Diabetic Nephropathy: Where Hemodynamics Meets Metabolism

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Key words
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Abstract

Diabetic nephropathy (DN), the most common cause of end stage renal disease in developed nations, is thought to result from interactions between metabolic and haemodynamic factors. Specific metabolically driven, glucose dependent pathways are activated within diabetic renal tissues. These pathways induce oxidative stress, polyol pathway flux, hexosamine flux and accumulation of advanced glycated end-products (AGEs).

Haemodynamic factors are also implicated in the pathogenesis of DN and include elevations of systemic and intraglomerular pressure and activation of various vasoactive hormone pathways including the renin-angiotensin aldosterone system (RAAS), endothelin and urotensin. These altered hemodynamics act independently and in concert with metabolic pathways, to activate intracellular second messengers such as protein kinase C (PKC) and MAP kinase (MAPK), nuclear transcription factors such as nuclear factor-κB (NF-κB) and various growth factors such as the prosclerotic cytokines, transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF) and the angiogenic, permeability enhancing growth factor, vascular endothelial growth factor, VEGF. Ultimately these molecular mechanisms lead to increased renal albumin permeability, and extracellular matrix accumulation, which results in increasing proteinuria, glomerulosclerosis and tubulointerstitial fibrosis.

In the past, the treatment of diabetic nephropathy has focused on control of hyperglycemia and the interruption of the RAAS with certain antihypertensive agents. Newer novel targets, some of which are linked to glucose dependent pathways, appear to be a major focus of new therapies directed against the development and progression of renal damage as a result of diabetes. It is likely that resolution of diabetic nephropathy will require synergistic therapies to target multiple mediators of this disease.

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Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease (ESRD), and accounts for significant morbidity and mortality in Western societies (Maisonneuve et al., 2000). The aetiology of the progressive structural and functional changes ultimately resulting in overt diabetic nephropathy is not fully elucidated. Recent advances in this area however, have provided clues to the specific mechanisms involved in the onset and progression of this disoder. Most likely, renal damage in diabetes is the result of an interaction between hemodynamic and metabolic abnormalities (Cooper, 2001), as evidenced by the major clinical determinants of diabetic nephropathy remaining hyperglycaemia (Stratton et al., 2000) and hypertension (Adler et al., 2000).

The vasoactive hormone angiotensin II (AII), plays a critical role not only in the regulation of systemic and glomerular hemodynamics, but also in glomerular hypertrophy and sclerosis. Indeed, therapeutic blockade of the renin-angiotensin system with angiotensin converting enzyme (ACE) inhibitors and angiotensin II type 1 receptor (AT1R) antagonists suppresses the development and progression of diabetic nephropathy in both type 1 and type 2 diabetic patients (Brenner et al., 2001, Lewis et al., 1993b, Mogensen et al., 2000b). During recent years, a myriad of data have emerged on the diverse effects associated with angiotensin II including growth stimulation, induction of fibrogenesis and immuno-modulation, which are clearly

beyond the classical vasopressor function described for this vasoactive peptide (Wolf, 1998).

Other vasoactive proteins such as endothelin (ET), nitric oxide (NO) and urotensin II (UII) have also recently gained attention in diabetic nephropathy. Suppression of the actions of endothelin with endothelin A receptor antagonists (Nakamura et al., 1995a) have shown beneficial outcomes in experimental models of diabetic nephropathy.

The metabolic abnormalities seen in the diabetic kidney are diverse. It is obvious from studies in diabetic patients, that strict glycaemic control is an important strategy to retard the progression of nephropathy (1998, Diabetes Control and Complications Trial Research Group, 1993), although clinically this not always attainable. There are four main pathways that have been implicated to explain how hyperglycaemia *per se* leads to the development of diabetic complications, including nephropathy. These include increased flux via either the polyol and hexosamine pathways, accumulation of advanced glycation end products and activation of protein kinase C (Brownlee, 2001, Sheetz and King, 2002) which are each discussed in detail later in this review. While manipulation of each of these individual pathways has shown benefit in experimental models, their role in the clinical treatment of diabetic nephropathy remains to be determined.

Furthermore, several *in vitro* and *in vivo* studies have implicated transforming growth factor-β (TGF-β), a fibrogenic cytokine, as a key effector molecule in promoting diabetic renal disease (Anderson et al., 1989, Cooper et al., 1988, Zatz et al., 1985). Moreover, other growth factors have been implicated in progression of diabetic nephropathy such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), and connective tissue growth factor (CTGF) in conjunction with intracellular signalling molecules such as mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB) and protein kinase C (PKC) (Flyvbjerg et al., 2002).

Haemodynamic factors

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Renin-angiotensin system (RAS)

Initial studies emphasised the role of haemodynamic factors in DN, in particular the potential role of inhibitors of the RAS as renoprotective agents in experimental diabetes (Anderson et al., 1989, Zatz et al., 1986). Micropuncture studies in diabetic rats revealed a range of intrarenal haemodynamic abnormalities including increased intraglomerular pressure, increased single nephron glomerular filtration rate (GFR) and preferential afferent versus efferent arteriolar vasodilatation (Zatz et al., 1986). This increase in intraglomerular pressure was considered to play a pivotal role in mediating progressive glomerular injury in a range of progressive renal diseases including diabetes (Hostetter et al., 1982). This hypothesis was based on studies where intraglomerular pressure was reduced by a range of treatments including ACE inhibitors (Zatz et al., 1986) and low protein diets (Zatz et al., 1985) in association with reduced renal injury. This central role for intraglomerular hypertension extended our understanding of the importance of haemodynamic factors in mediating renal injury in diabetes. It emphasised that determinants of progression of DN included not only systemic hypertension but also specific intrarenal haemodynamic changes, which were occurring even in the setting of a normal blood pressure (Zatz et al., 1986).

The hormone angiotensin II (AII), is traditionally recognised as regulating blood pressure, fluid and electrolyte balance. AII interacts with two specific receptor subtypes known as the AT1 and AT2 receptors. It is generally viewed that most of the actions of AII are via the AT1 receptor including its haemodynamic and prosclerotic effects (Carey and Siragy, 2003b). More recently, studies of the AT2 receptor have suggested that this receptor may also play a role in the diseased kidney (Cao et al., 2000b) although this issue has not been adequately characterised in the diabetic kidney. Diabetes induces changes in expression of the AII receptors in the kidney but the functional significance of these changes is not known (Burns, 2000b). Selective blockers of both receptor subtypes have been developed. However, long-term studies in DN have only been reported with blockers of the AT1 receptor subtype with these studies indicating that these agents are renoprotective in experimental and human DN (Allen et al., 1997, Andersen et al., 2000, Lacourciere et al., 2000, Remuzzi et al., 1993).

The coexistence of a complete intrarenal RAS (Carey and Siragy, 2003a) in conjunction with the known circulating RAS, has provided new insights into diabetic renal disease. Indeed, all components of the RAS are expressed in the normal kidney (Carey and Siragy, 2003a). The data, however, concerning the influence of diabetes *per se*, on the systemic and intrarenal RAS have been conflicting (Burns, 2000a). In general, plasma measurements of various components of the RAS are low or normal in diabetes (Leehey et al., 2000). However, it is well appreciated that the RAS also acts at a local level within the kidney. Measurements of the various renal components of the RAS by a range of techniques have been contradictory with reports of reduced, normal or decreased levels of renin and AII in the diabetic kidney (Anderson et al., 1993, Campbell et al., 1999, Kalinyak et al., 1993). In vitro studies in proximal tubular cells have described increased angiotensinogen expression in response to glucose (Zhang et al., 1999). In mesangial cells, glucose *per se* has also been reported to stimulate angiotensin II production in association with increased TGF-β1 production (Singh et al., 1999).

It is possible that rather than a change in the levels of these renal components, that diabetes may alter their distribution within the various kidney compartments. In experimental diabetes, Anderson et al have reported a redistribution of ACE to vascular and glomerular sites in diabetes (Anderson et al., 1993). We have documented a similar phenomenon in diabetic (mRen-2)27 transgenic rat, a model which involves the introduction of the murine renin gene into the rat genome (Mullins et al., 1990). This results in increased expression of various components of the RAS, particularly outside the kidney with subsequent development by these arts of an elevation in blood pressure. These diabetic rats have increased proximal tubular renin expression particularly in damaged tubules which is reduced at this site by an AT1 receptor antagonist, in the context of an increase in renin expression at the major site of synthesis, the juxtaglomerular apparatus (Kelly et al., 2000). This increase in proximal tubular renin is associated with a local increase in expression of AII. Further evidence for the local activation of the RAS in the tubular compartment has been suggested in studies demonstrating an early increase in proximal tubular renin in experimental diabetes (Zimpelmann et al., 2000). The authors postulated that this could contribute to a local increase in AII resulting ultimately in tubulointerstitial fibrosis in this model. These findings empha-

sise changes in the distribution of the RAS in the diabetic kidney, which may be important in mediating progressive renal injury.

Another important explanation for the disparity in the various measurements of both the systemic and intrarenal RAS in diabetes and the responsiveness of the diabetic kidney to blockade of the RAS may relate to increased sensitivity of the diabetic kidney to AII as has been suggested by several investigators (Burns, 2000b, Kennefick et al., 1996). In addition, recent long term clinical follow up of patients with diabetic nephropathy who are treated with ACEi has revealed the principle of "aldosterone escape". ACE inhibitors do not effectively suppress levels of the mineralocorticoid, aldosterone in the long term, and lead to suboptimal renoprotection as a result of ongoing prosclerotic effects. Indeed, these agents and others in this antihypertensive class may actually increase aldosterone levels, and thus may potentiate aldosterone's negative effects on the heart, blood vessels, and kidneys. One study of 45 patients with early type 2 diabetic nephropathy, treated with an ACE inhibitor for 40 weeks, showed that 40% of patients developed evidence of aldosterone escape and no longer showed maximal antiproteinuric effects of ACE inhibition (Sato and Saruta, 2003).

Although AII has haemodynamic effects both at the systemic level and intra-renally, it is becoming increasingly evident that AII has a range of non-haemodynamic effects relevant to progressive renal injury (Wolf and Ziyadeh, 1997). It has been difficult to separate the haemodynamic from nonhaemodynamic effects *in vivo* but the use of cultured cells has allowed investigators to explore these additional effects of AII. AII induces extracellular matrix accumulation, a hallmark of DN primarily via stimulation of the prosclerotic cytokine, TGF-β1 (Kagami et al., 1994). AII may also influence a range of other cytokines and in particular it may activate a range of intracellular mediators implicated in progressive renal injury such as PKC (Arendshorst et al., 1999, Nagahama et al., 2000) and the nuclear transcription factor, NF-κB (Ruiz-Ortega et al., 2000). Finally, AII may also influence pathways considered to be primarily metabolic, such as advanced glycations. Recent studies from our group in models of AII infusion, have demonstrated that AII can specifically manipulate the expression of AGE receptors and their renal and serum accumulation (Thomas et al., 2005). Conversely, the infusion of AGEs into normal rodents produces changes in the renin-angiotensin system reminiscent of those seen in our models of DN (Thomas et al., 2005).

AII also influences cell growth, proliferation and apoptosis via a range of pathways not yet fully defined (Bonnet et al., 2001, Shankland and Wolf, 2000). In particular, cell cycle regulation by may be linked to various changes in the diabetic kidney including renal hypertrophy (Shankland and Wolf, 2000). Indeed, the reduction in glomerular hypertrophy often observed with blockade of the RAS (Allen et al., 1997), supports a role for AII in cell cycle regulation. Studies in STZ diabetic rats treated with ACE inhibitors demonstrated reduced glomerular volume in addition to abolition of glomerular expression of the cyclin-dependent kinase inhibitors p^{16INK4} and p^{27Kip1} (Wolf, 2000). These actions of AII may be central to how haemodynamic pathways interact with metabolic and in particular glucose dependent factors in accelerating DN.

ACE inhibitors have clearly been shown to confer renoprotective effects, initially in experimental models but ultimately in human DN (Cooper, 1998). These beneficial effects relate not only to the capacity of these agents to reduce blood pressure but also to block AII formation itself. Although ACE inhibitors have other effects including inhibition of degradation of kinins, experimental studies using bradykinin and AT1 receptor antagonists indicate that the long-term renal protection afforded by these agents is primarily via inhibition of AII dependent pathways (Allen et al., 1997). The advent of selective AII receptor antagonists (Johnston, 1995) provides another approach for conferring renal protection in diabetic patients (Brenner et al., 2000, Rodby et al., 2000). Although ACE inhibitors confer a degree of renal protection, DN continues to progress relentlessly albeit at a slower rate (Lewis et al., 1993a). Indeed, it has been shown clinically (Mogensen et al., 2000a) and in experimental DN (Cao et al., 2001a, Wilkinson-Berka et al., 2001), that superior blockade of the RAS occurs with dual blockade of the system by combining ACE inhibition with AT1 receptor antagonism.

Other vasoconstrictors

Other vasoactive hormones have been postulated to play a role in mediating progressive renal injury (Johnston et al., 1998b). Intrarenal and systemic haemodynamics are maintained in homeostasis by a balance of vasoconstrictors and vasodilators. These substances include hormones which have differential effects on afferent and efferent arteriolar tone and thereby disparate actions on intraglomerular pressure. Important vasoconstrictors in addition to AII in diabetes are the endothelins (Benigni and Remuzzi, 1999) vasopressin (Burrell et al., 2000) and urotensin II (Langham et al., 2004). In addition, a range of vasodilatory substances including bradykinin, atrial natriuretic factor, certain prostaglandins and nitric oxide modulate glomerular vasomotor tone (Johnston et al., 1998a).

The ET system comprises three distinct endothelins 1-3, which signal via two receptors, the ET_A and ET_B receptors (Naicker and Bhoola, 2001). ET_A receptors are involved in vasoconstriction and cell proliferation through paracrine or autocrine release of ET-1, while ET_B receptors, which bind all ETs, mediate NO release and induce transient vasodilatation (Naicker and Bhoola, 2001). As for angiotensin II, studies examining ET-1 levels in diabetic kidneys have yielded conflicting results. In STZ diabetic rats, renal endothelin-1 expression has been reported to be increased (Hargrove et al., 2000, Nakamura et al., 1995b), suppressed (Hopfner et al., 1998), unchanged (Shin et al., 1995) or undetectable (Takahashi et al., 1991). In addition, various changes in endothelin receptor subtype regulation have been described (Fukui et al., 1993). Clinical studies have shown a more consistent trend toward elevation of plasma ET-1 in type 1 and type 2 diabetic patients, which correlated with disease progression (Schneider et al., 2002).

There is a lack of uniformity in the findings with respect to renoprotection and endothelin receptor blockade in diabetic and non-diabetic models of renal injury (Benigni et al., 1998, Benigni et al., 1996, Cao et al., 2000a, Orth et al., 1998). This may relate to the nature of the renal disease, sodium balance, pharmacological characteristics of the various receptor antagonists as well as the selectivity of these compounds for the ET_A and ET_B receptor subtypes (Jandeleit-Dahm et al., 2000). Previous studies in experimental diabetes, both in the m(Ren-2)27 by our group and in the STZ diabetic rat, have demonstrated that although the non-selective ET antagonist bosentan and PD142893 (which antagonise both ET receptor subtypes) reduced blood pressure and normalised GFR, no benefits on renal structural parameters were identified (Benigni et al., 1996, Kelly et al., 2000). However, other studies using selective ET_A receptor antagonists, such as FR139317 (Nakamura et al., 1995a) and LU224332 (Hocher et al.,

2001) have provided benefits both on renal structure and function in diabetic kidneys. Although there are clear renal benefits both in vitro and in vivo, the efficacy of ET blockade as a clinical target for diabetic nephropathy remains to be elucidated. The importance of ET_A receptor antagonism in diabetic nephropathy has been examined using a SPP 301. In a phase II study, this agent appeared to attenuate albuminuria in type 2 diabetic patients with hypertension and microalbuminuria (Dieterle et al., 2005). These positive findings have stimulated a larger phase III study in a type 2 diabetic population including those with more advanced renal disease.

Vasodilators

The formation and degradation of many vasoactive hormones rely on zinc dependent metallopeptidases, the most well known example being ACE. Other enzymes include firstly neutral endopeptidase (NEP), which plays an important role in the degradation of natriuretic peptides as well as bradykinin and secondly, endothelin converting enzyme (ECE), which is involved in generation of biologically active endothelin (Johnston et al., 1998a). A major target has been inhibition of both ACE and NEP leading to a lack of degradation of vasodilatory hormones and reduced formation of AII. This approach would theoretically lead to increased vasodilation resulting in greater hypotensive efficacy and potentially superior renoprotection. Such agents have now been explored in both preclinical and clinical contexts. In the subtotal nephrectomy model, several groups have reported renoprotective effects of the dual ACE/NEP inhibitor, omapatrilat (Cao et al., 2001b). In the diabetic SHR, our group has shown with another ACE/NEP inhibitor antihypertensive efficacy in association with beneficial effects on urinary albumin excretion (Davis et al., 2003b, Tikkanen et al., 1998). However, the high prevalence of angioedema with ACE/NEP inhibition using omapatrilat, particularly in African American subjects and the presence of persistent cough, attributable to its potent ACE inhibition have delayed and potentially halted further clinical development of this compound. Nevertheless, other ACE/NEP continue to be developed by some pharmaceutical companies.

Nitric oxide (NO) is a widely expressed signalling molecule, which plays a major role in most cellular and tissue functions throughout the body. NO is formed via conversion of L-arginine to L-citrulline, catalysed by nitric oxide synthase (NOS). There are three structurally distinct NOS isoforms, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (Komers et al., 1994) and their structure and function have been previously reviewed extensively (Hocher et al., 2001).

The status of the nitric oxide pathway in diabetes appears to be both organ and duration specific with evidence of nitric oxide deficiency in the macrovascular tree particularly with increasing duration of diabetes (Pieper, 1999). By contrast, it has been considered that in the diabetic kidney there may be increased nitric oxide levels and/or action (Komers et al., 1994, Tolins et al., 1993). Initial studies in the kidney suggested increased nitric oxide levels in the diabetic kidney, based on the relatively crude marker of urinary nitrate/nitrite production (Komers et al., 1994). Specific renal vascular studies have demonstrated impaired acetylcholine induced endothelial dependent relaxation. In that study NO dependent vasodilation was enhanced whereas vasodilation mediated by endothelium derived hyperpolarizing factor was severely impaired (De Vriese et al., 2000). Further studies suggesting increased NO in the diabetic kidney were based on experiments using non-selective inhibitors of NO synthase (NOS) which showed reductions in GFR and renal plasma flow in hyperfiltering, diabetic rats (Komers et al., 1994). More recent studies suggest that a major abnormality in the diabetic kidney is increased expression of endothelial NOS (Sugimoto et al., 1998, Veelken et al., 2000) with several groups reporting no change in inducible NOS (iNOS) (Soulis et al., 1997a, Veelken et al., 2000). This contrasts with initial studies suggesting a possible role for iNOS in diabetic nephropathy and in particular diabetic hyperfiltration. Indeed, administration of a selective iNOS inhibitor, L-imino-ethyl-lysine failed to influence renal function in experimental diabetic rats (Veelken et al., 2000). It remains to be determined if these effects of NO on early renal haemodynamic abnormalities are of any long-term relevance, particularly to the development of renal impairment and ultrastructural injury in diabetes. Our own studies using the non-specific inhibitor of NO synthase, L-NAME, did not clearly show renoprotection (Soulis et al., 1997a).

Metabolic Pathways

Advanced glycation

AGEs are generated as a result of a series of sequential biochemical reactions, some of which are poorly defined, resulting in the non-enzymatic glycation of free amino groups on protein, lipoproteins and nucleic acids by reducing sugars (Brownlee, 1994). This process is accelerated in the presence of oxygen with some AGEs can considered be 'glycoxidation' products (Fu et al., 1994). Some of the most commonly identified AGEs include carboxymethyllysine (Wells-Knecht et al., 1996) and pentosidine (Sell et al., 1991). AGE formation is increased in diabetes due to chronic hyperglycaemia although it has become increasingly evident that there is often disparity between good glycaemic control as measured by HbA_{1C} and circulating (Cohen et al., 2003) or skin AGE concentrations (Monnier et al., 1999). Indeed in the DCCT study, skin AGE accumulation was a more accurate predictor of progression to renal disease than HbA_{1C} (Monnier et al., 1999). In experimental DN there is renal and circulatory accumulation of AGEs (Soulis-Liparota et al., 1991). Renal filtration also plays a major role in the excretion of circulating AGEs (Forster et al., 2005, Miyata et al., 1998a) and therefore there is a strong correlation between declining renal function and AGE accumulation in most renal diseases (Miyata et al., 1998b, Miyata et al., 1997, Sebekova et al., 1999). Some studies have used exogenous intravenous administration of AGEs to mimic diabetic serum concentrations, which has been reported to induce complications (Vlassara et al., 1994). In fact, AGEs from dietary sources, of which between 50–80% are absorbed across the gut, are now considered important contributors to the circulating pool of AGEs in diabetes, as they are present at high concentrations in western style diets (Forster et al., 2005). Indeed, there are clear benefits of specific reduction of dietary AGE intake in both experimental DN (Uribarri et al., 2003) and in patients with DN (Koschinsky et al., 1997). This suggests that an important contribution to AGE induced damage may be via the interaction of circulating AGEs with receptors or binding proteins.

In early studies by Nicholls and Mandel in diabetic mice, renal AGE accumulation was detected which could be attenuated by either pancreatic islet transplantation or treatment with an inhibitor of AGE formation, aminoguanidine (Nicholls and Mandel, 1989). Subsequently, our group evaluated the role of aminoguanidine in diabetic rats and showed that reduced renal AGE

accumulation with this drug was associated with retardation in the development of albuminuria and attenuation of mesangial expansion (Soulis-Liparota et al., 1991). Since advanced glycation end products accumulate over time, we explored the role of early versus delayed introduction with aminoguanidine and its effects on renal injury in diabetes (Soulis et al., 1996). In a 32 week study, diabetic rats were randomised to receive aminoguanidine over the first or last 16 weeks of the study period. It was demonstrated that both treatments had similar effects on renal AGE accumulation, albuminuria and mesangial expression consistent with the hypothesis that the renal injury in diabetes is linked to the time of exposure to increased AGEs. Two double blinded placebo controlled randomised clinical trials with aminoguanidine (pimagedine) in combination with the ACE inhibitor ramipril have been performed in both type 1 (ACTION I) (Appel et al., 1999) and type 2 diabetic patients (ACTION II) with overt nephropathy. The primary end point in the ACTION I study, a reduction in the risk of doubling of serum creatinine above the ACE inhibition, was not achieved, although pimagedine reduced urinary protein excretion, serum triglycerides and LDL. Unfortunately, ACTION II was terminated early because of side effects (Thornalley, 2003).

Since aminoguanidine not only inhibits AGE formation but also has other actions including inhibition of iNOS (Corbett et al., 1992) and therefore associated side effects, we explored the role of a chemically related compound, ALT 946, which has less effect on iNOs but conferred the same degree of renal protection in experimental DN (Forbes et al., 2001). Pyridorin (pyridoxamine chloride, PM), which inhibits the conversion of AGE intermediates to AGEs, has also been shown to provide renoprotection in experimental diabetic nephropathy (Degenhardt et al., 2002). The clinical therapeutic potential of PM is currently under investigation in humans and is in phase II trials (http://www.biostratum.com). Other potent inhibitors of AGE formation, OPB-9195 (Nakamura et al., 1997, Yamamoto et al., 2001) and LR-90 (Figarola et al., 2003) have provided further support for the role of AGE blockade in diabetic nephropathy, although their clinical utility has not been specifically determined.

A number of studies have also shown that the insulin sensitizer metformin is beneficial in reducing diabetes-associated vascular risk beyond the benefits expected from its antihyperglycemic effect (1998). Clinical studies have shown that metformin has the ability to reduce toxic dicarbonyls and AGEs (Beisswenger and Ruggiero-Lopez, 2003). Additional studies including assessment in man of other potential cellular effects of metformin on AGE production are required to further elucidate the actions and role of metformin in diabetic nephropathy.

Other novel compounds also have the ability to reduce AGE accumulation in diabetes. Among these agents, the lipid-soluble thiamine derivative benfotiamine shows great promise. Studies have recently demonstrated that benfotiamine is able to block major biochemical pathways implicated in the pathogenesis of diabetic complications including the accumulation of AGEs (Hammes et al., 2003). Initially, this agent was reported to be useful in experimental diabetic retinopathy (Hammes et al., 2003). Similar end organ protection has also been reported by Thornalley et al. (Babaei-Jadidi et al., 2003) in STZ-induced diabetic nephropathy. It is thought that, as a consequence of hyperglycemia in diabetes, there is increased concentrations of the triosephosphate glycolytic intermediates glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetonephosphate (DHAP). These metabolites are considered to trigger processes such as mitochondrial oxidative stress and methylglyoxal formation, which facilitate the production of AGEs (Nishikawa et al., 2000). Consequently, an agent such as benfotiamine, that reduces the accumulation of triosephosphate intermediates could reduce these downstream pathways. Indeed this has been shown *in vivo* in various models of diabetic complications (Babaei-Jadidi et al., 2003, Nishikawa et al., 2000, Thornalley, 1998). Currently several clinical trials are either in progress or in development to assess this agent on renal parameters in both type 1 and type 2 diabetes.

More recently, alternative approaches to prevent renal AGE accumulation have been developed which involving the use of putative cross-link breakers, which cleave pre-formed AGEs (Vasan et al., 1996). Our group has demonstrated that delayed intervention following established DN with one of these cross-link breakers, ALT-711 (alagebrium) in STZ-induced diabetic rats, ameliorates diabetes associated increases in serum and tissue AGEs in association with reduced albumin excretion rate (AER), blood pressure, renal collagen accumulation, tubulointerstitial area and glomerulosclerosis (Forbes et al., 2003, Thallas-Bonke et al., 2004). Moreover, various cellular and molecular changes, such as the increased gene and protein expression of collagen IV, the cytokine TGF-β1, and α-smooth muscle actin (α-SMA) were reduced by ALT-711 treatment. Preliminary results from the DIAMOND (Distensibility Improvement and Remodelling in Diastolic Heart Failure) study also demonstrated reductions in left ventricular mass and improvement in left ventricular diastolic filling following treatment with ALT-711 (http://www.alteon.com/cross1.htm). This was manifested clinically by improvements in their NYHA class and quality-of-life. Future studies using this agent in patients with or at risk of overt diabetic nephropathy are now warranted and are in the planning stage.

The mechanisms of action whereby AGEs induce their effects have not been fully elucidated. Firstly, AGEs induce cross-link formation or steric hindrance, thereby directly affecting the function of various proteins. Secondly, they interact with a range of binding proteins, some of which have been isolated. A number of AGE binding proteins have been identified including RAGE, AGE-R1, AGE-R2, AGE-R3, lactoferrin, lysozyme, CD36 and macrophage scavenger receptors (Araki et al., 1995, Li et al., 1996, Li et al., 1995, Neeper et al., 1992) and the ERM family of proteins (McRobert et al., 2003). Some of these proteins are viewed to act as receptors and are involved in the transduction of various effects of AGEs.

RAGE has a central role in mediating the effects of AGEs in promoting the development of vascular disease in diabetes (Bierhaus et al., 2005). The engagement of RAGE by its ligands such as AGEs or S100 calgranulins induces inflammatory cell infiltration and activation of a range of adhesion molecules and cytokines (Basta et al., 2002, Hofmann et al., 1999). In the glomeruli of patients with diabetic nephropathy, RAGE expression is upregulated and positively correlates with AGE accumulation (Soulis et al., 1997b). The RAGE promoter contains nuclear factor-κB (NF-κB) binding sites that are active and are involved in the regulation of RAGE expression (Li and Schmidt, 1997). Ligand engagement of RAGE results in the generation of reactive oxygen species (ROS) and activation of NF-κB (Bierhaus et al., 2001, Hofmann et al., 1999). This ultimately leads to upregulation of a range of cytokines involved in mediating many of the structural changes seen in the diabetic kidney. RAGE knockout mice have decreased renal injury in response to diabetes (Wendt et al., 2003) and long-term administration of a RAGE neutralizing

antibody to type 2 diabetic *db/db*$^{(+/+)}$ mice confers renoprotection (Flyvbjerg et al., 2004). Furthermore, transgenic mice that have overexpression of RAGE develop accelerated renal disease in the setting of diabetes (Yamamoto et al., 2001). These studies underscore the significance of AGE-receptor-mediated pathways and in particular RAGE, in the pathogenesis of diabetic nephropathy.

Recently, it has been demonstrated that there are three distinct splice variants of RAGE. These are the full length receptor, the N-terminal variant that does not contain the AGE-binding domain and the Cterminal splice variant, soluble RAGE (sRAGE) which does not contain the transmembrane and effector domains (Malherbe et al., 1999, Yonekura et al., 2003). sRAGE, which is the extracellular portion of the RAGE receptor, binds to AGEs and thereby can block the interaction of various ligands with cell surface full-length RAGE (Wautier et al., 1996). Indeed, these actions of sRAGE acting as a functional antagonist to full-length RAGE, have been shown to confer therapeutic benefits in diabetic nephropathy (Wendt et al., 2003). Interestingly our own studies have shown that treatment of both experimental and DN in humans with ACE inhibitors not only reduced their circulating AGE levels but also increases their plasma sRAGE concentration (Forbes et al., 2005). Furthermore, it has recently been demonstrated that thiazolidinediones act at least in part via their ability to reduce endothelial expression of RAGE (Marx et al., 2004).

In diabetic NOD mice, the expression of the AGE receptors, R1, R2 and R3 has been characterised (He et al., 2000). There was a 6 fold increase in AGE-R3 with the development of diabetes. Interestingly, there was reduced AGE-R1 expression in the prediabetic NOD mice. It remains to be determined if the abnormality in this receptor which is implicated in AGE clearance contributes to delayed AGE removal thereby leading to early renal AGE accumulation providing an additional mechanism for development of DN in this model. Indeed, it has recently been demonstrated that mice transgenically over-expressing AGE-R1 are protected against the development of diabetic nephropathy. Recent in vitro studies in mesangial cells have also shown that decreases in the AGE-R1 receptor, induce the expression of RAGE and its signal transduction via NF-κB (Lu et al., 2004). Interestingly, genetic deletion of AGE-R3 is associated with accelerated glomerulopathy and proteinuria following the induction of diabetes (Pugliese et al., 2001). This is associated with an increase in RAGE but a decrease in AGE-R1 expression.

Other effects of AGEs appear to be the stimulation of various cytokines including TGFβ presumably via receptor dependent pathways including RAGE. Increased expression of renal TGF-β1 as well as PDGF and the extracellular matrix protein, type IV collagen in the diabetic kidney can be attenuated by aminoguanidine, particularly in the tubulointersitium (Kelly et al., 2001b). A similar effect has been reported in a model of type 2 diabetes, the OLETF rat, by a Japanese group which described reduced TGF-β1 and VEGF expression after 68 weeks of treatment with a novel AGE inhibitor, OPB-9195 (Tsuchida et al., 1999).

Aldose Reductase/Polyol Pathway

The accumulation of polyols within the kidney has been postulated to play a role in the development of DN (**Fig. 1**). Although this pathway has been most extensively investigated in terms of neuropathy, more limited exploration of this pathway has been performed in renal disease (Dunlop, 2000). Within the polyol pathway, glucose is reduced to sorbitol by aldose reductase (AR), an NADPH dependent aldo-keto reductase. Sorbitol accumulation is associated with depletion of myoinositol and changes in the cellular redox potential (Greene et al., 1987). The importance of polyols in activating pathways relevant to DN has been emphasised with the demonstration that aldose reductase inhibition is associated with reduced PKC activation and TGF-β1 production in human mesangial cells in response to glucose (Ishii et al., 1998). It has been proposed that oxidation of sorbitol by NAD^+ increases cytosolic NADH:NAD^+ ratio, thereby inhibiting the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in increasing triose phosphate (Williamson et al., 1993). As mentioned above, raised triose phosphate concentrations could increase the AGE precursor methylglyoxal, and diacylglycerol (DAG) activating PKC (**Fig. 1**).

Several studies have examined the effects of aldose reductase inhibition on functional and structural markers of experimental DN (Bank et al., 1989, Chang et al., 1991, Mauer et al., 1989, McCaleb et al., 1991, Soulis et al., 1995). The results are not consistent among the various studies and may reflect the different animal models, the measurements of renal injury and the classes of aldose reductase inhibitors used. In human DN, the effects of aldose reductase inhibition, in particular with ponalrestat, tolrestat and epalrestat , have also been conflicting and in general rather disappointing (McAuliffe et al., 1998, Passariello et al., 1993). The major effect appears to be a reduction in hyperfiltration (Pedersen et al., 1991) as was observed in some of the experimental studies (Bank et al., 1989).

Hexosamine pathway

It has been hypothesised that the hexosamine pathway, is involved in the development of diabetic complications (James et al., 2000, Nerlich et al., 1998, Schleicher and Weigert, 2000). In this pathway, glucose is converted to glucose-6-phosphate via hexokinase and subsequently to fructose-6-phosphate as part of the glycolytic pathway (**Fig. 1**). Fructose–6-phosphate is subsequently diverted from glycolysis to provide a substrate for the formation of proteoglycans and O-linked glycoproteins (Brownlee, 2001). This occurs under the control of the rate limiting enzyme glutamine:fructose–6-phosphate amidotransferase (GFAT), using glutamine as an amino donor. A series of additional reactions then occur ultimately leading to the generation of other glucosamines, which serve as precursors for amino sugars used for the synthesis of proteoglycans, glycolipids and glycoproteins. To explore this pathway investigators have overexpressed the enzyme GFAT (James et al., 2000) or directly inhibited this enzyme. It appears that increased flux through the hexosamine pathway is associated with PKC activation and increased TGF-β1 expression (Weigert et al., 2001). Indeed, glucose induced TGF-β1 expression and subsequent matrix production can be inhibited by GFAT blockade using either azaserine or an antisense oligonucleotide approach (Schleicher and Weigert, 2000).

Increased flux through the hexosamine pathway in DN may also be facilitated by glucose transporters such as GLUT-1 (Heilig et al., 1995). Initial studies showed that the effects of glucose on collagen production could be reproduced in mesangial cells overexpressing GLUT-1 despite exposure to normal glucose levels (Heilig et al., 1995). Subsequently it was demonstrated that glucose induces GLUT-1 expression and translocation in mesangial cells (Heilig et al., 1997), leading to increased aldose reductase expression as well as induction of PKCα, ultimately leading to increased matrix production (Henry et al., 1999). To further

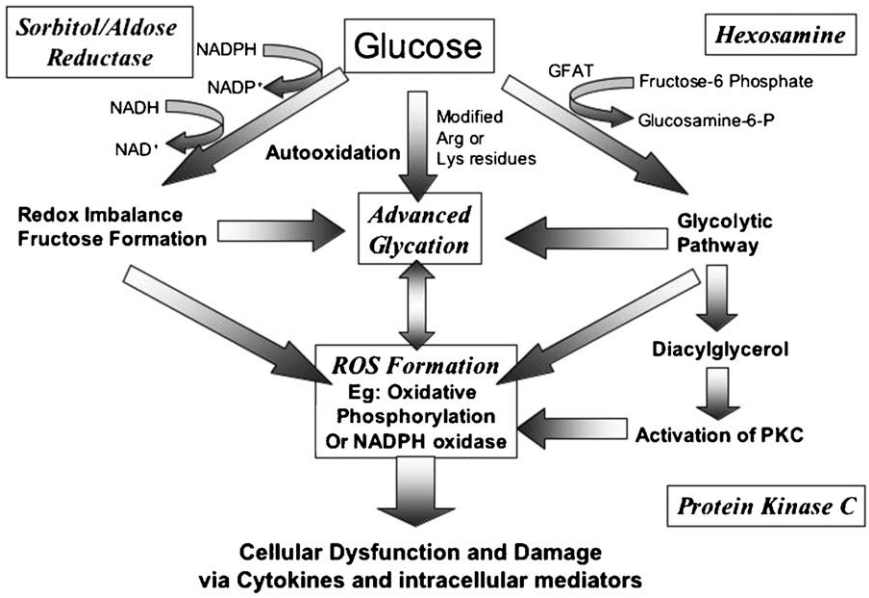

Fig. 1 Interactions between metabolic pathways in diabetic nephropathy

explore the role of GLUT-1 in mediating expression of matrix proteins such as fibronectin, cells were studied which expressed antisense GLUT-1 (Heilig et al., 2001). The in vivo relevance of this pathway remains to be determined but provides another target for the treatment of diabetic complications.

Oxidative Stress and Reactive Oxygen Species

Oxidative stress leading to the formation of reactive oxygen species (ROS) is recognised as a key component in the development of diabetic complications (◉ **Fig. 1**) (Brownlee, 2001, Nishikawa et al., 2000). ROS are directly cytotoxic and promote inflammation and fibrosis. In addition, oxidation of macromolecules including proteins, lipids, carbohydrates and DNA leads to molecular dysfunction.

There are a number of sources for the genesis of ROS in diabetes including autooxidation of glucose, transition metal catalysed Fenton reactions, mitochondrial respiratory chain deficiencies, xanthine oxidase activity, peroxidases, NO synthase and NAD(P)H oxidase (Baynes and Thorpe, 1999, Cameron and Cotter, 1999). The discussion within this article will be restricted to mitochondrial and NAD(P)H oxidase as sources of ROS. This is not, however, to down-play the importance of these other pathways. Indeed, xanthine oxidase activity is decreased in the presence of the AGE formation inhibitor, aminoguanidine (Courderot-Masuyer et al., 1999), while Cu^{2+} augmentation of ROS-mediated AGE formation is suggested *in vitro* (Ortwerth and James, 1999). In addition, synergistic actions between AGEs and endogenous nitric oxide synthase results in the apoptosis of neural cells by direct generation of ROS (Cellek et al., 2003).

The expression and activity of NAD(P)H oxidase represents one important source of oxidative stress in diabetes (Wautier et al., 2001). NAD(P)H consists of five subunits, the membranous p22phox and gp91phox (nox-1 and nox-4 homologues), the cytosolic subunits p47phox and p67phox and the regulatory G-protein rac-1 (Harrison et al., 2003). Upon stimulation, the cytosolic subunits assemble with the membranous subunits to form a functioning oxidase which uses NAD(P)H as an electron donor, resulting in the well characterised oxidative burst. Up-regulation of the cytosolic NAD(P)H oxidase subunit, p47phox, has been shown in experimental diabetes within the vasculature (Christ et al., 2002) and kidney (Onozato et al., 2002). Our own studies have demonstrated an increased expression of the membrane bound nox-4 subunit within the diabetic kidney (Forbes et al., 2002). Apocynin, which inhibits NAD(P)H oxidase assembly has been shown to confer beneficial effects on peripheral nerve perfusion in diabetic rodents (Cotter and Cameron, 2003) although the effects in the diabetic kidney are less well characterised (Asaba et al., 2005).

In vitro studies have suggested a role for dysfunction of the mitochondrial respiratory chain in the development of oxidative injury in diabetes (Brownlee, 2001). Diabetes is associated with a decrease in mitochondrial membrane potential leading to activation of oxidative pathways (Nishikawa et al., 2000). Patients with Friedreich ataxia (a genetic disorder due to frataxin mutations causing excessive mitochondrial ROS generation in association with down regulation of mitochondrial complex I) (Rotig et al., 1997) develop diabetes associated with accelerated microvascular complications and cardiomyopathy in some individuals. It should be noted that skin fibroblasts taken from patients with Friedreich ataxia, which are susceptible to oxidative stress and subsequent apoptosis, only respond to mitochondrial targeted antioxidants (Jauslin et al., 2003).

The elevated oxidative stress seen in diabetes is the result of an imbalance between ROS generation and endogenous anti-oxidant activity, including free radical scavengers and enzyme systems. Indeed, type 1 diabetic patients with nephropathy have abnormal antioxidant profiles (Hodgkinson et al., 2003). Antibodies to AGE-R2 and RAGE are also able to inhibit AGE-dependent depletion of cellular anti-oxidant systems such as glutathione peroxidase (Lander et al., 1997). Glycation of anti-oxidants such as Cu-Zn-superoxide dismutase (SOD-1) also contribute to the decline in anti-oxidant activity (Fujii et al., 1996) which may be related to specific SOD-1 gene mutations (Kato et al., 2000). While oxidative stress can augment the formation of AGEs through glycoxidation, AGEs can also lead to enhanced formation of free radicals, both directly through catalytic sites in their molecular structure (Yagihashi, 1997) and via stimulation of membrane-bound NAD(P)H oxidase through the RAGE receptor (Wautier et al., 2001). In rodent models, oxidative stress, induced following the hepatic infusion of diabetic erythrocytes can be prevented by pre-treatment with anti-RAGE IgG (Wautier et al., 2001).

Animal studies have shown that potent anti-oxidants may protect against the development of diabetic nephropathy (Jang

et al., 2000, Koo et al., 2002). However, human studies with less potent antioxidants such as α-tocopherol (Yagihashi, 1997) have been, in general, disappointing and this has been attributed partly to lack of penetration of these agents to target areas and the lack of specificity. Our own studies have demonstrated that oxidative stress is increased in diabetic animals in proportion to AGE accumulation (Forbes et al., 2002). There is also evidence in human diabetic glomerular lesions of increased oxidative stress and AGE accumulation (Suzuki et al., 1999), both of which may be attenuated by good glycaemic control (Odetti et al., 1996). Boldine, a mitochondrial free radical scavenger, has proven beneficial in experimental diabetes in combating mitochondrial free radical production and lipid peroxidation in the kidney (Jang et al., 2000). The utility of boldine and related agents as renoprotective agents may ultimately be related to their ability to combat oxidative stress, however this remains to be directly tested.

It remains to be determined if oxidative stress in purely an early event in hyperglycaemia induced vascular injury leading to the activation of other pathways or if oxidative stress is also implicated in downstream critical events for mediating vascular and in particular renal damage. To further explore this issue will require better markers of oxidative stress as well as the use of more specific inhibitors of this pathway.

Intracellular second messengers

Protein kinase C (PKC)

Evidence has accumulated over the last decade implicating PKC as an important mediator of diabetes induced vascular dysfunction (Inoguchi et al., 1992, Xia et al., 1994). Initial studies using various cell types including mesangial cells indicated that effects of hyperglycemia on mesangial dysfunction could be mimicked by phorbol esters, direct activators of PKC and could be attenuated by non-specific PKC inhibitors (Studer et al., 1993). Further studies identified that certain isoforms of this intracellular second messenger were preferentially activated in the diabetic kidney (Koya et al., 1997) and therefore these isoforms were considered as targets for development of inhibitors. A specific PKC β inhibitor, LY333531 (ruboxistaurin), was developed and in streptozotocin diabetic rats over 12 weeks this agent was shown to attenuate glomerular hyperfiltration, reduce albuminuria and decrease expression of TGFß and various extracellular matrix proteins (Ishii et al., 1996, Koya et al., 1997). More recently, this PKC inhibitor was administered to *db/db*$^{(+/+)}$ mice, a model of type 2 diabetes for 16 weeks (Koya et al., 2000) and in the STZ diabetic (mRen-2)27 rat (Kelly et al., 2003). In addition to normalising glomerular PKC activity in these models, urinary albumin excretion was reduced as was TGF-β1, fibronectin and type IV collagen expression. A number of clinical trials for various diabetic complications are in progress or just completed with ruboxistaurin. The results of one of these trials have been recently published evaluating the effect of this agent on albuminuria in type 2 diabetic patients although it was not significantly powered to show a definitive benefit between treated and untreated patient groups (Tuttle et al., 2005).

More recently, other PKC isoforms including PKCα have been implicated. Streptozotocininduced diabetic PKCα knockout mice have less albuminuria than wild type mice (Meier et al., 2003). Our own group has recently reported that treatment with the cross-link breaker ALT-711 attenuated the increased expression and translocation of PKCα in a series of *in vivo* and *in vitro* studies (Thallas-Bonke et al., 2004). It remains to be determined if this action of ALT-711 on PKCα expression and phosphorylation partly explain its renoprotective actions. As yet there are no specific PKC alpha isoform inhibitors suitable for clinical investigation to further examine the utility of such a therapeutic approach in diabetes.

Other studies have explored the effects of other treatments on renal PKC activation (Osicka et al., 2000). Interestingly, both the ACE inhibitor, ramipril and the inhibitor of AGE formation, aminoguanidine were associated with prevention of diabetes associated increases in PKC activation. Furthermore, the effects of these agents on PKC activity were also observed at other sites of vascular injury including the retina and mesenteric vascular bed (Osicka et al., 2001). These findings suggest that AGEs and AII may activate PKC *in vivo*. Furthermore, it is possible that PKC represents a critical downstream event in the pathogenesis of DN. Indeed various actions of cytokines implicated in diabetic complications such as VEGF are PKC dependent (Aiello et al., 1997). In addition, PKC modulates the effects of glucose including VEGF and TGF-β1 expression (Cha et al., 2000, Studer et al., 1997).

Nuclear factor kappa B (NF-κB)

NF-κB is a transcription factor composed of two subunits, the most common of which are the p50 and p65 subunits (Barnes and Larin, 1997). NF-κB is ubiquitously expressed and is stored in an inactive form bound to inhibitors in the cytoplasm. P50 is usually complexed with the p65 subunit and therefore has the ability to transactivate numerous genes including cytokines, adhesion molecules, NO synthase,

angiotensinogen and many other inflammatory and proliferative proteins implicated in the process of diabetic nephropathy (Barnes and Larin, 1997). NF-κB is activated by a range of stimuli including glucose (Pieper and Riazulhaq, 1997) and ROS (Nishikawa et al., 2000). This phenomenon appears to be PKC dependent (Pieper and Riazulhaq, 1997) and is able to be prevented by antioxidants (Nishikawa et al., 2000). AGEs are also involved in activation of NF-κB via a RAGE-dependent pathway leading to its translocation to the nucleus where it induces transcription (Yan et al., 1994). The promoter region of the RAGE gene also contains NF-κB binding sites (Li and Schmidt, 1997). This provides a pathway for perpetuation of injury since NF-κB is not only activated in response to RAGE, but RAGE is also activated by NF-κB. This is supported by studies in which mutations of both NF-κB like binding sites in the promoter region of RAGE led to stimulated promoter expression (Li and Schmidt, 1997).

The NF-κB inhibitor, pyrollidine dithiocarbamate (PDTC) has been studied in non-diabetic models of fibrosis and shown to confer renoprotection (Muller et al., 2000, Rangan et al., 1999), although its toxicity does not allow for direct translation to the clinical setting. More recently, we have performed a study investigating the role of activation of NF-κB, in particular the p65 subunit, in the pathogenesis of early renal macrophage infiltration in experimental diabetes (Lee et al., 2004). This study revealed early activation of NF-κB in the diabetic kidney, which could be modulated by interruption of the RAS (Lee et al., 2004). The diverse actions of NF-κB and the capacity of various factors such as AII and AGEs to activate this transcription factor (Ruiz-Ortega et al., 2000, Yan et al., 1994), are consistent with NF-κB playing a pivotal role in modulating diabetic complications. It is possible that NF-κB, like PKC, represents a major site of interaction between haemodynamic and glucose dependent pathways

in the development of diabetic vascular complications. However, approaches to inhibit NF-κB have not been explored fully in DN, most likely due to the intimate involvement of this transcription factor in a number of essential cellular processes.

MAP kinase

Mitogen-activated protein kinases (MAPK) are a major signalling system which translates extracellular signals to intracellular responses (Seger and Krebs, 1995). Three major subgroups have been identified, the ERK family, C-jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAPK. The MAPK cascades play a central role in a range of biological processes relevant to DN including cell growth, differentiation and apoptosis (Choi, 2000). Glucose has been reported to activate MAPK, and in particular p38 MAPK (Igarashi et al., 1999). This increase in MAPK has also been noted in glomeruli from diabetic rats (Dunlop and Muggli, 2000, Haneda et al., 1997), and the increase in MAPK in response to hyperglycaemia has been demonstrated to be PKC dependent (Haneda et al., 1997). Recently it has been hypothesised that MAPK may play a central role in the pathogenesis of diabetic complications (Tomlinson, 1999). In particular, various stimuli in addition to glucose activate these enzymes including AGEs (Lander et al., 1997, Simm et al., 1997), polyols (Kang et al., 1999) and PKC (Haneda et al., 1997). The activation of the angiotensinogen gene by glucose also appears to be p38 MAPK dependent (Zhang et al., 2000). The exact role of this family of kinases remains to be clarified but of additional relevance to DN is the finding that MAPK plays an important role in the TGF-β1 signalling pathway (Choi, 2000) and in particular in mediating the prosclerotic effect of this cytokine (Inoki et al., 2000). The exact role of this family of kinases is not clear but they appear to have relevance to diabetic nephropathy. Already these agents have been demonstrated to have a role in non-diabetic renal disease, and thus findings from studies using MAPK inhibitors in experimental diabetes are keenly awaited.

Cytokines

Transforming Growth Factor β (TGF-β)

Transforming growth factor beta is a superfamily with three mammalian isoforms. The major isoform, TGF-β1 is synthesised as an inactive or latent form, which subsequently is subjected to proteolytic cleavage leading to the generation of the active form. TGF-β1 binds to the type II receptor and subsequently binds to the type I receptor (Wrana et al., 1994) inducing phosphorylation and intracellular signalling involving the Smad proteins (Massague, 1998). As previously reviewed, these actions of TGF-β via the Smad pathway, are modulated by a range of signalling pathways including NF-κB and MAPK (Schiffer et al., 2000).

TGF-β1 is considered the pivotal cytokine in mediating collagen deposition in the kidney (Border and Noble, 1994, Isaka et al., 1993). Not only does it stimulate gene expression of various matrix proteins but it influences the matrix degrading enzyme pathways by inhibiting the synthesis of matrix metalloproteinases and stimulating the production of metalloproteinase inhibitors (TIMPs) (Border and Noble, 1994). In vitro studies have shown that a range of stimuli increase TGF-β expression (**Table 1**). These include hyperglycaemia, AGEs, stretch, AII, endothelin, lipids and various products of oxidative stress such as F_2 isoprostanes, all factors relevant to the progression of DN (Gruden et al., 1999, Herman et al., 1998, Jandeleit-Dahm et al., 1999, Montero et al., 2000, Rocco et al., 1992, Rumble et al., 1997, Wolf et al., 1995). Induction of TGF-β1 by glucose and glycated proteins appears to be a PKC dependent phenomenon (Chen et al., 2001). Studies by our group in proximal tubular epithelial to myofibroblast transdifferentiation (TEMT) in DN have demonstrated AGE/RAGE induced TEMT which is mediated by TGF-β1 (Oldfield et al., 2001) via smad phosphorylation (Li et al., 2003).

Table 1 ■■■

In humans and animal models, TGF-β mRNA and protein levels are significantly increased in the glomeruli and tubulointerstitium in type 1 and type 2 diabetes (Sharma et al., 1997, Yamamoto et al., 1993, Yamamoto et al., 1996). Ziyadeh et al have previously examined the effects of long-term administration of a neutralizing TGF-β antibody on the renal function and renal histology of diabetic *db/db* mice, (Ziyadeh et al., 2000) and STZ diabetic rats (Sharma et al., 1996). Although most of the benefits have been attributed to TGF-β1, Hill et al. have suggested that another isoform, TGF-β2, is closely linked to fibrogenesis in diabetic nephropathy (Hill et al., 2000). Consistent with this view, systemic delivery of CAT-152, a neutralizing anti-TGF-β2 antibody, during the acute stage of diabetic nephropathy reduced the rate of pathogenic fibrosis including attenuation of collagen type I synthesis and reduction in albuminuria in STZ-induced diabetic rats (Hill et al., 2000). Novel triarylimidazole and 2,4,5-pyridinylimidazole TGF-β receptor antagonists, have been developed by GlaxoSmithKline for the treatment of different fibrotic diseases including diabetic nephropathy (Sharma et al., 1996). These agents specifically inhibit the phosphorylation of smad2 or smad3 via blockade of activin-like kinase 5, the type I receptor for TGF-β. These agents may be useful in diabetic nephropathy although are not fully characterized as renoprotective agents in diabetes. However, TGF-β expression is decreased by the treatments such ACEi (Davis et al., 2003a), AT1R antagonists (Koga et al., 2002), antiglycation inhibitors (Forbes et al., 2003, Kelly et al., 2001a, Kim et al., 2000b) and statins in experimental diabetes. Therefore, TGF-β1 may not need to be specifically targeted to reduce it levels or action and no currently available therapies exist to directly target it in clinical DN.

Connective tissue growth factor

Another prosclerotic cytokine, connective tissue growth factor (CTGF) has been demonstrated to have increased renal and in particular, glomerular expression in diabetes (Murphy et al., 1999, Riser et al., 2000a). The synthesis of this peptide is thought to be stimulated by TGF-β1, hyperglycemia or cyclic mechanical stretch (Riser et al., 2000a), these stimuli all being relevant to the pathogenesis of DN. Furthermore, CTGF could induce its own expression providing a potential mechanism for inducing a perpetual cycle of progressive renal injury. Recently, it has also been reported that CTGF may potentiate some of the actions of TGF-β, by facilitating the binding of TGF-β to its type II receptor (Abreu et al., 2002). CTGF has been reported to be elevated in two different rodent models of DN (Riser et al., 2000b, Twigg et al., 2002). In addition, CTGF is also increased in both early and late diabetic nephropathy in humans (Ito et al., 1998). Interestingly, AGEs have been reported to increase CTGF expression, initially in

fibroblasts (Twigg et al., 2001a) but subsequently in mesangial cells (Twigg et al., 2001b). Indeed within our own study in STZ induced DN, the AGE inhibitor aminoguanidine ameliorated increases in CTGF (Twigg et al., 2002).

Currently FibroGen Inc. intends to initiate a Phase II study of FG-3019, an anti-CTGF antibody, in patients with idiopathic pulmonary fibrosis in 2005 (http://www.fibrogen.com/trials), but plans are also in place to consider an approach for diabetic nephropathy. Recently, Yokoi et al. demonstrated that the treatment with CTGF antisense oligonucleotide reduced ECM production in experimental obstructive nephropathy, ameliorating the development of interstitial fibrosis (Yokoi et al., 2004).

Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a cytokine whose major role in diabetes has been considered to be in the pathogenesis of diabetic retinopathy and in particular retinal neovascularisation (Aiello et al., 1994). However, it should be appreciated that vascular endothelial growth factor is highly expressed in the kidney, primarily in the glomerular podocytes but also in distal tubules and collecting ducts (Cooper et al., 1999). Its major receptor, VEGF-R2 is also expressed in the kidney, primarily on endothelial cells but also on cortical interstitial fibroblasts. A range of stimuli for VEGF expression and/or signaling, have been identified which may be relevant to DN. These include hyperglycemia, AGEs, PKC, mechanical stretch and AII (Gruden et al., 1997, Kim et al., 2000a, Lu et al., 1998, Williams et al., 1995).

Administration of neutralizing antibody to VEGF for 6 weeks has shown resolution of hyperfiltration and attenuation of albuminuria in experimental diabetes (De Vriese et al., 2001). Our own studies in the retina have suggested that ACE inhibitors reduce retinal VEGF expression in diabetic and non-diabetic models (Gilbert et al., 2000, Moravski et al., 2000). Indeed, it is possible that some of the renoprotective effects of ACE inhibitors are via effects on VEGF related pathways. Vascular endothelial growth factor receptor tyrosine kinase inhibitors (VEGFR-TKi) are newly developed compounds designed primarily to block the angiogenic effect of VEGF in oncology. As yet, no preclinical or clinical studies in diabetes have been published. Studies on the renal effects of VEGFR-TKi are currently being performed in animal models of type 1 and type 2 diabetes. Indeed, a recent preliminary report has shown that SU5416, a VEGFR-Tki, reduces albuminuria in *db/db* mice (Sung et al., J Am Soc Nephrol, 2004 15:720A), consistent with findings observed using neutralizing anti-VEGF antibodies (Flyvbjerg et al., 2002). In experimental DN, VEGF expression is also decreased by an inhibitor of AGE formation (Tsuchida et al., 1999) and with the AGE cross-link breaker, ALT-711 (Thallas-Bonke et al., 2004) further confirming the link between AGEs and VEGF expression.

A range of other cytokines have also been implicated in DN. These include the IGFs and EGF, their role having been reviewed in detail previously (Flyvbjerg, 2000).

Conclusion

Many of experimental studies described above have given us novel insights into the mechanisms responsible for diabetic nephropathy. Indeed, many of the agents utilised in these studies are currently being developed in various clinical trial programs. The knowledge we have today on the pathogenesis of diabetic nephropathy indicates that diabetes associated initiation and perturbation of various metabolic and hemodynamic pathways activate growth factors, cytokines and intracellular second messengers. Blockade of the RAS has been investigated extensively and is now considered first line treatment for diabetic nephropathy by many international organizations (Babaei-Jadidi et al.).

Newer treatments such as inhibition of the AGE pathway at the level of formation, degradation or receptor binding may be also be particularly useful in both the early and late stages of diabetic nephropathy. It is predicted that combination therapy with inhibitors of the RAS and the advanced glycation pathway may be superior to either therapy alone (Davis et al., 2003a). Finally, it is likely that recent insights into the pathogenesis of diabetic nephropathy will lead to a variety of new treatments with additional benefits not only in slowing but also possibly reversing the progression of renal dysfunction that occurs in this disorder.

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