L. Jones, University of Illinois, USA) (7).

**Immunoprecipitation of FLAG-Set7.** Cell lysates were prepared from cells expressing FLAG-Set7 or from cells transduced with pCX4neo as a control. $1 \times 10^7$ cells were washed twice with ice-cold PBS and extracted with 600 µl of lysis buffer (50mM Tris HCl, pH7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100 and proteinase inhibitor). Lysate were incubated with anti-FLAG M2 affinity gel (Sigma) for 2 hours at 4 °C. Immunoprecipitates were washed with TBS (50mM Tris HCl, pH7.5, 150mM NaCl) three times and then eluted from the gels with 3X FLAG peptide (Sigma), and analysed 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; 5 µg of recombinant histone H3 (Upstate), or 0.4 nmol of a biotin conjugated histone H3 peptide (amino acids 1-23), either K4 or R4 (Sigma), was used for methyltransferase reactions. Reactions were determined by spotting on P-81 paper (Upstate) and scintillation counting.

**Chromatin Immunoprecipitation (ChIP).** Cells were formaldehyde treated at 1% concentration for 10mins and then glycine treated at 0.15M for 10mins. Cell pellets were resuspended in SDS lysis containing 1%SDS, 10mM EDTA, 50mM Tris, pH 8.1 (Upstate) and a protease inhibitor cocktail (Roche). Cells were sonicated to shear chromatin to 700 – 300bp, resuspended in ChIP Dilution Buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1 and 167mM NaCl and 15µl salmon sperm DNA-protein A agarose (Upstate) was added and pre-cleared. To the soluble chromatin fraction the antibody of interest; H3K4m1 (Abcam ab8895), H3K4m2 (Upstate 07030), H3K4m3 (Upstate 07473), H3K9m1 (Abcam ab9045), H3K9m2 (Abcam ab7312-100), H3K9m3 (Abcam ab8898), Set7 (kind gift from Dr P Jones, Illinois at Urbana-Champaign), LSD1 (Upstate 05939), Suv39h1 clone MG44 (Upstate 05615) was added and incubated for a minimum of 8hrs. Immune complexes were collected with salmon sperm DNA-protein A agarose and subsequently washed and eluted with buffer (1%SDS, 0.1M NaHCO3). Protein-DNA cross-links were reversed overnight at 65°C and recovered by phenol-chloroform extraction and ethanol precipitated at –20°C. Analysis of ChIP DNA samples was performed by quantitative Reverse Transcriptase PCR (qPCR) of NFκB p65 promter region, sequences available on request. An 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 preparation. Cells were subsequently
treated with Turbo DNase (Ambion) that removed genomic DNA. First strand cDNA synthesis from the purified mRNA was performed using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. NFκB p65 gene was quantified using qPCR.

Reverse Transcriptase PCR. PCR amplification was performed using ABI Prism 7500 where 2pmol of each forward and reverse primer was added to a total of 20µl reaction containing 1x SYBR Green qPCR SuperMix-UDG and Rox Reference Dye (Invitrogen). Reactions were incubated for 50°C for 2mins, 95°C for 10mins followed by 50 cycles of 95°C for 15sec and 60°C for 1min.

In vivo studies

Animal model. Male apolipoprotein knock out (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 NHMRC of Australia. At week 7, mice were rendered diabetic via 5 daily intraperitoneal injections of streptozotocin (STZ) (MP Biomedicals, Eschwege, Germany) 55mg/kg/day, resulting in a model of insulin deficiency (11). ApoE diabetic mice animals were followed for 20 weeks. In over 90% of mice injected with these 5 daily injections of STZ, plasma glucose levels were >20mM 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 hyperglycaemia, after ~10 to 12 weeks these mice now had reduced plasma glucose levels (<15mM) despite no treatment which remained decreased till the time of sacrifice at week 20. This group was termed “previously hyperglycaemic” (HG→NG) and were compared to the control (NG→NG) and diabetic (HG→HG) mice. All these apoE KO mice received standard mouse chow and water ad libitum. Mice were culled by euthanasia using an intraperitoneal injection of Euthal (10mg/kg) (Delvet Limited, Seven Hills, Australia) followed by exsanguination via cardiac puncture. The excised aortae were placed in 10% neutral buffered formalin and quantitated for lesion area before being processed for subsequent immunohistochemical analysis. In a subset of animals, aortas were snap frozen in liquid nitrogen and stored at –70°C for subsequent RNA extraction.

Metabolic Parameters. Red blood cells were collected at sacrifice for measurement of glycated haemoglobin (GHb) by HPLC. Plasma glucose levels were measured using an automated system (Abbott Architect ci8200, Abbott Laboratories). At sacrifice, plasma lipids were measured by autoanalyzer (11).

Isolation of Total RNA, Synthesis of cDNA and Quantitative Real-Time PCR. Gene expression of NFκB p65, the adhesion molecule VCAM-1 and macrophage chemoattractant protein 1 (MCP-1) were assessed by real-time quantitative RT-PCR. This was performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7500, Perkin-Elmer Inc, PE Biosystems, Foster City, CA, USA), as previously utilized by our group (12). Briefly, whole aorta was homogenised using the Ultra-Turrax (Janke & Kunkel IKA, Labortechnik, Germany) in TRIZOL (Life Technologies Inc, Gaithersburg M.D., USA), and total RNA was isolated. cDNA was synthesised with a reverse transcriptase reaction using Superscript™ First Strand Synthesis System for RT-PCR (Life Technologies Inc) with random hexamers, dNTPs and total RNA extracted from mouse hearts. To assess genomic DNA contamination, controls without reverse transcriptase were included. Briefly, gene
specific 5'-oligonucleotide primer, 3'-oligonucleotide primer and MGB FAM-probe corresponding to each gene (sequences available upon request) were designed using the software program, ‘Primer Express’ (PE Applied Biosystems). The generation of amplicons was defined as the point during cycling when amplification of the PCR product is first detected above the threshold setting.

The RT-PCR reaction took place with 500nmol/L of forward and reverse primer and 50nmol/L of FAM/MGB probe and VIC™ 18S ribosomal probe, in 1x Taqman universal PCR master mix (PE Biosystems). Each sample was run and analyzed in triplicate. Gene expression was normalized to 18S mRNA with samples from the control (NG→NG) aorta used as the calibrator with a given value of 1 and all other groups were compared with this calibrator group.

**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 gene expression (3). Despite these and other recent advances, understanding the complexity of H3K4 and H3K9 methylation remains an important challenge. We show that transient hyperglycemia induces long-lasting activation of the NFκB-p65 gene (**Figure 1A**) and persistent changes in H3K4m1 (**Figure 1B**), which were not seen for H3K4m2 (**Figure 1C**) and H3K4m3 (**Figure 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 counter-acting cytokine, IL-10. Expression of 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 di-methylation or tri-methylation we postulated that the Set7 enzyme, previously reported to act as a histone mono-methyltransferase, albeit in non-endothelial cells, might be involved (13). Indeed, since 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 purified Set7 expressed from human vascular cells. These results show for the first time that expressed Set7 possesses histone methyltransferase activity, as assayed by its ability to methylate histone H3 (**Figure 1E**). To determine whether H3K4 is the unique methylation site of Set7 activity (14), histone methyltransferase activity of purified Set7 was assayed using H3K4 and H3R4 as substrate peptides (**Figure 1F**). 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.** Since Set7 appears to be the enzyme responsible for H3K4m1 in immortalised cancer cell types (14) and now confirmed in endothelial cells (Figure 1E and 1F) we subsequently tested the hypothesis that Set7 is mobilised to the p65 promoter to maintain the active transcriptional state. Immunopurified chromatin from BAECs exposed to transient hyperglycemic conditions was associated with sustained enrichment for Set7 on the p65 promoter ($P <0.05$) in response to glucose (Figure 1G). To exclude the possibility that the effects are a result of osmotic stress, cells were incubated in 30mM mannitol for 16 hours 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 (Figure 1H). ChIP analyses indicated no enrichment of the Set7 enzyme on the NFκB-p65 promoter consistent with persisting epigenetic marks being mediated by glucose (Figure 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 (wt) and knockdown (Set7KD) cells indicated robust silencing by the shRNA to Set7 in endothelial cells (Figure 2A). Transient hyperglycemia failed to induce NFκB-p65 gene expression in Set7KD cells (Figure 2B) suggesting that this specific histone methyltransferase is required for glucose mediated gene activity. We also confirmed that the erasure of NFκB-p65 gene expression is dependent on changes in histone methylation mediated by Set7 (Figure 2C). Parallel experiments using H3K4m2 antibody were also performed to illustrate the specificity of histone modification with no effect on H3K4m2 seen with Set7KD (Figure 2D). We also assessed IL-10 gene expression in Set7KD and wild type 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 p65 transcription and a component of hyperglycemic-memory. Soluble chromatin fractions derived from cross-linked HMECs were immunoprecipitated against SuV39 antibody and enrichment was compared to baseline glucose controls by realtime PCR quantitation. Chromatin immunopurification analysis indicates that the co-repressor SuV39h1 does not significantly change on the p65 promoter (Figure 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 hours and then returned to normoglycemic conditions.
for 2, 4 or 6 days and analysed for H3K9m1 (Figure 2F). ChIP measurements indicate that transient hyperglycemia causes a sustained reduction in both H3K9m2 (Figure 2G) and H3K9m3 (Figure 2H) on the NF\(\kappa\)B-p65 promoter.

**Transient hyperglycemia induces recruitment of the 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 or LSD1 and Jumonji C (JmjC) have recently been characterized as H3 demethylases (19-22). LSD1 is a nuclear amine oxidase that utilizes oxygen as an electron acceptor to reduce methylated lysine to form lysine (20). LSD1 demethylates H3K4m1 and H3K4m2 and 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 demethylate 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 (Figure 2E and 2F) prompted us to test the hypothesis that demethylation at histone H3 lysine 9 might be associated with the co-operative recruitment of LSD1 at the NF\(\kappa\)B 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 the LSD1 demethylase to the p65 promoter in response to hyperglycemia. Results shown in Figure 2I indicate that transient hyperglycemia enriches for the demethylase, LSD1, on the NF\(\kappa\)B-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 have examined changes in expression of p65 not only in a well validated model of diabetes associated atherosclerosis, the diabetic apo E KO mice (27) but also have evaluated these changes in a group of mice where there has been a degree of \(\beta\) cell recovery weeks after induction of diabetes, as has been previously reported with respect to streptozocin in rodents (28). Thus, this subgroup of mice have experienced a period of hyperglycemia followed by a significant period of near normoglycemia (HG\(\rightarrow\)NG), allowing us to determine if previous transient episodes of hyperglycemia can lead to sustained upregulation of genes implicated in diabetic vascular injury.

Glycated haemoglobin was increased significantly in the diabetic apoE KO group (17.6 ± 0.4%, n= 15) when compared to the control apoE KO group (4.7 ± 0.1%, n=15). Both these parameters were decreased in the previously hyperglycaemic group but were still elevated (6.4±0.8%, n=10), albeit modestly, when compared to the control apoE KO group (p<0.05). Body weight was significantly decreased in the diabetic apoE KO group (20.9 ± 0.4g) compared to control apoE KO mice (30.7 ± 0.3g, p<0.01). Mice in
the previously hyperglycemic group had a modest decrease in body weight (27.0±1.0g) when compared to 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 mM, diabetic 33.0±4.6mM*, previously hyperglycemic 15.5 ±1.3mM†; LDL cholesterol, control 10.4 ±0.6mM, diabetic 27.2 ±4.4mM*, previously hyperglycemic 10.5 ±2.8mM†, mean ± SEM shown, * p<0.01 vs Control, † 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 to control apoE mice (NG→NG) (Table 1). Importantly, the greater than 3-fold increase in plaque area was also seen in the previously hyperglycemic (HG→NG) mice. When plaque area was quantified in the 3 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 3 sites in diabetic mice and a similar increase at these sites in the previously hyperglycemic mice (Figure 3). Aortic mRNA levels for NFκB p65 were significantly upregulated in diabetic apoE KO mice when compared to 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 2 important mediators of atherosclerosis, particularly in the diabetic setting which are known to be NFκB dependent, vascular adhesion molecule, VCAM-1 (11), and macrophage chemoattractant protein 1, 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 have 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 knock-down of the Set7 methyl-lysine writer. Second, the consequent increase in activating epigenetic marks is inversely 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. While 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 disease in type 1 and type 2 diabetes. For example, the identification of a long-term impact on cardiovascular events as a result of previous periods of altered glycemic control in the DCCT study has emphasized this phenomenon of “metabolic memory” (2).

More recently, the long-term outcomes, in particular cardiovascular outcomes, from the UKPDS suggest that the period of intensive glycemic control that occurred more than a decade ago and initially did not demonstrate statistically significant reductions in macrovascular disease, now shows clear benefits on a range of cardiovascular endpoints including mortality (30). It remains to be determined as to the underlying explanation for these persistent, ongoing injurious effects to diabetic vessels as a result of previous hyperglycemia. However, the recent demonstration by several groups of various epigenetic changes as a result of hyperglycemia (3; 26; 31-34) and in particular in this study, certain changes in enzymes involved in histone methylation and demethylation emphasize the potential long-term effects of glucose on processes linked to gene expression and activation. This study focused on histone methylation changes in response to transient hyperglycemia, illustrating that distinct H3K4 and H3K9 methylation patterns on the NFκB-p65 gene co-exist to regulate gene expression and these methyl-marks are probably associated with the activity of methyl-writing and -erasing enzymes on the promoter. Interestingly, other evidence suggests that H3K4m2 is increased on genes activated by hyperglycemia, which was 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 employed an extensively characterized model of diabetic 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; 35; 36). Despite a reduction in plasma glucose and lipid levels at sacrifice in these previously hyperglycemic mice, p65 gene upregulation persisted, as did the increase in gene expression of various NFκB 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 which 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 diabetic complications.

ACKNOWLEDGEMENTS

We thank Dr Peter L Jones (University of Illinois at Urbana-Champaign, USA) for the Set7 antibody, and, Dr Audrey Koitka and Dr Anna Watson for their technical assistance.
with the in vivo studies. The authors acknowledge grant support from the Juvenile Diabetes Research Foundation International (JDRF), the National Health and Medical Research Council (NHMRC) of Australia and the National Heart Foundation (NHF) of Australia. Dr Assam El-Osta, Dr Mark Cooper and Dr Anna Calkin are recipients of a Career Development Award, a Senior Principal Research Fellowship and a Doherty Post-Doctoral fellowship respectively from the NHMRC. Dr C Tikellis and Dr A Balcerczyk are recipients of fellowships from the JDRF and the Foundation for Polish Science (FNP).

Disclosures
None

REFERENCES

Figure 1. Ambient and prior hyperglycemia sustains increased activating H3K4m1 associated Set7 enrichment on the NFkB-p65 gene in aortic endothelial cells.

A, Ambient and prior hyperglycemia sustains increased NFkB-p65 gene expression in BAECs. RNA was extracted, and NFkB-p65 mRNA levels were quantified by real-time RT-PCR (qRT-PCR) and normalized to the level of 18S. *P < 0.05 vs LG 16 hr group. Mono-methylation is the predominant H3K4 mark following transient hyperglycemia. BAECs were exposed to glucose and soluble chromatin were immunopurified at the indicated times using B, H3K4m1, C, H3K4m2 and D, H3K4m3 antibodies and qPCR was used to measure the level of enrichment on the NFkB-p65 promoter (-400bp from the +1 transcription start site). Error bars represent SEM. Samples were analyzed in triplicate, and data are presented as means ± SEM. *P < 0.05 vs LG 16 hr group, **P < 0.01 vs LG 16hr group. E, Set7 associated histone methyltransferase activity of human flag tagged Set7 (Flag-Set7) over-expressed in endothelial cells and immunoprecipitated using anti-flag antibody. Substrate used to determine methyltransferase activity was recombinant histone H3. F, Lysine 4 of histone H3 (H3K4) is a major methylation site for the Set7 enzyme. Flag-Set7 was immunoprecipitated by anti-flag antibody in over-expressed flag-Set7 or control pCX4neo human endothelial cells. Substrates used to determine methyltransferase activity were synthesized histone H3K4 and mutant H3R4 peptides. G, Aortic endothelial cells were exposed to glucose and soluble chromatin were immunopurified at the indicated times with Set7 antibody and qPCR was used to measure the level of enrichment on the NFkB-p65 promoter. *P < 0.01 vs LG 16 hr group. H, The osmolyte, mannitol (MN) does not increase NFkB-p65 mRNA levels. Samples were analyzed in triplicate, and data are presented as means ± SEM. I, Mannitol does not increase Set7 association to the NFkB-p65 promoter. Samples were analyzed in triplicate, and data are presented as means ± SEM.
Figure 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 HMEC’s 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 Set7 enzyme rescinds H3K4m1 mark on the NFκB-p65 promoter. Samples were analyzed in triplicate, and data are presented as means ± SEM. *P < 0.05 vs LG 16 hr group.
D, Set7 KD does not change the H3K4m2 mark on the NFκB-p65 promoter. HMEC’s were exposed to glucose and soluble chromatin were immunopurified at the indicated times for E, SuV39h1, F, H3K9m1, G, H3K9m2, H, H3K9m3 and I, LSD1 antibodies and qPCR was used to measure the level of enrichment on the NFκB-p65 promoter. Error bars represent SEM. Samples were analyzed in triplicate, and data are presented as means ± SEM. *P < 0.05 vs LG 16 hr group.
Hyperglycemic memory and epigenetic persistence

![Graphs showing qPCR for NF-kB p65 promoter after H3K9me3 ChIP and LSD1 ChIP (fold change)]
Figure 3. Plaque area remained increased in the previously hyperglycemic mice. Atherosclerotic plaques in aorta staining in red with picro sirus red solution in (A) control apoE KO, (B) diabetic apoE KO and (C) previously hyperglycaemic apoE KO mice.
**Table 1.** Total and arch plaque area was quantitated as a percentage area of aorta stained red with Sudan IV in control apoE KO, diabetic apoE KO and previously hyperglycaemic apoE KO mice. Data are expressed as mean±SEM. *P<0.05 compared to control apoE KO group.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total</th>
<th>Arch</th>
<th>Thoracic</th>
<th>Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control apoE KO (NG→NG)</td>
<td>10</td>
<td>3.0±0.5</td>
<td>8.0±0.5</td>
<td>1.2±0.3</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>Diabetic apoE KO (HG→HG)</td>
<td>10</td>
<td>13.0±1.0*</td>
<td>24.4±1.0*</td>
<td>8.7±1.0*</td>
<td>8.5±0.7*</td>
</tr>
<tr>
<td>Previously hyperglycaemic apoE KO (HG→NG)</td>
<td>6</td>
<td>11.4±1.9*</td>
<td>21.3±2.1*</td>
<td>8.0±2.7*</td>
<td>8.2±1.8*</td>
</tr>
</tbody>
</table>
Table 2. mRNA expression of NFκB-p65, MCP-1 and VCAM in control apoE KO, diabetic apoE KO and previously hyperglycaemic apoE KO mice. Data are expressed as mean±SEM. * $P<0.05$ compared to control apoE KO group, †$P<0.05$ compared to diabetic apoE KO group.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>NFκB-p65</th>
<th>MCP-1</th>
<th>VCAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control apoE KO (NG→NG)</td>
<td>5</td>
<td>1.0±0.2</td>
<td>1.1±0.2</td>
<td>1.0±0.2</td>
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<tr>
<td>Diabetic apoE KO (HG→HG)</td>
<td>5</td>
<td>2.5±0.9*</td>
<td>3.2±1.1*</td>
<td>3.2±0.5*</td>
</tr>
<tr>
<td>Previously hyperglycaemic apoE KO (HG→NG)</td>
<td>4</td>
<td>2.7±1.1*</td>
<td>3.7±0.9*</td>
<td>1.9±0.06*†</td>
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</tbody>
</table>