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SYMPOSIUM

Biologic Variability in Plasma Glucose, Hemoglobin A1c, and Advanced Glycation End Products Associated with Diabetes Complications

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Abstract

Plasma glucose plays a key role in the complications of diabetes mellitus. Hemoglobin A1c (HbA1c) and circulating concentrations of advanced glycation end products (AGEs) are central to diabetes clinical care and pathophysiology. However, there is evidence for variation between individuals in the relationship of plasma glucose to both these measures and to specific complications. The glycation gap (GG) and hemoglobin glycation index represent tools for quantitating the variability in the relationship between plasma glucose and HbA1c useful for identification of underlying mechanisms. Recent evidence demonstrates the heritability of HbA1c, the GG, and AGEs, yet not of glycated serum proteins. There has been tremendous effort devoted to identifying the heritable basis of types 1 and 2 diabetes; however, studies on the heritable contributors to these mediators of glucose effect on complications are only beginning. New evidence for normal biologic variation in the distribution of glucose into the red blood cell (RBC) intracellular compartment and RBC lifespan in people with and without diabetes represent candidates for heritable mechanisms and contributors to the rise in HbA1c with age. Taken as a whole, genetic and mechanistic evidence suggests new potential targets for complications prevention and improvement in complications risk estimation. These observations could help tilt the risk–benefit balance in glycemic control toward a more beneficial outcome.

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Introduction

Powerful homeostatic mechanisms ensure that the internal "milieu" is maintained within a range near a set point that maximizes the functional elements of that cell, tissue, or organism. To achieve these goals, complex regulatory genes, cells, and mediators are activated. All vertebrates have these components at a level of complexity unparalleled in other living things, perhaps because there is a relationship between degrees of regulation and increasing levels of multicellular complexity. It follows that those systems that sustain homeostasis

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Abbreviations: (ADAG) A1c-derived average glucose, (AGE) advanced glycation end product, (CGM) continuous glucose monitoring, (CML) carboxymethyl-lysine, (DirecNet) Diabetes Research in Children Network, (DZ) dizygotic, (eAG) estimated average glucose, (FOS) Framingham Offspring Study, (GG) glycation gap, (GSP) glycated serum protein, (HbA1c) hemoglobin A1c, (HGI) hemoglobin glycation index, (MZ) monozygotic, (NHANES) National Health and Nutrition Examination Survey, (RBC) red blood cell, (T1DM) type 1 diabetes mellitus, (T2DM) type 2 diabetes mellitus

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are coded in the genome to maintain an evolutionary stable strategy and, therefore, that the given set point is itself genetically determined. Glucose, as one critical metabolic substrate for energy within cells, must be tightly regulated. As glucose shows biological diversity in blood concentrations (i.e., set points) for given individuals, that biological variation is, itself, probably genetically determined. In general, when disturbances of glucose are mild, the homeostatic response is correspondingly low-key. But when the change is severe or sustained, then the response can develop into a pathological process responsible for major human disease, including diabetes.

Blood Glucose Levels Are Strongly Inherited

Evidence from twin and family studies indicate that variation in glucose metabolism is genetically determined. Strikingly, genome-wide association studies identified several genetic loci associated with the risk of a major glucose metabolic disturbance, i.e., diabetes. The primary characteristics of type 2 diabetes mellitus (T2DM) include insulin resistance, relative insulin deficiency, and hyperglycemia.[1–4] The disease develops due to genetic predisposition and environmental factors such as altered food habits, obesity, and sedentary lifestyle. The basis of the disease is altered homeostasis of glucose metabolism through inadequate insulin secretion. Common gene variants consistently associated with T2DM include genes involved in insulin secretion such as calpain 10,[5,6] PPAR-γ coactivator 1,[7] Glu23Lys potassium inward rectifying channel (KCNJ11),[8,9] hepatocyte nuclear factor-4 alpha,[10–13] the glucose transporter (GLUT2),[14–16] and transcription factor 7-like 2 gene (TCF7L2).[17–19] Obesity, which is associated with insulin resistance, is also a major risk factor responsible for the development of T2DM and is potentially linked through disease risk genes such as Pro12Ala PPAR-γ,[20–22] FTO,[23] and retinol binding protein.[24] Estimates of high heritability of fasting blood glucose in some populations in some settings suggest that this as a suitable endpoint/marker for the identification of additional genes,[25] subject to the constraints raised by Simonis-Bik and colleagues regarding the susceptibility of fasting glucose to environmental factors.[26] The strongest association of fasting glucose has been with MTNR1B.[27]

Implications

Human genes were likely programmed in an energy-depleted environment, such as ancestral man might have encountered in the savannah ("thrifty genotype")[28] or that a fetus might encounter in the presence of placental insufficiency ("thrifty phenotype").[29] In either case, such a thrifty response would become maladaptive in an energy-replete environment. A genotype, after all, is only as good as the environment it finds itself in. One way whereby the environment influences genetically determined glucose homeostasis is through nongenetic events such as epigenetic factors. For example, epigenetic events influence adaptation of the cell to the intracellular energy status. A study revealed how transient hyperglycemia can induce long-lasting activating epigenetic changes (histone methylation) in the promoter of the nuclear factor B subunit p65 in endothelial cells in mice without diabetes, with increased p65 gene expression. Both the epigenetic changes and the gene expression changes persist for at least 6 days thereafter.[30] These results highlight the dramatic and long-lasting effects that short-term hyperglycemic spikes can have on vascular cells and suggest that transient spikes of hyperglycemia may be a hemoglobin A1c (HbA1c)-independent risk factor for diabetes complications.

Blood Glucose is Associated with Glycated Hemoglobin

Early studies illustrated a relationship between blood glucose, over a 3-month period, and HbA1c levels. That relationship held, even when studying large number of patients over many years.[31–33] However, the correlation was never perfect, and the question arose as to why it was not.[34–37] Such an imperfect correlation might reflect either diversity in what each reflects or inaccuracy of each measurement or both. Potential resolution of the latter came with improved assays and the international standardization of HbA1c.[38–40] Reducing blood glucose sampling distribution bias using continuous glucose monitoring (CGM) suggested that biometric issues could be, in part, resolved. This supported the belief of some that, if HbA1c could be expressed as an estimated average glucose (eAG), then patients with diabetes could converse with clinicians in a common "currency."[41]

The A1c-derived average glucose (ADAG) study was conducted to establish the best estimate of the relationship between mean blood glucose and HbA1c to permit use of eAG.[42] The design of the ADAG study was to measure continuous glucose levels for 48 h every month for 4 months and at least a 7-point profile for 3 days per week using a handheld glucose meter. The acceptance criterion was that the mean glucose of ≥90% of subjects in the study be within 15% of the eAG derived from the regression line relating mean glucose to HbA1c. The variance (r^2)

accounted for by the "weighted" glucose, HbA1c was 0.84, and exactly 90% of patients were within 15%. Setting aside the issue of what a weighted glucose might mean and why normal subjects were included in the analysis, even this study illustrates the imperfect relationship between HbA1c and blood glucose.[43] As also noted in another study from the Diabetes Research in Children Network (DirecNet) Study Group involving 47 children with type 1 diabetes mellitus (T1DM), utilizing CGM almost continuously for between 3 and 6 months, sampling once every minute, an r value of just 0.68 was found (variance r^2 accounted for 0.46), leading the investigators to conclude that expressing HbA1c as mean glucose was "tenuous at best."[44] Some of the differences between the ADAG and DirecNet results could be methodological: (1) ADAG used laboratory HbA1c and calibrated CGM using HemoCue (which has higher precision), while DirecNet used point-of-care HbA1c determinations and calibration of CGM with routine self-glucose monitoring; and (2) ADAG selected for subjects at relatively stable glycemic control, while control was more variable in the DirecNet subjects. But since other studies have shown that HbA1c can vary with hemoglobinopathy, race, age, pregnancy, and renal dysfunction, it seems reasonable to conclude that there is a good but imperfect relationship between blood glucose and HbA1c.[45]

Implications

The relationship between blood glucose and HbA1c implies that HbA1c might be related to diabetic microvascular and macrovascular complications, and so it is.[46–51] But the relationship between blood glucose and complications is likely to be complicated, involving cell stress, reactive oxygen species, protein glycation, advanced protein glycation, and endothelial dysfunction.[52,53] It follows that the relationship between HbA1c and diabetes complications is unlikely to be direct but instead serves as a surrogate marker of risk.

Glycated Hemoglobin is Strongly Inherited

A classic twin study of normal identical (monozygotic [MZ]) and nonidentical (dizygotic [DZ]) twin pairs demonstrated that genetic factors could influence HbA1c levels. Interclass correlations (r) were higher in MZ (r = 0.77) compared with DZ (r = 0.53) twin pairs, suggesting a substantial genetic effect with additive genetic effects (heritability), explaining 62% of population variance in HbA1c, the remainder being attributable to the influence of unique environment (23%) and age (14%).[54] This is similar to but slightly lower than the heritability of 74% for HbA1c reported by Simonis-Bik and associates.[26] Multivariate modeling indicated that genetic factors also have a substantial influence on fasting glucose levels (51%). However, HbA1c heritability could not be explained by genes in common with fasting glucose. In patients with T1DM, HbA1c levels were correlated in MZ twins concordant for diabetes (r = 0.68) but also in MZ twins discordant for the disease (r = 0.52). These significant correlations for HbA1c in both concordant and discordant pairs indicate a diabetes-independent familial effect. Thus HbA1c levels are substantially genetically determined and independent of the genes influencing fasting glucose. Even in T1DM, familial (i.e., diabetes-independent) factors influence protein glycation, implying that familial factors may explain, in part, the risk for microvascular complications, distinct from blood glucose levels. Age is one factor related to HbA1c levels. In the Framingham Offspring Study (FOS) and the National Health and Nutrition Examination Survey (NHANES) cohorts, HbA1c levels were positively associated with age in subjects without diabetes; in multivariate analysis, this was independent of fasting and 2 h plasma glucose,[55] although that does not fully exclude any possible role of variation in glycemic control. Linear regression revealed comparable increases in HbA1c per year in the nondiabetes populations (0.014 and 0.010 U, respectively), using both cross-sectional and longitudinal data. The 97.5 percentiles for HbA1c were 6.0 and 5.6 for persons without diabetes aged less than 40 years in FOS and NHANES, respectively, compared with 6.6 and 6.2 for persons 70 years or older. In a genome-wide analysis, Pare and coworkers found a novel linkage of glycated hemoglobin to the hexokinase 1 locus (HK1), which could be mediated through hemoglobin glycation, glucose metabolism, and/or diabetes;[56] also, linkage was found to sites previously associated with T2DM: GCK, SLC30A8, and G6PC2.

Implications

Hemoglobin A1c is subject to biological variation, including unidentified genetic factors. Implicit in current HbA1c interpretation is negligible variation in either red cell turnover in people who are hematologically normal or in the relationship between plasma glucose and red blood cell (RBC) glucose concentration to which hemoglobin is directly exposed. However, it would require only modest variation, a standard deviation of the order of 15% in either of these measures, to have a clinically important effect. Evidence presented later for such variation implies clinically relevant biological variation in HbA1c levels.

Glycated Hemoglobin is Associated with Glycation Gap

Persistent differences between HbA1c and blood glucose in subjects without diabetes have been ascribed to differences in "high glycator" and "low glycator" subsets.[35,36] This observation led to efforts to fractionate the variance in HbA1c to determine whether components of it are more closely related to glycemic control, while others remain constant despite variations in glycemic control. One strategy has been to identify glycated serum proteins (GSPs) using the measure fructosamine (i.e., resulting in a measure identified as the glycation gap [GG], previously called the glycosylation gap[57]) and, in the other instance, by the mean of capillary blood glucose measured throughout the day (yielding a measure referred to as the hemoglobin glycation index [HGI][58,59]). Cohen and colleagues reported that the GG is reproducible over time despite variation in glycemic control reflected in HbA1c and GSPs; GG correlated with the development of diabetic nephropathy in this retrospective study.[57] In a prospective study, the higher the GG, the greater the subsequent frequency of diabetic retinopathy.[60] McCarter and associates[59] found that HGI, like GG, was reproducible over time and that retinopathy and nephropathy risk were predicted by the HGI determined on numerous extended capillary glucose profiles throughout the duration of the Diabetes Control and Complications Trial. Use of these analytic approaches is subject to a number of limitations, requiring careful consideration of possible interference from the dependence of serum fructosamine on serum protein metabolism, of mean capillary glucose on sampling frequency and distribution and monitoring technique, and of the imprecision of HbA1c techniques.

Implications

The association between HGI and complications and HbA1c and complications could be mutually dependent or independent. Separating the two would be difficult, as both are correlated and, indeed, the one is derived from the other. The Diabetes Control and Complications Trial presented statistical models suggesting that subjects with similar levels of HbA1c had a higher risk of retinopathy progression in the conventional treatment group than in the intensive treatment group.[61] That analysis has been cited to support the hypothesis that specific patterns of glucose variation, in particular, postprandial hyperglycemia, contribute uniquely to an increased risk of microvascular complications above and beyond that explained by the level of HbA1c.[62,63] Additional analyses by Lachin and coworkers showed that virtually all (96%) of the beneficial effect of intensive versus conventional therapy on progression of retinopathy is explained by the reductions in the mean levels of HbA1c, similarly for other outcomes.[64] Further, subjects within the intensive and conventional treatment groups with similar levels of HbA1c over time have similar risks of retinopathy progression, especially after adjusting for factors on which they differ. It was concluded that HbA1c explained virtually all difference in risk of complications between the intensive and conventional groups and that a given level of HbA1c has similar effects within the two treatment groups. While the authors concluded that other components of hyperglycemia can only explain a small part of the differences in risk between intensive and conventional therapy over time, it is difficult to segregate the impact of hyperglycemia associated with HbA1c from that associated with HGI or GG since, in the analysis, HbA1c and HGI could be interchangeable.

Glycation Gap is Strongly Inherited

Evidence from both healthy twins and twins with diabetes indicates that HbA1c levels are genetically determined, which provides an independent line of evidence that HbA1c is, in part, determined by factors other than glycemic control. Given the two independent lines of evidence showing HbA1c variance and heritability,[26,54] a logical question that arises is whether the heritable components of HbA1c are associated preferentially with the GG fraction or the GSP fraction of the HbA1c variance, as this would narrow the range of mechanisms involved and inform candidate gene studies. Researchers therefore conducted a classic twin study using healthy female MZ and DZ twins without diabetes.[65] The predicted HbA1c was based on the regression line between HbA1c and GSP in a separate population spanning the pathophysiologic range. Glycation gap was more strongly correlated between MZ (r = 0.65) than DZ (r = 0.48) twins, adjusted for age and body mass index, i.e., 69% of population variance in GG is heritable, while the remaining 31% is due to unique environmental influences. In contrast, GSP was similarly correlated between MZ and DZ twins, hence not genetically determined. Glycation gap was strongly correlated to HbA1c (r = 0.48), attributable mostly to genetic factors. About one-third of the heritability of HbA1c is shared with GG; the remainder is specific to HbA1c.

Implications

There are two key implications of that study. First, it demonstrates a contrast between GSPs, which are not inherited, and HbA1c, which is inherited. This observation limits the range of candidate mechanisms that could

account for the heritability of HbA1c. Second, it demonstrates that a measure, the GG, that captures the variation of HbA1c in a population beyond that which is attributable to blood glucose variation also captures a proportion of the heritability of HbA1c based on quantitative genetic modeling. This second observation could not have been predicted given that GG is derived from both HbA1c and GSPs and that the former is inherited while the latter is not. The present work illustrates how measures of HbA1c variance from blood glucose control, including the GG and Chalew's "hemoglobin glycosylation index,"[66] are compatible with each other and internally consistent. They demonstrate that these measures in normal individuals capture different aspects of the same clinically important biological phenomenon, namely, that a HbA1c of a given value may not necessarily have the same clinical meaning in two different individuals with diabetes, even if a reduction of HbA1c by one percentage point does.

Hemoglobin A1c is a measure of glycemic control determined in the intra-RBC space, while glycation of serum proteins reflects a process in the extracellular compartment. By inference, the GG reflects the variance in HbA1c determined by processes in both the extracellular and intracellular compartments compared with those unique to the extracellular compartment. This study implicates genetically determined mechanisms active in the intracellular RBC compartment in the determination of HbA1c levels, potentially through the modification of glycation or deglycation of hemoglobin.

Potential Biologic Sources of the Glycation Gap

Demonstration of the GG led us to two investigative strategies: (1) establishment of a method to test the assumption by determining the distribution of a labeled glucose analog across the RBC membrane under controlled conditions *in vitro* and (2) application of state-of-the-art methods for RBC *ex vivo* labeling with biotin and re-infusion which permit more precise determination of RBC survival and quantitation of *in vivo* glycation rate. Using the labeled glucose analog technique, researchers found variation in glucose concentration inside compared to outside at steady state among individuals with and without diabetes. In a cross-sectional study, the ratio concentration inside/concentration outside increased with HbA1c and with the GG, but not with the corresponding serum fructosamine concentration.[67] While this is strictly a correlation, it strongly implies that the higher the intra relative to extra-cell glucose concentration, the higher the HbA1c. While the mechanism remains elusive, if glucose entry into cells subject to diabetes complications is predominantly GLUT1-dependent, GG could potentially serve as a surrogate marker of risk for intracellular glucose-related complications.

Using the biotinylated RBC technique, researchers were able to show that the glycation of hemoglobin is indeed linear with time in people with diabetes at steady state glycemic control, a widely held assumption that had not previously been demonstrated *in vivo*.[68] This property means that the clinically determined HbA1c will be linearly related to the RBC survival. High-precision RBC survival curves demonstrated curvilinear rather than linear RBC disappearance. This makes the traditional method for calculating red cell survival invalid and necessitated a new approach translating the curvilinearity into a "mean RBC age," with values roughly half those of traditionally reported survivals.[69] In subjects without and with diabetes, RBC mean cell age ranged from 38–59 days and 39–56 days, respectively. The linearity of glycation with time demonstrated in these experiments means that differences in RBC age (i.e., duration of hemoglobin exposure to circulating glucose) should result in corresponding differences in HbA1c at a given mean glucose concentration.

Implications

In other words, given three hematologically normal people with identical mean blood glucose differing in RBC survival across the range observed, if the one with the most typical RBC mean cell age has HbA1c 8.0%, having the shortest survival could lower the HbA1c to 6.4% and having the longest could raise it to 9.6%.[68] The tight fit of the biotinylated RBC curves to the data and the similarity of mean cell age in a subset of the population suggest that this range of RBC survival is fairly commonly observed in the general population. However, intrasubject reproducibility of RBC survival, as outlined, needs to be confirmed.

Blood Glucose is Associated with Advanced Glycation End Products

The formation of advanced glycation end product (AGE) by glycation and oxidation alters the functional property of matrix proteins and mediates sustained cellular changes by binding to AGE receptors. Advanced glycation end product formation has been implicated in microvascular and macrovascular disease associated with diabetes.[70,71] Heterogeneity of AGE implies that many products could be measured to estimate AGE formation. Of them, pentosidine and N^{ε} carboxymethyl-lysine (CML) are the

best characterized, CML can serve as a biomarker of oxidative stress resulting from sugar and lipid oxidation, and CML elevations are associated with diabetes and renal dysfunction.[72–75] It follows from these observations that AGEs are formed in the context of hyperglycemia but that the relationship between blood glucose levels and CML levels is not a simple one.

An Advanced Glycation End Product Can be Inherited

The formation of AGE is predominantly endogenous, despite also being derived from exogenous sources such as food,[76] so levels may be genetically determined. Genetic factors do, indeed, determine serum CML levels, at least in normal subjects. In a study of normal MZ and DZ twins, it was found that additive genetic effects (heritability) explained 74% of the population variance, while the remainder was due to unique environment, with no contribution from shared environment.[77] In these same normal twins, genetic factors influenced both fasting glucose levels (heritability, 51%) and HbA1c (heritability, 62%), but CML heritability could not be explained by genes in common with either. In striking contrast, a study of MZ twins discordant for T1DM found that twin serum CML levels were high, irrespective of diabetes status, with only limited heritability (unpublished personal observations).

Implications

The low heritability for serum CML levels in T1DM does not imply that these factors have no additive genetic variance. A low heritability means that, of all the observed variation, a small proportion is caused by variation in genotypes; it does not mean that additive genetic variance is small. Since we have previously shown that there is a strong heritability of serum CML levels in normal healthy subjects, it follows that a genetic effect on AGE levels, through AGE formation, AGE removal, or both, can be overridden in the context of disease or maladaption.

Summary

In summary, blood glucose is subject to powerful homeostatic forces. An evolutionary stable strategy has assured that homeostasis is maintained by genetic factors, ensuring that glucose metabolism is strongly inherited. It follows that surrogate markers of blood glucose levels are themselves genetically determined, though it is unclear whether this genetic effect is due to a direct effect of the glucose (e.g., protein glycation determining HbA1c or AGE levels) or an indirect effect, because glycation or deglycation rates are themselves genetically determined. Certainly, the genetic influence of HbA1c is not wholly dependent on genes responsible for fasting glucose. In either event, HbA1c is closely linked to vascular risk, especially microvascular risk. Components of this risk association will be glucose dependent and others glucose independent; the concept of GG aims to capture elements of this source of variation. HbA1c and glycation gap are genetically determined independent of fasting glucose. Reevaluation of some of the long-held assumptions about the relationship between HbA1c and plasma glucose raises new questions regarding potential new targets for intervention and new opportunities to refine complications risk assessment.

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