Impact of Glycemic Variability on Chromatin Remodeling, Oxidative Stress, and Endothelial Dysfunction in Patients With Type 2 Diabetes and With Target HbA_{1c} Levels

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Intensive glycemic control (IGC) targeting HbA_{1c} fails to show an unequivocal reduction of macrovascular complications in type 2 diabetes (T2D); however, the underlying mechanisms remain elusive. Epigenetic changes are emerging as important mediators of cardiovascular damage and may play a role in this setting. This study investigated whether epigenetic regulation of the adaptor protein $p66^{Shc}$, a key driver of mitochondrial oxidative stress, contributes to persistent vascular dysfunction in patients with T2D despite IGC. Thirty-nine patients with uncontrolled T2D (HbA_{1c} >7.5%) and 24 age- and sex-matched healthy control subjects were consecutively enrolled. IGC was implemented for 6 months in patients with T2D to achieve a target HbA_{1c} of ≤7.0%. Brachial artery flow-mediated dilation (FMD), urinary 8-isoprostaglandin $F_{2\alpha}$ (8-isoPGF$_{2\alpha}$), and epigenetic regulation of $p66^{Shc}$ were assessed at baseline and follow-up. Continuous glucose monitoring was performed to determine the mean amplitude of glycemic excursion (MAGE) and postprandial incremental area under the curve (AUCpp). At baseline, patients with T2D showed impaired FMD, increased urinary 8-isoPGF$_{2\alpha}$, and $p66^{Shc}$ upregulation in circulating monocytes compared with control subjects. FMD, 8-isoPGF$_{2\alpha}$, and $p66^{Shc}$ expression were not affected by IGC. DNA hypomethylation and histone 3 acetylation were found on the $p66^{Shc}$ promoter of patients with T2D, and IGC did not change such adverse epigenetic remodeling. Persistent downregulation of methyltransferase DNMT3b and deacetylase SIRT1 may explain the observed $p66^{Shc}$-related epigenetic changes. MAGE and AUCpp but not HbA_{1c} were independently associated with the altered epigenetic profile on the $p66^{Shc}$ promoter. Hence, glucose fluctuations contribute to chromatin remodeling and may explain persistent vascular dysfunction in patients with T2D with target HbA_{1c} levels.

The prevalence of type 2 diabetes (T2D) is extremely high, with ∼415 million people affected worldwide. Most importantly, this number is expected to rise to 642 million by the year 2040 (1,2). In the constellation of diabetes-related comorbidities, cardiovascular disease (CVD) carries the largest burden (3,4). Although a consistent body of evidence has unmasked a major pathophysiological role of hyperglycemia in the development and progression of vascular complications (5), the attempt to control CVD progression in T2D with intensive glycemic control (IGC) has been a disappointment for a long time (6). Indeed, ACCORD (Action to Control Cardiovascular Risk in Diabetes), ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation), and VADT (Veterans Affairs Diabetes Trial) have almost unanimously reported that IGC with a significant reduction of HbA_{1c} is not able to improve

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cardiovascular outcomes in patients with long-standing hyperglycemia (7,8). Recently, EMPA-REG OUTCOME (BI 10773 [Empagliflozin] Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients), LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results—A Long Term Evaluation), and SUSTAIN-6 (Trial to Evaluate Cardiovascular and Other Long-term Outcomes With Semaglutide in Subjects With Type 2 Diabetes 6) have shown a remarkable benefit of empagliflozin, liraglutide, and semaglutide on cardiovascular outcomes. However, cardiovascular benefit of these emerging antidiabetic agents was also largely explained by nonglycemic factors, namely blood pressure reduction, osmotic diuresis, and anti-inflammatory effects (9). Furthermore, a growing body of experimental evidence suggests that the hyperglycemic environment is somehow remembered in the vascular system (6,10). Whether this phenomenon occurs in patients with T2D remains to be elucidated. Epigenetic modifications are emerging as important players in CVD (11,12). Indeed, alterations of the epigenome may significantly affect the expression of oxidant and inflammatory genes (13). Major mechanisms of epigenetic regulation are DNA methylation of cytosine-phosphate-guanine (CpG) dinucleotide sequences and acetylation of histone proteins. DNA methylation is an important repressor of gene expression, whereas acetylation of histone tails favors an open chromatin, leading to active transcription (11). Reduced DNA methylation and increased histone acetylation represent an adverse epigenetic pattern that leads to dysregulation of genes with detrimental effects for cellular homeostasis (14). The adaptor protein $p66^{Shc}$ is a key driver of mitochondrial oxidative stress and vascular damage in experimental diabetes (15–17). Indeed, diabetic $p66^{Shc-/-}$ mice are protected against hyperglycemia-induced endothelial dysfunction and vascular redox changes (16). We have shown that epigenetic remodeling of $p66^{Shc}$ is responsible for the persistence of endothelial dysfunction in diabetic mice despite glycemic control with insulin (18). Consistent with these findings, other investigators reported that transient spikes of hyperglycemia trigger inflammation through chromatin changes that persist even after restoration of normoglycemia (19,20). Additional evidence indicates that glucose fluctuations may also exert detrimental effects on the vasculature as a result of their ability to derail pathways implicated in cardiovascular homeostasis. In patients with T2D, glycemic variability during postprandial hyperglycemic swings have been reported to exert a triggering effect on oxidative stress compared with chronic sustained hyperglycemia (21). Whether glucose fluctuations affect chromatin structure and activity in humans is largely unknown. In the current study, we postulate that glycemic variability causes persistent epigenetic remodeling of $p66^{Shc}$ and vascular dysfunction in patients with T2D. Understanding the relationship among glucose variability, $p66^{Shc}$-related epigenetic changes, and vascular disease in the clinical setting may have major implications for the development of mechanism-based therapeutic strategies in patients with T2D.

RESEARCH DESIGN AND METHODS

Population

Between January and December 2012, we recruited 39 consecutive patients with uncontrolled T2D (HbA_{1c} >7.5%) in the outpatient service of Sant'Andrea and Agostino Gemelli University Hospitals in Rome, Italy. Exclusion criteria were overt atherosclerotic vascular disease as well as other relevant comorbidities. Individuals with an estimated glomerular filtration rate of <60 mL/min/1.73 m^2 were also excluded. Twenty-four healthy subjects of similar age and sex were recruited during the same period. Control subjects were not taking medications, and their blood pressure was <130/80 mmHg, LDL cholesterol <160 mg/dL, and fasting plasma glucose <100 mg/dL. A medical history was taken from all participants followed by anthropometric measurements and blood and urine sampling. The study was carried out according to the ethical principles stated in the Declaration of Helsinki. The protocol was approved by local ethics committee (Sant'Andrea Hospital and Agostino Gemelli University Hospital, Rome, Italy) and in accordance with institutional guidelines, and all participants were aware of the investigational nature of the study and gave written consent.

Study Protocol

After enrollment, an IGC program was implemented in all patients with diabetes aiming to achieve an HbA_{1c} level ≤7% in accordance with current recommendations (22,23). All participants were subjected to follow-up visits at months 1, 3, and 6. Glycemic control was assessed by serial HbA_{1c} determinations during the study. Patients with T2D received diet and lifestyle counseling, glucose monitoring equipment, and antidiabetic medications. In addition to lifestyle modifications, the antidiabetic treatment at baseline was uptitrated and/or new glucose-lowering agents added if HbA_{1c} levels remained >7% or if >50% of premeal and postmeal capillary glucose readings were >100 mg/dL or 140 mg/dL, respectively. All drug combinations were allowed, and medications were reduced only in the presence of adverse effects or major hypoglycemic events (requiring intervention of a third party). The following analyses were performed at baseline in both study groups: *1*) epigenetic changes of $p66^{Shc}$ promoter in isolated peripheral blood monocytes (DNA methylation and histone 3 [H3] acetylation), *2*) 24-h urinary excretion rates of 8-isoprostaglandin $F_{2\alpha}$ (8-iso$PGF_{2\alpha}$), and *3*) brachial artery flow-mediated dilation (FMD). After 6-months of the IGC program, patients with T2D repeated the same tests and performed continuous glucose monitoring (CGM) to assess markers of glycemic instability, such as the mean amplitude of glycemic excursion (MAGE) and postprandial incremental area under the curve (AUCpp) of blood glucose levels.

Isolation of Peripheral Blood Monocytes

Blood was collected in a BD Vacutainer CPT Mononuclear Cell Preparation Tube–Sodium Heparin (BD Biosciences, Franklin Lakes, NJ) and centrifuged for 20 min at 1,800*g*

at room temperature. The turbid white layer above the Ficoll-paque density gradient containing the mononuclear blood cells was transferred to a clean tube and washed twice with PBS. Subsequently, monocytes were isolated by using magnetic CD14-coated beads and magnetic activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) (24).

Real-time PCR

PCR experiments for $p66^{Shc}$ gene were performed with a TaqMan Gene Expression Assay kit and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). A $p66^{Shc}$ predesigned primer (Hs01050695_g1; Applied Biosystems) and TATA-box binding protein (TBP) (Hs00427620_m1; Applied Biosystems) as endogenous control for normalizing RNA concentration were used.

Real-time PCR for SIRT1 (forward: 5′-GCCGGAAACAATACCTCCAC-3′; reverse: 5′-ACCCCAGCTCCAGTTAGAAC-3′) and DNMT3b (forward: 5′-AGTGACACGGGGCTTGAATA-3′; reverse: 5′-CTTCACGGTTCCAACAGCAA-3′) were performed in an Mx3000P PCR cycler (Stratagene) with SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich, St. Louis, MO). TBP (forward: 5′-CGTGGCTCTCTTATCCTCATG-3′; reverse: 5′-GCCCGAACCGCCGAATATA-3′) were used as endogenous controls for normalizing RNA concentration. Differences in cycle threshold (Ct) values between test gene and endogenous controls (TBP, ΔCt) were calculated and used for statistical analysis.

Analysis of DNA Methylation by Methylation-Specific PCR

Genomic DNA (gDNA) from peripheral blood monocytes was obtained by using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. One microgram of gDNA was denatured, and unmethylated cytosines were converted to uracil in the denatured samples (Cells-to-CpG Bisulfite Conversion Kit; Applied Biosystems). CpG methylation was quantified by methylation-specific real-time PCR by using 100 ng bisulfite-converted gDNA as the template and methylation-specific primers for CpG in the human $p66^{Shc}$ promoter. Because the $p66^{Shc}$ promoter region from −100 to +40 base pairs is rich in CpG island, a specific primer for methylated (forward: 5′-GTTTAGGTTTATTGTATGGGGTAGC-3′; reverse: 5′-CCTTCCTATCCTAATTAAACACTCG-3′) and unmethylated DNA (forward: 5′- TTAGGTTTATTGTATGGGGTAGTGG-3′; reverse: 5′-TTCCTATCCTAATTAAACACTCAAA-3′) located in this specific region (−45 to +45 base pairs) was predesigned. The methylation level of the $p66^{Shc}$ promoter was calculated by using the methylation index as previously reported (25).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed by using the Magna ChIP Assay Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. ChIP was performed with 10 μg anti-acetylated H3 antibody (06-599; Millipore) and an equivalent amount of mouse IgG as negative control. Washes and elution of the immunoprecipitant DNA were performed according to the Magna ChIP protocol (Millipore). ChIP quantifications to $p66^{Shc}$ promoter were performed by real-time PCR (forward 1: 5′- ATTGCCTCATTCTCACCCTTG-3′; reverse 1: 5′-GCCAAGAGGAAGAGCAAAGC-3′; forward 2: 5′-GCAGATGTGTCTTCTGATCTCTCTGT-3′; reverse 2: 5′-TGAGAATGAGGCAATCAGGGTCC-3′; forward 3: 5′-CACCAGCTTTGCTCTTCCTCTTG-3′; reverse 3: 5′-ACAGTAAGCCTGGGCCATTAGC-3′).

Assessment of Urinary 8-isoPGF$_{2\alpha}$ Levels

Both at baseline and at follow-up, 24-h urinary samples were collected and incubated with the antioxidant 4-hydroxy-TEMPO (1 mmol/L) and immediately stored at −80°C until analyses for 8-isoPGF$_{2\alpha}$. Levels of isoPGF$_{2\alpha}$ were assessed by using a commercially available kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions.

FMD of the Brachial Artery

Endothelial-dependent vasodilation was assessed as dilation of the brachial artery in response to increased blood flow. Two expert sonographers (L.C. and G.R.) carried out the examinations with Doppler echocardiography (26,27). Endothelium-independent vasodilation was elicited by the administration of a low dose (25 μg) of sublingual glyceryl trinitrate (GTN). Recording time frames were 10 min for FMD studies (1 min for baseline, 5 min of ischemic period, 4 min for assessing changes in diameter after reactive hyperemia) and 6 min for GTN-mediated dilation (1 min for baseline, 5 min for assessing changes in diameter after GTN administration) (26,27).

CGM

Subcutaneous interstitial glucose levels were monitored on an ambulatory basis over 3 consecutive days by using a second-generation CGM system (iPro2; Medtronic). The sensor was inserted on day 0 and removed on day 3 at midmorning. The data were downloaded to a computer for evaluation of glucose variations, but these calculations were limited to data obtained on days 1 and 2 to avoid bias as a result of both insertion and removal of the sensor (insufficient stabilization of the monitoring system). The characteristic glucose pattern of each patient was calculated by averaging the profiles obtained on study days 1 and 2. MAGE and AUCpp were calculated as previously reported (21).

Statistical Analysis

The normality of continuous variables was assessed by the Kolmogorov-Smirnov test. All normally distributed variables are expressed as mean (SD), unless otherwise stated. Data not normally distributed are shown as median (interquartile range [IQR]). Comparisons of continuous variables between control subjects and patients with T2D were performed by using unpaired two-sample *t* and Mann-Whitney *U* tests, as appropriate, whereas comparisons of variables at enrollment and 6-month follow-up in the T2D group were done by paired *t* or Wilcoxon test, as indicated. Multiple comparisons between normally distributed variables were performed by one-way ANOVA followed by

Bonferroni correction. Non-Gaussian variables were compared by the Kruskal-Wallis test followed by Dunn's post hoc test. Between-variable correlations were assessed by Spearman test. Multiple linear regression models were built to explore the independent link between glycemic markers (AUCpp, MAGE, and HbA_{1c}) and epigenetic changes of the $p66^{Shc}$ promoter. Regressions were adjusted for potential confounders, namely age, sex, BMI, and antidiabetic treatment. $P < 0.05$ was considered statistically significant. We calculated the number of patients with T2D patients required for the study to reject the null hypothesis 99% of the time (one-tailed type II error rate of 0.01) when $r \geq 0.80$ with a two-tailed type I error at the 0.05 level of significance (28). All statistical analyses were performed by using GraphPad Prism version 5.0 and SPSS version 20 software.

RESULTS

Study Population

Clinical and laboratory characteristics of patients with T2D and control subjects are shown in Table 1. No significant age and sex differences existed between groups, whereas blood glucose, HbA_{1c} levels, BMI, waist circumference, and triglyceride levels were increased in the T2D group. In the T2D group, the mean disease duration was 6.9 (5.6) years. At baseline, 38 patients with T2D (97.5%) were receiving glucose-lowering treatment with hypoglycemic agents, insulin, or their combination, and 1 (2.6%) patient was not taking any glucose-lowering drug. After enrollment, an IGC program was implemented in all patients with T2D aiming to achieve an $HbA_{1c} \leq 7\%$ (Fig. 1*A*). Comparison of clinical and laboratory data in patients with T2D at baseline and follow-up are shown in Table 2. Median HbA_{1c} decreased from 7.8% (IQR 7.5–8.5%) to 6.6% (6.3–7.0%; $P < 0.001$). No major hypoglycemic events (requiring intervention) were observed during follow-up. Anthropometric parameters (body weight/BMI), and other cardiovascular risk factors (blood pressure, lipids) did not change throughout the study.

Effects of Glycemic Control on Endothelial Function and Oxidative Stress

Baseline endothelial function, as assessed by FMD of the brachial artery, was significantly impaired in patients with T2D compared with control subjects (median 4.9% [IQR 3.7–5.8%] vs. 8.5% [7.3–9.8%]; $P < 0.001$) (Fig. 1*B*). Endothelium-independent dilatation to nitroglycerine was comparable in the two groups (11.85% [10.9–13.6%] vs. 11.80% [8.5–14.8%]; $P = 0.69$), and no differences were observed in arterial diameter as well as in resting or hyperemic flow (data not shown). Patients with T2D also showed higher 24-h urinary excretion rates of 8-isoPGF$_{2\alpha}$, an in vivo marker of oxidative stress (360.9 [351.2–411.5] vs. 155.8

Table 1—Demographics, laboratory parameters, and medications of the study population

Characteristics	Control subjects (n = 24)	Patients with T2D (n = 39)	P value[a]
Age (years)	44.8 (12.7)	50.9 (11.6)	0.06
Women, n (%)	14 (58.3)	15 (38.5)	0.19[b]
Diabetes duration (years)	—	6.9 (5.6)	NA
BMI (kg/m^2)	23.4 (2.9)	29.2 (4.1)	<0.001
Waist circumference (cm)	81.1 (12.3)	100.7 (14.3)	<0.001
Current smokers, n (%)	6 (25.0)	4 (10.3)	0.16[b]
Blood pressure			
Systolic (mmHg)	125.0 (112.0–137.3)	130.0 (120.0–140.0)	0.13[c]
Diastolic (mmHg)	80.0 (70.0–85.0)	80.0 (75.0–80.0)	0.99[c]
Glucose (mg/dL)	81.0 (77.0–88.0)	153 (137.0–189.0)	<0.001[c]
HbA_{1c} (%)	5.0 (4.8–5.3)	7.8 (7.5–8.5)	<0.001[c]
Total cholesterol (mg/dL)	182.4 (23.5)	180.2 (42.2)	0.84
LDL cholesterol (mg/dL)	101.9 (20.9)	103.8 (37.6)	0.85
Triglycerides (mg/dL)	95.0 (72.8–120.0)	105.5 (87.8–151.8)	0.044[c]
HDL cholesterol (mg/dL)	50.0 (44.3–68.8)	49.0 (41.0–55.0)	0.21[c]
Baseline medications, n (%)			
Statins	—	18 (46.2)	NA
ACE inhibitors/ARBs	—	17 (43.6)	NA
Diuretics	—	14 (35.9)	NA
β-Blockers	—	8 (20.5)	NA
Hypoglycemic agents	—	31 (79.5)	NA
Insulin	—	9 (23.1)	NA

Data are mean (SD) or median (IQR) unless otherwise indicated. SI conversions: To convert glucose to mmol/L, multiply by 0.0555; total LDL and HDL cholesterol to mmol/L, multiply by 0.0259; and triglycerides to mmol/L, multiply by 0.0357. ARB, angiotensin receptor blocker; NA, not applicable. [a]Reported P values are from unpaired two-sample t tests, unless otherwise stated. [b]From χ^2 test. [c]From Mann-Whitney U test.

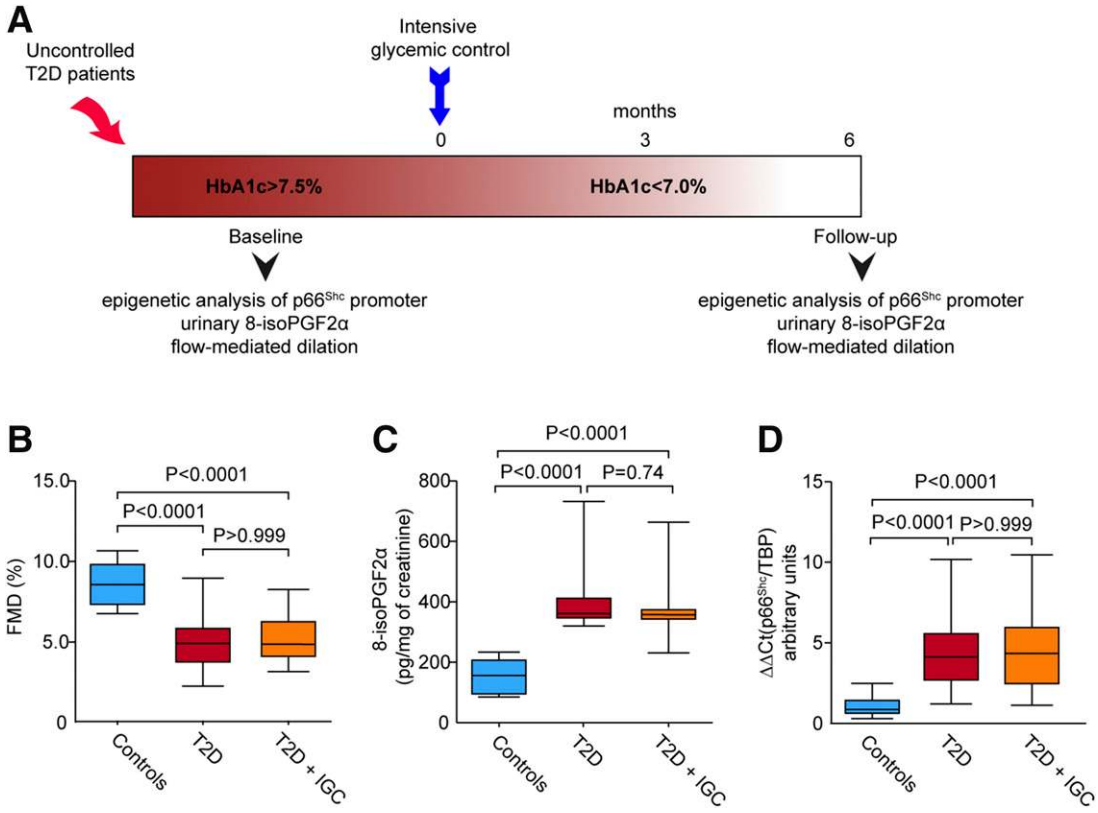

Figure 1—IGC does not affect endothelial dysfunction, oxidative stress, and $p66^{Shc}$ upregulation. *A*: Schematic showing the study design. Thirty-nine patients with uncontrolled T2D (HbA_{1c} >7.5%) were consecutively enrolled in an outpatient setting. Patients were assigned to IGC for 6 months with the aim of achieving an HbA_{1c} target of ≤7.0%. Brachial artery FMD, 24-h urinary excretion rates of 8-$isoPGF_{2\alpha}$, and $p66^{Shc}$-related epigenetic changes in peripheral blood monocytes were assessed at baseline and follow-up. *B*: Box plots show median values of FMD of the brachial artery in control subjects (baseline) and patients with T2D at baseline and follow-up (T2D + IGC). *C*: Median 24-h urinary excretion rates of 8-$isoPGF_{2\alpha}$. *P* values for FMD and 8-$isoPGF_{2\alpha}$ refer to Kruskal-Wallis test followed by Dunn's post hoc test. *D*: Real-time PCR showing gene expression of the mitochondrial adaptor $p66^{Shc}$ in the various groups. Data are mean (SD). *P* values refer to one-way ANOVA followed by Bonferroni correction.

[95.2–206.3] pg/mg of creatinine; $P < 0.001$) (Fig. 1*C*). Of note, 6-month IGC did not improve FMD (4.8% [4.1–6.2%]; $P = 0.16$) and 8-$isoPGF_{2\alpha}$ (357.5 [342.9–373.7] pg/mg of creatinine; $P = 0.10$) compared with baseline values, respectively (Fig. 1*B* and *C*).

Persistent $p66^{Shc}$ Upregulation

The $p66^{Shc}$ gene expression was significantly higher in peripheral blood monocytes isolated from patients with T2D than in control subjects (4.87 [2.91] vs. 1.00 [0.55] arbitrary units [AU]; $P < 0.001$) (Fig. 1*D*). Linear regression models showed that $p66^{Shc}$ mRNA levels were independently associated with 8-$isoPGF_{2\alpha}$ urinary excretion and FMD, regardless of potential confounders (Supplementary Table 1). We found that upregulation of $p66^{Shc}$ was not reverted by IGC (4.77 [2.88] AU; $P = 0.39$ vs. baseline), suggesting that persistent $p66^{Shc}$ overexpression may contribute to ongoing oxidative stress and vascular dysfunction in this setting (Fig. 1*D*).

Epigenetic Remodeling of $p66^{Shc}$ Promoter

Bisulfite analysis of transcriptionally active regions of $p66^{Shc}$ promoter revealed that DNA methylation was significantly reduced in peripheral blood monocytes isolated from patients with T2D compared with control subjects (43.5% [19.3%] vs. 100% [0.37%]; $P < 0.001$), and IGC was not able to reverse such a detrimental signature (49.2% [22.7%]; $P = 0.53$ vs. baseline) (Fig. 2*A*). To understand the mechanisms of reduced CpG methylation, we assessed the expression of the methyltransferase DNMT3b, an important methyl-writing enzyme involved in the maintenance of DNA methylation (12). DNMT3b expression was inhibited in patients with T2D compared with control subjects, and such downregulation was not affected by IGC (Fig. 2*B*). Furthermore, ChIP experiments revealed that the interaction between DNMT3b and $p66^{Shc}$ promoter in control subjects was strongly reduced in patients with T2D, and IGC could not rescue such an interaction (Fig. 2*C*). This latter finding confirms that DNMT3b-dependent methylation of $p66^{Shc}$ promoter is suppressed in patients with T2D and remains unchanged despite reduction of HbA_{1c} levels.

Because DNA hypomethylation triggers gene expression by clustering with histone acetylation (12), we determined the acetylation status of H3 on $p66^{Shc}$ promoter. H3 acetylation

Table 2—Baseline and follow-up clinical characteristics and glucose-lowering medications of patients with T2D

	Patients with T2D		
	Baseline (n = 39)	Follow-up (n = 39)	P value[a]
Weight (kg)	83.6 (13.2)	83.1 (12.7)	0.27
BMI (kg/m^2)	29.2 (4.1)	29.0 (4.0)	0.23
Blood pressure			
Systolic (mmHg)	130.0 (120.0–140.0)	122.5 (120.0–132.5)	0.13[b]
Diastolic (mmHg)	80.0 (75.0–80.0)	80.0 (70.0–80.0)	0.13[b]
Glucose (mg/dL)	153.0 (137.0–189.0)	136.5 (125.8–157.0)	0.002[b]
HbA_{1c} (%)	7.8 (7.5–8.5)	6.6 (6.3–7.0)	<0.001[b]
HbA_{1c} (mmol/mol)	31 (29–34)	62 (58–69)	<0.001[b]
Total cholesterol (mg/dL)	180.2 (42.2)	182.5 (42.8)	0.28
LDL cholesterol (mg/dL)	103.8 (37.6)	108.3 (37.2)	0.14
Triglycerides (mg/dL)	105.5 (87.8–151.8)	107.0 (77.5–150.5)	0.45[b]
HDL cholesterol (mg/dL)	48.9 (11.6)	50.1 (12.8)	0.16
Diabetic treatment, n (%)			
Metformin	30 (76.9)	29 (74.4)	1.0[c]
Secretagogues	8 (20.5)	7 (17.9)	1.0[c]
DPP-4 inhibitors	4 (10.3)	9 (23.1)	0.22[c]
GLP-1 agonists	3 (7.7)	7 (17.9)	0.31[c]
Acarbose	1 (2.6)	1 (2.6)	1.0[c]
Insulin	9 (23.1)	15 (38.5)	0.22[c]
Combination of glucose-lowering drugs, n (%)			
0	1 (2.6)	0 (0)	1.0[c]
1	25 (64.1)	16 (41.0)	0.07[c]
2	9 (23.1)	16 (41.0)	0.15[c]
3	4 (10.3)	7 (17.9)	0.52[c]

Data are mean (SD) or median (IQR). SI conversions: To convert glucose to mmol/L, multiply by 0.0555; total LDL and HDL cholesterol to mmol/L, multiply by 0.0259; and triglycerides to mmol/L, multiply by 0.0357. [a]Reported P values are from paired two-sample t test, unless otherwise stated. [b]From Wilcoxon test. [c]From χ^2 test.

bound to p66^{Shc} promoter was increased in patients with T2D compared with control subjects (345.4 [185.4] vs. 100 [37.0] AU; $P < 0.001$) (Fig. 3*A*). Such a posttranslational mechanism of active transcription was not erased by IGC (354.4 [206.2] AU; $P = 0.90$ vs. baseline) (Fig. 3*A*). Accordingly, we found that the chromatin-modifying enzyme SIRT1 involved in H3 deacetylation (29) was downregulated in patients with T2D regardless of glycemic control (Fig. 3*B*). ChIP analysis showed that SIRT1-dependent deacetylation of p66^{Shc} promoter was markedly

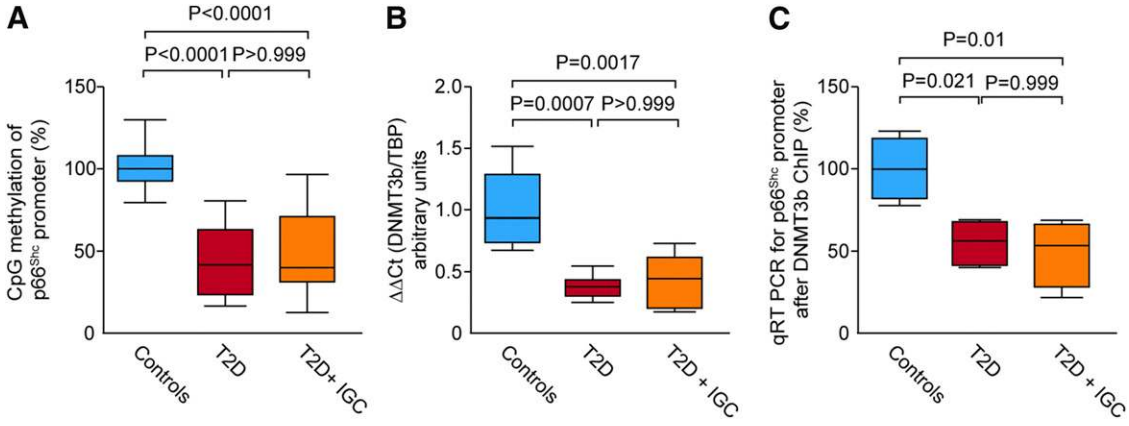

Figure 2—Persistent demethylation of p66^{Shc} promoter. *A*: Bisulfite analysis showing CpG methylation of p66^{Shc} promoter in control subjects and patients with T2D before and after IGC. *B*: Quantitative real-time (qRT) PCR showing gene expression of the methyltransferase DNMT3b in the three groups. *C*: Interaction between DNMT3b and p66^{Shc} promoter as shown by ChIP assay. All determinations were performed in peripheral blood monocytes. Data are mean (SD). P values refer to one-way ANOVA followed by Bonferroni correction.

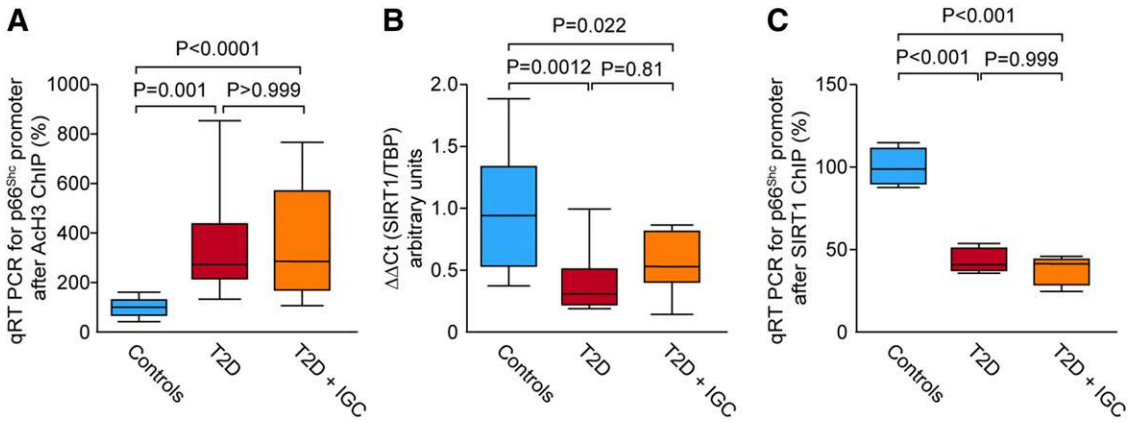

Figure 3—SIRT1-dependent histone acetylation despite glycemic control. *A*: Acetylation of H3 (AcH3) bound to $p66^{Shc}$ promoter. *B*: Quantitative real-time (qRT) PCR showing gene expression of the deacetylase SIRT1. *C*: ChIP assay reveals the interaction between SIRT1 and $p66^{Shc}$ promoter in the three groups. All determinations were performed in peripheral blood monocytes. Data are mean (SD). *P* values refer to one-way ANOVA followed by Bonferroni correction.

reduced in patients with T2D and not affected by IGC (Fig. 3*C*). To further strengthen these findings, we investigated the correlation between these epigenetic changes and $p66^{Shc}$ gene expression. Both DNA methylation ($r = -0.51$; $P < 0.009$) and H3 acetylation ($r = 0.48$; $P < 0.01$) significantly correlated with $p66^{Shc}$ gene transcription within the T2D cohort.

Glycemic Variability and Adverse Epigenetic Signatures

The results obtained so far suggest that the reduction of HbA_{1c} levels is unable to reprogram the adverse chromatin pattern underlying persistent $p66^{Shc}$ transcription. Hence, we investigated whether glycemic excursions rather than HbA_{1c} explain the epigenetic pattern of $p66^{Shc}$ promoter. At follow-up, CGM was performed in patients with T2D to assess MAGEs and AUCpp. After 3-day monitoring, mean values of MAGE and AUCpp were 84.9 (29.8) mg/dL and 457.6 (842) mg/dL · h, respectively. Only subjects with values >50th percentile of MAGE and AUCpp showed an adverse epigenetic remodeling of $p66^{Shc}$ promoter (Fig. 4*A* and *B*). By contrast, the epigenetic profile did not differ between patients with HbA_{1c} above and below the median value (Fig. 4*C*). Linear regression models adjusted for age, sex, BMI, and glucose-lowering treatment confirmed that MAGE and AUCpp were independently associated with adverse epigenetic signatures on $p66^{Shc}$ promoter (Supplementary Table 2). Taken together, these findings indicate that glycemic variability promotes chromatin changes, leading to persistent vascular dysfunction despite IGC (Fig. 5).

DISCUSSION

This study demonstrates that the reduction of HbA_{1c} levels cannot inhibit the overexpression of mitochondrial adaptor $p66^{Shc}$, resulting in persistent oxidative stress and endothelial dysfunction. Several lines of evidence support these conclusions. First, epigenetic changes of $p66^{Shc}$ promoter, namely DNA hypomethylation and H3 acetylation, promote gene transcription in patients with T2D, and IGC does not reverse them. Second, persistent downregulation of methyl-writing DNMT3b and acetyl-erasing SIRT1 enzymes favors the adverse chromatin remodeling responsible for continuous $p66^{Shc}$ upregulation. Finally, AUCpp and MAGE but not HbA_{1c} are independently associated with these epigenetic signatures.

Experimental studies in human endothelial cells have suggested that transient hyperglycemia elicits long-lasting epigenetic changes of oxidant and inflammatory genes, which may account for sustained cellular damage despite restoration of normoglycemic conditions (19,30). Consistently, we showed that adaptor protein $p66^{Shc}$ is upregulated in diabetic mice and that such overexpression is not affected by restoration of normoglycemia. This phenomenon was associated with sustained generation of mitochondrial reactive oxygen species (ROS), reduced nitric oxide availability, and persistent endothelial dysfunction (18). In the current experimental model, we found that $p66^{Shc}$ upregulation was triggered by hypomethylation of CpG dinucleotides and H3 acetylation of the promoter. Whether these mechanisms are active in patients with T2D is unknown.

The current study investigated whether $p66^{Shc}$ is involved in persistent ROS generation and endothelial dysfunction in patients with T2D despite IGC. We show that targeting $HbA_{1c} \leq 7.0\%$ did not revert diabetes-induced $p66^{Shc}$ overexpression, oxidative stress, and endothelial dysfunction. Epigenetic changes of DNA/histone complexes may explain persistent upregulation of $p66^{Shc}$. Indeed, DNA hypomethylation and H3 acetylation were found on the $p66^{Shc}$ promoter of patients with T2D, and IGC did not affect such detrimental chromatin modifications. That these epigenetic signatures are functionally linked is well established. Demethylation of CpG dinucleotides favors histone acetylation and chromatin accessibility to transcription factors (11). Persistent downregulation of chromatin-modifying enzymes methyltransferase DNMT3b and deacetylase SIRT1 may contribute to the epigenetic pattern

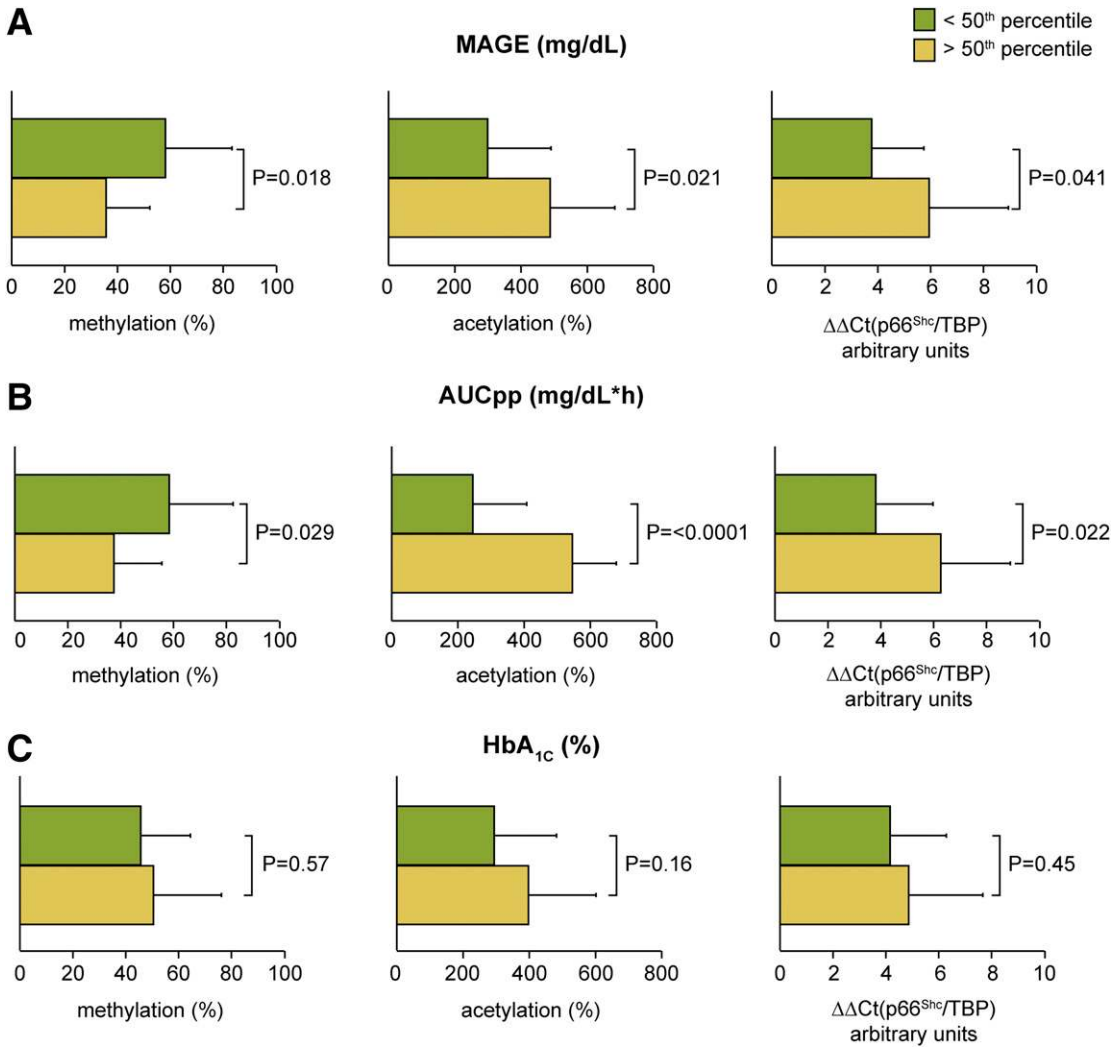

Figure 4—Glycemic variability but not HbA_{1c} is associated with adverse epigenetic signatures. $p66^{Shc}$ promoter methylation, H3 acetylation, and $p66^{Shc}$ mRNA levels in patients with T2D above and below median values of MAGE (*A*), AUCpp (*B*), and HbA_{1c} (*C*). Data are mean (SD). *P* values refer to Student *t* test.

observed on $p66^{Shc}$ promoter of patients with T2D, even after IGC. In agreement with other in vitro studies (17,24,30), reprogramming of DNMT3b and SIRT1 blunts $p66^{Shc}$ expression by resetting a condensed chromatin (25,31). Although DNMT3b and SIRT1 are well-established regulators of $p66^{Shc}$ transcription (25,31), we cannot exclude that other chromatin modifiers may co-occur to modulate $p66^{Shc}$ expression under hyperglycemic conditions. Future studies that use unbiased approaches may help with reaching a definite conclusion on this important aspect.

Of note, we found that restoration of HbA_{1c} target levels does not suppress the epigenetic changes of the $p66^{Shc}$ gene. Although HbA_{1c} is a reliable marker of glycemic control, it may explain only <25% of the risk of developing diabetic complications (32). Indeed, HbA_{1c} does not correlate with glycemic variability when adjusted for mean blood glucose (33). Experimental evidence suggests that transient spikes of hyperglycemia may be considered an independent risk factor (19). In this regard, significant emphasis has been given to the relationship between postprandial hyperglycemia and cardiovascular complications (34). Glycemic variability can be quantified by MAGE and AUCpp (35). MAGE has been conceived to measure the mean of the difference between consecutive peaks and nadirs and to provide a reliable estimate of glycemic instability (21). On the other hand, AUCpp is a well-established marker that reflects meal-related hyperglycemic swings. Previous work has shown that postprandial glucose levels are significantly prolonged in patients with T2D compared with healthy control subjects and may trigger ROS generation, reduced nitric oxide bioavailability, and endothelial damage (21,36–39). Of note, postprandial hyperglycemia is an independent risk factor for micro- and macrovascular complications in patients with T2D (40–42). In this regard, the Study to Prevent NIDDM has shown that decreasing

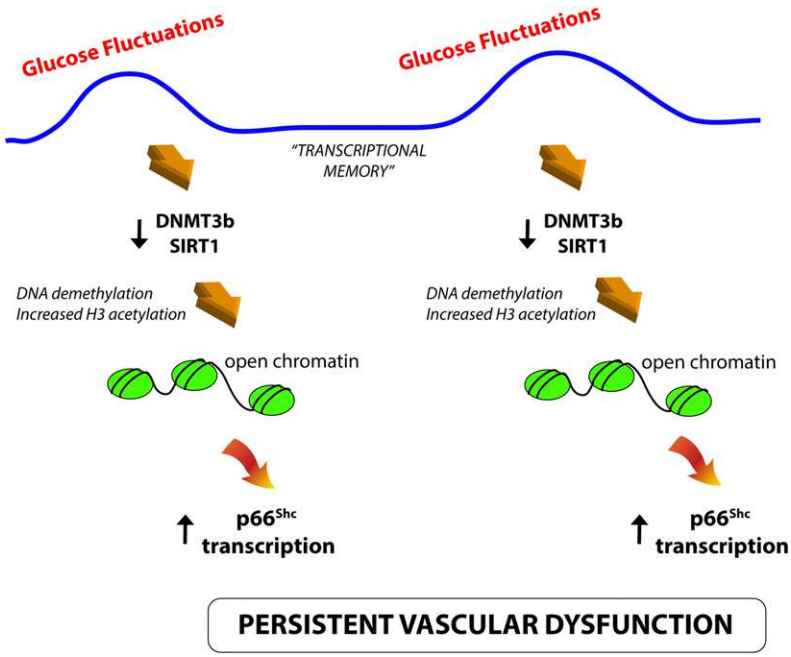

Figure 5—Role of glycemic variability in persistent vascular dysfunction. In patients with T2D with target HbA_{1c} values, continuous glucose fluctuations cause downregulation of chromatin-modifying enzymes DNMT3b and SIRT1 and subsequent epigenetic changes, namely reduced DNA methylation and increased H3 acetylation. Such epigenetic marks favor an open chromatin, leading to enhanced $p66^{Shc}$ transcription, oxidative burst, and persistent vascular dysfunction despite IGC. Therefore, glycemic variability maintains an epigenetic-driven transcriptional memory that may contribute to the progression of diabetic vascular complications in this setting.

postprandial hyperglycemia is associated with a 49% relative risk reduction in the development of cardiovascular events (hazard ratio 0.51 [95% CI 0.28–0.95]; P = 0.03) in high-risk subjects with impaired glucose tolerance (43). Although a growing body of evidence supports the association between glycemic excursions and vascular damage, no previous studies have investigated the possible mechanisms that underlie this relation in patients with T2D. Moreover, the current study is the first to our knowledge to link glucose fluctuations with modifications of the epigenetic repertoire in humans. Previous work in human endothelial cells demonstrated that transient hyperglycemic spikes increase chromatin accessibility through epigenetic changes that favor a proatherosclerotic phenotype (19). We were prompted by this background to explore the possible interconnections among glycemic fluctuations, chromatin remodeling, oxidative stress, and endothelial dysfunction. We observed that epigenetic signatures of $p66^{Shc}$ promoter are strongly associated with markers of glycemic instability (AUCpp and MAGE), regardless of relevant confounders such as age, sex, BMI, and diabetic treatment. By contrast, HbA_{1c} is unable to discriminate subjects with and without $p66^{Shc}$ epigenetic remodeling. This observation suggests that a well-established marker of chronic sustained hyperglycemia may not detect gene-activating events elicited by glucose fluctuations. On the basis of these findings, tailoring glucose-lowering strategies only on the level of HbA_{1c} may leave patients with T2D exposed to a substantial burden of glycemic peaks and nadirs, which perpetuate the epigenetic changes responsible for dysregulation of vascular oxidative pathways. The use of glucose-lowering drugs that specifically suppress glycemic variability, namely α-glucosidase inhibitors, GLP-1 receptor agonists, and SGLT2 inhibitors, might contribute to erasing detrimental epigenetic modifications and restoring vascular homeostasis. The reduction of glucose fluctuations may have contributed to the beneficial cardiovascular effects of liraglutide, semaglutide, and empagliflozin in the LEADER, SUSTAIN-6, and EMPA-REG OUTCOME trials, respectively (44).

The current study has some limitations. The epigenetic changes of $p66^{Shc}$ were assessed in peripheral blood monocytes from patients with T2D. However, an increasing body of evidence supports the concept that molecular changes in circulating mononuclear cells mirror early alterations in endothelial vasomotor function. In this regard, a strong correlation between oxidative stress in mononuclear cells and endothelium-dependent vasorelaxation in patients with T2D has been reported (45). These findings suggest that epigenetic changes observed in circulating cells may represent a reliable indicator of endothelial dysfunction and inflammation. Although the current analysis was conducted in a relatively small cohort and further studies are needed to confirm these results, we have unmasked a deleterious link between glucose fluctuations and chromatin remodeling and vascular dysfunction. These results provide insights into why targeting HbA_{1c} failed to improve cardiovascular outcomes in T2D and may set the stage for further investigations to exploit the impact of glycemic

variability on the entire epigenetic landscape and its implications for CVD phenotypes (46). Although we could not directly prove a casual relationship between glycemic variability and epigenetic changes of $p66^{Shc}$ promoter, previous experimental work demonstrated that glucose oscillations induce epigenetic signatures in human endothelial cells as well as in diabetic mice (19,47). Moreover, Quagliaro et al. (30) showed that glucose excursions compared with chronic sustained hyperglycemia induce the activation of protein kinase C, a master regulator of $p66^{Shc}$ activity. Future clinical investigations specifically targeting glycemic variability will be invaluable to support the biological link between glucose fluctuations and epigenetic changes. Therefore, in the midst of a global diabetes epidemic, this work encourages efforts to assess and minimize glycemic variability as an HbA_{1c}-independent trigger of adverse chromatin alterations and may offer an attractive perspective to reducing the staggering cardiovascular burden of diabetes.

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