Minireview

Evolving concepts in advanced glycation, diabetic nephropathy, and diabetic vascular disease

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Abstract

Advanced glycation endproducts (AGEs) have been postulated to play a role in the development of both nephropathy and large vessel disease in diabetes. However, it is still not clear which AGE subtypes play a pathogenetic role and which of several AGE receptors mediate AGE effects on cells. This review summarises the renoprotective effect of inhibitors of AGE formation, including aminoguanidine, and of cross-link breakers, including ALT-711, on experimental diabetic nephropathy and on mesenteric vascular hypertrophy. It also demonstrates similar effects of aminoguanidine and ramipril (an angiotensin converting enzyme inhibitor) on fluorescent and immunoassayable AGE levels, renal protein kinase C activity, nitrotyrosine expression, lysosomal function, and protein handling in experimental diabetes. These findings indicate that inhibition of the renin angiotensin system blocks both upstream and downstream pathways leading to tissue injury. We postulate that the chemical pathways leading to advanced glycation endproduct formation and the renin angiotensin systems may interact through the generation of free radicals, induced both by glucose and angiotensin II. There is also evidence to suggest that AGE-dependent pathways may play a role in the development of tubulointerstitial fibrosis in the diabetic kidney. This effect is mediated through RAGE and is TGF-β and CTGF-dependent.

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Abbreviations used: AGE, advanced glycation endproduct; NOS, nitric oxide synthase; PTB, N-phenacylthiazolium bromide; ACE, angiotensin converting enzyme; ERM, ezrin, radixin, and moesin; AG, aminoguanidine; RAM, ramipril.

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Diabetes is associated with a 2–3-fold increase in the incidence of macrovascular disease and most patients with diabetes develop evidence of microvascular disease, despite current methods of treatment if the duration of follow-up exceeds 10 years. In patients with type 2 diabetes, the United Kingdom Prospective Diabetes Study showed a linear relationship between long-term glycaemic control and macrovascular disease, with the incidence of myocardial infarction doubling as A1c levels increased from 5.5 to 10.5% [1]. By contrast, the incidence of microvascular endpoints increased 10-fold in the same range of A1c levels.

Improvement in glycaemic control has been shown to decrease the rate of development of diabetic microangiopathy in proportion to the decrement in A1c levels. This provides strong evidence for the role of hyperglycaemia as a pathogenetic factor in the microvascular complications of diabetes. By contrast, the hypothesis that tight glycaemic control would likewise reduce the risk of macrovascular complications of diabetes such as myocardial infarction has so far eluded clinical proof [2]. However, recent studies have shown that interrupting advanced glycation endproduct (AGE)1 signalling in atherosclerosis-prone mice by infusing a soluble form of RAGE can prevent the development of atheroma and can also arrest the progression of already established atherosclerosis in diabetic apolipoprotein E null mice [3]. Furthermore, a recent human study has shown that the AGE cross-link breaker ALT-711 improves total...
arterial compliance in elderly subjects with vascular stiffening [4]. These data suggest a novel therapeutic approach to vascular disease associated with ageing, diabetes, and isolated systolic hypertension. They identify a new therapeutic target in diabetic vascular disease, which could be termed “post-glycaemic” control, since glycaemic control as currently practised has not been shown to influence the incidence of diabetic macrovascular disease.

**Experimental diabetic nephropathy: AGE inhibitor studies**

The process of advanced glycation has been considered as a possible contributor to the pathogenesis of diabetic vascular disease for over 15 years [5]. Some 10 years ago, our own group showed that inhibition of advanced glycation with aminoguanidine ameliorated both the structural and functional aspects of experimental diabetic nephropathy in parallel with an attenuation of the diabetes-related increase in tissue levels of AGEs [6]. When commenced at the time of onset of diabetes, aminoguanidine ameliorated the increase in albuminuria (Fig. 1) and fractional mesangial volume [6] and late intervention with aminoguanidine resulted in a decrease in albuminuria proportional to the duration of treatment [7]. These experiments supported the existence of a pathogenetic role for AGEs in diabetic nephropathy. However, some doubts remained about the interpretation of the initial data since aminoguanidine has other actions apart from inhibition of AGE formation in the kidney [8]. This implied that aminoguanidine decreased renal carboxymethyl-lysine levels, since the latter is the main AGE epitope reacting with the antiserum. However, it is still not possible to differentiate specific pathogenetic roles for various categories of advanced glycation products which can be classified according to early, intermediate, and late, glycoxygenation versus anaerobic glycation products, glucose-, fructose- or triose phosphate-derived products and extracellular versus intracellular advanced glycation products. Nevertheless, it has been possible to differentiate long-term effects of extracellular AGEs which are associated with cross-link formation in long-lived protein such as collagen from short-term effects of AGEs which are related to activation of cell surface receptors such as RAGE [9].

One of the most important and readily made distinctions between AGEs is whether they fluoresce or not. For instance, carboxymethyl-lysine is a major epitope of most AGE immunoassays and does not fluoresce. By contrast, pentosidine, argpyrimidine, and cross-links are fluorophores, which may contribute to tissue or serum fluororescence measurements. It follows that aggregate AGE measurements by immunoassay may give different results to fluorescence assays in different tissues depending on the AGE composition at that site and also in response to specific interventions which may affect some but not all AGEs. Conversely, biochemical assays of specific AGEs may yield different results to AGE levels measured by immunoassay, fluorescence assay or HPLC [10].

In a 32-week study, aminoguanidine and ALT-946 \[N-(2-acetamidoethyl)hydrazine carboxydiamide HCL\], a chemically related compound with minimal inhibitory effect on iNOS, were compared in experimental diabetic nephropathy [11]. Diabetic animals were randomly allocated to aminoguanidine, ALT-946 or vehicle therapy from 0 to 32 weeks, with an additional group receiving

![Fig. 1. Serial data for albuminuria (mg/24h) over the 32-week study period for control (C), diabetic (D), and aminoguanidine-treated diabetic (D + AG) streptozotocin-rats. Results expressed as geometric mean ±/− tolerance factor. *p < 0.01 versus control, †p < 0.01 versus diabetic [6].](image-url)
delayed intervention with ALT-946 from 16 to 32 weeks. Treatment with ALT-946 was more potent than aminoguanidine in preventing AGE formation. Diabetes-related increases in albuminuria were attenuated in all treatment groups including the delayed intervention group. In the same study, glomerular staining for AGEs was approximately halved by aminoguanidine and ALT-946. These results indicated that the renoprotective effects of AGE inhibitors were not dependent on inhibition of nitric oxide synthesis.

**In vivo studies using AGE inhibitors and cross-link breakers**

Since the initial experiments with aminoguanidine, our group has performed a number of other intervention studies with agents which either inhibit AGE formation or promote the breakdown of AGE cross-links. In general, these agents have shown renoprotective effects which are similar to those of aminoguanidine, even though they have different biochemical mechanisms of action. Since the effect of aminoguanidine as an inhibitor of nitric oxide synthase could potentially explain some of its vascular and renoprotective effects, it was important to study AGE inhibitors, which did not influence NOS activity. This was performed in studies with new inhibitors of AGE production which did not affect nitric oxide synthesis.

In a 3-week study in streptozotocin diabetic rats, treatment with 2,3 diaminophenazine attenuated mesenteric vascular hypertrophy in a similar fashion to aminoguanidine and both agents reduced the formation of AGEs in the mesenteric artery, glomeruli, and renal tubules as measured by radioimmunoassay and also by immunohistochemistry [12].

Our group has also studied the in vivo effects of two AGE cross-link breakers. The first study showed that diabetes-related mesenteric vascular hypertrophy and increases in mesenteric vascular AGE levels were prevented by N-phenacylthiazolium bromide (PTB) given for 3 weeks from the time of induction of diabetes. By contrast, delayed intervention with PTB after 3 weeks did not attenuate diabetes-related mesenteric vascular hypertrophy at 6 weeks, even though AGE immunostaining in the medial layer was reduced compared with the untreated group [13]. In studies by other investigators using the streptozotocin-diabetic rat model, delayed intervention with a stable derivative of PTB (ALT-711) attenuated the diabetes-related decrease in large artery compliance [14]. In that study, ALT-711 treatment was associated with an increase in collagen digestibility with pepsin, implying a decrease in collagen cross-linking.

Recent studies by our group have shown that ALT-711 prevents diabetes-induced increases in renal AGE levels as measured by immunostaining [15] and in addition, that ALT-711 halves albumin excretion rate in diabetic animals studied over 32 weeks [16]. The above evidence is consistent with the concept that AGE accumulation in the kidney and in blood vessels in diabetes is not merely a result or an association of evolving diabetes vasculopathy. Instead, the data obtained with interventions using inhibitors of AGE formation and with AGE cross-link breakers support the involvement of AGEs in the causation of diabetic nephropathy.

**Cross-talk with other pathways: common mechanisms?**

With respect to diabetic nephropathy, both metabolic and haemodynamic factors may play a pathogenetic role [17]. Hyperglycaemia and hypertension share intracellular signalling mechanisms, which increase the expression of growth factors and cytokines such as TGF-β, CTGF, VEGF, and levels of the adhesion molecule VCAM 1 (Fig. 2). Fig. 2 presents a hypothesis about the interactions of hyperglycaemia and hypertension with growth factors and cytokines. TGF-β and CTGF mediate the effects of AGEs and angiotensin II predominantly on the glomerulus leading to mesangial expansion. By contrast, RANTES and MCP-1 mediate the effects of filtered protein predominantly on renal tubules, leading to tubulointerstitial fibrosis and inflammation. Protein kinase C is a likely mediator of altered intrarenal albumin processing which contributes to diabetes-related albuminuria and its effects are exerted on both the glomerulus and the renal tubules. It was traditionally thought that hyperglycaemia alone was responsible for the above process. However, recent evidence supports a similarity in the renal responses to tissue injury associated with raised glucose levels and increased blood pressure.

These observations raise the possibility that interactions may exist between hyperglycaemia and hypertension, even though they are clinically considered as separate entities and are treated by agents which are considered to have different mechanisms of action. Our group has therefore investigated the possibility that
cross-talk exists between the advanced glycation pathway and other pathways mediating tissue injury in diabetes such as the renin angiotensin system.

**Advanced glycation and the renin angiotensin system**

Our group has previously assessed the relative roles of advanced glycation, oxidation, and aldose reductase inhibition in the development of diabetic nephropathy in the Sprague–Dawley rat [7] and subsequently assessed the relative contribution of advanced glycation and NOS inhibition to aminoguanidine-mediated renoprotection in diabetic rats [18]. These studies indicated that aminoguanidine functioned primarily as an inhibitor of AGE formation in vivo. We then performed two studies comparing aminoguanidine and the angiotensin converting enzyme (ACE) inhibitor ramipril, noting that aminoguanidine does not affect blood pressure levels and neither intervention affects blood sugar levels. The hypothesis was that an inhibitor of the AGE system could be distinguished from an inhibitor of the renin angiotensin system on the basis of their upstream effects, even though they have similar downstream effects on albuminuria and renal structure.

**Aminoguanidine and ramipril: novel inhibitors of protein kinase C**

Recent studies by our group examined whether diabetes-related albuminuria may be mediated by a common mechanism involving protein kinase C [19]. In 12–24 week studies, treatment with either aminoguanidine or ramipril prevented the increase in albuminuria associated with experimental diabetes [20] and normalised both renal and serum fluorescent (Fig. 3) and immunodetectable (Fig. 4) AGE levels [20]. Both interventions also prevented the diabetes-related increase in glomerular protein kinase C activity at 24 weeks (Fig. 5).

Neither drug affected glycaemic control nor glomerular filtration rate but ramipril reduced systolic blood pressure whereas aminoguanidine did not [19]. These data indicated that albuminuria may be modulated independently of glycaemic control and blood pressure, supporting the concept that glomerular protein kinase C
activity plays a central role in the development of diabetic nephropathy. These results are consistent with the in vitro findings of other investigators on the importance of protein kinase C as a modulator of glomerular injury in diabetes [21,22]. However, the new data also indicated that inhibition of protein kinase C activity in the glomerulus is not a property confined to specific PKC inhibitors [19,23] but instead is shared with other renoprotective agents such as inhibitors of AGE formation and ACE inhibitors.

**Aminoguanidine and ramipril: effects on renal albumin processing**

Recent evidence indicates that albumin is degraded to small fragments through post-filtration processing in normal subjects [24]. This means that immunoassayable (intact) albumin normally represents less than 10% of total albumin-derived products excreted in urine. This raises the possibility that increases in immunoassayable albuminuria may be derived by changes in post-filtration processing without changes in glomerular filtration of albumin. The normal role of the glomerulus and tubule in the filtration and reabsorption of albumin remains undefined. The mechanisms by which renal tubules reabsorb and process filtered albumin and the amount of protein presented to the renal tubules in health and disease remain a subject of controversy.

In the streptozotocin diabetic rat, the percentage of intact albumin in urine, as measured by size exclusion chromatography, increased in parallel with increasing levels of albuminuria throughout a 24-week study [19]. This was consistent with a modulating role for protein kinase C in the post filtration lysosomal processing of albumin by the kidney. Diabetes-related changes in renal lysosomal processing were also studied in streptozotocin diabetic rats using another parameter of lysosomal function [25]. In that study, the percentage desulphation of intravenously injected [3H]dextran sulphate was used as a marker of lysosomal sulphatase activity. Renal sulphatase activity was measured in diabetic rats treated with aminoguanidine or ramipril for 12 weeks and was found to be decreased in diabetic rats compared with control rats. This was partially prevented by both aminoguanidine and ramipril [25].

The striking similarity of the effects of aminoguanidine and ramipril on protein kinase C activity in the above studies is supported by other studies, which show that protein kinase C is modulated by AGE receptors as well as by the AT1 receptor pathway. Thus, AGE receptor interaction has been shown to induce PKC activation in cultured cells [26] and angiotensin-II binding to the AT1 receptor in proximal tubule cells has been shown to activate phospholipase C and D which in turn increase diacylglycerol production and protein kinase C activity [27].

**Oxidative stress: a link between glycation and the renin angiotensin system?**

To explore further the possibility of cross-talk between AGE inhibitors and renin angiotensin system inhibitors at the level of oxidative stress and AGE formation, a second comparison of the effects of aminoguanidine and ramipril was performed on AGE and nitrotyrosine levels in the kidney of diabetic rats [20]. Renal AGE levels were increased after 12 weeks in diabetic rats when quantitated by immunostaining for CML and also by AGE fluorescence (370/440 nm) and these increases were prevented by treatment with aminoguanidine and ramipril to a similar degree. Two to 3-fold increases in immunostaining for nitrotyrosine were observed in glomeruli and in the apical region of proximal tubule cells of diabetic animals (Fig. 6) and

![Fig. 6. Morphometric analysis of nitrotyrosine immunostaining in glomerular (left panel) and tubular samples (right panel) at 12 weeks of diabetes expressed as proportional area (%). C, control; D, diabetic; AG, aminoguanidine; RAM, ramipril. *p < 0.001 versus control and # p < 0.001 versus diabetic [20].](image-url)
these were attenuated equally by aminoguanidine and ramipril [20]. Since nitrotyrosine is a marker of protein nitrosylation associated with oxidative stress, this may be the common link which explains the similar effects of aminoguanidine and ramipril on immunosayable glycoxidation products (predominantly carboxymethyl-lysine) in the kidney.

Evidence of increased oxidative stress and increased carbonyl modification of proteins has been previously described in diabetic glomerular lesions [28]. In the above study, the results obtained with aminoguanidine were as expected. However, the finding that ramipril was equally effective in preventing the accumulation of AGEs and increases in nitrotyrosine levels was surprising. The implication is that ramipril reduces angiotensin-mediated oxidative stress in the kidney. This supports previous in vitro studies, which have shown that angiotensin II promotes oxidative stress and that this is inhibited by angiotensin receptor blockade [29].

In vitro evidence (using cultured bovine aortic endothelial cells) suggests that increased generation of reactive oxygen species in diabetes occurs in mitochondria [30]. Whether increased generation of reactive oxygen species in response to diabetes also occurs in renal mitochondria is not yet known. However, increases in diabetes-related oxidative stress may occur in other cellular sites, mediated by glucose itself or by AGEs and their receptors. A non-mitochondrial pathway involving AGEs and RAGE has been shown to induce reactive oxygen species through stimulation of membrane bound NADPH oxidase [31]. In a study by Wautier et al. [31] activation of NADPH oxidase has been shown to occur in human endothelial cells exposed to AGEs on red blood cells from subjects or to specific AGEs. This was blocked by soluble RAGE, indicating that the generation of oxidative stress was linked to a cell surface AGE–RAGE interaction. In a study by our group, increased gene expression of the membrane bound gp91phox subunit of NADPH oxidase was observed in the diabetic kidney but this could not be prevented by either ramipril or aminoguanidine treatment [32]. In addition, neither drug influenced the expression of RAGE nor NF-κB. These in vivo results suggest that mitochondrial generation of oxidative stress may be more important than reactive oxygen species generation at extra-mitochondrial sites as reflected by NADPH oxidase activity. However, it is important to note that although oxidative stress may contribute to AGE formation, AGEs themselves may induce oxidative stress through interaction with their receptors and during AGE formation.

Our group has also investigated the effects of experimental diabetes on renal cortical nitrotyrosine levels (a marker of oxidative damage to proteins by peroxynitrit radicals) [32]. Immunohistochemical staining for nitrotyrosine was increased in proximal tubules of diabetic rats. These new data suggest that oxidative stress in diabetes may have both mitochondrial and extra-mitochondrial origins (Fig. 7). Further elucidation of the role of AGEs and oxidative stress in the causation of diabetic nephropathy awaits the development of specific inhibitors of oxidative stress, which are compatible with in vivo use.

A recent in vitro study has elucidated the biochemical mechanisms, which mediate the decrease in AGE formation observed with renin angiotensin system inhibitors [10], Olmesartan, an angiotensin receptor blocker, and temocaprilat, an ACE inhibitor, decreased the formation of pentosidine and carboxymethyl-lysine when incubated with glucose and plasma from patients with diabetes and varying degrees of renal impairment. This effect was shared by all tested angiotensin receptor blockers and ACE inhibitors but not by the calcium channel blocker nifedipine. Unlike inhibitors of advanced glycation such as aminoguanidine or pyridoxamine, renin angiotensin system inhibitors did not trap reactive carbonyl precursors for AGEs such as glyoxal or methylglyoxal. Instead, the renin angiotensin system inhibitors were shown to possess free radical scavenging activity and to decrease the formation of reactive carbonyl AGE precursors in vitro. It was concluded that renin angiotensin system inhibitors exert their effects mainly on the pre-Amadori and only to a limited extent on the post-Amadori formation of AGEs [10].

In summary, the above data demonstrate a new pathway to explain the renoprotective effects of renin angiotensin system inhibitors, independent of haemodynamic mechanisms. However, it is still not clear if the inhibition of AGE formation by renin angiotensin inhibitors is a major or minor component of the so-called specific renoprotective effects of these agents, which are considered independent of their blood pressure lowering actions.

**Cell surface AGE receptors**

Prior to the discovery of AGE binding proteins, the main biological effects of AGEs were considered to be extracellular and related to the cross-linking of long-
lived proteins such as collagen [5]. The discovery of cell surface AGE receptors such as RAGE [33], macrophage scavenger receptors [34], a lactoferrin-like receptor [35], and AGE receptors R1, R2, and R3 [36] raised the possibility that these receptors may modulate biological effects of AGEs by activating the cellular expression of growth factors and cytokines. Our group showed that the AGE products and their receptors co-localise in the kidney [18]. Further studies showed that AGE binding sites are present in rat renal proximal tubules [8]. In this study, there was a significant increase in 125I-AGE binding in diabetic kidneys in association with elevated renal AGE levels. This diabetes-related increase in AGE binding was prevented by aminoguanidine therapy. Whether these binding sites represent clearance receptors or whether they participate in the development of nephropathy in a pathogenetic sense remains to be determined.

In support of the role of RAGE as a pathogenetic factor in diabetic nephropathy, a study by another group has documented the development of advanced nephopathy in diabetic mice over-expressing RAGE [37]. In that study, transgenic mice that over-express RAGE in vascular cells were cross-bred with mice, which developed spontaneous type 1 diabetes. This transgenic model demonstrated some of the features of human diabetic nephropathy including enlargement of the kidney and glomeruli, increased levels of albuminuria, mesangial expansion, and glomerulosclerosis and increased serum creatinine compared with diabetic littermates lacking the RAGE transgene [37]. All of the above features of diabetic nephropathy were prevented by treatment with the thiazolidine derivative OPB-9195, an inhibitor of advanced glycation.

In addition to extracellular AGE binding proteins, our group has recently shown that the amino-terminal domain of the ezrin, radixin, and moesin (ERM) proteins bind AGEs [38]. The ERM proteins function as a link between the cytoplasmic tail of membrane proteins and cytoplasmic actin filaments and also regulate kinases (such as Rho kinase, focal adhesion kinase, and phosphatidylinositol 3-kinase). ERM proteins also modulate membrane ion transport proteins. These results raise the possibility that intracellular ERM binding sites for AGES may play a role in the development of diabetic complications.

**AGEs and transdifferentiation**

In a further study by our group, the specific binding of 125I-AGE BSA was studied in cell membranes prepared from rat proximal tubular cells [15]. The binding site was identified as RAGE and exposure to AGES induced dose-dependent epithelial-myofibroblast transdifferentiation. These effects were blocked by neutralising antibodies to RAGE or to TGF-β. In diabetic rats, the AGE cross-link breaker ALT-711 reduced transdifferentiation and also reduced tubular AGE and TGF-β expression. The potential in vivo implications of this study are that AGEs promote the development of tubulointerstitial fibrosis by binding to RAGE and that the effect is mediated by TGF-β.

These studies indicate that AGEs and their receptors play an important role in the development of diabetic nephropathy. In addition, preliminary evidence suggests that endogenous ligands for RAGE such as the S100/calgranulin polypeptides promote inflammatory reactions in the diabetic kidney through a separate and additional mechanism to that described for AGES [37].

**Conclusion and perspectives**

Progress in the area of AGES and diabetic nephropathy will require identification of specific AGES with proven pathogenic links to the development of diabetic nephropathy. Initial studies examining the localisation and quantification of specific AGES in renal biopsy tissues from subjects with diabetic nephropathy support this pathogenic link [39]. This means that positive results obtained using aggregate AGE assays described above will need to be replicated using biochemical assays of specific AGES in both the experimental and clinical settings.

In contrast to animal data, results of intervention by monotherapy of human diabetic nephropathy have been generally less effective. Although hyperglycaemia is a prerequisite and the major initiator of diabetic nephropathy, raised blood pressure is the dominant modulator of disease progression. These two risk factors should therefore be considered concomitantly in strategies for intervention. This suggests that combination therapy using glycation inhibitors, inhibitors of the renin angiotensin system, antioxidants, protein kinase C inhibitors, and inhibitors of the action and secretion of prosclerotic cytokines such as TGF-β will be needed to ameliorate or arrest the progression of diabetic nephropathy in humans.

The relative contribution of mitochondrial versus non-mitochondrial sources of oxidative stress requires further in vivo exploration, since the results of pharmacological intervention may differ according to the subcellular target site. It is possible that varying tissue specific response to interventions reflects regional differences in the expression of oxidative stress as well as modulators of AGE effects such as RAGE and ERM proteins.

Since only 1 in 3 patients develops diabetic nephropathy and it is not currently possible to identify predisposed patients, intervention strategies for human diabetic nephropathy are likely to require agents which...
attenuate the progression of established disease at the stage of microalbuminuria or later. Theoretically, the AGE cross-link breakers may have an advantage over inhibitors of AGE formation. However, it has not been possible to show clear differences between these two groups of agents either at a biochemical or a clinical level. The results from future clinical studies will reveal if the new generation of inhibitors of AGE formation or cross-link breakers exhibits similar efficacy and safety profiles to that seen in experimental diabetic models.

References