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NF-kappaB signalling in chronic kidney disease

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1. ABSTRACT

The mammalian NF-kappaB signalling pathway is an important intracellular transcription factor system that is induced in response to diverse extracellular stimuli. The hallmark of NF-kappaB activation is the nuclear translocation of dimeric Rel protein transcription factors, which regulate hundreds of kappaB-dependent genes that are involved in inflammation, immunity, apoptosis, cell proliferation and differentiation. In addition, cell-surface receptors (TNFR, Toll-like and angiotensin II, type 1 receptors), inhibitory kappaB kinases (IKK proteins), I kappaB proteins and factors regulating the post-translational modification of the Rel proteins (acetylation, phosphorylation), are other intracellular components that regulate NF-kappaB activation. Over the last decade, *in vitro* studies, animal models and human studies have provided evidence that upregulation of the canonical (RelA/p50) NF- kappaB isoform (in tubular epithelial cells, podocytes, mesangial cells, macrophages) has a pathogenic role in mediating chronic inflammation in chronic kidney disease (CKD). This review will examine current evidence regarding NF- kappaB isoforms and their potential role in the treatment of kidney failure due to CKD.

2. INTRODUCTION TO THE NF-KAPPAB SYSTEM

Since the initial discovery in 1986 by Sen and Baltimore, the inappropriate activation of the nuclear factor (NF)-kappaB transcription factor family has been linked to almost all human diseases, including cancer, atherosclerosis, arthritis, diabetes and stroke (1). Over the last decade, NF-kappaB has also been closely examined as a potential drug-target and biomarker, in chronic kidney disease (CKD). Like other areas of biomedical research, the interest in NF-kappaB in Nephrology has arisen because it is ubiquitous in almost all cell types including that of the kidney; it is rapidly induced in response to diverse extracellular stimuli without the need for *de novo* protein or mRNA synthesis; and it is a transcription factor that regulates an entire network of pro-inflammatory and apoptosis-regulating genes (2, 3). In this regard, modulation of NF-kappaB was viewed as a therapeutic opportunity to overcome the theoretical problems of cytokine and chemokine redundancy and indeed, several studies have shown that components of the NF-kappaB system are aberrantly expressed in humans with CKD (4, 5). Furthermore, experiments using animal models (Figure 1 and Table 1) supported the hypothesis that NF-kappaB has a pathogenic role in the cellular events that underlie the progression of humans with CKD (6-9, 2, 3).

The purpose of this review is to provide an update on the potential therapeutic role of NF-kappaB in CKD. We will begin with an overview of NF-kappaB biology, and then examine evidence regarding NF-kappaB in CKDs intertwined with potential approaches to fill the missing gaps in our current knowledge about the kidney. The reader is also referred to several excellent papers about NF-kappaB and the kidney, as well as two recent journal issues celebrating the 20th anniversary of NF-kappaB (10-14, 3, 15). Finally, recent legal battles regarding the patenting of NF-kappaB modulation might also be of interest to readers (16).

2.1. Components of the NF-kappaB system

2.1.1. NF-kappaB transcription factor family (complexes of Rel protein dimers)

2.1.1.1. Overview

All together there are over 85 components that make up the NF-kappaB signalling pathway (17). From a therapeutic targeting viewpoint, one might conceptualise the NF-kappaB signalling pathway in the shape of an hourglass (Figure 2), with upper chamber comprising the many cell-surface activators along with their adaptor/intermediary proteins responding to diverse extracellular stimuli; the middle component representing the potential therapeutic bottlenecks, comprising the IκB kinases (IKK), Ikappa B proteins and the NF-kappaB complexes (Rel/NF-kappaB protein dimers); and the lower chamber representing the multitude of genes transcribed.

The NF-kappaB complex is the centrepiece and the key component of the NF-kappaB signalling pathway. Although NF-kappaB is often referred as a singular term, it more correctly denotes a heterogenous group of transcription factors which, by definition, induce the transcription of multiple genes that specifically contain a 10-base pair κB-responsive sequence in their promoter region (5'-GGGRNYYYCC-3'; R=purine, Y=pyrimidine). To date, at least, two hundred and fifty genes have been catalogued to fulfil this criteria (see extensive database maintained by Dr. T. Gilmore at www.nf-kb.org), and the majority of the inflammatory gene repertoire is NF-κB-dependent (17-21). In quiescent mammalian cells, NF-kappaB exists in the cytosol in a dormant state either as hetero- or homodimers of Rel/NF-kappaB proteins bound to IkappaB proteins (cytoplasmic inhibitory proteins, that prevent nuclear translocation and DNA-binding (see Section 2.1.2.) (2, 3). The degradation of IkappaB proteins instigated by IkappaB kinases in response to the extracellular stimuli, leads to NF-κB nuclear translocation and DNA-binding.

2.1.1.2. Structure and function of Rel/NF-kappaB proteins

Five mammalian proteins of the Rel/NF-kappaB family have been cloned and characterized. These include, p50 (also called NF-kappaB1), p52 (also called NF-kappaB2), RelA (also called p65), RelB and c-Rel which are shown schematically in Figure 3A. Single gene knockout mice (RelA$^{-/-}$, p50$^{-/-}$, p52$^{-/-}$, RelB$^{-/-}$ and c-Rel$^{-/-}$) have revealed the tissue-specific roles of Rel/NF-kappaB proteins in immunity, inflammation and cell survival (22-24). RelA$^{-/-}$ die on embryonic day 14 to 15, due to widespread TNFα-mediated hepatocyte apoptosis and liver degeneration (which also affects macrophages and fibroblasts), indicating a specific role for RelA in hepatocyte survival during development (25). Blockade of TNF signalling rescues embryonic lethality in RelA$^{-/-}$ mice (26, 27). In contrast to its role in hepatocyte survival, RelA has an anti-proliferative function in normal epidermal cells (28, 29). In comparison to RelA, p50$^{-/-}$, p52$^{-/-}$, RelB$^{-/-}$ and c-Rel$^{-/-}$ mice develop normally but have defects in immune cell function (24, 30).

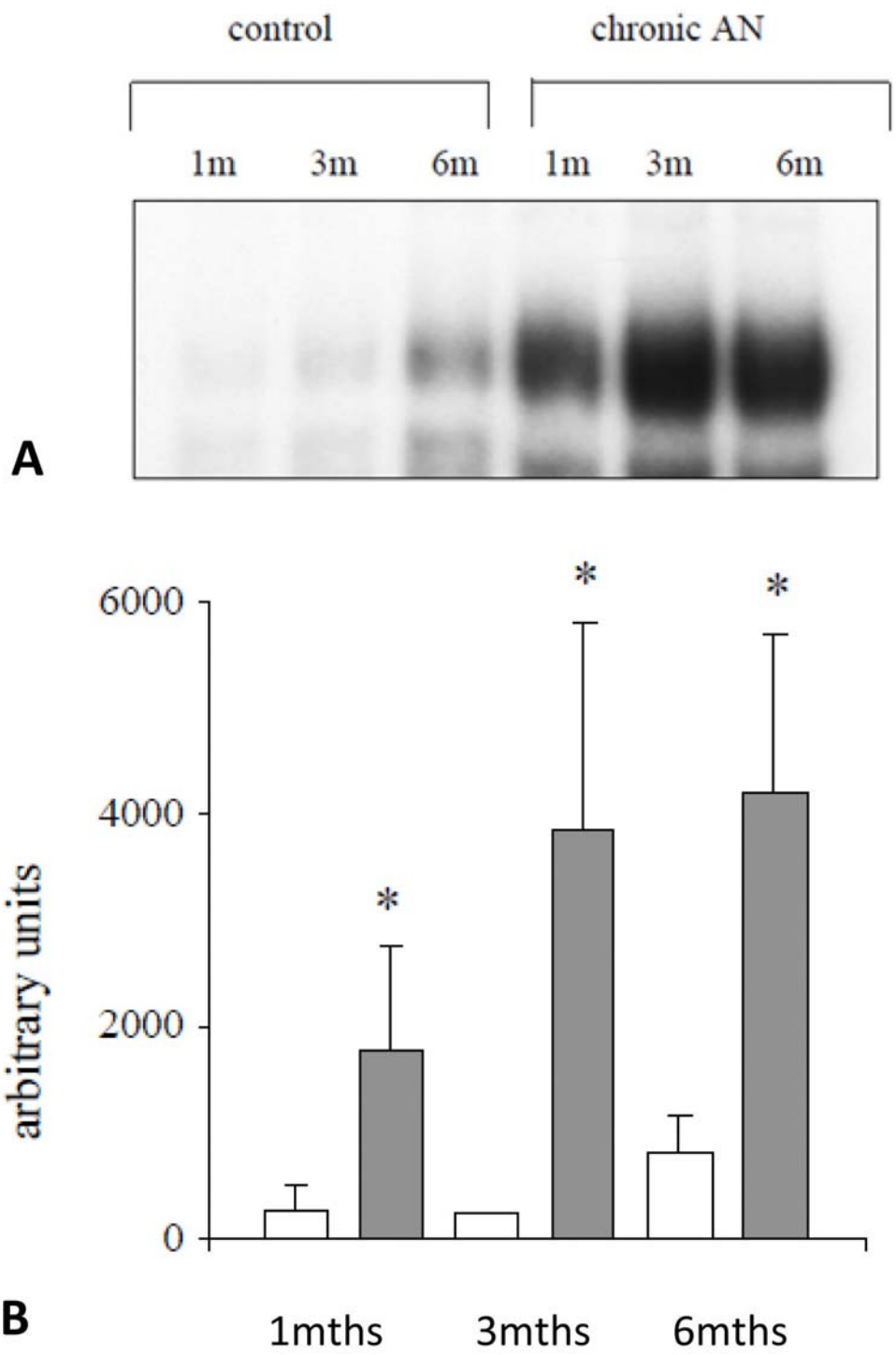


Figure 1. The timecourse of NF-kappaB activation in chronic adriamycin nephropathy: Adult male Wistar rats received two injections of doxorubicin hydrochloride (2 mg/kg) (n=15) or saline (n=15) three weeks apart. Kidneys were examined 1, 3 and 6 months after the second injection. Renal cortical NF-kappaB activation, as assessed by DNA-binding activity by EMSA (panel A), was increased as early as 1 month following disease induction, and remained elevated for up to 6 months persistent disease. The longterm upregulation of NF-kappaB paralleled progressive increase in proteinuria, focal segmental glomerulosclerosis, interstitial inflammation and fibrosis. Densitometric analysis is shown in Panel B. Data expressed as mean+SE (*P<0.0.5 compared to the control group).

Table 1. Animal models of chronic kidney disease (CKD) in which NF-kappaB DNA binding activity is altered

Model	NF-kappaB activation	Species	Reference
Proliferative Models of CK			
Nephrotoxic nephritis	Increased	Rat	6
Anti-thymocyte	Increased	Rat	7
Immune complex nephritis	Increased	Rat	123
Non-proliferative models of CKD			
Protein-overload nephropathy	Increased	Rat	8
Heymann Nephritisearly	Increased	Rat	177
Heymann Nephritis chronic	Increased	Rat	9
Remnant kidney	Increased	Rat	9
Adriamycin Nephropathy	Increased	Rat	180
Tubulointerstitial Disease models			
Ureteral obstruction	Increased	Rat	211
Ureteral obstruction	Increased	Mouse	174
Allogenic renal transplant	Increased	Rat	187
Acute renal ischaemia	Increased	Rat	212
Uric acid nephropathy	Increased	Rat	214
Angiotensin-induced	Increased	Rat	214
Miscellaneous models			
Aging	Increased	Rat	215
LPS[1]-induced	Increased	Rat	188
TNF[2]-induced	Increase	Rat	188

Abbreviations: [1]lipopolysaccharide; [2]tumor necrosis factor

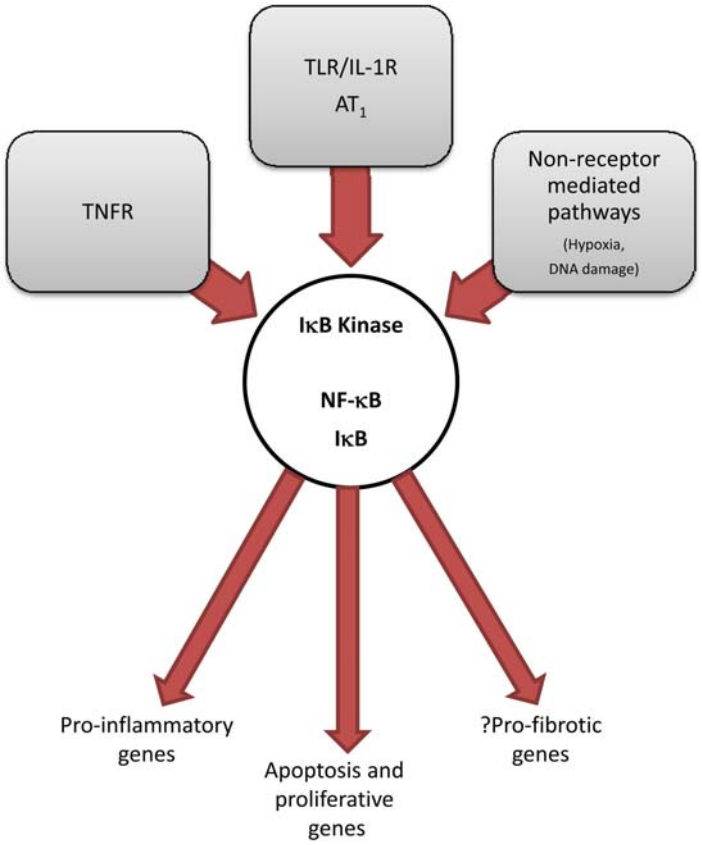


Figure 2. Simplified overview of the intracellular NF-kappaB signalling system*:* The upper components of the NF-kappaB system consist of a number of cell-surface receptors (tumor necrosis factor receptor, TNFR; Toll-like receptors, TLR; IL-1 receptor, IL-1R; angiotensin II, type 1 receptor, AT_1) and non-receptor mediated pathways which converge on the central components: IkappaB kinase (IKK), NF-kappaB complexes and IkappaB proteins. The latter represent potential targets for therapy. Activation of IKK lead to phosphorylation and degradation of IkappaB protein, which results in the nuclear localisation of NF-kappaB complexes and the differential induction of kappaB-dependent genes (predominantly regulating inflammation and cellular growth/survival). The role of NF-kappaB in directly modulating genes with a predominant profibrotic function are not well defined.

The innate functions of Rel/NF-kappaB proteins have confounded the ability to use these knockout mouse strains as tools to understand the specific role of NF-kappaB *in vivo* in kidney disease models. Although the use of conditional knockout mice studies may overcome this limitation (discussed in Section 5), it is noteworthy that even this approach is not foolproof, because individual Rel/NF-kappaB proteins can also affect non-NF-kappaB pathways (31-33). The knockout studies also highlight the potential toxicity that may be associated with chronic and untargeted pharmacological inhibition of NF-kappaB *in vivo*.

2.1.1.3. NF-kappaB isoforms and mechanisms of formation

In vivo, pairs of Rel/NF-kappaB protein monomers unite to form unique NF-kappaB dimer complexes (defined as isoforms), which have distinct transcriptional and DNA-sequence specificity as well as cell/tissue-specific distributions (22, 34). All Rel/NF-kappaB proteins can form either homo- or heterodimers, with the exception of RelB which is unable to homodimerize or heterodimerize to c-Rel and RelA (30). Of the 15 possible NF-kappaB isoforms, at least 12 are known to potentially bind DNA and regulate gene transcription. These include, RelA/RelA, RelA/p50, RelA/c-Rel, RelB/p52, (RelB/p100), RelB/p50, c-Rel/c-Rel, c-Rel/p50, p50/50, p50/Bcl3, p52/52, p52/Bcl3 (17, 30, 35). In most cells, the prototypical NF-kappaB isoform is a heterodimer consisting of RelA and p50, and this is often designated "classical NF-κB". The RelA/p50 complex is the most common, most potent gene transactivator and to date the most extensively studied NF-κB isoform in the kidney and most other tissues. In contrast, RelB/p52 isoforms exhibit nuclear localization and may represent constitutive NF-kappaB activity in many tissues (30). The mechanics of why and how Rel/NF-kappaB proteins dimerize and come to form specific NF-kappaB isoforms with unique biochemical and physiological roles are not clear (Tables 3 and 4) (20).

At present, more detailed and specific knowledge about the role of each of the NF-kappaB isoforms in intrinsic kidney cells is required (36). One approach to do this involves bioinformatic analysis of transcriptomes and gene networks regulated by transcription factor modules, and this is currently in progress for a number of kidney diseases, using human clinical samples (37). Furthermore, in addition to studying individual Rel/NF-kappaB proteins using conditional knockout mice, it is also necessary to investigate the functional roles of specific intact NF-kappaB complexes *in vivo* in animal models of CKD, perhaps, by mutating the phosphorylation site that promotes the dimerization of a specific Rel/NF-kappaB complex or its binding to an IkappaB protein; by the use of RNA aptamers or decoy oligonucleotides against a specific NF-kappaB isoform in a cell- and time-specific manner; and/or by the use of NF-kappaB reporter assays *in vivo* in whole animals using green fluorescent protein (38-42).

2.1.2. Cytoplasmic inhibitors of NF-kappaB: IkappaB proteins/rel protein precursors

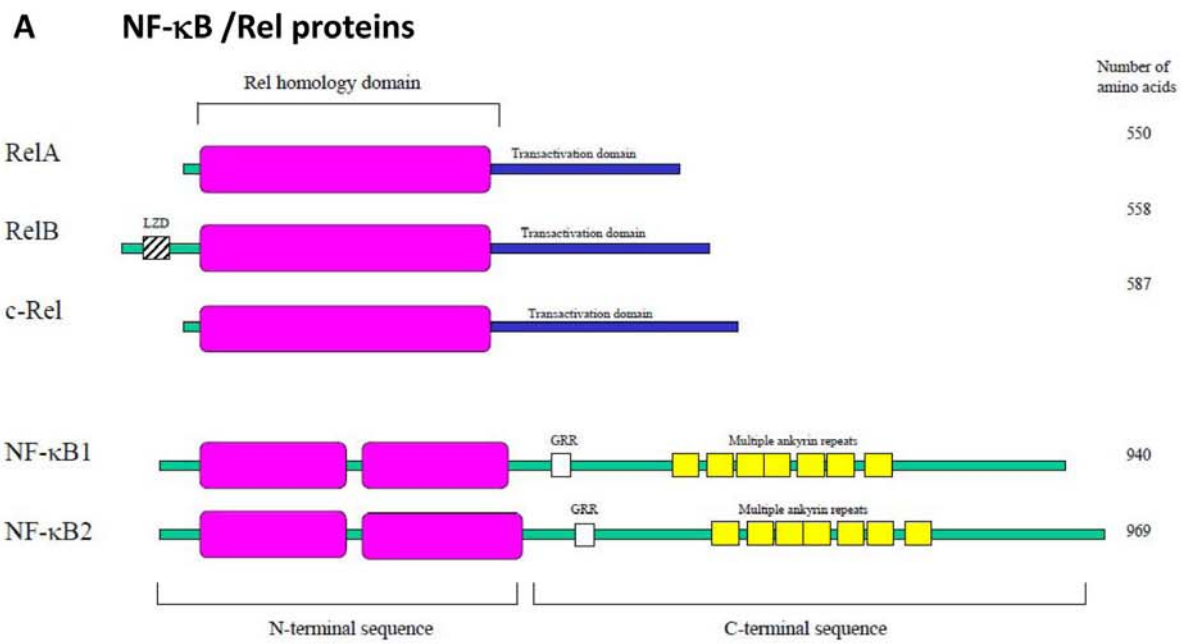
The cytoplasmic inhibitors of NF-kappaB are stringent regulators of NF-kappaB nuclear-cytoplasmic shuttling and consist of either the IkappaB (*i*nhibitors of NF-kappaB) protein family (IkappaBalpha, IkappaBbeta and IkappaBepisolon are the three main members; also IkappaBgammą and Bcl-3) or the NF-kappaB/Rel protein precursors (p100 and p105) (22, 23, 43) (see Figure 3A and B). With the exception of Bcl3 and IkappaBalpha, IkappaB proteins are currently only known to exist in the cytosol (22, 23, 43). Bcl-3 is also a member of the IkappaB family but it is localized in the nucleus and acts as a positive transcriptional coactivator for p50 and p52 homodimers (2). Each IkappaB protein differs in the number of ankryin repeats, and this partly determines its binding specificity and effect on NF-kappaB isoforms. IkappaBalpha is the predominant regulator of RelA/p50 whereas IkappaBepisolon has a selective role in the regulation of RelA homodimers and c-Rel/RelA heterodimers. In addition, differences in the degradation and synthesis of IkappaB proteins also determine their functional characteristics (24). As discussed, both p100 and p105 contain ankyrin repeats like that found in IkappaB proteins and can therefore function as cytoplasmic inhibitors of NF-kappaB complexes (44).

Consistent with the central role of IkappaBalpha in regulating RelA/p50, IkappaBalpha $^{-/-}$ mice are affected by a severe widespread inflammatory dermatitis with increased expression of pro-inflammatory cytokines and die in the early postnatal period (day 7 to 10) (21). The generation of transgenic mice expressing a degradation- or phosphorylation-resistant mutant IkappaBalpha or IkappaBbetaβ is another approach to assess the *in vivo* function of IkappaB proteins (IkappaB super-repressor transgenic mice). These mutant proteins function as dominant inhibitors of NF-kappaB activation (24). In such a system, the targeted over-expression of a IkappaBalpha mutant specifically in epidermal cells, using a keratin promoter, resulted in TNF-dependent hyper-proliferation, thus confirming this functional role for RelA in the skin, as described earlier (24, 29, 45). However, a limitation of using the IkappaB super-repressor, is that it can interact and alter the activity of other signalling pathways, such as p53. Therefore, ideally different techniques should be undertaken to fully verify a molecular-based hypothesis of a specific NF-kappaB isoform in the kidney (37).

2.2. Activation of the NF-kappaB system

2.2.1. Linking the extracellular environment to intracellular NF-kappaB activation

Activation of the NF-kappaB system occurs in response to (Figure 4): (i) ligand binding to cell-surface receptors, such as the Toll-like/IL-1 receptor family (TLRs; which bind pathogen-associated microbial peptides), the TNF receptor family (which bind TNF, lymphotoxin-beta1, BAFF-R, CD40), the T- and B-cell receptor, angiotensin II receptor, type 1 (AT_1) and the integrins (which respond to changes in extracellular matrix composition); as well as (ii) intracellular 'stressors', that are poorly defined and do not involve receptor-ligand binding, and may include certain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NF-κB /Rel proteins
Rel homology domain
Number of amino acids
RelA
Transactivation domain
550
RelB
LZD
Transactivation domain
558
c-Rel
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GRR
Multiple ankyrin repeats
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NF-κB2
GRR
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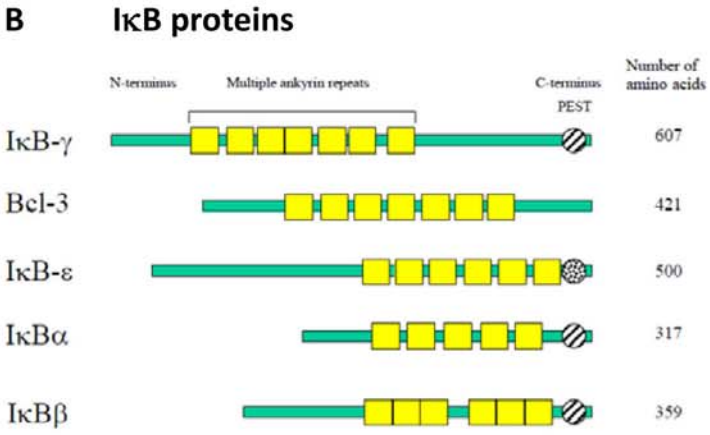
N-terminal sequence
C-terminal sequence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N-terminus
Multiple ankyrin repeats
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IκBβ
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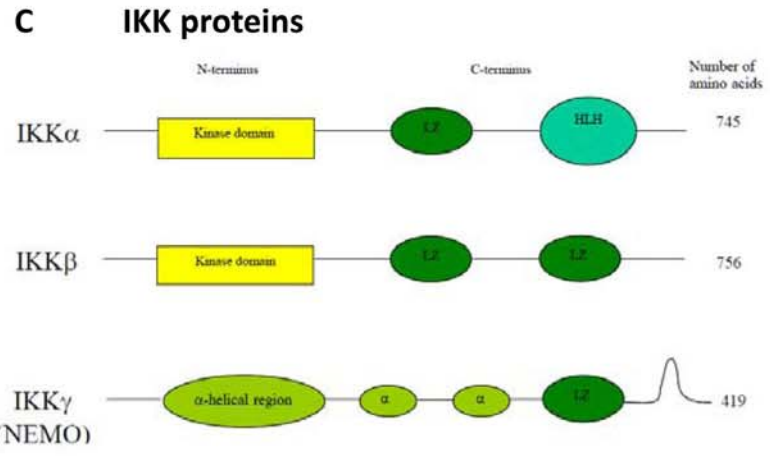
C
IKK proteins
N-terminus
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Number of amino acids
IKKα
Kinase domain
LZ
HLH
745
IKKβ
Kinase domain
LZ
LZ
756
IKKγ (NEMO)
α-helical region
α
α
LZ
419

Figure 3. Structure of the core components of the NF-kappaB signalling pathway: A. NF-kappaB/Rel proteins: All have in common a 300-amino acid conserved N-terminus (known as the Rel homology domain (RHD)). The RHD contains important coded information which enables dimerization with other Rel members; interaction with IkappaB in the cytoplasm; entry into the nucleus (the nuclear localisation sequence); and sequence-specific DNA binding (40). The Rel/NF-kappaB proteins can be subdivided into two groups, according to differences in the sequences of their C-terminus: (i) Group I proteins (RelA, RelB and c-Rel), in which the C-terminal region contains the transactivating domain (TAD), which is essential for NF-kappaB-mediated gene transactivation. Additionally, RelB contains a leucine zipper domain (LZD) in the N-terminal sequence (22, 23); (ii) Group II proteins (p50 and p52) are produced as precursor proteins (p105 and p100 respectively) which are cleaved in the cytosol, by a ubiquitin-proteasome pathway, to produce the smaller mature peptides (p50 and p52). The N-terminal end contains the mature peptide (p50 or p52) and the C-terminal domains contain seven repeats of a conserved 33 amino acid sequence, called ankyrin repeats (also present in IkappaB proteins). The N-terminus and C-terminus of Group II proteins are linked by a flexible glycine-rich region (GRR), which functions to allow intramolecular inhibition of the p50/p52 portion by the IkappaB-like C-terminal portion; B. IkappaB proteins (IkappaBalpha, IkappaBbeta, IkappaBepisolon, IkappaBgamma, Bcl-3): All have in common a conserved core of five to seven ankyrin repeat-motifs (similar to the Group II Rel/NF-kappaB proteins), an N-terminal regulatory domain and a C-terminal domain that contains a proline, glutamic acid serine and threonine domain (PEST) (22, 23, 43). The ankyrin repeats are the regions which mask the nuclear localisation sequence of Rel/NF-kappaB proteins to prevent the importation of NF-kappaB dimers into the nucleus. C. IkappaB kinase proteins (IKKalpha, IKKbeta, IKKgamma); IKKalphą (85 kDa) and IKKbetą (87 kDa) share 52% homology with each other and contain protein-kinase domains at their N-terminals, and leucine-zippers (LZ) and helix-loop-helix motifs (HLH) in their C-terminal regions (22, 23, 43). IKKgammą (also known as NF-kappaB Essential Modifier, NEMO) is an unrelated structural/regulatory subunit which receives and integrates signals from upstream signalling pathways and receptor-associated proteins.

Table 2. Members of the mammalian NF-kappaB system

Protein	Gene	MW[1]	Rel/NF-kappaB partners
Rel/NF-kappaB proteins			
p50/105	*NFKB1*	50/105	p50, RelA
p52/100	*NFKB2*	52/100	p52, RelB
RelA	*relA*	65	RelA, p50, c-Rel
RelB	*relB*	66	RelA, c-Rel
c-Rel	*c-rel*	68	RelA
IkappaB proteins			
IkappaBalpha	*Med-3*	37	p50/RelA
IkappaBbeta	*Iκbβ*	41	P50, RelA, c-Rel
IkappaBepisolon	*Iκbε*	45	RelA homodimers, RelA/c-Rel
IkappaBgamma	*Iκbγ*	70	p50/p52 homodimer
Bcl3[2]	*Bcl3*	45*	p50/p52 homodimer

Abbreviations: [1]molecular weight Other: [2]Bcl-3 exists in the nucleus only (all other IkappaB proteins are cytoplasmic distribution)

Table 3. List of known NF-kappaB isoforms

NF-kappaB isoforms	IkappaB partner	Selected target genes
RelA/RelA	IkappaBepisolon	*IL[1]-8, type-VII collagen, ICAM1[2]*
RelA/p50	IkappaBalpha	*IkappaBalpha, IL[1]-8, IL[1]-6, TNF[3]-alpha, GM-CSF[4], HIV1[5]*
RelA/c-Rel	IkappaBepisolon	*uPA[6], IL2R[7], CCL[8]2*
RelB/p50 RelB/p52[13]	p105 p100	*MDC[9]* *CCL[8] 19, 21 and 22*
c-Rel/c-Rel	c-Rel/c-Rel	*IL8*
c-Rel-p50	c-Rel-p50	*IL[1]-12 p40 and p35, IL2Ra[7]*
P50/p50	P50/p50	*TNF[3] alpha, IL[1] 6, MHC[10]-associated-invariant chain*
P50/Bcl3	P50/Bcl3	*BCL[11] 2*
P52/p52 P52/Bcl3	P52/p52 P52/Bcl3	*MHC-associated invariant chain* *Cyclin D1, BCL2*

Abbreviations: [1]interleukin, [2]intercellular adhesion molecule-1, [3]tumor necrosis factor, [4]granulocyte monocyte-colony stimulating factor, [5]human immunodeficiency virus 1, [6]urokinase-type plasminogen activator, [7]interleukin-2 receptor alpha, [8] CC chemokine ligand, [9]macrophage derived chemokine, [10]major histocompatibility complex, [11]B-cell lymphoma leukemia.
Other: [12]Product of the non-canonical NF-κB pathway. Modified from (35).

stimuli such as DNA damage and involve mitochrondrial production of reactive oxygen species (30, 46-48). Each of the above pathways have a unique set of associated adaptor/intermediary proteins and kinases, which almost universally converge and lead to the degradation of IkappaB proteins, and a full discussion of each is beyond the scope of the present review (30). However, angiotensin II and TLRs will be briefly discussed below and plasma proteins in Section 4.2., because of the distinctiveness of these stimulants to kidney disease.

2.2.1.1. Angiotensin II

Increased levels of angiotensin II in the kidney as well as the serum are characteristic features of almost all progressive CKDs (reviewed in 49, 50). There is substantial *in vitro* and *in vivo* evidence that binding of angiotensin II to AT_1 receptors leads to the activation of the NF-kappaB complexes as well as kappaB-dependent genes in intrinsic renal cells and the kidney (49, 50). The activation of NF-kappaB mediates the proinflammatory and growth-promoting (proliferation

Table 4. DNA binding specificity of distinct NF-kappaB isoforms

NF-kappaB isoforms	kappaB motifs	Source
RelA/p50	5'GGG RNN TCC C-3'	
p50	5'GGG ACT TTC C-3' [1,2] 5'GGG ATT TCC C-3' [1]	Mouse Igkappa chain enhancer HIV[1] LTR MHC[2] class I h-2K enhancer
p52	5'GGG ATT TCC C-3'	
RelA	5'GG ACT TTC C-3' [2] 5'GGA AAT TTC C-3'	*MDC*[3] *CCL*[4] *19, 21 and 22*
RelA/c-Rel	5'HGG ARN YYC C-3' 5'GGG AAT TCC C-3'	*IL*[5] *8*

Abbreviations: [1]human immunodeficiency virus, [2]major histocompatibility complex, [3]macrophage derived chemokine, [4]CC chemokine ligand, [5]interleukin. Note: H = A, C or T; N = any base; R = purine; Y = pyrimidine RelA homodimers have low affinity for these sites Underlined segment shows the preferred binding site of RelA

and hypertrophy) effects of angiotensin in intrinsic kidney cells (49-52). The intermediate signalling mechanisms that transduce AT_1 receptors to NF-kappaB signal activation are not well characterized but involve either the canonical or non-canonical pathways (49, 50). AT_2 receptors also bind to angiotensin II and mediate the upregulation of classical kappaB-dependent genes, such as monocyte chemoattractant protein-1 (CCL2 in systematic nomenclature), but data regarding the involvement of NF-kappaB through this route is conflicting (53, 54).

2.2.1.2. TLRs

The TLRs have attracted a great deal of interest in recent years, because of their fulfilment as transducers of tissue injury and autoimmunity to the NF-kappaB pathway (see review by Anders and Schlondorff, 55). They consist of a family of thirteen single-pass transmembrane proteins that recognize specific pathogen-associated molecular peptides (PAMPs). The ligands for TLRs are diverse and include not only microbial proteins (such as LPS for TLR4) but also synthetic agents (such as deoxynucleotides for TLR9) and endogenous substances. The latter are particularly relevant to the pathogenesis of tissue injury and CKD as they consist of extracellular matrix proteins (hyaluronan fragments, fibronectin), fibrinogen, lipoproteins, Tamm-Horsfall protein as well as molecules released by dying cells (self-RNA and DNA, HMGB1, heat-shock proteins) (55-57). The cytoplasmic domains of TLRs are combined with adaptor proteins (Myeloid Differentiation Primary Response protein (MyD)88 or TRIF), which are essential for the activation of the distal signalling pathways, including NF-kappaB (30, 56, 57). Intrinsic renal cells, such as mesangial cells, tubular epithelial cells (TECs), collecting duct epithelial cells and glomerular endothelial cells express TLR1 to TLR4 but may lack TLR7 (55, 58). TECs also express TLR6 and TLR9, and mesangial cells contain TLR6 (59-61). The functional significance of TLRs in NF-kappaB signalling in renal disease has been addressed in animal models (62, 63). In MRL *lpr* mice, synthetic oligodeoxynucleotides against TLR7 and TLR9 reduced chronic renal inflammation and the production of NF-kappaB - dependent genes (TNF and IL-6) (62). Moreover, inhibition of TLR4 (predominantly *via* MyD88) modulated the up-regulation of kappaB-dependent pro-inflammatory genes and suppressed TEC apoptosis in mice with renal ischaemia, confirming their role as important transducers of innate immunity to the NF-kappaB system (63).

2.2.2. Convergence of proximal signal transduction pathways onto IkappaB degradation

NF-kappaB inducing stimulants can activate isoform-specific NF-kappaB complexes through at least three different pathways (designated the canonical, non-canonical and atypical pathways). The canonical and non-canonical pathways are the main mechanisms of NF-kappaB activation.

2.2.3. Canonical NF-kappaB activation (NEMO-IKKbeta-IkappaB-dependent)

The canonical (or classical) pathway is the most the well studied model of NF-kappaB activation in mammalian cells. It is induced rapidly (usually within minutes) in response to pro-inflammatory stimuli, such as PAMPs and cytokines (IL-1 and TNF) and leads to the activation of the prototypical NF-kappaB isoform, RelA/p50, bound to IkappaBalpha. In quiescent cells, the nuclear localisation signal of RelA is predominantly bound to IkappaBalpha, maintaining cytoplasmic retention. Within minutes of ligand binding to the TNFR (about 10 minutes), TLR or T-cell receptor (about 45 minutes), a number proximal signal transduction pathways converge onto the IkappaB kinase (IKK) complex. The latter is a large (500 to 900 kDa) multi-protein cytosolic complex consisting of two catalytic subunits (IKKalpha and IKKbeta) bound to multiple copies of IKKgamma (also known as NF-kappaB Essential Modifier, NEMO). Activation of IKK complex in the canonical pathways is characterised by ubiquitination and phosphorylation of IKKgamma and phosphorylation of two serine residues in IKKbeta (56). These events lead to the phosphorylation of IκBα at serines 32 and 26 (or IkappaBbeta at serines 19 and 23), which tag IkappaBalpha as a substrate for the Skp1/Cul1/F-box protein-B-TrCP ubiquitin ligase complex (ubiquitination at lysines 21 and 22), and rapid degradation via a non-lysosomal, ATP-dependent 26S proteolytic complex composed of a 700-kD proteasome. The canonical pathway probably also applies to activation of the RelA/RelA and RelA/c-Rel isoforms, which are predominantly bound to IkappaBepisolon.

2.2.3.1. Regulation of the canonical pathway

Termination of RelA/p50 activation occurs by a regulatory feedback loop involving the induction of IkappaBalpha, which is a kappaB-dependent gene. Re-synthesis of new IkappaBalpha occurs within 60 minutes, and serves as a mechanism to terminate the activation of RelA/p50. In contrast to IkappaBalpha, IkappaBbeta and

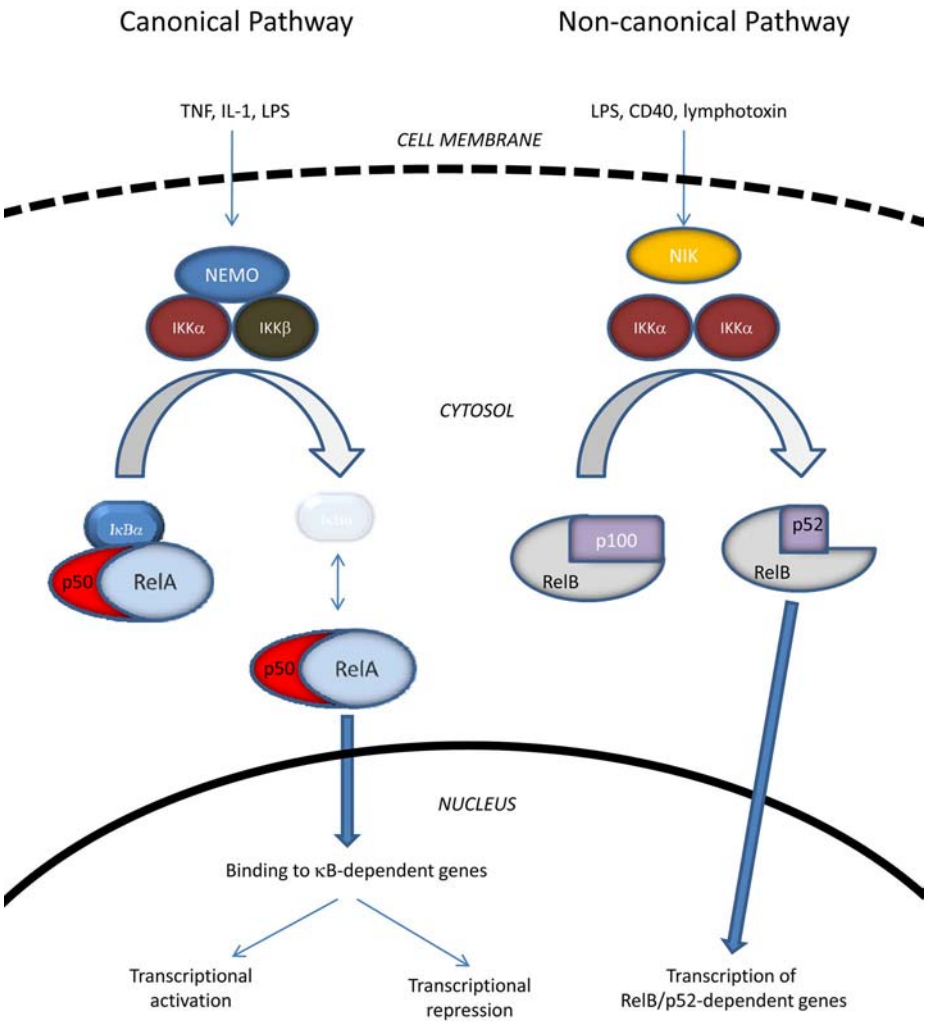

Figure 4. Overview of the canonical and non-canonical pathways of NF-kappaB activation: 1. Canonical (or classical) pathway (NEMO-IKKbeta-IkappaB-dependent): Induced by pro-inflammatory stimuli, such as pathogen-activated microbial peptides (e.g. lipopolysaccharide, LPS) and cytokines (e.g. IL-1 and TNF) which lead to phosphorylation of the IKK complex (IKKbeta, IKKalpha and IKKgamma, the latter also known as NF-kappaB Essential Modifier, NEMO), phosphorylation and degradation of IkappaBalpha which allows RelA/p50 NF-kappaB complexes to enter the nucleus and induce or repress the activation of a multitude of genes. The latter depends on the nature of the complexes entering the nucleus, post-translational modification of NF-kappaB complexes and presence of other transcription factors. 2. Non-canonical pathway (NIK-IKKalpha-p100-dependent): Induced by specific stimuli (CD40, lymphotoxin-β receptors, B-cell activating factor of the TNF family, LPS). This leads to the induction of the NF-kappaB inducing kinase (NIK) which then activates IKKalpha homodimers leading to the phosphorylation of p100 and its proteolytic cleavage to p52 by ubiquitin-dependent processing by the 26S proteasome. The active RelB/p52 heterodimer then enters the nucleus and mediates gene transcription.

IkappaBepisolon are not kappaB-dependent genes. Thus the IkappaBbeta, and IkappaBepisolon proteins are degraded over longer period of time (2 hours) and may contribute to more persistent activation of RelA-p50/NF-kappaB (more than 20 hours), which is particularly relevant to chronic disease processes. In addition, genes encoding c-Rel, RelB, p100 and p105 also contain kappaB-promoter elements suggesting that the composition of NF-kappaB complexes and therefore pattern of gene transcription can change over time (30, 44). Finally, NF-kappaB inducing stimuli may autophosphorylate IKKbeta at a carboxy terminal serine cluster, which reduces IKK activity (64), and this may also serve as an additional autoregulatory mechanism to prevent the sustained activation of the canonical pathway (64).

2.2.4. Non-canonical NF-kappaB activation (NIK-IKKalpha-p100-dependent)

In contrast to the canonical pathway, the non-canonical (or alternative) pathway results in the stimulus-specific activation of a RelB/p52 NF-kappaB complex from

an inactive RelB/p100 dimer. In addition, activation of the non-canonical pathway activation tends to occur more slowly (over hours to days) providing chronic NF-kappaB activation (20). Specific stimuli, such as CD40, lymphotoxin-beta receptors, B-cell activating factor of the TNF family, LPS and latent membrane protein-1 of the EBV, are typical activators of the non-canonical pathway. Activation of the non-canonical pathway by these stimuli begins with the induction of the NF-kappaB inducing kinase (NIK) which then activates IKKalpha homodimers leading to the phosphorylation of p100 and its proteolytic cleavage to p52 by ubiquitin-dependent processing by the 26S proteasome. The active RelB/p52 heterodimer then enters the nucleus and mediates gene transcription.

Recent studies suggest that the non-canonical pathways are activated in *db/db* mice with type 2 diabetes (see Section 5.3.) (36). In addition, certain stimuli, such as LPS and angiotensin II, are able to induce the canonical as well as non-canonical pathways therefore resulting in different mixtures of NF-kappaB complexes and transcription of specific genes. The non-canonical pathway does not lead to the activation of p50 from p105, which is produced in the absence of cell stimulation by co-translational mechanisms (44).

2.2.5. Atypical NF-kappaB activation

2.2.5.1. IKK-independent pathways of activation

Although the majority of NF-kappaB activation probably occurs via the canonical pathways requiring IKKbeta, two additional pathways, independent of IKK have been reported: (i) CK2 pathway: Stimulation with short-wavelength ultraviolet (UV-C) light or expression of Her2/Neu oncogene leads to casein kinase II (CK2)-dependent phosphorylation of IkappaBalpha at the C-terminal site (rather than serines 32/36 in the canonical pathway) and NF-kappaB activation. With relevance to the kidney, CK2 is over-expressed in the kidney of rats with anti-glomerular basement membrane disease, and its inhibition with anti-sense oligodeoxynucleotides or CK2-specific inhibitors (emodin and 4', 5,7-trihydroxyflavone) reduced renal injury and proliferation (but not apoptosis) and the production of NF-kappaB-dependent pro-inflammatory genes (65); (ii) Tyrosine kinase pathway: Stimulation with hypoxia or hydrogen peroxide led to activation of tyrosine kinases (c-Src, Syk) which phosphorylate IkappaBalpha at tyrosine residue 42 leading to its degradation and resulting in NF-kappaB activation (44).

2.2.5.2. Alternative IKK-dependent pathways

As discussed earlier, genotoxic stimuli (such as due to UV-C and chemotherapeutic drugs, including etoposide) may activate NF-kappaB, but which it may involve alterations to IKKs (for example, sumoylation of NEMO) that are different from the canonical and non-canonical pathways (44).

2.2.6. Molecular phylogeny of NF-kappaB signalling

NF-kappaB signalling is absent in *C. Elegans* but otherwise is highly conserved in species as diverse as *drosophila* and humans (66). Although there are species-specific variations in the genomic sequences of NF-kappaB/Rel proteins (67), the basic signalling mechanisms of the pathway appear to be conserved amongst mammals. For instance, the amino acids (serine 32/36 and tyrosine 42) known to be phosphorylated before activation of NF-kappaB are conserved in mouse, pig and humans, suggesting that the mechanisms of IkappaBalpha degradation are similar manner in these species (68).

2.3. Cellular effects of NF-kappaB activation

Through the positive and negative transcriptional regulation of multiple genes (18), the activation of NF-kappaB has a myriad of potential cellular effects. It is well known that NF-kappaB mediates inflammation and interstitial macrophage accumulation, through the up-regulation of pro-inflammatory cytokines, chemokines, cell surface receptors, vasoactive peptides, complement proteins and colony stimulating factors (18). To prove this function *in vivo* NF-kappaB, Carlsen and co-workers developed transgenic mice that express a luciferase reporter whose transcription is NF-kappaB-dependent (69). This was necessary because, quantitation of NF-kappaB activity in tissue samples from animals does not distinguish between transcriptionally active and inactive NF-kappaB complexes. Using this system, the activity of NF-kappaB was measured non-invasively by real-time imaging and found to be constituitively present in lymphatic tissue. Following treatment with either TNF, IL-1beta or LPS increased luminescence was detected in a tissue-specific fashion, with the greatest expression in skin and lymphatic tissue and moderate activity in the kidney and other organs (69). In addition to inflammatory genes, NF-kappaB also regulates genes that control cell survival, proliferation, immortality, angiogenesis, epithelial-mesencyhmal transition and fibrosis (44). In the case of apoptosis, as discussed earlier, RelA has cell-specific roles in hepatocytes and the epidermal cells. These differences may be mediated by the differential regulation of apoptotic regulatory genes that encode proteins such as c-FLIP, cIAP1, cIAP2, A20, GADD45b, MnSOD and ferratin heavy chains (28).

2.4. Regulation of NF-kappaB transcriptional selectivity

Comparative genomic analyses have revealed that the number of protein-encoding genes does not correlate with the morphological and behavioural complexity of an organism (70, 71). In other words, the nematode worm has two-thirds of the genes that a human possess (20 000 vs. 30 000) but the biological complexity of the organisms are magnitudes apart. One hypothesis to explain this observation is the complexity of gene transcriptional regulation is a more important factor for explaining animal diversity than the absolute number of genes possessed (70). The mechanisms behind the transcriptional specificity of NF-kappaB isoforms are also one of the most important questions that need to be addressed in NF-kappaB biology. That is, although NF-kappaB plays a central role in the transcription of hundreds of pro-inflammatory genes, how is it that not all κB-promoter sites are activated upon NF-kappaB nuclear translocation. For instance, stimulation of human proximal TECs with TGF-beta1 induced the

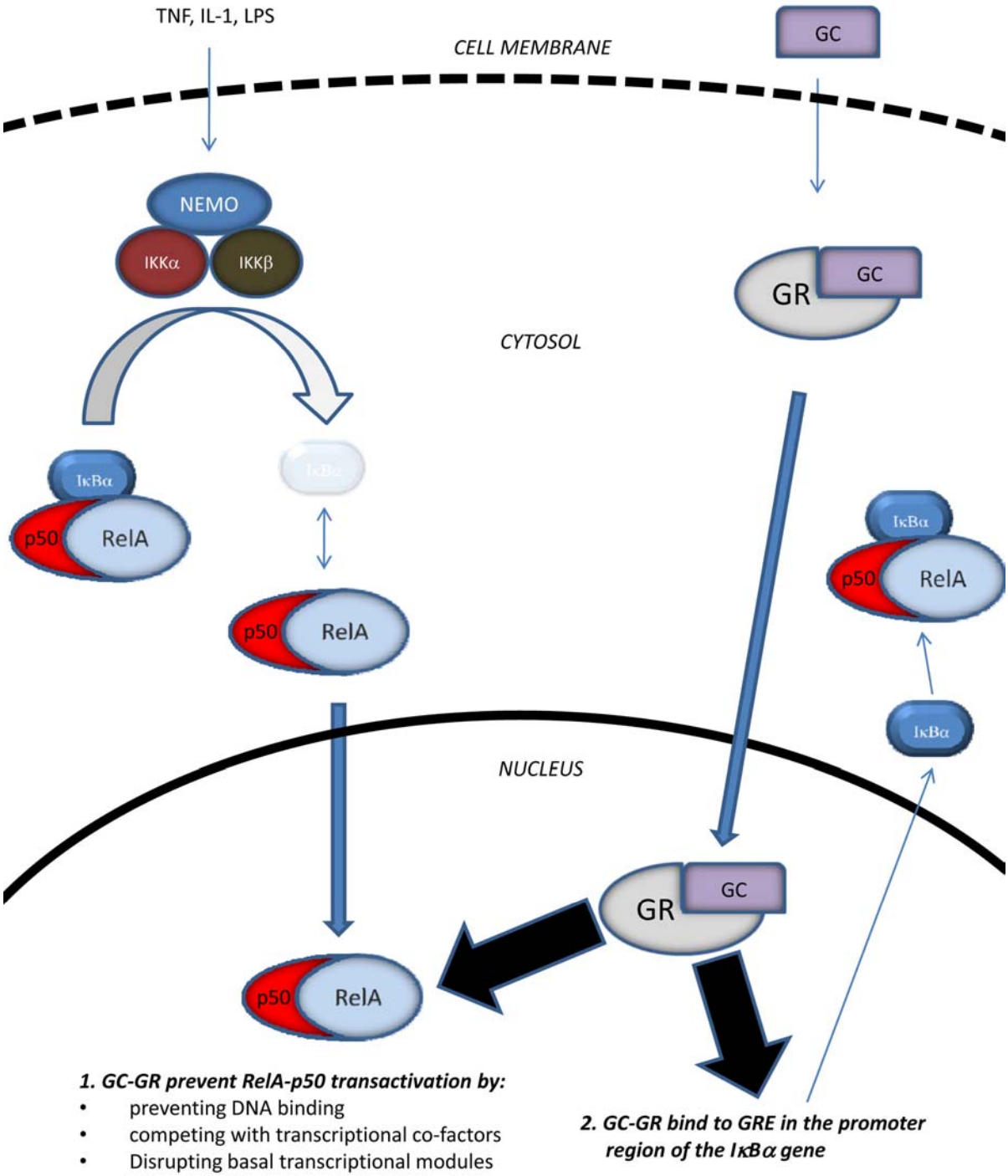

Figure 5. Molecular mechanisms of glucocorticoids (GCs) on NF-kappaB activation*:* Many of the anti-inflammatory effects of GCs are attributed to their ability to inhibit NF-kappaB activation in T cells and monocytes (197, 208, 209). GCs pass through the cell membrane and bind and activate the glucocorticoid receptor (GR) in the cell cytoplasm. NF-kappaB activation is reduced by: (i) Direct interactions of GC-GR with the RelA/p50 complex which prevents DNA binding; competition with transcription co-factors and/or prevention of transactivation; (ii) alternatively, GC-GR bind to GC response element (GRE) in the promoter region of the IkappaBalpha gene, resulting in increased synthesis and binding to NF-kappaB complexes in the cytosol. In contrast to these effects on T cells and monocytes, the actions of GCs on NF-kappaB activation in intrinsic renal cells are less consistent (139, 197, 210). In mesangial cells, GCs promote apoptosis (139) and reduce cytokine production (210), in part through NF-kappaB, whereas in TECs NF-kappaB signalling is glucocorticoid-resistant (197). Figure modified with permission from references (197, 208).

expression of IL-8, but reduced CCL2 and C3/C4 protein expression, even though CCL2 and C4 are both kappaB-dependent genes (72). Similarly in co-culture studies of TECs, T-cell contact induced CCL2 and interferon inducible protein (IP)-10, but not regulated upon activation, normal T-cell and expressed (RANTES) (CCL5 in systematic nomenclature), even though all are kappaB-dependent CC chemokine genes (73). There are many other NF-kappaB-transcriptional conundrums like this in Nephrology alone, which will be discussed later.

Some of the postulated mechanisms regulating the selectivity of gene transactivation by NF-kappaB (which are stimulus- and cell-specific) consist of the following: (i) the formation of specific NF-kappaB isoforms; (ii) the presence of other transcription factors (23, 34, 74, 75); (iii) variations in post-transcriptional RNA processing of κB-dependent genes (37, 76); (iv) post-translational modifications of Rel/NF-kappaB proteins (phosphorylation, acetylation, prolyl isomerization) which may determine the the potency of gene transactivation (77, 78); and/or (v) autoregulation of IkappaB proteins (IkappaBalpha, p100, p105).

3. EVIDENCE OF ABERRANT NF-kappaB EXPRESSION IN HUMAN CKD

The first question to ask about NF-kappaB is whether its expression is altered in human CKD. A number of clinical studies have been undertaken to address this hypothesis, and the methods used have typically included immunohistochemistry of individual NF-kappaB/Rel protein and/or southwestern immunohistochemistry of DNA-binding NF-kappaB dimers, to localise activation of NF-kappaB in paraffin sections of kidneys from patients with CKD. Because, it is difficult to develop antibodies against transcription factors due to their low-level of expression (79), southwestern immunohistochemistry has been used to detect NF-kappaB in these studies. In this technique, double-stranded oligonucleotides of kappaB-responsive elements, labelled with digoxigen are reacted with paraffin sections. Binding between NF-kappaB isoforms and the digoxigenin-labelled DNA probe are then recognised by an antibody directed against-digoxigenin (79). In control kidneys (usually samples of normal kidney tissue obtained from nephrectomies performed for renal carcinoma), NF-kappaB, p50 and RelA were only weakly detectable in the glomerulus and tubulointerstitium, using these methods (80-82).

3.1. Diabetic kidney disease

3.1.1. Immunohistology of NF-kappaB

Diabetic kidney disease is the most common cause of end-stage kidney failure. By southwestern immunohistochemistry, activated NF-kappaB was present in the kidneys of patients with overt type 2 diabetic nephropathy (n=11) and completely absent in normal control kidneys (83). The NF-kappaB complex was mainly detected in cortical TECs, and to a lesser extent in some glomerular cells (mainly podocytes and some parietal and endothelial cells) as well as renal interstitial cells (mainly mononuclear) (83, 84). A similar pattern of expression was detected for the Rel/NF-kappaB proteins, p50 and RelA (83-85). In serial sections, the NF-kappaB-positive TECs also expressed angiotensin II and NF-kappaB-dependent chemokines (CCL2, CCL5). The number of NF-kappaB-positive TECs was positively correlated with interstitial inflammation and proteinuria (83, 84). In another study, TECs recovered from the urine of patients with type 2 diabetes, had an increased expression of RelA and IL-6, and this was associated with the increased urine excretion of carboxymethyllysine (CML, an advanced glycation end-product) (85). RelA was also detected in mesangial and endothelial cells, podocytes and TECs, where it mirrored the up-regulation of the mitogen-activated protein kinase pathway (80).

3.1.2. Microarray mRNA analysis of NF-kappaB members and dependent genes

More recently, Schmid and co-workers undertook a bioinformatic approach to determine the molecular signature of progressive diabetic nephropathy, using mRNA generated from the tubulointerstitial compartment of thirty-two patients with diabetic nephropathy compared to a control group (living kidney donors n=12; cadaveric kidneys n=5 and minimal change disease n=7) (37, 86). Focusing on NF-kappaB-based transcriptional pathways, they identified that 27 percent (predominantly belonging to the canonical pathway) of the 85 known members of the NF-kappaB signalling pathway, and 59% (138 genes) of the 232 compiled NF-kappaB target genes (as documented in www.nf-kb.org), had increased mRNA expression compared to the control groups. Of the 138 genes, 39% were strongly induced in progressive diabetic nephropathy, and these included chemokines, cytokines and adhesion molecules (86).

3.1.3. Peripheral blood mononuclear cell expression of NF-kappaB

NF-kappaB DNA binding activity and RelA expression was also found to be elevated in *ex vivo* peripheral blood mononuclear cells (PBMCs) isolated from 33 to 43 patients with either type 1 or type 2 diabetes (87, 88). In these studies, the patients taking angiotensin converting enzyme inhibitors were excluded, as the authors reported (no data shown) that they reduce NF-kappaB activation in PBMCs in humans (87). NF-kappaB activation was most marked in those patients who had diabetic nephropathy, and by univariate analysis the expression was positively correlated with proteinuria, plasma markers of lipid peroxidation (malondialdehyde and 4-hydroxy-3- (E)-nonenal) and elevations in the plasma HbA1c (87, 88). The activation of NF-kappaB could be suppressed by short-term treatment (3 to 14 days) with the antioxidant, alpha-lipoic acid, or in a separate study, an eight week treatment with vitamin E (87-89). It was hypothesised that sustained PBMC NF-kappaB activation may protect mononuclear cells from apoptosis, and thereby contribute to tubulointerstitial injury in diabetic nephropathy (87).

3.1.3.1. Genetic polymorphisms of NF-kappaB predisposing to diabetic kidney disease

Increasing evidence suggests that polymorphisms of various genes, particularly those of the renin-angiotensin-aldosterone system, may be associated an increased risk for the progression of CKD (90, 91). In this regard, in a study of 211 patients with type 2 diabetes, a significant increase in the homozygous AA genotype of the NFKB1A gene was detected when compared to the control group (highest in those without renal disease) whereas no relationship was present for the CA repeat polymorphism of the NFKB1 gene (92). In addition, the methionine-to-valine substitution at codon 55 (M55V) variant of SUMO4 (a small ubiquitin modifier that conjugates IkappaBalpha and negatively regulates NF-kappaB activation), upregulated NF-kappaB activation and was associated with the severity of nephropathy in 430 type 2 diabetic patients (93).

Taken together, these clinical studies (using a range of histological and genomic approaches) strongly support the hypothesis that NF-kappaB signalling is upregulated in humans with diabetic kidney disease.

3.2. Chronic glomerulonephritis

3.2.1. IgA Nephropathy

Renal NF-kappaB (as determined by southwestern immunohistochemistry) was increased by about 2 to 3-fold in patients with IgA nephropathy or IgA-negative mesangioproliferative glomerulonephritis (n=28), compared to control groups (94). The increase in NF-kappaB was observed in mesangial cells and most marked in areas of proliferation. In addition, NF-kappaB was evident in atrophic tubules and infiltrating cells, where it correlated with degree of tubulointerstitial injury. The up-regulation of NF-kappaB in IgA nephropathy correlated positively with the tubulointerstitial expression of CCL2, ICAM-1 and GM-CSF (94). In contrast, in another study of seven patients with IgA nephropathy with minimal proteinuria, NF-kappaB activation, was similar to control kidneys, and detected in glomerular mesangial and glomerular epithelial cells (82). A potential reason for the discrepant findings between these studies may be the differences in the degree of proteinuria in two cohorts. The patients in the study showing no elevation in NF-kappaB had minimal proteinuria in comparison to the other study (1.1. g/day). In support of this possibility, Hong and co-workers found that glomerular NF-kappaB activation was highest in patients with more severe IgA deposition, proteinuria and haematuria in patients with post-transplant IgA nephropathy (95). Therefore, there are some inconsistencies in whether NF-kappaB is increased in humans with IgA nephropathy, which require further investigation.

3.2.2. Minimal change disease and membranous nephropathy

In nephrotic glomerular diseases, Mezzano and co-workers predicted that NF-kappaB activation would be a marker of renal disease progression, because of *vitro* data showing that NF-kappaB activation was increased in TECs by exposure to nephrotic-range concentrations of plasma proteins (10, 82). In their study, the immunohistochemical expression of p50, RelA and NF-kappaB was increased mainly in the tubules of proteinuric patients (membranous nephropathy, n=20; minimal change disease, n=14), but not in non-proteinuric chronic glomerular disease (CGD) (a subset of patients with IgAN, n=6) (82). In addition, a significant correlation between proteinuria and NF-kappaB activation was detected. However, an unexpected observation of this study was that only patients with membranous nephropathy had elevated mRNA levels of kappaB-dependent (CCL2, CCL5) genes in TECs, even though tubular NF-kappaB was increased in all proteinuric patients (minimal change disease plus membranous nephropathy) (82). In another study, NF-κB was also found to be strongly induced in glomerular cells in minimal change disease and membranous nephropathy (81). RelA was the predominant (if not exclusive) NF-kappaB/Rel protein expressed in podocytes, whereas endothelial cells, mesangial cells and parietal epithelial cell expressed both RelA and p50 (81). The number of RelA- and NF-kappaB-positive podocytes correlated with the severity of proteinuria and upregulation of TNFalpha and IL-beta1 protein (81).

These results therefore support the hypothesis that proteinuria is an inducer of NF-kappaB in intrinsic renal cells in humans with nephrotic glomerular diseases. However, they also highlight that immunohistochemical assessment alone is insufficient to explain the mechanisms regulating differential gene expression by NF-kappaB (as discussed in Section 2.3.) and the functional activity of gene promoters *in vivo (*69).

3.2.3. Immune-mediated inflammatory renal disease (crescentic glomerulonephritis and lupus nephritis)

In a study of patients with crescentic glomerulonephritis (n=34, three-quarters ANCA-related), NF-kappaB-positive nuclei were increased nearly 6-fold in necrotizing and crescentic glomerular lesions, TECs and interstitial mononuclear cells, in comparison to the control group (thin basement membrane disease, minimal change disease) (96). Glomerular NF-kappaB activation correlated with the number of monocytes/macrophages in the glomerulus and urinary macrophage inflammatory protein-1α (CCL3 in the systematic nomenclature for chemokines) levels, and decreased in a cohort (n=12) who were treated with glucocorticoids and underwent a second biopsy (96).

The activation of NF-kappaB (p50 and RelA) was also examined in patients with Classes III to V lupus nephritis (n=31) (81). RelA and p50 were increased in glomerular endothelial and mesangial cells, podocytes (RelA mainly) and infiltrating cells (81). As in nephrotic glomerular diseases, the expression of NF-kappaB (predominantly RelA) in podocytes correlated with proteinuria and TNFalpha/IL-beta1 co-localization (81). In a second study, the same authors found that glomerular NF-kappaB (and the expression of inducible nitric oxide synthase, an NF-kappaB-dependent gene) correlated with the induction of glomerular cell apoptosis in lupus nephritis whereas tubular NF-kappaB correlated with TEC proliferation (97). In addition, the tubular expression of

RelA was positively correlated (r=0.51, P=0.01) with the serum creatinine, but not with proteinuria (97).

These studies suggest that NF-kappaB is induced in intrinsic renal cells in humans with immune-mediated renal diseases, and may mediate proliferation as well as inflammation.

3.3. Effects of renal impairment on NF-kappaB activation in circulating monocytes

In CKDs, it has been postulated that the systemic alterations associated with chronic uraemia (even with mild reductions in glomerular filtration rate such as less than 30 and 60 ml per minute per 1.7.3m^2 of body-surface area) are linked to inflammation, insulin-resistance and oxidative stress, and may contribute to the heightened incidence of cardiovascular disease as well as renal injury progression (98, 99). Indeed, patients with kidney failure undergoing chronic haemodialysis have increased or altered levels of NF-kappaB-DNA binding activity in circulating peripheral blood mononuclear cells (100, 101). These data therefore suggest that the activation of NF-kappaB in circulating monocytes in uraemic patients may be a molecular link mediating not only renal injury, but possibly also cardiovascular disease.

4. ACTIVATORS AND PATHWAYS OF NF-kappaB ACTIVATION IN RENAL CELLS

4.1. Glomerular cells

4.1.1. Podocytes

The progressive loss of podocytes is thought to play a key role in the pathogenesis of focal segmental sclerosis glomerular lesion (which is one of the earliest histological manifestations of generic CKD) (102), and contribute to the genesis of proteinuria in progressive renal diseases (103). As discussed earlier, human studies, show that NF-kappaB and RelA are upregulated in podocytes in nephrotic glomerular disease that are characterized by podocytopenia. *In vitro* studies using murine podocytes suggest that a myriad of factors could upregulate NF-kappaB in podocytes under these circumstances including components of plasma proteins, angiotensin II, reactive oxygen species as well as bacterial components. For example, NF-kappaB was upregulated in podocytes exposed to either Shiga-toxin or protein-overload (IgG and albumin) and mediated the increase in endothelin-1 production (104, 105). Reactive oxygen species also upregulated GM-CSF in murine podocytes in a NF-kappaB dependent manner (106). Angiotensin II upregulated the expression of TLR4 in murine podocytes (as well as mesangial cells but not in TECs) (107).

With relevance to diabetes, advanced glycation end-products (CML) induced CCL2 and reactive oxygen species expression in murine podocytes by activation of the soluble receptor for advanced glycation end-products (RAGE) through an NF-kappaB-dependent mechanism that could be suppressed by a statin (108, 109). Hyperglycaemia also directly upregulated NF-kappaB in murine podocytes, and this could be suppressed by poly (ADP-ribose) polymerase inhibitors (110).

In contrast to inducing proinflammatory gene expression, NF-kappaB may also control “life” and “death” genes in podocytes (103). In murine podocytes, exposure to TGF-beta1 caused apoptosis by suppressing RelA/p50 through a Smad7-dependent mechanism (111). Separate experiments revealed that Smad7, but not TGF-beta1, specifically reduced NF-kappaB/Rel reporter gene activity and was also able to prevent the induction of NF-kappaB by other stimulants, such as TNFalpha (111). These data contrast with the role of NF-kappaB on podocyte survival in HIV nephropathy, a disease characterized by podocyte proliferation and de-differentiation, compared to classical primary nephrotic glomerular diseases and diabetes (103). Here, HIV gene expression is mutually dependent on persistent NF-kappaB activation *in vivo* (112, 113). In immortalized podocyte cell lines derived from HIV-transgenic mice, NF-kappaB directly mediated the expression of the pro-apoptotic system, Fas and Fas ligand (114). It is not clear if the differences in podocyte survival between TGF-beta1 stimulation and HIV gene expression are due to variations in NF-kappaB isoform synthesis. At least in murine podocytes expressing the HIV genome, p50, p52 RelA, RelB and c-Rel were all present, and p50/RelA was the predominant activated NF-kappaB isoform, with IkappaBalpha the main regulatory IkappaB protein. In contrast, alterations in IkappaBbeta or the non-canonical pathway were not detected (113, 114).

4.1.2. Mesangial cells

Mesangial cell injury leads to proliferation, proinflammatory gene expression and matrix deposition, all of which are critical processes in the two most common causes of CKD - diabetic kidney disease (115) and IgA nephropathy (116). Not surprisingly, the mesangial cell has been the most frequently studied intrinsic renal cell in NF-kappaB signalling research, with over 150 references to date and a previous review devoted to this topic specifically (117). Human mesangial cells contain all Rel proteins (118, 119). NF-kappaB activation is expressed constitutively in mesangial cells (120), and increased in response to a wide range of stimulants, including cytokines (IL-1beta, TNFalpha), immunoglobulins (including IgA), cyclic adenosine 3',5'-monophosphate, angiotensin II, aldosterone, mechanical stretch, high glucose, monoclonal light chains, contact with platelets, advanced glycosylation endproducts, C-reactive protein, TGF-beta1, soluble products of activated macrophages, anti-DNA antibodies and adiponectin (121-135). Mesangial cell-induced NF-kappaB activation positively regulated the gene expression of proinflammatory mediators (CCL2, iNOS, matrix metalloproteinase, intercellular adhesion molecule-1) and proliferation through activation of the PI3K/Akt pathway (125, 133, 136, 137). On the other hand, as in podocytes, inhibition of NF-kappaB induction, for example with exposure to 25 mM glucose or with corticosteroids, may lead to apoptosis in mesangial cells (138, 139). Though, in contrast to the podocyte, the latter could be beneficial in certain renal diseases (139).

4.1.3. Glomerular endothelial and parietal epithelial cells

Glomerular endothelial cells are of great importance in the pathogenesis of CKD (140). They regulate glomerular capillary lengthening, vascular thrombosis and pro-inflammatory gene production. The role of NF-kappaB in glomerular endothelial cells, however, has received much less attention than the other intrinsic glomerular cells. *In vitro* studies suggest that the activation of NF-kappaB mediated apoptosis and the transendothelial leukocyte migration through the local transcription of chemokine genes (141, 142). In contrast, chronic shear stress suppressed NF-kappaB activation (143), which might predict a possible adverse effect on cell survival (as seen in podocytes), though this remains to be tested. Although, NF-kappaB activation is increased in parietal epithelial cells *in vivo*, the role of NF-kappaB in these cells *in vitro* has (to our knowledge) not been published, though this is likely to change in the future with improved techniques to isolate this cell becoming available (144).

4.2. Cortical tubular epithelial cells

4.2.1. Pro-infammatory gene expression and stimulants

The cortical TEC is an active producer of proinflammatory proteins, and plays a central in the pathogenesis of tubulointerstitial inflammation and fibrosis in CKD (145). Several stimulants that classically induce NF-kappaB in other cell types do the same in TECs. However, the study of the effects of plasma proteins on NF-kappaB activation in TECs, is unique to research in Nephrology, and deserves discussion.

It is well known that nephrotic-range proteinuria is an indicator of poor renal prognosis (10). One hypothesis (the proteinuria hypothesis), to explain these clinical observations is that glomerular injury/capillary wall hypertension leads to the increased presence of plasma proteins in the tubular lumen, and that these cause TEC injury and activation (10). *In vitro,* the exposure of TECs to nephrotic-range concentrations of plasma proteins has been shown to upregulate proinflammatory cytokines and chemokines (146, 147). In the LLC-PK_1 cell line (a porcine TEC line), DNA binding activity to the classical kappaB-site on the murine Igkappa enhancer region, was induced in nuclear extracts within 30 minutes of exposure to nephrotic range concentrations of bovine serum albumin (BSA, 10 to 30 mg/ml) (146). Supershift analysis demonstrated that the nucleoprotein complex consisted of p50/RelA, p50/c-Rel, RelA/RelA and RelA/c-Rel heterodimers. The early upregulation of NF-kappaB preceded the induction of the kappaB-dependent chemokine. Prophylactic treatment with the antioxidant NF-kappaB inhibitor, pyrrolidine dithiocarbmate (PDTC), reduced albumin-induced activation of CCL5 (by 20 to 63%). In primary cultured rat TECs, continuous exposure to BSA (2.5. to 15 mg/ml) also induced NF-kappaB activation. NF-kappaB activation (p50/RelA, p50/p50, p50/c-Rel) was maximal at 8 hours, and sustained for at least 16 hours following continuous exposure.

These alterations in NF-kappaB were correlated with a reduction in IkappaBalpha protein expression (evident within 30 minutes of exposure), and re-synthesis occurred 4 to 8 hours later. In contrast, IkappaBbeta protein levels were reduced at 2 hours and did not recover during the time-points of the study. Again, in this study, NF-kappaB activation was correlated with the up-regulation of a kappaB-dependent chemokine, CCL2. Prophylactic treatment with anti-sense oligonucleotides to RelA or mutation of kappaB-binding sites on the rat CCL2 promoter, prevented albumin-induced CCL2 mRNA expression (147). In the NRK52E rat TEC line, albumin-induced NF-kappaB activation was was maximal at 30 minutes and declined by 60 minutes of continuous exposure (148). NF-kappaB up-regulation was suppressible by lysine (an inhibitor of protein and albumin uptake), an angiotensin type II receptor antagonist and/or an endothelin-1 antagonist (148). In contrast, in parallel experiments, Zafiriou and colleagues demonstrated that LDL and albumin induced both CCL2 and TGFbeta1 in HK-2 TECs, but only that of albumin was dependent on NF-kappaB (149).

The mechanisms and proximal signal transduction pathways by which serum proteins induce NF-kappaB activation in TECs (particularly the potential role of the multi-ligand receptors, cubilin and megalin, as well the TLRs) are not known. One possibility is that the accumulation of excess proteins within the endoplasmic reticulum (ER) membrane, causes release of calcium and generation of reactive oxygen species (ROS) resulting in the degradation of IkappaB and NF-kappaB nuclear translocation (10). The latter, designated the ER response, was first noted in T-cells overloaded with excess viral proteins (150), and more recently, thought to be involved in other processes such as cholesterol loading of macrophages (151). Alternatively, protein-induced NF-kappaB activation in TEC may be the consequence of cellular injury and generation of ROS, caused by excess lysosomal swelling and rupture, with cytoplasmic spillage of noxious enzymes.

In addition to plasma proteins, pro-inflammatory cytokines (IL-1beta, TNFalpha, platelet activating factor; CCL2; IL-17; interferongamma, growth factors (such as PDGF)), anigotensin II, glycated albumin, hyperglycaemia, hypoxia and receptor ligands (CD-40 ligand) activate NF-kappaB and induce pro-inflammatory gene expression in TECs (152-156). In cultured mouse cortical TECs, intermediate molecular weight (but not high molecular weight) fragments of hyaluronan increased NF-kappaB and AP-1 activation, followed by the up-regulation of kappaB-dependent adhesion molecules (ICAM-1 and VCAM-1) (157). Bacterial and viral products such as LPS; human immunodeficiency virus and leptospira as well as nephrotoxins also cause NF-kappaB activation in TECs (158-161).

4.2.2. Role of NF-kappaB in TEC survival and proliferation

The loss of TECs through apoptosis contributes to tubular atrophy, which is a consistent histopathological feature of CKD and predictor of poor renal prognosis (162). *In vitro* studies suggest that NF-kappaB activation may be pro- or anti-apoptotic, depending on the cell type, stimulus

and kappaB-dependent genes that are induced (23). Inhibition of NF-kappaB activation in LLC-PK_1 cells by a transdominant IκBα adenovirus, exacerbated TNFalpha-induced cytoxicity and apoptosis (163). Furthermore, prevention of cadmium-induced TEC apoptosis, required the upregulation of the multi-drug resistance P-glycoprotein through a kappaB-dependent mechanism (161). A similar anti-apoptotic role for NF-kappaB was in TECs exposed to doxorubicin (164). Furthermore, induction of NF-kappaB in TECs by CD40L induced the expression of both pro-inflammatory (reactive oxygen species) as well as anti-apoptotic genes (heme oxygenase 1) (165). The foregoing data, therefore raise the question as to whether it will be possible to separate the pro-inflammatory responses of NF-kappaB from its effects on apoptosis. NF-kappaB may also mediate the proliferation of TECs, possibly through the up-regulation of cyclin D1 in response to epidermal growth factor and albumin (166 and unpublished observations).

4.3. Renal fibroblasts

Collagen-secreting alpha-smooth muscle actin (SMA)-positive fibroblasts (myofibroblasts) are the principal producers of extracellular matrix in renal fibrosis (167-169). The mechanisms of myofibroblast accumulation in the kidney are primarily due to the migration, proliferation and activation of local fibroblasts in response to chronic inflammation (approximately 50% of the total population), and secondarily due to trans-differentiation from TECs (approximately 35% of the total population) (170). The remainder of fibroblast accumulation could also be due to transdifferentiation from endothelial cells and/or circulating blood-borne fibrocytes (171). The direct role of NF-kappaB in the fibroblast accumulation in CKD is currently unclear. As a key regulator of chronic inflammation one can hypothesise that NF-kappaB indirectly regulates fibroblast accumulation through paracrine effects of infiltrating inflammatory models (172), as shown in animal models of ureteric obstruction using inhibitors of angiotensin or TNF recepetor mice (discussed in Section.5.4.) (172-174). However, on the other hand, the direct role for NF-kappaB in fibroblasts in CKD has not been definitively delineated. Data from other organs suggests a potential role for NF-kappaB. For example, overactivity of NF-kappaB reduced the ability of fibroblasts to form a myofibroblast phenotype in neonatal rat lung fibroblasts and in the liver, NF-kappaB promoted the proliferation of human myofibroblastic hepatic stellate cells (175, 176).

5. INSIGHTS REGARDING NF-kappaB FROM ANIMAL MODELS OF CKD

5.1. Podocytopenia-associated proteinuria

The progressive loss of podocytes is one of the factors involved in the pathogenesis of proteinuria (103), but the specific role of NF-kappaB in this process remains inconclusive. In the autologous phase of rodent Heymann nephropathy, NF-kappaB (p50) was increased in podocytes and proteinuria was reduced by the administration of PDTC, possibly through a reduction in MMP-9 (177). Multiple aetiological agents (puromycin, LPS, immunological) also upregulated B7-1 *via* TLR4 and mediated actin skeletal organization and proteinuria *in vivo (*178). On the other hand, in toxin-induced rat models of focal segmental glomerulosclerosis induced by either puromycin or adriamycin, pharmacological studies suggest that NF-kappaB does not have a role in mediating proteinuria (179, 180).

5.2. Hypertension-induced renal injury

Hypertension is strongly associated with progression to end-stage kidney failure, and the role of the vasoactive peptides (angiotensin) in this process are well-known (10, 50, 91). The significance of NF-kappaB in hypertension-induced renal injury was recently addressed in a novel *in vivo* study by Henke and co-workers (181). In this study, hypertension was induced in mice with a degradation-resistant mutant IkappaBalpha protein that was conditionally expressed only in endothelial cells, using a Tie-1 reporter and Cre/Lox approach. At equivalent levels of blood pressure, mice in which NF-kappaB was suppressed in endothelial cells had partial reductions in proteinuria, tubulointerstitial fibrosis, vascular sclerosis and interstitial inflammation as well as attenuation of renal expression of TNFalpha and VCAM-1. These studies highlighted for the first time the important role of hypertension-induced endothelial cell NF-kappaB activation in directly mediating the cellular events associated with CKD progression.

Interestingly, some evidence suggests that NF-kappaB could also directly mediate hypertension. In rats with CKD induced by 5/6 nephrectomy, chronic inhibition of NF-kappaB with PDTC reduced systemic, but not intraglomerular hypertension (182). The mechanisms of these effects and whether this might be due to alterations of vasoactive peptides by NF-kappaB (endothelin and angiotensin) remains unclear.

5.3. Hyperglycaemia-induced renal injury

The expression of NF-kappaB has been examined in a number of animal models of diabetes. Recent data by Starkey and colleagues, reveal unexpected alterations in the canonical and non-canonical pathways, and their study is an example of the current need for further basic data regarding NF-kappaB in kidney diseases (36). These authors undertook a detailed examination of NF-kappaB/Rel protein composition in cytosolic and nuclear fractions of the kidney in 3-month *db/db* mice. Interestingly, the amount of canonical NF-kappaB/Rel proteins (RelA, p50, IkappaBalpha, IkappaBbeta), were unchanged in the cytosol but reduced in the nuclear fraction. In contrast, non-canonical NF-kappaB/Rel proteins (NIK, RelB, IKKalpha) were increased in the cytosolic fraction but reduced in the nuclear fraction. DNA-binding activity, however, was increased and consisted of predominantly p52-containing proteins (36).

5.4. Sterile renal inflammation

Sterile renal inflammation without the presence of infectious agents is a common feature of all types of progressive CKD, and is a consequence of parenchymal cell injury. Consistent with its known pro-inflammatory effects, inhibition of NF-kappaB activation with

Table 5. Differential effects of ACEI, AT_1 RA and AT_2 RA in animals with unilateral ureteric obstruction (173)

	ACEI [1]	**AT_1 RA[2]**	**AT_2 RA[3]**
	Enalapril	SC-51316	PD-123319
NF-kappaB inhibition	Yes	Yes	Yes
Interstitial monocyte	↓↓	No Δ	No Δ
Interstitial myofibroblast	↓↓	↓↓	↑
Interstitial collagen	↓↓	↓↓	↑
CCL2 mRNA	↓	↓	?
ICAM-1 mRNA	No Δ	No Δ	?
VCAM-1 mRNA	↓	No Δ	?
Tubular apoptosis	?	?	↓↓

Abbreviations: [1]ACEI, angiotensin converting enzyme inhibitor; [2]AT_1 RA, angiotensin II type 1 receptor antagonist; [3]AT_2 RA, angiotensin II type 2 receptor antagonist

angiotensin II inhibitors (ACEI, AT_1 RA) or other pharmacological agents (L-arginine, *N*-acetylcysteine, PDTC) was correlated with a reduction in interstitial monocyte accumulation in several animal models of CKD, including unilateral ureteric obstruction (UUO), immune complex nephritis, remnant kidney model, adriamycin nephropathy and rats transgenic for the human renin and angiotensin genes (52,123,180,183-185). Interestingly, pharmacological inhibitors of ACE and angiotensin II, type 1/2 receptors may differ in with respect to their effects on tubulointerstitial injury, despite the collective inhibition of NF-kappaB activation (Table 5) (173). Again, it would be interesting to see if these agents have specific effects on the formation of selected NF-kappaB isoforms (53, 184, 186).

More direct *in vivo* evidence supporting a pro-inflammatory role for NF-kappaB in CKD has been provided through specific molecular approaches and pharmacological inhibition. The prophylactic molecular inhibition of NF-κB activation with decoy oligonucleotides (against the kappaB-binding site) reduced interstitial monocyte accumulation in rats with allogenic renal transplants and crescentic glomerulonephritis (187,188). Similarly, Takase and colleagues reported that prophylactic adenovirus-mediated gene transfer of a truncated IkappaBalpha prevented interstitial monocyte accumulation in rats with protein overload nephropathy (189). By drug-inhibition, an 8-week treatment with an orally active IKKbeta inhibitor (AS602868) reduced the number of CD45+ interstitial cells and renal injury as well as serum levels of CCL5, in a murine model of chronic HIV nephropathy (190).

In a recent study, Wilson and co-workers examined the cell-specific effects of NF-kappaB activation specifically in macrophages by transfecting them with a dominant-negative IkappaBalpha repressor (191). *In vitro,* transfected macrophages had reduced production of nitric oxide, IL-12 and TNFalpha in response to LPS, whereas IL-10 production was enhanced. Furthermore, in a rat model of nephrotoxic serum nephritis, a single injection of transfected macrophages into the renal artery 24 hours after induction of disease led to a reduction in glomerular necrosis, glomerulosclerosis and crescent formation on day 6; and a transient early reduction in proteinuria without an effect on glomerular cellularity (191). These data suggest an important role for macrophage-associated NF-kappaB activation in mediating inflammatory glomerular injury associated with hypertension.

5.5. Renal fibrosis

Many of the models in which NF-kappaB inhibition showed an anti-inflammatory effect, were also associated with improvements in renal fibrosis. For example, inhibition of NF-κB activation (with either enalapril; TNFR-null mice or AT_1-null mice) in the UUO model reduced interstitial collagen IV deposition and alpha-SMA accumulation (173,174,184). Moreover, chronic treatment with PDTC for more than 90 days also attenuated renal fibrosis in the remnant kidney model (182). More direct evidence using molecular methods to suppress NF-kappaB activation have also showed the same the results (181,187-189, 191). Inhibition of NF-kappaB activation by Smad7-gene therapy also reduced injury in rats with remnant kidney and crescentic glomerulonephritis (192,193). However, it remains unclear as to whether these anti-fibrotic effects were because of a reduction in inflammation or a consequence of a direct effect on fibrogenesis, such as extracellular matrix protein remodelling, myofibroblast survival and/or changes in epithelial-mesenchymal cell transition (194). The specific modulation of NF-kappaB in myofibroblasts using conditional and non-conditional knockout studies are needed to answer these questions in CKD models (172,195).

5.6. Renal cell apoptosis

The divergent effects of NF-kappaB on survival in cultured renal cells are also revealed in *in vivo* models of CKD. As in the cell culture studies of podocytes, TGFbeta1 transgenic mice develop progressive glomerulosclerosis preceded by podocyte apoptosis (111). The latter was associated with upregulation Smad7 in damaged podocytes along with the concurrent inhibition of NF-kappaB (111). In contrast, HIV-transgenic mice develop severe focal glomerulosclerosis in which persistent NF-kappaB activation has pro-apoptotic properties, due to the up-regulation of Fas and Fas ligand in podocytes and other renal epithelia induces apoptosis (114).

6. THERAPEUTIC APPROACHES TO MODULATE NF-kappaB IN HUMANS WITH CKD

More than 750 synthetic and naturally occurring compounds currently exist that, in part, mediate their effects through the modulation of NF-kappaB activation (3, 196). Inhibitors may be classified as antioxidants, proteasome and protease inhibitors, IkappaBalpha

phosphorylation and/or degradation inhibitors and miscellaneous agents (196). Many of the listed pharmacological componds include agents proven for clinical use as renoprotectants in humans with CKD (10,16,50). These agents include ACE inhibitors and AT_1 receptor antagonists, glucocorticoids (GCs), HMG CoA reductase inhibitors and erythropoietin-stimulating agents (16, 40, 50, 197-199). Some evidence is already available that confirms that these conventional agents modulate NF-kappaB (albeit indirectly) *in vivo* in humans with diabetic kidney disease (89).

Although, one can therefore speculate that conventional therapy that patients are currently already receiving modulate NF-kappaB, it remains to be seen whether specific and targeted modulation of the NF-kappaB pathway will be beneficial to humans with CKD. Specific inhibitors of NF-kappaB are not yet in clinical use, but are in development (196). As discussed, several experimental approaches are available to specifically manipulate renal NF-kappaB activation *in vivo*, including administration of phosphorothioate antisense oligonucleotides (although this is associated with nephrotoxicity); transfection of a *cis*-element decoy oligonucleotide that binds to a specific kappaB-binding site or an IkappaBalpha mutant; the use of RNA aptamers or peptides that bind to NF-kappaB isoforms and prevent them binding to duplex DNA (40, 187-189, 200-202). However, these approaches are not readily suitable for modulation of NF-kappaB in humans with CKD. Perhaps, the most promising approach to manipulate NF-kappaB *in vivo* at present include inhibitors of IKK, which are presently in preclinical development (190). Another potential approach is to modulate inflammation and fibrosis by manipulation of TLRs using specific inhibitors (57, 203, 204).

7. CONCLUSION AND PERSPECTIVES

This review has attempted to highlight the current complexity of the NF-kappaB signalling pathway. There is no doubt that NF-kappaB isoforms have a central role in the pathogenesis of chronic inflammation and cell survival associated with CKD. However, there are several gaps in our understanding of them in the kidney at the present time, and there is still much work to do before NF-kappaB modulatory therapies can be taken to the clinic to treat the patient with CKD. First, the cell- and isoform- specific biological roles of NF-kappaB in CKD are only beginning to be understood and the direct role of NF-kappaB in mediating fibrosis needs further study (181, 191). Second, non-specific NF-kappaB blockade might reduce proinflammatory gene expression but promote the apoptosis of podocytes or TECs, and a detailed understanding of the cross-talk that occurs between signalling pathways induced by specific isoforms would be useful (205). Thirdly, given its important role in normal immune function, ideally NF-kappaB should be modulated in a cell- and time-specific manner. In this regard, new methods of intracellular drug-delivery are becoming a reality and could overcome such challenges in a chronic disease process such as CKD (206). The next decade therefore requires further fundamental research to be undertaken to address how to "fine-tune" the NF-kappaB signalling pathways. Studies using conditional murine knockouts and development of more detailed experimental questions along with bioinformatic analysis of the genes affected by these molecular manipulations will be very important. In addition, identification of specific targets identified through these approaches will allow the development of specific pharmacological and molecular inhibitors that are able to be used safely in humans with CKD (16, 207).

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