Amadori-glycated albumin in diabetic nephropathy: Pathophysiologic connections

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Amadori-glycated albumin in diabetic nephropathy: Pathophysiologic connections. Nonenzymatic glycation of proteins represents a major mechanism by which hyperglycemia leads to diabetic renal disease. Recent research has shown that Amadori-modified albumin, the principal glycated protein in plasma, elicits pathobiologic effects in cultured renal cells that are identical to those of high ambient glucose. When added to the incubation media of glomerular mesangial and endothelial cells, glycated albumin stimulates protein kinase C (PKC) activity, increases transforming growth factor-β (TGF-β) bioactivity, and induces gene overexpression and enhanced production of extracellular matrix proteins. These cellular events, whereby PKC-mediated TGF-β activation leads to increased matrix expression, are inextricably linked, and they form the central tenets of a pathophysiologic connection between glycated proteins and diabetic nephropathy. In vivo studies further corroborate the role of glycated proteins in the pathogenesis of diabetic nephropathy. Reduction or neutralization of glycated albumin in the db/db mouse model of type 2 diabetes significantly ameliorates the proteinuria, renal insufficiency, mesangial expansion, and overexpression of matrix proteins. In human type 1 diabetes, the plasma-glycated albumin concentration is independently associated with the presence of nephropathy. Abrogating the biologic effects of increased glycated albumin has novel therapeutic potential in the management of renal complications in diabetes.

Large-scale clinical studies have established that hyperglycemia, the defining metabolic abnormality in diabetes mellitus, increases the risk for diabetic renal disease [1, 2]. Although these studies did not define the responsible mechanisms, research in the past decade has shown that hyperglycemia has deleterious effects on kidney cell function. Renal cell lines grown in high glucose media exhibit activation of intracellular signaling pathways [3], up-regulation of the transforming growth factor-β (TGF-β) system [4], and a consequent overproduction of extracellular matrix molecules [5] that leads to glomerulosclerosis, tubulointerstitial fibrosis, and renal failure. Additional work has shown that factors in the diabetic milieu other than hyperglycemia are equally damaging. Prominent among these are circulating nonenzymatically glycated proteins.

Glycated proteins arise from a condensation reaction, driven by the ambient glucose concentration, in which a free sugar covalently attaches to a protein at reactive –NH₂ groups. Glycation proceeds through the formation of a labile Schiff base adduct, which then undergoes an intramolecular Amadori rearrangement to become a stable glucose-modified protein. The reaction occurs slowly, and the degree and duration of hyperglycemia influence the amount of glycated protein. Amadori-modified proteins may further evolve through a series of spontaneous rearrangement, dehydration, and polymerization reactions to become advanced glycation end products (AGEs). The accelerated formation of glycated proteins in diabetes has prompted research into their role in the pathogenesis of diabetic complications. Recent work has defined Amadori products, which comprise the overwhelming majority of glucose-modified proteins in plasma, as substantive contributors to the development of diabetic nephropathy.

EFFECTS OF GLYCATED PROTEINS IN THE KIDNEY

Earlier work demonstrated that glycated proteins influence renal function and have distinct renal handling. Infusion of Amadori-modified plasma proteins into normal rats induces glomerular hyperfiltration [6], an early functional feature of diabetes, and repeated injections of glycosylated proteins into normal mice produce “pseudo-diabetic” renal glomerular changes [7]. Glycated proteins alter the permeability properties of the glomerular capillary wall [8] and are preferentially transported across the glomerular filtration barrier and into the mesangial space [9]. They appear in the urinary space in greater amounts as evidenced by their increased clearance rates from the bloodstream, but final glycated protein concent-
trations in urine actually decrease relative to those of nonglycated proteins as patients with diabetes progress from normoalbuminuria to frank proteinurias [10]. This suggests preferential tubule cell uptake of the glycated forms of protein, which may have adverse consequences in the tubulointerstitial compartment and in the renal microvasculature. Endothelial cells preferentially take up circulating glycated proteins, which may lead to endothelial dysfunction and contribute to diabetic microangiopathy [11].

**Glycated protein receptors**

Putative mesangial and endothelial cell receptors recognize the Amadori-glycated form of albumin [12]. Ligand binding studies conducted on phenotypically stable, nontransformed mesangial cells have demonstrated selective binding of glycated albumin in a dose-dependent and saturable manner and are consistent with the existence of more than one affinity class of glycated albumin receptors [12]. In competition experiments, a monoclonal antiglycated albumin antibody specifically inhibits binding of glycated albumin to the mesangial cell receptor, without interfering with binding of nonglycated albumin to mesangial cells [12]. Although further work needs to be done to characterize the receptors for glycated albumin, it is reasonable to surmise that activation of this receptor by ligand binding may transduce important downstream pathobiologic effects.

**Glycated albumin increases protein kinase C activity**

Several studies have shown that the protein kinase C (PKC) system is up-regulated in the diabetic state. PKC activity, measured by the cytosol-to-membrane shift of various classical PKC isoforms, is increased in mesangial and endothelial cells incubated in high glucose media [13–16]. Glomeruli from diabetic rodents also display elevated PKC activity and cytosol-to-membrane translocation of diacylglycerol-sensitive PKC isoforms [17, 18]. Based on a series of 14C-radiolabeling experiments, the relationship between hyperglycemia and PKC activation has been ascribed to an increased intracellular metabolism of glucose that promotes de novo formation of diacylglycerol, the major endogenous activator of PKC [19]. Our work has suggested additional mechanisms. Notably, Amadori-glycated albumin, independent of glucose, can trigger PKC activation in glomerular cells [20].

We assayed PKC activity in two renal cell types after incubation with concentrations of glycated albumin that are found in diabetic subjects. Cells incubated with equal concentrations of nonglycated albumin served as the controls, and experiments were performed in normal glucose media to avoid the confounding effects of high glucose on the PKC system. Both rat and mouse mesangial cells exhibited significant increases in the overall PKC activity, measured by the extent of phosphorylation of a PKC-specific substrate, and in the PKC-β1 isoform activity, measured by the degree of cytosol-to-membrane translocation of this isoform [20]. We have performed experiments of similar design with rat glomerular endothelial cells and shown that glycated albumin also stimulates PKC activity in this cell type independently of high glucose concentrations [21].

**Glycated proteins activate the TGF-β system**

TGF-β, a hypertrophic and prosclerotic cytokine, plays a pivotal role in mediating the morphologic changes characteristic of diabetic nephropathy [22]. The biologic effects of TGF-β in kidney cells, which include cell hypertrophy and stimulation of extracellular matrix production, closely resemble those of hyperglycemia [23]. We have reported that proximal tubule epithelial cells, mesangial cells, and renal interstitial cells and fibroblasts cultured in high ambient glucose express significantly increased TGF-β1 mRNA levels and bioactivity [24–27]. The TGF-β system in turn stimulates collagen synthesis in these cells [4, 27]. To examine whether glycated albumin, independent of high ambient glucose, modulates the TGF-β system in renal cells, we exposed cultured mouse mesangial cells to normal glucose media containing glycated albumin at concentrations found in clinical specimens [28]. Northern blot analysis revealed that glycated albumin significantly stimulated TGF-β1 mRNA levels by approximately twofold compared with nonglycated albumin. It also induced a concomitant twofold increase in the mRNA expression of the type II TGF-β receptor, the primary signaling receptor for TGF-β. Extrapolated to the in vivo situation, these results suggest that glycated albumin, which has a circulating half-life of about 2 weeks, may continue to influence the TGF-β system long after restoration of normoglycemia. Further, glycated albumin was nearly as potent as high glucose in stimulating expression of both TGF-β1 and its receptor. Additionally, glycated albumin in high glucose media caused an even greater increase in the TGF-β1 and type II TGF-β receptor mRNAs [28]. Thus by stimulating the TGF-β system at both the ligand and receptor levels, glycated albumin can amplify the pathobiologic effects of TGF-β and can conspire with hyperglycemia to sustain these effects.

**Glycated proteins stimulate extracellular matrix synthesis**

Mesangial expansion due to overproduction of extracellular matrix proteins causes gradual obliteration of glomerular capillary lumens and a progressive decline in effective filtration surface area [29]. Given the central importance of the mesangium in diabetic nephropathy, we first examined the responses of mesangial cells to glycated proteins [30]. The primary endpoint in these studies was expression of type IV collagen, the predomi-
nant constituent of the expanded mesangial matrix in diabetes. Mouse mesangial cells incubated for 72 h in media containing glycated serum proteins and a normal glucose concentration displayed increased α1(IV) collagen mRNA, an effect that was exaggerated in high glucose media [31]. Inclusion of a monoclonal antibody (A717), site-specific for Amadori glucose adducts in glycated albumin, prevented the increase in type IV collagen production, indicating that glycated albumin was primarily responsible for the observed effect due to glycated serum proteins. Subsequent experiments established that glycated serum and glycated albumin significantly increased the expression of another matrix protein, fibronectin, and that this effect was also accentuated in high glucose media [28].

In keeping with the paradigm that glycated albumin contributes substantially to structural and functional abnormalities associated with diabetic renal disease, we more recently studied the effects of this glycated protein on the production of matrix by glomerular endothelial cells [21, 32]. Compared with nonglycated albumin, glycated albumin significantly increased the expression of fibronectin and type IV collagen, effects that were prevented by the A717 antiglycated albumin monoclonal antibody. Thus, the glomerular endothelium, strategically positioned between the circulation and the other glomerular elements, responds to an elevated glycated protein concentration in ways that promote glomerular pathology.

Glomerular epithelial cells also respond to glycated proteins with increased extracellular matrix synthesis. Cells exposed to glycated fetal bovine serum (FBS) compared with those exposed to nonglycated FBS show increased laminin protein and basement membrane matrix, but unchanged type I collagen and fibronectin proteins [33]. Glycated FBS also decreases collagenase activity, suggesting that in glomerular epithelial cells, glycated proteins promote extracellular matrix accumulation in part through inhibition of matrix degradation.

**Mediators of glycated protein-induced extracellular matrix**

Activation of the PKC signaling pathway by glycated proteins has been causally linked to increased extracellular matrix expression [20]. Incubation with glycated albumin activates PKC in cultured rat and mouse mesangial cells, and when overall PKC activity is blocked by GF 109203X, the glycated albumin-evoked increase in type IV collagen production is prevented. Likewise, when PKC-β activity is specifically blocked by LY-379196, the increase in type IV collagen synthesis is prevented to the same degree, consistent with the notion that the β isof orm plays a predominant role among the PKC isotypes [34, 35]. Glomerular endothelial cells also respond to glycated albumin with increased PKC activity. As in mesangial cells, generalized PKC inhibition by GF 109203X prevents the increased type IV collagen production [21].

The TGF-β system also mediates the glycated protein-induced increases in extracellular matrix production [28]. The increase in fibronectin expression by mesangial cells incubated with glycated serum or glycated albumin is prevented when the cells are concurrently treated with a neutralizing anti-TGF-β antibody [28]. Similarly, the elevation in type IV collagen production by glomerular endothelial cells incubated with glycated albumin is attenuated in the presence of neutralizing anti-TGF-β antibodies [21]. Further, inhibition of PKC with GF 109203X prevents stimulation of both TGF-β and type IV collagen proteins, suggesting that PKC may indirectly signal increased extracellular matrix production by stimulating the TGF-β system [36]. The presence of several PKC-responsive activated protein (AP-1) binding sites on the promoter of the TGF-β1 gene supports this hypothesis [37, 38].

**Glycated proteins in animal models of diabetic nephropathy**

Animal studies have confirmed that the in vitro effects of glycated proteins are relevant in vivo, and that they play an important role in the pathophysiology of diabetic renal disease. The db/db mouse represents an animal model of type 2 diabetes that develops glomerulopathy closely resembling that found in the human disease [39, 40]. As in humans, glycated albumin levels are two- to threefold higher in diabetics than in nondiabetic controls. Chronic administration of Fab fragments of the monoclonal antibody, A717, significantly lowers the plasma glycated albumin concentration in the db/db mouse [41]. After 8 weeks of treatment, the A717-treated db/db mice had significantly less urinary albumin excretion than the db/db mice treated with irrelevant IgG, nearly achieving the normoalbuminuric state of the nondiabetic db/m controls [41]. The antibody treatment protocol also remarkably improved glomerular pathology. Glomeruli from IgG-treated db/db controls showed diffuse mesangial expansion that encroached on the normal capillary network, whereas the glomeruli from A717-treated db/db mice showed considerably less mesangial expansion. Northern blot analysis revealed that the gene expression of both α1(IV) collagen and fibronectin were significantly increased in db/db mice compared with that in db/m mice [41]. Additional experiments demonstrated that the lowering of plasma glycated albumin in db/db mice by A717 delayed the development of renal insufficiency, assessed by the level of azotemia and the creatinine clearance [42].

**Glycated proteins in human diabetic nephropathy**

Few human studies have examined the role of Amadori-glycated proteins in the progression of diabetic re-
nal disease. In early reports, correlations were sought between serum and urine levels of glycated proteins and the severity of albuminuria, but these studies reached conflicting conclusions about the contribution of glycated proteins to the genesis of diabetic proteinuria [10, 43–46]. However, a more recent study that addressed the question from a different perspective found that the localization of glycated proteins in glomeruli paralleled the severity of tissue damage in diabetic nephropathy [47]. The largest and most recent human study examined the association between plasma levels of Amadori albumin and nephropathy in type 1 diabetes [48]. Not surprisingly, plasma levels of glycated albumin in these patients were higher than in nondiabetic subjects. More interestingly, the 199 patients with type 1 diabetes in this study who had nephropathy had significantly higher levels of Amadori albumin than did the 192 diabetic subjects without nephropathy. This difference remained even after adjustments for age, gender, body mass index, smoking, duration of diabetes, level of creatinine, glycemic control, blood lipid profile, blood pressure, and the presence of retinopathy or cardiovascular disease. This independent association between the plasma-glycated albumin level and diabetic nephropathy, while not proving cause and effect, supports a role for Amadori-modified albumin in the development of diabetic microvascular complications in humans.

CONCLUSIONS

Several lines of evidence point to a causal relationship between increased nonenzymatic glycation of serum proteins and the development of diabetic nephropathy. The cell biology changes that underlie the structural abnormalities characterizing the diabetic renal glomerulus have been elucidated in glomerular mesangial and endothelial cells incubated with Amadori-glycated albumin. Highly specific monoclonal antibodies that block the biologically active epitope of glycated albumin prevent these changes in cultured renal cells and ameliorate the mesangial matrix expansion, proteinuria, and renal insufficiency in diabetic mice. The mechanisms by which glycated serum proteins induce pathobiologic events in the renal glomerulus are incompletely defined but may relate to receptor-induced events followed by PKC-mediated TGF-β activation (Fig. 1). Given that increased glycated albumin levels potently stimulate renal extracellular matrix production, therapeutic strategies that negate the effects of glycated albumin are a logical approach to the treatment of diabetic nephropathy.

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REFERENCES


Kikkawa R, Haneda M, Uzu T, Koya D, Sugimoto T, Shigeta S-44


