This could represent an important finding if this genetic variant protects the fetus from impaired kidney development *in utero*, particularly if the mother has a vitamin A deficiency. However, this work provides only one piece of the puzzle in the search for causative genetic variants.

While one SNP in *ALDH1A2* was associated with the two outcomes, 18 other SNPs in this gene were also genotyped. Furthermore, the authors considered 28 SNPs in two other retinoic acid metabolism genes (*CYP26A1* and *CYP26B1*). One would expect two of the 47 SNPs to generate a *P* value less than 0.05 by chance alone. But the same SNP was associated with two different outcomes. Does that strengthen the inference?

Perhaps the deck was already stacked for such a finding. Imagine you are looking for a weather-predicting coin. You have 47 coins (representing the SNPs) that you flip at midnight on 113 days (representing the subjects). Your aim is to see whether heads in one of the coins is associated with cloudy skies at 10 AM and rain in the next 24 hours. Just your luck, one of the coins is associated with both outcomes. However, the positive correlation between clouds and precipitation tilts the odds in favor of such a finding—just as an association between retinoic acid and nephrogenesis may create dependency between the genetic association results.

In their discussion of the finding, El Kares *et al.*[1] make a strong case for rs7169289 being a causative SNP for nephrogenesis: the SNP was associated with kidney volume in both of the RET^{1476} subgroups, it is in a 3′ Sp1 binding site, and the gene is important in retinoic acid metabolism. However, similar arguments could be made for the other 46 SNPs tested—that is why those SNPs were selected to begin with. The associated SNP could also be in linkage disequilibrium with one or more truly causative SNPs.

A stringent correction for multiple testing (such as a Bonferroni correction) requires that various associations being tested are independent. This may be true for genome-wide association studies that include thousands of SNPs not preselected for a particular function. However, when variants in 'biologically plausible' genes are being examined, the usual Bonferroni correction is too conservative. Chapman and Whittaker have compared several proposed methods for examining multiple SNPs in a gene or region that maximize power while accounting for multiple testing.[2] Even with such methods, an individual researcher is facing an uphill battle when the number of SNPs is large and the sample size is relatively small. Replication is the best way to strengthen the causative interpretation of such results, and El Kares *et al.*[1] have included the full results for all of their SNPs in supplemental tables to aid future researchers.

Another limitation is that fetal genetic polymorphisms are not the only determinants of kidney development. The uterine environment is influenced not just by maternal environmental exposures (including dietary intake), but also by the mother's genetic variations. Unfortunately, separating the influence of the maternal and fetal genomes is difficult, as mother and child share 50% of their genes. Mother and child polymorphisms have usually been studied separately, but there are available methods for joint analysis in a case-control setting.[3,4]

DISCLOSURE

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The sweet side of HIF

Volker H. Haase[1,2]

Hypoxia-inducible factors (HIFs) are oxygen-sensitive transcription factors that mediate cellular adaptation to hypoxia. Depending on the type of injury, activation of HIF signaling in renal cells may be renoprotective or promote fibrosis. Isoe and colleagues demonstrate that hyperglycemia activates HIF-1 in mesangial cells via carbohydrate response element binding protein (ChREBP), thus providing a novel link between alterations in systemic glucose homeostasis and HIF-regulated gene expression.

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Key mediators of cellular adaptation to hypoxia are hypoxia-inducible factors (HIFs), basic helix-loop-helix transcription factors that consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIFs regulate energy metabolism, angiogenesis, erythropoiesis, cellular differentiation, extracellular matrix turnover, and other biological processes, primarily by transactivation of oxygen-sensitive genes following binding to specific regulatory DNA sequences, so-called hypoxia-response elements. Increased HIF expression has been found in animal models of chronic kidney disease (CKD)

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and in renal biopsy material from patients with diabetic nephropathy and other forms of renal disease.[1–3] Relative hypoxia, which is the major stimulus that causes HIF activation, can be detected in CKD tissues irrespective of etiology and is thought to result from a combination of structural and functional changes that include decreased peritubular blood flow associated with glomerular injury, capillary rarefaction, vasoconstriction, luminal narrowing of atherosclerotic vessels, increased oxygen demand from hyperfiltration and tubular hypertrophy, limited oxygen diffusion as a consequence of extracellular matrix expansion, and renal anemia.[1] HIF activation in this setting has been shown to promote fibrogenesis and to negatively impact disease outcome on the basis of cell type-specific gene ablation studies.[2] There are several possible mechanisms by which HIF may exert its profibrotic effect. These include direct transcriptional regulation of gene products that control extracellular matrix turnover; functional cooperation with transforming growth factor-β1, a potent profibrotic factor; promotion of epithelial to mesenchymal transition; and modulation of renal inflammation. In contrast to HIFs' profibrotic role in renal epithelial cells, there is experimental evidence in animal models that systemic administration of cobalt chloride, possibly through HIF activation, is beneficial in diabetic nephropathy and other forms of CKD.[4,5]

Although hypoxia is a major stimulus for HIF activation, stabilization of the oxygen-sensitive HIF α-subunit can occur in the absence of significant hypoxia. This notion has significant implications for the pathogenesis of renal injury, as activation of HIF responses could modulate disease activity at an early stage, when tissue oxygenation is not impaired. Classic examples of oxygen-independent HIF activation are inherited renal cancer syndromes, which are associated with genetic defects that result in an inability to degrade HIF-α—for example, von Hippel–Lindau disease and hereditary leiomyomatosis and renal cancer syndrome (HLRCC).[6] Normoxic HIF stabilization can also be achieved pharmacologically by inhibition of 2–oxoglutarate-dependent dioxygenases (prolyl-4–hydroxylase domain (PHD) proteins), which function as intracellular oxygen sensors. PHDs hydroxylate specific proline residues within the oxygen-dependent degradation domain of HIF-α, thus enabling interaction with the von Hippel–Lindau–E3 ubiquitin ligase complex, which in turn targets HIF-α for proteasomal degradation (reviewed by Kaelin and Ratcliffe[7]). Several signaling molecules, some with key roles in the pathogenesis of CKD, have been shown to induce stabilization of HIF-α under normoxia. These include nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor-α, interleukin-1, angiotensin II, and growth factors such as epidermal growth factor and insulin and insulin-like growth factors, which either inhibit HIF prolyl-hydroxylation directly or indirectly (for example, NO and ROS are direct inhibitors) or increase HIF-α levels through phosphatidylinositol-3-kinase-dependent mechanisms (growth factors).

Given the wide range of HIF-regulated biological functions, normoxic stabilization of HIF-α is likely to modulate disease activity at an early stage before the occurrence of significant hypoxia. An example of this is the stabilization of glomerular HIF-1α in the early phases of diabetic nephropathy in *db*/*db* mice.[8] Isoe and colleagues[9] (this issue) now identify a carbohydrate response element (ChRE) in the proximal *HIF1A* promoter that regulates *HIF1A* transcription in mesangial cells in response to glucose *in vitro* and *in vivo* (Figure 1). Exposure of mesangial cells to high-glucose culture conditions or to hyperglycemia *in vivo* resulted in increased *HIF1A* (and also *EPAS1*/*HIF2A*) transcription and HIF-1α stabilization, which appeared to be independent of PHD activity. This response, which is cell type-specific, as *HIF1A* transcription cannot be induced in renal tubular epithelial cells, depends on the presence of carbohydrate response element binding protein (ChREBP), which binds to the HIF-ChRE localized between nucleotides −254 and −243. ChREBP is a glucose-responsive basic helix-loop-helix transcription factor of approximately 100 kDa, also termed MondoB or Williams–Beuren syndrome critical region gene 14 (WBSCR14), that dimerizes with Max-like protein X (Mlx) to transactivate glucose-sensitive genes, such as liver-type pyruvate kinase, fatty acid synthase, and acetyl-CoA carboxylase 1. In the liver, ChREBP functions as a regulator of *de novo* lipid synthesis in response to elevated serum glucose, irrespective of insulin levels. Its nuclear translocation is regulated by the formation of xylulose-5-phosphate and protein phosphatase 2A activity (reviewed by Uyeda and Repa[10]) (Figure 1).

A recent report indicates that ChREBP in pancreatic islet β-cells is a negative regulator of ARNT, the constitutively expressed β-subunit of HIF heterodimers.[11] This finding predicts an impairment of HIF-mediated transcriptional responses under high-glucose conditions. Whether ARNT is also glucose-regulated in mesangial cells has not been investigated. Isoe and colleagues show that several genes known to be HIF targets, including *vascular endothelial growth factor* (*VEGF*), *plasminogen activator inhibitor-1* (*PAI-1*), *connective tissue growth factor* (*CTGF*), *glucose transporter 1* (*GLUT-1*), *hexokinase 2*, and *adrenomedullin*, are upregulated in normoxic mesangial cells when cultured in high-glucose-containing medium.[9] Although the authors do not formally demonstrate that HIF-1 binds to the respective hypoxia-response elements using chromatin immunoprecipitation analysis, they found that interfering RNA directed against *HIF1A* reduced *CTGF* and *PAI-1* expression to control levels (expression levels under normal glucose conditions). ChREBP knock-down phenocopied HIF-1α knock-down, supporting the notion that the ChREBP–HIF axis is responsible for the upregulation of these genes under high-glucose conditions. It is important to mention here that CTGF and PAI-1 are strong promoters of fibrosis, and further studies using genetic models are needed to investigate to what degree hyperglycemic activation of HIF-1 in mesangial cells contributes to the development of diabetic glomerulopathy and matrix deposition.

Isoe *et al.*[9] make the important observation that high glucose induces mesangial *VEGF* expression through presumed HIF-1 transactivation (the role of HIF-1

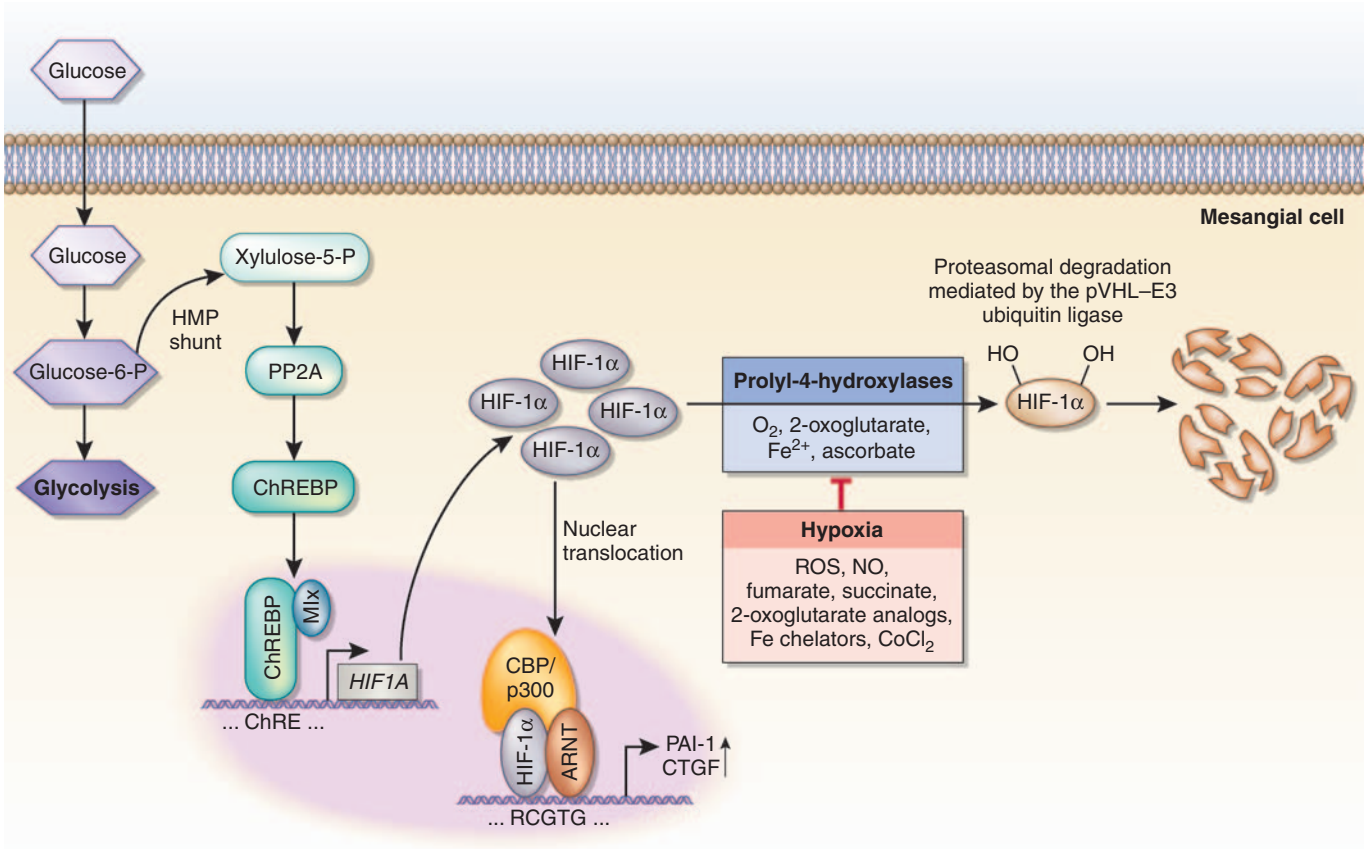

Figure 1 | Glucose regulates *HIF1A* transcription via ChREBP in mesangial cells. Under normoxia, HIF-1α is hydroxylated by prolyl-4-hydroxylases and targeted for proteasomal degradation by the pVHL–E3 ubiquitin ligase complex. When prolyl-4-hydroxylation is inhibited—for example, in the absence of molecular oxygen—HIF-1α is not degraded and translocates to the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1α/ARNT heterodimers bind to the HIF consensus binding site, RCGTG, followed by transactivation of target genes. Nitric oxide, reactive oxygen species, the Krebs cycle metabolites succinate and fumarate, cobalt chloride, and iron chelators such as desferrioxamine inhibit HIF prolyl-4-hydroxylation in the presence of oxygen. In normoxic mesangial cells, hyperglycemia results in increased HIF-1α protein levels and increased expression of HIF-regulated genes (for example, *PAI-1* and *CTGF*) irrespective of oxygen levels. Increased glucose flux results in the conversion of glucose-6-phosphate to xylulose-5-phosphate by the hexose monophosphate shunt pathway. Xylulose-5-phosphate activates protein phosphatase 2A, which in turn dephosphorylates carbohydrate response element binding protein (ChREBP), allowing for its nuclear translocation. In mesangial cells ChREBP binds to the proximal *HIF1A* promoter, stimulating its transcription. The proposed mechanism for ChREBP activation in mesangial cells is based on studies with hepatocytes. CBP, CREB-binding protein; $CoCl_2$, cobalt chloride; CTGF, connective tissue growth factor; Fe^{2+}, ferrous iron; HMP, hexose monophosphate; Mlx, Max-like protein X; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PP2A, protein phosphatase 2A; ROS, reactive oxygen species.

in its glucose-mediated induction is not directly examined), which not only has significant implications for the pathogenesis of diabetic nephropathy, but is also important for understanding HIF transactivation function in the setting of diabetes, which appears to be cell type-dependent. In contrast, at least in the context of hypoxia, HIF-mediated *VEGF* induction in certain diabetic tissues has been shown to be impaired or absent. For example, dermal fibroblasts from diabetic patients fail to induce *VEGF* under hypoxia, resulting in poor wound healing, and reduced tubulointerstitial *VEGF* expression was found in renal biopsy material from patients with diabetic nephropathy and evidence of capillary rarefaction, which is associated with the presence of relative hypoxia.[12,13] The inability to induce *VEGF* under hypoxic conditions in these settings may involve glucose-dependent alterations of HIF-α protein stability[14] or may result from a covalent, methylglyoxal-based modification of p300, a coactivator of HIF that interacts with the C-terminal transactivation domain, resulting in reduced transcriptional activity and decreased VEGF levels.[12] Importantly, HIF-1α itself was found to be modified by methylglyoxal, which reduces its ability to dimerize with ARNT, thereby negatively impacting HIF signaling.[15] Given these findings, it is likely that the mesangial HIF transcriptional complex is also subject to similar hyperglycemia-induced protein modifications. Whether these changes affect the pattern of HIF-dependent glomerular gene expression, and to what degree they impact the pathogenesis of diabetic renal disease, remain to be defined. Interestingly, PHD3, a known HIF-1 target, does not respond to high glucose.[9] PHD3 is a HIF prolyl-4-hydroxylase that is highly hypoxia-inducible and is involved in targeting HIF-α for degradation following reoxygenation.[7] The unresponsiveness of PHD3 to high glucose indicates that hyperglycemia-induced expression of HIF targets is likely to

involve additional regulatory mechanisms that modulate HIF responses in a gene-dependent manner.

In summary, the study by Isoe and colleagues[9] provides novel insights into the regulation of HIF signaling in mesangial cells exposed to hyperglycemia and links systemic glucose homeostasis directly to HIF-dependent gene expression via ChREBP. The study also nicely illustrates that activation of HIF signaling can occur in the absence of significant hypoxia. Although a time course was not presented, the study by Isoe and colleagues[9] furthermore suggests that HIF activation can occur at an early stage during disease evolution before overt diabetic nephropathy is present. The pathogenetic role of early mesangial HIF activation in this setting, however, remains to be investigated. With regard to therapeutic intervention, inhibition of HIF for the treatment of diabetic nephropathy may not be feasible, given its role in the regulation of a wide spectrum of hypoxia responses, which include renal erythropoietin production and activation of certain renoprotective pathways. A more viable approach could be the pharmacologic targeting of specific HIF-regulated gene products that have key roles in the pathogenesis of diabetic nephropathy. There is certainly need for detailed genetic studies in rodents that examine cell type- and context-dependent functions of HIF. These studies will ultimately tell whether and to what degree glucose-induced activation of HIF signaling in mesangial cells contributes to the pathogenesis of diabetic nephropathy.

DISCLOSURE

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