Reviews

Interaction of metabolic and haemodynamic factors in mediating experimental diabetic nephropathy

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Abstract

Diabetic nephropathy seems to occur as a result of an interaction of metabolic and haemodynamic factors. Glucose dependent pathways are activated within the diabetic kidney. These include increased oxidative stress, renal polyol formation and accumulation of advanced glycated end-products. Haemodynamic factors are also implicated in the pathogenesis of diabetic nephropathy and include increased systemic and intraglomerular pressure and activation of various vasoactive hormone pathways including the renin-angiotensin system and endothelin. These haemodynamic pathways, independently and with metabolic pathways, activate intracellular second messengers such as protein kinase C and MAP kinase, nuclear transcription factors such as NF-κB and various growth factors such as the prosclerotic cytokine, TGF-β and the angiogenic, permeability enhancing growth factor, VEGF. These pathways ultimately lead to increased renal albumin permeability and extracellular matrix accumulation which results in increasing proteinuria, glomerulosclerosis and tubulointerstitial fibrosis. Therapeutic strategies involved in the management and prevention of diabetic nephropathy include currently available treatments such as intensified glycaemic control and antihypertensive agents, particularly those which interrupt the renin-angiotensin system. More novel strategies to influence vasoactive hormone action or to inhibit various metabolic pathways such as inhibitors of advanced glycation, specific protein kinase C isoforms and aldose reductase are at present under experimental and clinical investigation. It is predicted that multiple therapies will be required to reduce the progression of diabetic nephropathy. [Diabetologia (2001) 44: 1957–1972]

Keywords Protein kinase C, nephropathy angiotensin II, endothelin, glycation, VEGF, TGFβ, CTGF, hypertension, nephrin.

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As the prevalence of diabetic nephropathy increases it has become increasingly urgent to determine the pathophysiological mechanisms responsible for this disorder. This will allow more rational strategies to be developed to prevent, retard or reverse this condition. The major clinical associations with diabetic nephropathy (DN) are hyperglycaemia and hypertension [1, 2]. Our own group has been exploring the links between metabolic and haemodynamic factors in mediating diabetic renal disease (Fig. 1) [3]. This seems to involve a range of growth factors as reviewed recently [4], intracellular signalling molecules and nuclear transcription factors that lead to the classical functional and structural hallmarks of DN, albuminuria and extracellular matrix accumulation. This
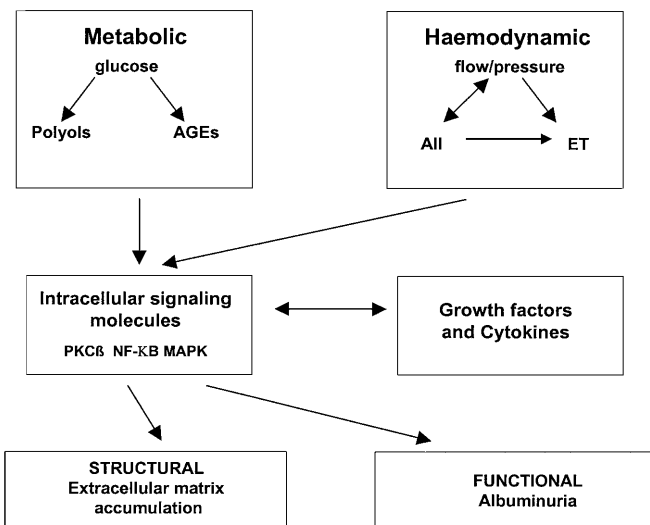
Fig. 1. Schema outlining interactions among haemodynamic factors, metabolic pathways, cytokines and intracellular signalling molecules in mediating diabetic nephropathy. Adapted and revised from [36]

review will summarise important pathways implicated in DN, describe potential sites of interaction among these pathways and explore therapeutic options, both current and pending, which interrupt these pathophysiological processes within the kidney.

Much of the work described below has used experimental models of diabetic nephropathy, particularly in rodents. This includes not only the use of genetic models of diabetes but specifically models which involve the induction of diabetes with pancreatic beta-cell specific toxins such as streptozotocin [5]. These models show most of the functional and structural features of DN including increases in GFR, albuminuria and glomerular ultrastructural injury including glomerular hypertrophy, mesangial expansion and glomerular basement membrane thickening. Initially most studies were carried out in models of Type I (insulin-dependent) diabetes mellitus but increasingly experiments are also being carried out in models of Type II diabetes such as the obese Zucker rat [6] and various genetic models of type II diabetes in the mouse [7]. When compared to human DN these models lack considerable tubulointerstitial injury or renal impairment. Other models however, have been evaluated recently and show some of these additional abnormalities [8, 9].

Haemodynamic factors

A range of studies by our group showed the importance of systemic hypertension in accelerating renal injury in diabetes [10]. In a model combining genetic hypertension with streptozotocin diabetes, the diabetic spontaneously hypertensive rat (SHR), comparisons on both functional and structural markers of DN were made with normotensive Wistar Kyoto rats with diabetes. Hypertension was associated with increased albuminuria and a further increase in glomerular basement membrane thickening. Furthermore, a range of studies using different antihypertensive agents in diabetic SHR were associated with attenuation of the rise in albuminuria and improvement in various glomerular morphological parameters [11].

Renin-angiotensin system (RAS). The landmark studies by Brenner's group emphasised the role of haemodynamic factors in DN, particularly the potential of inhibitors of the RAS as renoprotective agents in experimental diabetes [12]. Micropuncture studies in diabetic rats showed a range of intrarenal haemodynamic abnormalities including increased intraglomerular pressure, increased single nephron GFR and preferential afferent compared with efferent arteriolar vasodilatation [12]. This increase in intraglomerular pressure was considered to play a pivotal role in mediating progressive glomerular injury in a range of renal diseases including diabetes [13]. This hypothesis was partly based on findings whereby intraglomerular pressure was reduced by a range of treatments including ACE inhibitors [12] and low protein diets [14] associated 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 intrarenal haemodynamic changes which could occur even in the setting of a normal blood pressure [12].

To further explore the role of the RAS in mediating progressive renal injury, a number of studies were done on the Ren 2 rat, a model which introduces the murine renin gene into the rat genome [15]. These animals show increased expression of various components of the RAS particularly outside the kidney and develop systemic hypertension. Diabetes was induced in heterozygous Ren 2 rats and this was associated with rapid development of glomerular and tubulointerstitial changes and a decline in renal function within 12 weeks [8]. These functional and structural changes could be attenuated by agents which block the RAS including ACE inhibitors and angiotensin II (AII) receptor antagonists [8, 16]. Despite a similar reduction in systemic blood pressure, endothelin receptor blockade, did not confer a similar degree of renal protection to agents which interrupt the RAS suggesting that much of the damage in this model is AII dependent [16].

The status of the RAS in diabetes remains controversial. In general, plasma measurements of various components of the RAS are low or normal in diabe-

tes [17]. However, it is well appreciated that the RAS also acts locally within the kidney with all components including enzymes and receptors present within the kidney. Measurements of the various components of the RAS within the kidney by a range of techniques have been contradictory with reports of reduced, normal or decreased expression of renin and AII in the diabetic kidney [18–20]. In vitro studies in proximal tubular cells have described increased angiotensinogen expression in response to glucose [21]. In mesangial cells, hyperglycaemia has also been reported to stimulate angiotensin II production in association with increased TGF-β production [22].

It is possible that rather than a uniform change in these components within the kidney there is primarily a change in the distribution of these proteins within the various compartments of the kidney. For example, in experimental diabetes a redistribution of ACE to vascular and glomerular sites in diabetes was reported [18]. In the model of subtotal nephrectomy, which has functional and structural similarities to DN, we observed increased proximal tubular renin expression which was attenuated by ACE inhibitor treatment [23]. More recently, we have documented a similar phenomenon in diabetic Ren 2 rats with renin expression particularly in damaged tubules which was reduced at the site by an AII receptor antagonist in the context of an increase in renin expression at the major site of synthesis, the juxtaglomerular apparatus [16]. This increase in proximal tubular renin was associated with increased expression of AII, the effector peptide of the RAS which mediates a range of haemodynamic and non-haemodynamic effects (see below). Further evidence for the local activation of the RAS in the tubular compartment has been suggested in studies measuring tubular renin by RT-PCR. In those studies there was an early increase in proximal tubular renin in experimental diabetes [24]. The authors postulated that this could contribute to a local increase in AII resulting in tubulointerstitial fibrosis in this model. These findings emphasise changes in the distribution of the RAS in the diabetic kidney which could 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 could relate to increased sensitivity of the diabetic kidney to AII [25].

Although AII has haemodynamic effects both systemically and within the kidney, it is becoming increasingly evident that AII has a range of non-haemodynamic effects relevant to progressive renal injury [26]. It has been difficult to separate the haemodynamic from its non-haemodynamic 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 through stimulation of the prosclerotic cytokine, TGFβ [27]. Indeed, a range of studies has shown that inhibition of the RAS in both non-diabetic and diabetic models of renal injury is associated with reduced renal TGFβ expression, particularly in the tubulointerstitium [28, 29]. AII could also influence a range of other cytokines and in particular it could activate a range of intracellular mediators implicated in progressive renal injury such as protein kinase C [30] and the nuclear transcription factor, nuclear factor kappa B (NF-$\varkappa$B) [31].

AII also influences cell growth, proliferation and apoptosis via a range of pathways not yet fully defined [32, 33]. In particular, AII-induced effects on cell cycle regulation could be relevant to various changes in the diabetic kidney including renal hypertrophy [32]. The reduction in glomerular hypertrophy which is often observed with blockade of the RAS [34] might partly relate to effects on cell cycle regulation. For example, treatment of diabetic rats with ACE inhibitors was associated not only with reduced glomerular volume but also abolition of glomerular expression of the cyclin-dependent kinase inhibitors p^{16INK4} and p^{27Kip1} [35]. These actions of AII might be central to how haemodynamic pathways interact with metabolic and 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 [36]. These beneficial effects relate to the capacity of these agents to reduce blood pressure and to block AII formation. Although ACE inhibitors have other effects including inhibition of degradation of kinins, experimental studies using bradykinin and angiotensin II receptor antagonists indicate that the long-term renal protection given by these agents is mostly via the inhibition of AII dependent pathways [34]. The advent of selective AII receptor antagonists has now allowed investigators to explore the specific role of AII in mediating renal injury and provides a new approach for conferring renal protection in diabetic patients.

AII interacts with two specific receptors known as the angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptor subtypes. It is generally viewed that most of the actions of AII are via the AT1 receptor including its haemodynamic and prosclerotic effects. More recent studies of the role of the AT2 receptor have suggested that this receptor could also play a role in the kidney [37] although this issue has not been adequately characterised in the diabetic kidney. Changes in expression of the AII receptors in the diabetic kidney have been reported but the functional significance of these changes is not known [25]. Selective blockers of both receptor subtypes have been developed. However, long-term studies have only reported on blockers of the AT1 receptor subtype indi-

cating that these agents are renoprotective in experimental and human DN [34, 38–40].

Although ACE inhibitors afford a degree of renal protection, DN continues to progress relentlessly albeit at a slower rate [41]. It has been suggested that a more effective blockade of the effects of AII will occur by both blocking its synthesis with an ACE inhibitor and reducing its action via blockade at the receptor with an AII receptor antagonist [42]. In recent experimental studies, we documented that this combination in diabetic SHR and in diabetic Ren2 rats was associated with increased hypotensive efficacy and less albuminuria [43, 44]. Similar effects on blood pressure and renal functional parameters of the combination have been reported in various stages of human DN [42].

Other vasoactive hormones. In addition to the RAS, various other vasoactive hormones have been postulated to mediate progressive renal injury [45]. Intrarenal as well as systemic haemodynamics are influenced by a balance of vasoconstrictors and vasodilators. These substances include hormones which have different effects on afferent and efferent arteriolar tone, thereby having disparate actions on intraglomerular pressure. Important vasoconstrictors to consider in addition to AII within the kidney are the endothelins [46] and vasopressin [47]. In addition, a range of vasodilatory substances including bradykinin, atrial natriuretic factor, certain prostaglandins and nitric oxide modulate glomerular vasomotor tone [48].

The formation and degradation of many of these vasoactive hormones rely on zinc dependent metallopeptidases, the most well known example being ACE. Other enzymes include neutral endopeptidase (NEP) which plays an important role in the degradation of natriuretic peptides as well as bradykinin and endothelin converting enzyme (ECE) which is involved in endothelin formation [48]. Because these zinc dependent metallopeptidases have significant homology, pharmaceutical companies have developed molecules which act to inhibit more than one of these enzymes [49]. A major target has been the inhibition of ACE and NEP leading to reduced degradation of vasodilatory hormones and reduced formation of the vasoconstrictor hormone, AII. This approach would theoretically lead to increased vasodilation resulting in greater antihypertensive efficacy and potentially superior renoprotection than would be obtained with ACE inhibition alone. Such agents have now been explored in preclinical and clinical contexts. In the subtotal nephrectomy model, several groups have reported renoprotective effects of the dual ACE/NEP inhibitor, omapatrilat [50]. In a short-term study in the diabetic SHR, our group has shown with another ACE/NEP inhibitor antihypertensive efficacy in association with beneficial effects on urinary albumin excretion [51].

A number of selective antagonists to receptors of the vasoconstrictor hormones, endothelin and vasopressin have also been developed [46, 47]. The role of these agents as renoprotective therapies in diabetes remains to be clarified. Endothelin-1 expression has been reported to be increased in the diabetic kidney [52]. Various changes in endothelin receptor subtypes have also been reported [53]. The findings are not consistent in terms of renoprotection with endothelin receptor blockade in diabetic and non-diabetic models of renal injury [54, 55]. This could relate to the nature of the renal disease, sodium balance, pharmacological characteristics of the various receptor antagonists and the selectivity of these compounds for the endothelin type A (ET_A) and the endothelin type B (ET_B) receptor subtypes [56].

Nitric oxide. Although exploration of haemodynamic pathways in diabetes has focussed on vasoactive hormones, one cannot exclude a role for nitric oxide, a potent vasodilator which influences glomerular vasomotor tone [57]. The status of the nitric oxide pathway in diabetes seems to be organ and duration specific with evidence of nitric oxide deficiency in the macrovascular tree with increasing duration of diabetes [58]. In contrast, it has been considered that in the diabetic kidney there are increased nitric oxide concentrations and/or action [59, 60].

Initial studies in the kidney suggested increased nitric oxide concentrations in the diabetic kidney, based on the relatively crude marker of urinary nitrate and nitrite production [59]. Further studies on this increase of nitric oxide were based on experiments using nonselective inhibitors of nitric oxide synthase (NOS) which showed reductions in GFR and renal plasma flow in hyperfiltering diabetic rats [68]. Recent studies suggest that a major abnormality in the diabetic kidney is increased expression of endothelial NOS [61, 62] with several groups reporting no change in inducible NOS (iNOS) [61, 63]. This contrasts with initial studies suggesting a possible role for iNOS in diabetic nephropathy and diabetic hyperfiltration in particular. Indeed, in a recent study, administration of a selective iNOS inhibitor, L-imino-ethyl-lysine failed to influence renal function in diabetic rats [61].

The situation could be even more complex in diabetes evident from differing results on the various nitric oxide synthase isoforms from several studies. This could relate to different techniques for assessing the various nitric oxide synthase isoforms. For example, using RT-PCR, no changes in either endothelial or inducible nitric oxide synthase were observed [64]. However, when localisation of endothelial nitric oxide synthase was assessed, specific upregulation was observed in the afferent arteriole which was postulated to be involved in the development of diabetes associated glomerular hyperfiltration [62]. Evaluation of neuronal nitric oxide synthase has suggested

reduced expression of this isoform in the macula densa as assessed histochemically using NADPH diaphorase [65, 66].

It remains to be determined if these effects of nitric oxide on early renal haemodynamic abnormalities are of any long-term relevance, particularly to the development of renal impairment and ultrastructural injury in diabetes. Our studies using the nonspecific inhibitor of nitric oxide synthase, L-NAME, did not clearly show renoprotection [63]. Of interest, there was a tendency for a reduction in renal advanced glycated end products (AGE) formation and this could partly relate to the role of nitric oxide in inducing oxidative stress via production of peroxynitrite [67].

Metabolic pathways

Advanced glycation. AGEs are generated as a result of a series of sequential biochemical reactions, some of which are poorly defined, involving nonenzymatic glycation of protein and lipids [68]. This process also involves oxidation reactions; therefore AGEs can be considered 'glycoxidation' products [69]. These AGEs are increased in diabetes due to chronic hyperglycaemia. Some of these AGEs have been identified chemically and include carboxymethyllysine [70] and pentosidine [71]. In experimental diabetic nephropathy there is renal accumulation of AGEs [72]. These AGEs were evaluated initially, based on their characteristic fluorescence [72]. In studies on diabetic mice, renal AGE accumulation was found which could be attenuated by either pancreatic islet transplantation or treatment with an inhibitor of AGE formation, aminoguanidine [73].

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 [72]. Since advanced glycation end products accumulate over time, we explored the role of early versus delayed introduction of aminoguanidine in influencing renal injury in diabetes [74]. 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 shown that both treatments were associated with 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.

Since aminoguanidine inhibits AGE formation and also has other actions including inhibition of iNOS [75], we have recently explored the role of a chemically related compound, ALT 946, which has less effect on iNOs [76]. Over the 32 week study period, ALT 946 conferred similar if not superior reno-
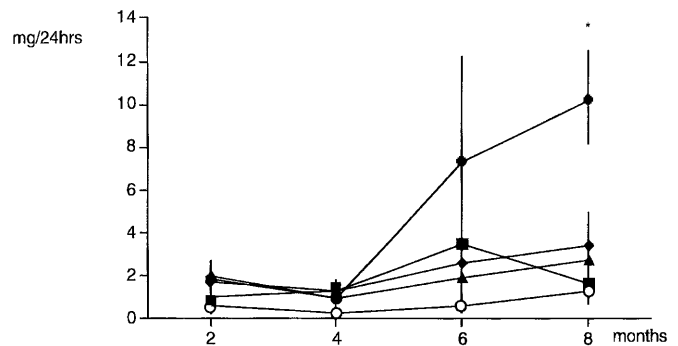

Fig. 2. Serial measurements of albuminuria (y axis) are shown at 8-week intervals for Control (○), Diabetic (●), Diabetic + aminoguanidine (◆), Diabetic + ALT-946 (▲) and Diabetic + ALT-946 received for only the last 16 weeks of the study (■). Results are shown as geometric means and tolerance factors. Adapted from [76]

protection to aminoguanidine consistent with the view that the renoprotective effects of aminoguanidine are due to its action as an inhibitor of AGE formation (Fig. 2). More recently, an alternative approach to prevent renal AGE accumulation has been developed which involves the use of cross-link breakers which cleave pre-formed AGEs [77]. Although these agents have been shown to have effects on the vasculature in diabetes [78], their specific role in diabetic nephropathy is not well defined.

The mechanisms of action whereby AGEs induce their effects have not been fully elucidated. Firstly, AGEs induce cross-link formation, thereby directly affecting the function of various proteins including the collagens. 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 receptor for advanced glycation end-products (RAGE), AGE-R1, AGE-R2, AGE-R3, lactoferrin, lysozyme and the macrophage scavenger receptor [79–82]. Some of these proteins are considered to act as receptors and are involved in the transduction of various effects of AGEs. For example, AGEs interact with the receptor RAGE to stimulate cytokines and to activate NF-$\varkappa$B [83–84]. Other binding proteins such as AGE-R1 could be more important in the clearance of AGEs [85].

The specific role of these receptors in renal injury in diabetes is not yet known. In other diabetic complications such as atherosclerosis it has been postulated that the receptor RAGE could be particularly important with evidence that recombinant soluble RAGE reduces plaque formation in a model of diabetes associated atherosclerosis [86]. Several groups have characterised the distribution of these receptors in the diabetic kidney [87, 88]. RAGE has been shown to be expressed in various renal cell types including the podocyte and tubular epithelial cells [87, 88]. Recently it has been suggested that in the kidney

RAGE mRNA is expressed in the glomerulus [87] and it is possible that tubular RAGE represents filtered receptor.

In diabetic NOD mice, the expression of the AGE receptors, R1, R2 and R3 has been characterised [85]. There was a sixfold increase in AGE-R3 with the development of diabetes and a reduced AGE-R1 expression in the prediabetic NOD mice. It is not clear if the abnormality in this receptor, which is implicated in AGE clearance, contributes to delayed AGE removal thereby leading to early renal AGE accumulation and providing an additional mechanism for development of diabetic nephropathy in this model. We investigated the regulation of AGE binding proteins in the diabetic kidney using autoradiographic techniques incubating kidney sections with radiolabelled exogenous AGEs [89]. These experiments confirmed upregulation of AGE binding sites in the diabetic kidney particularly in proximal tubules which was attenuated by aminoguanidine treatment. This would suggest that AGEs could modulate certain AGE binding proteins. The chemical identity of these AGE binding proteins has not been determined but does not seem to be RAGE or any of the other AGE receptors cloned previously.

A major effect of AGEs appears to be the stimulation of various cytokines, including TGFβ, presumably via the receptor dependent pathways which include RAGE. In a recent study, the increased expression of renal TGFβ1 as well as platelet derived growth factor (PDGF) and the extracellular matrix protein, type IV collagen in the diabetic kidney was attenuated by aminoguanidine in the tubulointersitium [90]. A similar effect has been reported in a model of Type II diabetes, the OLETF rat, by a Japanese group which described reduced TGFβ1 and vascular endothelial growth factor (VEGF) expression after 68 weeks of treatment with a novel AGE inhibitor, OPB-9195 [91].

Polyols. The accumulation of polyols in the kidney has been suggested to be involved in the development of diabetic nephropathy [92]. Although this pathway has been investigated extensively in terms of neuropathy, more limited exploration of this pathway has been done in renal disease [93]. Renal polyol accumulation has been reported in diabetic nephropathy and inhibition of this phenomenon has been observed with various aldose reductase inhibitors. In the polyol pathway, glucose is reduced to sorbitol by the enzyme aldose reductase. Sorbitol accumulation is associated with depletion of myoinositol and changes in the cellular redox potential [94]. The importance of polyols in activating pathways relevant to diabetic nephropathy has been emphasised with the demonstration that aldose reductase inhibition is associated with reduced protein kinase C (PKC) activation and TGFβ1 production in human mesangial cells in response to glucose [95].

In experimental diabetes, several studies have examined the effects of aldose reductase inhibition on various functional and structural markers of diabetic nephropathy [92, 96–98]. The results of various studies are not consistent and could reflect the different animal models, the measurements of renal injury and the aldose reductase inhibitors used. In human diabetic nephropathy, the effects of aldose reductase inhibition have also been conflicting and overall rather disappointing [99]. The major effect appears to be a reduction in hyperfiltration [100] as was observed in some of the experimental studies [100].

Hexosamine pathway. It has been hypothesised that the hexosamine pathway is involved in the development of diabetic complications [101, 102]. In this pathway, glucose is converted to glucose-6-phosphate via hexokinase and subsequently to fructose-6-phosphate as part of the glycolytic pathway. Under control of the rate limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT), glucosamine-6-phosphate is formed 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 to amino sugars used for the synthesis of proteoglycans, glycolipids and glycoproteins. To explore this pathway investigators have overexpressed the enzyme GFAT [102] or directly inhibited this enzyme. It seems that increased flux through the hexosamine pathway is associated with PKC activation and increased TGFβ expression [103]. Indeed, glucose induced TGFβ expression and subsequent matrix production can be inhibited by GFAT blockade using either azaserine or an antisense oligonucleotide approach [101].

Glucose transporters. It has been suggested that glucose transporters, in particular GLUT-1, could play a part in inducing extracellular matrix production in response to hyperglycaemia [104]. The 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 concentrations [104]. Subsequently it was shown that glucose induces GLUT-1 expression in mesangial cells [105]. Furthermore, this increase in GLUT-1 expression leads to increased aldose reductase expression as well as the PKC alpha isoform leading to increased matrix production [106]. To further explore the role of GLUT-1 in mediating expression of matrix proteins such as fibronectin, cells which expressed antisense GLUT-1 were studied [107]. These experiments revealed that glucose did not induce fibronectin expression in the setting of reduced GLUT-1 providing further evidence for this glucose transporter playing an important part in promoting extracellular matrix production. In streptozotocin diabetes various changes in GLUT-1 and other glucose transporters

have been described in the kidney [108]. The in vivo relevance of this pathway in particular, if GLUT-1 represents another target for the treatment of diabetic complications, has yet to be determined.

Oxidative stress and reactive oxygen species. Oxidative stress is widely recognised by some investigators as a key component in the development of diabetic complications [69]. It is not known, however, if it is an important early link between hyperglycaemia and complications or if it is a consequence of the primary pathogenic mechanisms. A recent study showed that inhibition of reactive oxygen species (ROS) in cultured bovine endothelial cells interferes with multiple independent pathways of hyperglycaemic damage, namely PKC activation, NF-κB activation and the formation of advanced glycation end products [109]. Other studies have shown that hyperglycaemia itself could contribute to oxidative events by forming glycation products that can propagate free radicals [69]. The lipid soluble classical anti-oxidant butylated hydroxytoluene (BHT) has been used [110]. These studies showed reductions in renal AGE accumulation and decreases in oxidative stress as measured by TBARS, a relatively crude marker of oxidative stress.

The status of oxidative stress in diabetic nephropathy and other complications is still controversial [111]. Much of the work is in vitro and appears contradictory. In a recent study [109], ROS generation activated AGE dependent pathways in cultured bovine endothelial cells. In contrast, others reported that an AGE/RAGE interaction led to the generation of ROS [83]. Another study also showed that AGEs can induce oxidative stress and activate PKC in neonatal mesangial cells [112]. The link between ROS and PKC also seems to be bi-directional with evidence that ROS generation in certain contexts is PKC dependent [113]. For example, in a study on proximal tubular cells, glucose induced generation of lipid peroxides was attenuated by PKC inhibition [114]. In contrast, ROS have been shown to mediate glucose induced activation of PKC [109] and to generate vasoactive hormones such as endothelin [115] providing another potential site of interaction between haemodynamic and metabolic pathways in diabetic nephropathy. It remains to be determined if oxidative stress is solely 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 renal damage. To explore this issue further 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 over the last decade has implicated PKC as an important mediator of diabetes-induced vascular dysfunction [116]. Initial studies using various cell types, including mesangial cells, indicated that effects of hyperglycaemia on mesangial dysfunction could be mimicked by phorbol esters, direct activators of PKC, and could be attenuated by non-specific PKC inhibitors [117]. Further studies identified that certain isoforms of this intracellular second messenger were preferentially activated in the diabetic kidney [118] and therefore these isoforms were considered as targets for development of inhibitors. A specific PKCβ inhibitor, LY333531, was developed and in streptozotocin diabetic rats treated with this agent for over 12 weeks was shown to attenuate glomerular hyperfiltration, reduce albuminuria and decrease expression of TGFβ and various extracellular matrix proteins [118, 119]. More recently, this PKC inhibitor was administered to *db/db* mice, a model of Type II diabetes, for 16 weeks [120]. In addition to normalising glomerular PKC activity, urinary albumin excretion was reduced as was TGFβ1, fibronectin and type IV collagen expression. These recent findings confirm the potential utility of this class of agents in diabetic nephropathy.

Investigators have explored the effects of other treatments on renal PKC activation [121]. Of note, 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 [122]. These findings suggest that AGEs and AII could activate PKC in vivo. Furthermore, it is possible that PKC represents a critical downstream event in the pathogenesis of diabetic nephropathy. Indeed, various actions of cytokines implicated in diabetic complications such as VEGF are PKC dependent [123]. In addition, PKC modulates the effects of glucose including VEGF and TGFβ expression [124, 125].

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 [126]. 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 thus has the ability to activate numerous genes including cytokines, adhesion molecules, nitric oxide synthase, angiotensinogen and many other inflammatory and proliferative proteins implicated in the process of diabetic nephropathy [126]. NF-κB is activated by a range of stimuli including glucose [127]. This

Table 1. Stimuli and inhibitors of TGFβ expression

Stimuli	Inhibitors	Reference
Glucose	Insulin	[142]
Stretch	?Antihypertensive agents	[144]
AII	ACE inhibition/AT1 receptor blockade	[29, 158]
AGEs	Aminoguanidine	[90, 143]
PKC	PKCβ_2	[118, 120]
Isoprostanes	?Anti-oxidants	[148]
?Lipids	HMG CoA reductase inhibitors	[147]
Glycosaminoglycans	Heparin	[155]
Hexosamines (e. g. glucosamine-6-phosphate)	?GFAT inhibitors	[101]
TGFβ (autocrine)	TGFβ neutralising antibodies/Decorin	[141]
Endothelin	?Endothelin antagonists	[146]
Thrombospondin-1	?	[150]

GFAT, glutamine: fructose-6-phosphate amidotransferase; HMG CoA reductase, Hydroxy methyl glutaryl CoA reductase

phenomenon seems to be PKC dependent [127] and can be prevented by antioxidants [123]. AGEs are also involved in activation of NF-$\varkappa$B via a RAGE-dependent pathway leading to its translocation to the nucleus where it induces transcription [83]. The promoter region of the RAGE gene also contains NF-$\varkappa$B binding sites [128]. This provides a pathway for perpetuation of injury not only because NF-$\varkappa$B is activated in response to RAGE, but RAGE is also activated by NF-$\varkappa$B. This is supported by studies in which mutations of both NF-$\varkappa$B like binding sites in the promoter region of RAGE led to stimulated promoter expression [128].

The NF-$\varkappa$B inhibitor, pyrollidine dithiocarbamate (PDTC) has been studied in non-diabetic models of fibrosis and shown to confer renoprotection [124]. However, approaches to inhibit NF-$\varkappa$B have not been explored in diabetic nephropathy. The diverse actions of NF-$\varkappa$B and the capacity of various factors such as AII and AGEs to activate this transcription factor [31, 83] are consistent with NF-$\varkappa$B playing a pivotal role in modulating diabetic complications. It is possible that NF-$\varkappa$B like PKC represents a major site of interaction between haemodynamic and glucose dependent pathways in the development of diabetic vascular complications.

MAP kinase. Mitogen-activated protein kinase (MAPK) is a major signalling system which transduces extracellular signals to intracellular responses [130]. Three major subgroups have been identified, the extracellular signal regulated kinase (ERK) family, C-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and p38 MAPK. The MAPK cascades play a central part in a range of biological processes relevant to diabetic nephropathy including cell growth, differentiation and apoptosis [131]. Glucose has been reported to activate MAPKs including p38 MAPK [132]. This increase in MAPK has also been noted in glomeruli from diabetic rats [133, 134], and the increase in MAPK in response to hyperglycaemia has been shown to be PKC dependent [134]. Recently it has been hypothesised that MAPK could play a central role in the pathogenesis of diabetic complications [130]. Various stimuli in addition to glucose activate these enzymes including AGEs [135], polyols [136] and PKC [134]. The activation of the angiotensinogen gene by glucose also appears to be p38 dependent [137]. The exact role of this family of kinases is not clear but, of relevance to diabetic nephropathy, MAPK was found to play an important role in the TGFβ signalling pathway [131] and in mediating the prosclerotic effect of this cytokine [138].

Cytokines

Transforming growth factor β (TGFβ). Transforming growth factor betas are part of a superfamily with three mammalian isoforms. The major isoform, TGFβ1 is synthesised as an inactive or latent form which is subject to proteolytic cleavage leading to the generation of the active form. TGFβ binds to the type II receptor and subsequently binds to the type I receptor which is then phosphorylated to induce intracellular signalling involving the Smad proteins [139]. These actions of TGFβ via the Smad pathway are modulated by a range of signalling pathways including NF-$\varkappa$B and MAPK [140].

TGFβ1 is considered the pivotal cytokine in mediating collagen formation in the kidney [141]. 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) [141]. In vitro studies have shown that a range of stimuli increase TGFβ 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 diabetic nephropathy [142–148]. Induction of TGFβ1 by glucose and glycated proteins seems to be a PKC-dependent phenomenon [149]. A recent study has suggested that glucose-induced effects in increasing conversion of latent TGFβ to the active form could involve thrombospondin-1 in mesangial cellls [150]. A range of novel proteins has been identified which is stimulated by TGFβ1 and increased in the diabetic kidney [151, 152]. These include gremlin, an antagonist of bone morphogenic proteins [151] and LSGK, a serine and threonine kinase, implicated in renal tu-

bular sodium transport [152]. Indeed, TGFβ seems to be an important site of interaction between haemodynamic and metabolic pathways, playing a pivotal role in the synergy between hypertension and hyperglycaemia in mediating diabetic nephropathy. It is likely that this multi-functional cytokine has a range of direct as well as indirect effects which mediate many of the functional and structural features of diabetic nephropathy. Therefore it is a prime candidate for developing appropriate antagonists in the prevention and treatment of diabetic nephropathy.

In experimental and human diabetic nephropathy, TGFβ1 expression is increased [153]. Because TGFβ1 is synthesised as a latent peptide, it cannot be assumed that gene or protein expression is a marker of increased TGFβ1 activity. Our group evaluated the expression of a matrix protein which is selectively induced by TGFβ, a β-inducible gene H3 [154]. This protein is expressed in the kidney and there is evidence of upregulation in the diabetic kidney consistent with increased TGFβ bioactivity in this context [154]. Further evidence of a functional role for TGFβ in mediating various structural hallmarks of diabetic nephropathy such as glomerular hypertrophy and glomerulosclerosis has been obtained from studies using neutralising antibodies in diabetic mice [9]. Administration of TGFβ antibodies to these mice led to improvement in renal function and reduced extracellular matrix accumulation. Proteinuria was not affected suggesting that this functional marker of diabetic nephropathy might not be TGFβ-dependent and that the pathogenesis of albuminuria and glomerular structural injury in diabetic nephropathy is not necessarily identical.

Various therapeutic approaches which confer renoprotection as outlined previously such as agents which interrupt the renin-angiotensin system, inhibitors of advanced glycation, hydroxymethylglutaryl (HMG)CoA reductase inhibitors, heparin and inhibitors of specific PKC isoforms are associated with reduced renal TGFβ1 expression in the context of less glomerular and tubulointerstitial injury [29, 90, 120, 147, 155]. Although most studies have focussed on TGFβ1, the status of the other TGFβ isoforms as well as the TGFβ receptors in experimental diabetes has also been evaluated [156, 157]. There is upregulation of TGFβ2, TGFβ3 and the TGFβ RII receptors in the diabetic kidney. Each protein appears to have its own spatial and possibly temporal distribution within the diabetic kidney with changes observed in the glomerulus and also in the tubulointerstitium. Furthermore, the various TGFβ receptors seem to be reduced by renoprotective treatments such as ACE inhibitors [158]. This effect on TGFβ receptors could represent a major mechanism of action of agents which interrupt the RAS.

Although the TGFβ family and specifically TGFβ1 is considered the major prosclerotic cytokine implicated in the progression of renal disease; other cytokines, some of which are generally considered to be primarily proliferative such as PDGF [159] could also act in a prosclerotic manner. Increases in PDGF gene expression have been reported in the diabetic kidney [90, 160]. Investigation of the role of PDGF per se in progressive renal injury has been assisted by the development of PDGF receptor antagonists [161]. Such a compound, STI-571, a tyrosine kinase inhibitor has been shown to confer a degree of protection in a model of renal injury which is considered to be partly PDGF-dependent, the anti Thy1 model of glomerulonephritis [162]. Whether this class of compound also confers renal protection in diabetes has yet to be determined.

Another prosclerotic cytokine, connective tissue growth factor (CTGF) has been shown to have increased renal and glomerular expression in diabetes [163]. The synthesis of this peptide is stimulated by TGFβ, hyperglycaemia or cyclic mechanical stretch [163], with all these stimuli relevant to the pathogenesis of diabetic nephropathy. Furthermore, CTGF could induce its own expression providing a potential mechanism for inducing a perpetual cycle of progressive renal injury. The induction of CTGF by hyperglycaemia seems to be partly TGFβ-dependent. The importance of this growth factor in diabetic nephropathy and its relation to TGFβ expression has not been fully characterised. Of interest, AGEs have recently been reported to increase CTGF expression, albeit not in a renal context [164].

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 [165]. VEGF is highly expressed in the kidney, primarily in the glomerular podocytes but also in distal tubules and collecting ducts [166]. Its major receptor, VEGF-R2 is also expressed in the kidney, primarily on endothelial cells but also on cortical interstitial fibroblasts. Our group has explored gene and protein expression of VEGF and its receptor VEGF-R2, in the diabetic kidney [166]. To further address the status of VEGF receptors in the diabetic kidney, we have utilised in vitro and in vivo autoradiographic techniques using radio-labelled VEGF. VEGF expression is increased in the diabetic kidney. Renal VEGF receptor-2 expression seems to be increased early in experimental diabetes but this increase is not sustained after 32 weeks of diabetes. A similar pattern was observed when assessing VEGF binding sites in the kidney using autoradiographic techniques (Fig. 3). The functional significance of these changes in VEGF and its receptor in the diabetic kidney is not known. It is not certain if the VEGF pathway is implicated in the development of albuminuria in diabetes or if renal angiogenesis is an important phenomenon in the diabetic kidney. Of
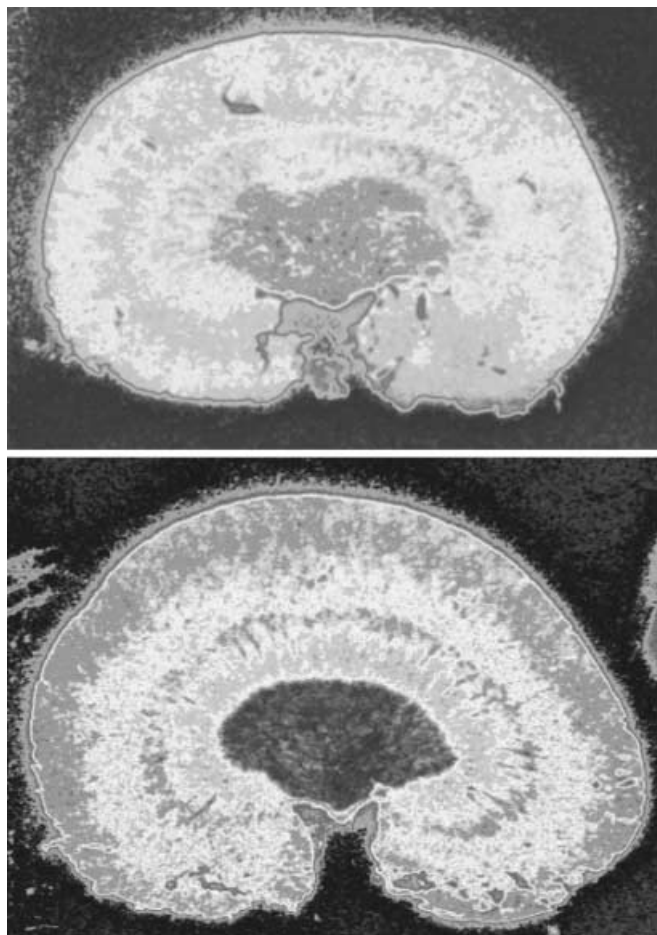
Fig. 3. ^{125}I-VEGF binding in a control kidney (upper panel) and in a kidney from a rat with 3 weeks of streptozotocin diabetes (lower panel) rats is indicated semiquantitatively in pseudocolorized computer images (blue-nil, green-low, yellow-moderate, red-high). Adapted from [166]

interest, administration of neutralising antibody to VEGF for 6 weeks was associated with resolution of hyperfiltration and attenuation of albuminuria [167].

A range of stimuli for VEGF expression and/or signalling have been identified which could be relevant to diabetic nephropathy. These include hyperglycaemia, AGEs, PKC, mechanical stretch and AII [168–171]. Our studies in the retina suggest that ACE inhibitors reduce retinal VEGF expression in diabetic and non-diabetic models of retinal disease in association with a decrease in retinal albumin permeability and neovascularisation [172, 173]. In experimental diabetic nephropathy, VEGF expression is decreased by an inhibitor of AGE formation [91].

A range of other cytokines have also been implicated in diabetic nephropathy. Epidermal growth factor (EGF) has been reported to be increased in the diabetic kidney [174]. However, the pathophysiological relevance of this finding needs to be ascertained. A large body of evidence now exists for various changes in the Growth Hormone-IGF pathway in the dia-

betic kidney [175–177] as well as data indicating that inhibition of this pathway by various approaches influences renal ultrastructural injury as reviewed recently [4].

Nephrin

A major determinant of proteinuria seems to be the glomerular slit diaphragm. Recent advances in the nature and molecular properties of the slit diaphragm have included the identification of the protein, nephrin which is a transmembrane protein of the Ig superfamily that acts as an adhesion receptor and signalling protein [178, 179]. Our group recently observed a deficiency in glomerular nephrin in SHR after 32 weeks of diabetes [180] (Fig. 4). A similar deficiency in nephrin has been reported in other models of renal disease including Passive Heymann nephritis [181] as well as in acquired human nephrotic syndrome [182]. In our studies, administration of the AII antagonist, irbesartan, to diabetic SHR restored the reduced nephrin content in the diabetic kidney [180]. These changes occurred in association with attenuation of albuminuria. Similar preservation of glomerular nephrin content has been noted by investigators in another model of renal disease using both ACE inhibitors and AII antagonists in association with the prevention of proteinuria and renal injury [181]. These investigators suggested that the renoprotection afforded by agents which block the RAS could involve effects on nephrin assembly. However, it is not certain if the changes in nephrin are causative or secondary to renal disease. Over the last few years other slit diaphragm proteins have been isolated and it is anticipated that this aspect of renal research will be a major area of ongoing investigation [183].

Outlook

The experimental studies described above have provided insights into the mechanisms responsible for diabetic nephropathy and led to the development of appropriate clinical trials. Blockade of the RAS has been investigated extensively and is now considered a first line treatment for diabetic nephropathy by many international organizations [184]. The Action 1 trial evaluating aminoguanidine in diabetic nephropathy was instituted, based on preclinical findings of this agent. Preliminary findings from that study suggest beneficial effects on proteinuria and a trend towards preservation of renal function, although this did not reach statistical significance (www.Alteon Pharma/pimag1.htm.com). It is anticipated that over the next few years more novel agents such as PKC inhibitors and vasopeptidase inhibitors will be investigated in clinical trials. Nevertheless, despite a range
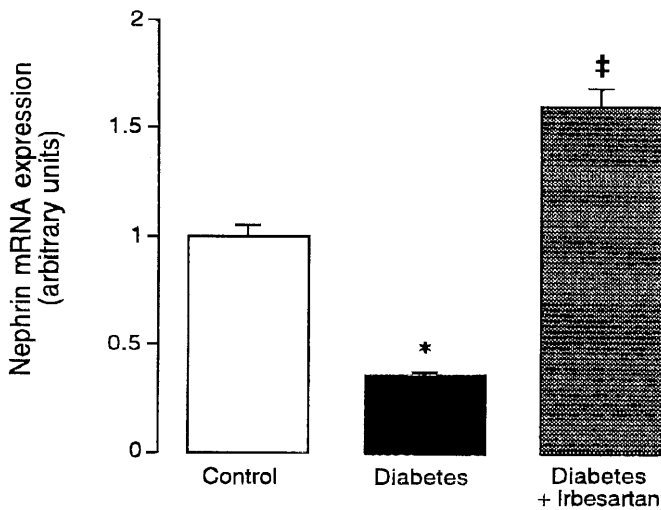
Fig. 4. Measurement of renal nephrin gene expression by RT-PCR in control SHR, diabetic SHR and diabetic SHR treated for 32 weeks with the AII antagonist, irbesartan. Adapted from [180]

of new treatments for diabetic nephropathy, the mainstay of therapy involving optimisation of glycaemic control and aggressive antihypertensive therapy remains the best approach to retard the progression of diabetic nephropathy [36].

As further studies identify important sites of interaction between these metabolic and haemodynamic pathways and pivotal downstream events, it will be possible to develop a more targeted approach for the prevention and treatment of diabetic nephropathy. This will be greatly facilitated by the development of improved models of diabetic nephropathy and newer approaches such as microarray technology [185] and proteomics which will assist in identifying novel targets for ongoing research and development, as part of a rational strategy for the management of diabetic nephropathy.

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