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Metabolic memory: mechanisms and implications for diabetic vasculopathies

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In the past decades, a persistent progression of diabetic vascular complications despite reversal of hyperglycemia has been observed in both experimental and clinical studies. This durable effect of prior hyperglycemia on the initiation and progression of diabetic vasculopathies was defined as "metabolic memory". Subsequently, enhanced glycation of cellular proteins and lipids, sustained oxidative stress, and prolonged inflammation were demonstrated to mediate this phenomenon. Recently, emerging evidence strongly suggests that epigenetic modifications may account for the molecular and phenotypic changes associated with hyperglycemic memory. In this review, we presented an overview on the discovery of metabolic memory, the recent progress in its molecular mechanisms, and the future implications related to its fundamental research and clinical application.

diabetic vascular complications, metabolic memory, oxidative stress, inflammation, epigenetic modifications

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1 The discovery of metabolic memory

Diabetes mellitus is one of the most common chronic diseases in the world today. It was estimated that approximately 285 million adults (aged 20–79 years) were suffering from diabetes around the globe in 2010, and this number may rapidly increase to 439 million by 2030 [1]. In this regard, reducing the burden of diabetes remains to be a major challenge for both clinical doctors and bench scientists.

Although the advent of insulin therapy in 1922 prolonged life expectancy in diabetes patients, micro- and macrovascular complications have gradually become the most serious manifestations in patients with long-standing diabetes [2]. Microvascular complications are disabling and more diabetes-specific, whereas macrovascular complications are life-threatening and primarily caused by accelerated atherosclerosis. The devastating consequences of the long-term complications led to a series of intensive studies and vigorous debates over their pathoetiology. Although the underlying mechanisms seemed to differ between micro- and macrovasculopathies, hyperglycemia was generally proposed as the culprit in the last century. This is also known as the "glucose hypothesis" [3].

To test this hypothesis, Diabetes Control and Complications Trial (DCCT) was designed and conducted from 1982 to 1993. It was a controlled clinical trial in 1441 subjects (aged 13–39 years) with recently diagnosed type 1 diabetes mellitus (T1DM), comparing intensive insulin therapy (INT) with conventional insulin therapy (CNT). While INT aimed at achieving glucose levels as close to the nondiabetic range as safely possible, CNT aimed to maintain safe asymptomatic glucose control. In DCCT, the median HbA_{1c} level was 7% in INT compared with 9% in CNT. At study end, improved glycemic control was associated with a lower

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incidence and progression of retinopathy, nephropathy, and neuropathy, suggesting that early and intensive insulin therapy was beneficial for ameliorating diabetic microvascular complications [4].

In light of the successful completion of the DCCT, an observational follow-up study of the DCCT cohort, the Epidemiology of Diabetic Interventions and Complications (EDIC) study, was initiated to investigate the long-term effects of the original DCCT INT on the development of microvascular complications, as well as on the incidence of macrovascular complications that were not observed in DCCT due to its relatively short duration. During EDIC, more beneficial intensive regime was advised to CNT group as the new standard of care, resulting in a narrowing and then disappearance of the differences in HbA_{1c} maintained during DCCT [3]. Surprisingly, despite the loss of separation in HbA_{1c} as early as the first year of EDIC, the first four years of the EDIC follow-up demonstrated a further widening of the difference in microvascular outcomes (retinopathy and nephropathy) [5], and the beneficial effects of former DCCT INT on microvascular complications persistently exist even when EDIC enters its third decade [6–8]. In addition, former DCCT INT was also found to be associated with macrovascular complications, represented by a substantial reduction in the risk of cardiovascular diseases [9].

Similarly, the United Kingdom Prospective Diabetes Study (UKPDS), another controlled trial which recruited 5102 subjects (aged 25–65 years) with newly diagnosed T2DM from 1977 to 1991 and followed the cohort for about 10 years, also demonstrated persistent benefits of prior intensive diabetes management on both micro- and macrovascular complications [10,11]. During the trial phase, subjects were assigned to receive either conventional dietary treatment, aimed at maintaining fasting plasma glucose level <15 mmol L^{-1} (CNT), or intensive glycemic control with insulin or a sulfonylurea, aimed at maintaining a fasting plasma glucose level <6 mmol L^{-1} (INT). In the trial phase of UKPDS, the median HbA_{1c} level was 7.0% in INT compared with 7.9% in CNT. This difference correlated with a 25% risk reduction in the development of microvascular complications, in spite of insignificant correlation in the incidence of macrovascular complications [10]. During the following 10-year observational phase, no attempts were made to maintain previously assigned therapies and between-group differences in HbA_{1c} were lost after the first year. Despite disappearance of the difference, however, a persistent reduction in the incidence of microvascular complications and a significantly lower incidence of macrovascular complications (myocardial infarction and all-cause mortality) were observed in INT compared with CNT [11]. Since these clinical trials and subsequent epidemiological studies with both T1DM and T2DM respectively revealed a long-lasting memory-like phenomenon strongly associated with prior hyperglycemia, this durable effect of early glycemic control on the initiation and progression of diabetic vascular complications were, then, collectively termed as the "metabolic memory" phenomenon [3].

In fact, long before the above mentioned large-scale clinical trials, phenomena resembling metabolic memory had already been described in cell culture and animal models of diabetes as early as in the 1980s. Engerman et al. [12] showed that dogs subjected to poor glycemic control (PC) for 2.5 years and then treated with insulin to maintain good glycemic control (GC) for another 2.5 years still developed incipient diabetic retinopathy. In contrast, dogs treated with insulin within two months of the development of diabetes and maintained GC for subsequent five years exhibited significantly lesser signs of retinopathy. Shortly afterward, Roy et al. [13] further showed that transient hyperglycemic stimulation could persistently increase the expression of fibronectin and collagen IV mRNA in cultured human endothelial cells exposed to PC and followed by GC. Consistently, Hammes et al. [14] found that, while islet transplantation in rats within six weeks of diabetes significantly slowed the development of diabetic retinopathy and prevented retinal vessel occlusion, transplantation 12 weeks after the development of diabetes only partially slowed the progression towards retinopathy and failed to prevent vessel occlusion. Although these pioneer work jointly implied a novel concept of hyperglycemic memory, not enough attention was given to these basic scientific researches until their clinical relevance was established in large-scale trials, such as DCCT/EDIC and UKPDS, in the early 21st century.

During the investigation of EDIC, mechanistic studies have already been performed in DCCT/EDIC cohorts [15–17]. For instance, Genuth et al. [15] reported that the earlier measurements of glycated collagen and advanced glycation end product (AGE) levels strongly correlated with the risk of progression of retinopathy and nephropathy from the end of the DCCT to 10 years later. In addition, glycated collagen, rather than AGEs, explained more of the variation in the 10-year progression of these complications. Furthermore, the predictive effect of HbA_{1C} disappeared after adjustment for glycated collagen and AGEs. Hence, these results collectively indicated that glycation and subsequent AGE formation may be the driving force for the persistent progression of diabetic microvascular complications [15]. Ever since, metabolic memory and its underlying mechanisms have attracted broad interest.

2 The mechanisms of metabolic memory

2.1 Oxidative stress plays central roles in the occurrence of metabolic memory

Previously, five major mechanisms have been demonstrated to mediate hyperglycemia-induced tissue damage: (i) increased flux of glucose through the polyol pathway; (ii)

increased intracellular formation of AGEs (advanced glycation end products); (iii) increased expression of the receptor for AGEs and its activating ligands; (iv) activation of protein kinase C (PKC) isoforms; and (5) overactivity of the hexosamine pathway [18]. Moreover, several lines of evidence have suggested that all of these mechanisms are activated by a single upstream event: mitochondrial overproduction of reactive oxygen species (ROS) and subsequently exacerbated intracellular oxidative stress [19]. Therefore, tremendous focus has been laid on the pivotal roles of oxidative stress in the development of metabolic memory.

In 2007, two research groups respectively demonstrated the central roles of persistent oxidative stress in the cellular memory of hyperglycemia [20,21]. Ihnat et al. [20] showed that, in ARPE-19 retinal cells in culture, as well as in the retina of diabetic rats, markers of oxidative stress and mitochondrial damage (e.g., PKC-β, NADPH oxidase p47phox, PARP, 3-nitrotyrosine, and Bax) remained upregulated for one week of GC after being exposed for two weeks of PC. Surprisingly, while blocking sources of extra-mitochondrial ROS production only reduced some of the persistently induced stress markers, overexpression of the mitochondrial respiratory chain uncoupling protein UCP2 or α-lipoic acid during the normalization period reduced the induction of all the stress proteins, indicating that persistence of oxidative stress, especially mitochondrial ROS overproduction, may account for the memory phenomenon.

Consistently, Kowluru et al. [21] reported that peroxynitrite accumulation was significantly increased in rat retinal microvasculature in response to six months of PC and failed to normalize after six additional months of GC. In the meantime, the activity of manganese superoxide dismutase (MnSOD), the enzyme responsible for scavenging mitochondrial superoxide, remained inhibited in the same rat retina and the total antioxidant capacity was subnormal 6 months after cessation of PC. Successively, Kowluru and colleagues performed a series of studies in rat retina with similar experimental settings (2–6 months of PC followed by 2–6 months of GC). It was shown that, not only the structure and biogenesis of mitochondria remained persistently impaired in spite of reversal of hyperglycemia [22–26], but the genes and biomarkers of inflammation and apoptosis were also closely associated with metabolic memory [27,28].

A recent study done by Zheng et al. [29] further extended our understanding on oxidative stress-mediated metabolic memory in diabetic retinopathy. In bovine retinal capillary endothelial cells (BRECs) and the retina of diabetic rats treated with two weeks of hyperglycemia and one week of normoglycemia, hyperglycemia-induced Bax and NF-κB remained elevated after returning to normoglycemia. Small interfering RNA-mediated SIRT1 knockdown had increased sensitivity to hyperglycemia stress, whereas SIRT1 overexpression or activation by metformin inhibited the increase of mitochondrial ROS overproduction and ultimately suppressed the expression of Bax and NF-κB, via the SIRT1/LKB1/AMPK/ROS/PARP pathway. Furthermore, hyperglycemia was found to induce PARP activation, which in turn may downregulate SIRT1, implying a potential auto-feedback loop amplifying mitochondrial ROS overproduction and exacerbating intracellular oxidative stress.

2.2 Epigenetic mechanisms contribute to the chronicity of metabolic memory

In the last decade, with a surge in the discovery of epigenetic modifications induced by variations in glucose levels, oxidative stress-mediated metabolic memory was demonstrated to be mediated by epigenetic mechanisms. Epigenetic modifications refer to a variety of stable and heritable patterns that regulate gene expression without altering the DNA sequence. In general, epigenetic modifications consist of three types of regulation: (i) transcriptional inhibition by hypermethylation of CpG dinucleotides at gene promoters; (ii) transcriptional activation or inhibition through acetylation and/or methylation at histone tails; and (iii) post-transcriptional inhibition via microRNAs. As an important complement to the classic control of gene expression, epigenetic mechanisms modulate a variety of long-lasting environment-gene interactions during the development of diabetes and its vascular complications [30].

In the context of diabetic retinopathy, all the three types of control have been found to be directly associated with metabolic memory [31–34]. For instance, in a study performed by Zhong et al. [32], streptozotocin-induced diabetic rats were maintained in PC for two months and followed by GC for two additional months. It was shown that initial PC upregulated trimethyl histone H4 lysine 20 (H4K20me3), acetyl histone H3 lysine 9 (H3K9), and NF-κB p65 at the promoter and enhancer regions of *Sod2*, the nuclear gene encoding MnSOD, in isolated retinal endothelial cells. Subsequent reversal of hyperglycemia was unable to prevent these increases, resulting in decreased expression of MnSOD. Moreover, SUV420h2, one of the prime enzymes for the trimethylation of H4K20, were found to mediate the increased H4K20me3 at *Sod2* and thus contribute to the occurrence of metabolic memory. In another study of the same research group [34], monomethyl H3K4 (H3K4me1), dimethyl H3K4 (H3K4me2), and lysine-specific demethylase-1 (LSD1) were examined at *Sod2* in a similar experimental setting (PC for 3 months followed by GC for 3 months). Initial hyperglycemia reduced H3K4me1 and -me2, and increased the binding of LSD1 at *Sod2*. These changes remained unaffected after reinstitution of normoglycemia, but were reversed by LSD1-siRNA in rat retinal endothelial cells. Retina from human donors with diabetic retinopathy also had decreased H3K4me2 and increased LSD1 at *Sod2*. Collectively, since H3K4me1 and H3K4me2 are active markers, and H4K20me3 is a repressive epigenetic marker of gene transcription, these results imply that the metabolic

memory phenomenon of continuously decreased retinal MnSOD may be regulated by a complex array of post-transcriptional histone modifications. Despite advances in diabetic retinopathy, however, we are still at the very beginning to understand the underlying mechanisms of metabolic memory in other microvascular complications, such as diabetic nephropathy [35] and neuropathy [36,37].

In parallel with microvascular complications, huge progress has also been made in oxidative stress-mediated metabolic memory on the macrovasculature. In human aortic endothelial cells (HAECs) exposed to high glucose and aortas of diabetic mice, activation of the mitochondrial adaptor $p66^{Shc}$ by PKC-βII persisted after returning to normoglycemia (3 d of hyperglycemia followed by 3 d of normoglycemia). Persistent $p66^{Shc}$ upregulation and mitochondrial translocation were associated with continued ROS production, reduced nitric oxide (NO) availability, and apoptosis. Moreover, $p66^{Shc}$ activation accounted for the persistent elevation of AGEs. Of note, the overexpression of $p66^{Shc}$ was found to be epigenetically regulated by promoter CpG hypomethylation and general control nonderepressible 5 (GCN5)-induced acetylation at H3. $p66^{Shc}$-derived ROS production maintained PKC-βII upregulation and PKC-βII-dependent inhibitory phosphorylation of endothelial nitric oxide synthase (eNOS) at Thr-495, leading to a vicious cycle despite restoration of normoglycemia. More importantly, *in vivo* gene silencing of $p66^{Shc}$, performed at the time of glucose normalization, ameliorated persistent endothelial dysfunction and vascular apoptosis [38]. Hence, $p66^{Shc}$ is the key effector promoting the acceleration of diabetic macrovascular complications, and it may serve as a novel therapeutic target to reduce the deleterious effect of metabolic memory.

In another macrovascular cell model of metabolic memory, human umbilical vein endothelial cells (HUVECs) were incubated in high glucose for 14 d followed by normal glucose for 7 d. The effects of gliclazide and glibenclamide were added to the culture media early (first 14 days), late (last 7 days), or throughout the study to evaluate markers of oxidative stress and apoptosis. It was observed that gliclazide applied early or throughout the study partially improved endothelial cell apoptosis by reducing oxidative stress, while glibenclamide showed no relevant effect, implying that the increase in ROS production may be a primary, yet reversible biological event following hyperglycemia [39]. Accordingly, whether the downstream targets of ROS production may also confer metabolic memory on vascular cells is attracting growing attention.

Indeed, El-Osta et al. [40] demonstrated that the increased expression of NF-κB p65 gene after 16 h of hyperglycemia was retained for up to 6 d following reinstitution of normoglycemia in human microvascular endothelial cells (HMEC-1). In addition, a similar trend was also observed in the isolated aorta of non-diabetic mice subject to four sequential injections of high glucose solution at 2-h intervals. The sustained expression of the NF-κB p65 was associated with both upregulation of an active marker (H3K4m1) and downregulation of repressive markers (H3K9m2 and H3K9m3) of gene transcription, as a result of altered recruitment of relevant histone methyltransferases (SET7 and SUV39h1) and demethylases (LSD1) to the p65 promoter. In a follow-up study, the authors [41] also showed that the NF-κB target genes may also subject to sustained epigenetic modifications (e.g., SET7-mediated H3K4m1) in response to prior hyperglycemia. Based on these findings, it can be speculated that transient high glucose insults may simultaneously or hierarchically induce persistent epigenetic changes on a variety of genes that work in concert with each other to maintain the detrimental memory effects of hyperglycemia in either micro- or macrovascular endothelial cells.

In addition to endothelial cells, other cell types in macrovasculature, such as macrophages and vascular smooth muscle cells (VSMCs) were also associated with epigenetically-regulated metabolic memory. For example, Natarajan and colleagues [42] showed that the expression of inflammatory genes, monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), were increased in VSMCs isolated from 9–11 weeks old male diabetic *db*/*db* mice, and retained even after up to eight weeks of normoglycemic *in vitro* culture. This memory phenomenon was mediated by sustained decreases in H3K4me3 levels and SUV39h1 occupancy at their promoters. Furthermore, the same research group [43] demonstrated that increased miR-125b in response to hyperglycemic stimulation could promote the expression of MCP-1 and IL-6 by targeting SUV39h1 mRNAs. It remains unclear whether miR-125b would sustain after reversal of hyperglycemia and continuously contribute to the occurrence of metabolic memory. Nonetheless, this cross-talk regulation between different types of epigenetic regulations strongly implies that dynamically-changed microRNAs can exert crucial roles in fine-tuning the functions of long-lasting epigenetic codes (e.g., histone modifications and DNA methylations) and thus may synergistically modulate the development of diabetic vascular complications.

Taken together, previous studies have well recognized mitochondrial ROS generation as a unifying mechanism for the pathogenesis of diabetes and its chronic complications [18,19]. Since the discovery of metabolic memory in both preclinical [12–14] and clinical [3–9,11] studies, transient hyperglycemia-induced persistent ROS overproduction has been closely associated with the memory phenomenon in both micro- [20–29] and macrovascular [38,40] complications . Recently, stable and heritable epigenetic machinery, such as DNA methylations and histone modifications, added a new layer of gene regulation, not only to oxidative stress-related pathways [31–34,38,39] but also inflammation-associated signalings [40–42]. Furthermore, dynami-

cally-changed microRNAs have been proposed to coordinate the formation of cellular memory, and thus may contribute to the chronicity of the disease [43]. These novel findings extended our understanding about the underlying mechanisms of metabolic memory in diabetic vasculopathies.

3 Clinical perspectives and future implications

During the last decades, studies have found numerous biomarkers, such as HbA_{1c} [44–46], skin collagen glycation [15], plantar fascia thickness [47], AGEs [48,49], oxidized low-density lipoprotein (oxLDL) [49], nitric oxide metabolic pathway products (NOx) [49], as well as H3K9 acetylation in monocyte [17], to predict the progression of diabetic vascular complications and the occurrence of metabolic memory. Recently, circulating microRNAs have been associated with diabetes and its vascular complications [50]. For instance, miR-126, a highly endothelium-enriched microRNA, has been shown to be packaged into circulating microparticles and delivered to neighboring endothelial cells to further modulate their cellular functions. Additionally, miR-126 was significantly reduced in endothelium-derived microparticles and endothelial progenitor cells from diabetic patients, which, in turn, contributed to the initiation of diabetic vasculopathies [51,52]. Although the functional roles of circulating microRNAs (e.g., miR-126) in metabolic memory are still obscure, with growing understanding on their extracellular communication machinery, circulating microRNAs may hold a great potential as diagnostic markers for monitoring the progression of diabetic vascular complications.

In terms of therapeutics, the discovery of metabolic memory strongly supports the adoption of a more aggressive treat-to-target approach, instead of waiting for treatment failure. Although some anti-diabetes drugs (e.g., metformin) and antioxidant substances (e.g., MnSOD) have been shown to partially reverse metabolic memory at the experimental level, whether these chemicals may also bring benefits in real clinical practice still warrants thorough investigation. Interestingly, a latest effort made by Natarajan et al. taken the discovery of microRNAs to a higher level in the context of diabetic vasculopathies. Previously, miR-192 has been demonstrated as a master up-stream regulator in diabetic nephropathy, because it presides over a cascade of events leading to glomerular hypertrophy [53]. By treating diabetic mice with a locked nucleic acid (LNA)-modified inhibitor of miR-192 (LNA-anti-miR-192) from the onset of diabetes up to 17 weeks, Putta et al. [54] found a concomitant decreased gene expression in collagen, TGF-β, and fibronectin, and ameliorated renal fibrosis in diabetic nephropathy, indicating that LNA-miR-192 may serve as an ideal tool of pharmaceutical intervention against the formation of diabetic vascular complications. However, whether LNA-miR-192, as well as other microRNA inhibitors, may actually exert similar effects against the progression of metabolic memory needs to be answered.

On the other hand, results from the Outcome Reduction With Initial Glargine Intervention (ORIGIN) trial, the first large-scale study specifically designed to test the effects of early insulin therapy on patients with recent-onset diabetes and high cardiovascular risk, showed no benefit over comparator therapies (sulfonylureas and metformin), both for micro- and macrovascular diabetic complications [55]. Meanwhile, the STENO-2 and a more recent observational study on the role of blood glucose control have shown the crucial roles of controlling lipids, hypertension, and hypercoagulability on macrovascular outcomes [56–58]. These findings may jointly imply that considerable difference may exist in the mechanisms of microvascular complications developed from T1MD and T2MD, and that although hyperglycemia may be directly responsible for microvascular complications, other risk factors may also be very crucial for macrovascular complications. Therefore, despite the benefit of early aggressive glycemic control on preventing metabolic memory, challenges remain to find appropriate drugs and time points of intervention for each individual patient with overt and clinical diabetes. Of importance, emerging evidence suggests that micro- and macrovascular dysfunctions may interact with each other to further amplify the potential impact of metabolic memory [59–61]. Thus, future studies should also regard both micro- and macrovasculopathies as a whole, so as to acquire a more comprehensive view on the underlying mechanisms of their pathogenesis, and to develop more feasible and effective clinical strategies against the occurrence and persistence of hyperglycemia-induced metabolic memory.

In summary, the discovery of metabolic memory and novel insights into its potential mechanisms have brought diabetes-related scientific research and clinical practice into a new era. It could be hypothesized that metabolic memory is a newly-formed disease homeostasis that stores past hyperglycemic stress in the form of altered macromolecule contents (e.g., ROS and AGEs) and epigenetic signatures (DNA methylations and histone modifications) and becomes a sustained source of cellular hormesis. As a result, it may persistently drive the development of diabetic vasculopathies long after the reinstitution of normoglycemia. Notably, the hyperglycemic memory of macrovasculature is not only a direct result of prior hyperglycemic stimulation, but also indirect consequences of secondary biochemical and hemodynamic changes. Taken together, more light should be shed on metabolic memory-related systemic and translational medical researches so as to reduce the incidence of diabetic micro- and macrovasculopathies in the coming future.

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