

REVIEW

Calcineurin inhibitors: a double-edged sword

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Abstract

Recently, research has directed its interests into identifying molecular pathways implicated in calcineurin inhibitor (CNI)-induced renal fibrosis. An emerging body of studies investigating calcineurin (CnA) activity has identified distinct actions of two main ubiquitously expressed isoforms: CnA α and CnA β . CNIs have the capacity to inhibit both of these CnA isoforms. In the kidney, CnA α is required for development, whereas CnA β predominantly modulates the immune response and glomerular hypertrophic signaling powered by activation of the transcription factor, nuclear factor of activated T lymphocytes (NFAT). Interestingly, data have shown that loss of CnA α activity contributes to the expression of profibrotic proteins in the kidney. Although this finding is of great significance, follow-up studies are needed to identify how loss of the CnA α isoform causes progressive renal damage. In addition, it is also necessary to identify downstream mediators of CnA α signaling that assist in upregulation of these profibrotic proteins. The goal of this review is to provide insight into strides taken to close the gap in elucidating CnA isoform-specific mechanisms of CNI-induced renal fibrosis. It is with hope that these contributions will lead to the development of newer generation CNIs that effectively blunt the immune response while circumventing extensive renal damage noted with long-term CNI use.

calcineurin inhibitors; calcineurin isoforms; renal fibrosis

INTRODUCTION

The use of calcineurin inhibitors (CNIs) in the prevention of organ graft rejection posttransplantation has dramatically improved graft and patient survival since its introduction into clinical practice in 1980 (1, 2). Yet despite the advantages of graft and patient survival, solid organ transplant recipients face consequences of long-term immunosuppression on a variety of organ systems, particularly the kidneys. Detailed histopathological analyses describe irreversible damage by CNI treatment with fibrotic lesions noted within the vessels (arteriolar hyalinosis) (3), glomeruli (glomerulosclerosis) (4, 5), and between tubular epithelial cells (tubulointerstitial fibrosis). Mechanisms for this phenomenon remain unclear, and there are currently no therapeutic strategies in place to mitigate this phenomenon. Even though physiological roles of calcineurin signaling have been investigated extensively, less is known about specific functions of the main isoforms. Particularly, it remains better to be understood how calcineurin signaling and fibrotic pathways intersect in the kidneys and which calcineurin isoforms serve as targets of CNI therapy.

This review provides clues for future studies that address this gap in knowledge. First, this article briefly provides an overview of CNI discovery and integration into clinical practice, followed by relevant studies demonstrating CNIs to be nephrotoxic. Then, the focus will address the growing body of knowledge characterizing the presence of multiple active calcineurin isoforms, which have been found to signal

independently of each other. Next, evidence will be introduced demonstrating that loss of the alpha catalytic isoform of calcineurin (CnA α) promotes expression of key fibrogenic proteins in the kidney. Lastly, this review article will conclude with insight inspiring future studies identifying the renal CnA isoform responsible for CNI nephropathy. Moreover, identification of downstream mediators of CnA α signaling will advance understanding of how calcineurin signaling and fibrogenic pathways intersect in the kidneys. This endeavor, once completed, will advocate for the development of future CNIs that preserve CnA α function, ultimately reducing renal side effects due to CNI toxicity.

CALCINEURIN INHIBITORS: MEDICINE OF THE EARTH

With its incorporation into clinical practice in the early 1980s, CNIs quickly became the cornerstone of immunosuppressive therapy post organ transplantation. The first and prototype of its class, cyclosporine A (CsA), was isolated from the soil fungus *Tolypocladium inflatum* in 1970 by Sandoz Laboratory (now Novartis) scientists, Dr. Sandor Lazary and Dr. Jean-Francois Borel (6). Successful preliminary human studies fast-tracked its immediate widespread use in renal transplant recipients in 1980 (7). Upon dosage adjustments, initial reports citing its lethal side effects decreased with posttransplantation survival rates increasing dramatically. In 1985, *Streptomyces tsukubaensis*, another soil microbe,



provided an additional immunosuppressive compound for scientists at Fujisawa Pharmaceuticals in Japan. Its initial name FK-506 was later renamed Tacrolimus (acronym Tsukuba macrolide immunosuppressive). Owing to its milder range of side effects and better efficacy, it largely outpaced CsA in success, with majority of solid organ transplant recipients placed on tacrolimus postoperatively (8, 9).

CALCINEURIN INHIBITORS: MECHANISM OF IMMUNOSUPPRESSION

Studies attributed CsA and tacrolimus' immunosuppressive effects to diminished T cells activity via inhibition of calcineurin (6, 10). Calcineurin is a Ca^{2+} -dependent serine/threonine protein phosphatase that is a critical component of immune system regulation. To achieve their immunosuppressive effect, CNIs bind with high affinity to a class of cytosolic protein receptors called immunophilins: cyclophilins in the case of CsA and the FK-binding protein-12 in the case of tacrolimus (Fig. 1) (11). CNI-immunophilin complexes then bind calcineurin to sterically inhibit its intrinsic phosphatase activity (11). In T cells, calcineurin inhibition prevents dephosphorylation and subsequent activation of the transcription factor nuclear factor of activated T cells (NFATc). NFAT inactivity leads to decreased synthesis of interleukin-2 (IL-2), a key cytokine required for T cell activation in amplification of the immune response (9, 12).

TROUBLE IN PARADISE: RENAL DAMAGE

The initial success of CNIs in transplant medicine was quickly followed up with early clinical and animal studies supporting evidence of CNI nephropathy, leading to renal failure

in some cases (2, 13). In one group of patients, CNI nephropathy was observed as early as 3 mo postoperatively (14), with increased prevalence in 20%–25% of patients at 1 yr (14, 15) and 50% at 2 yr. In a cohort of patients assessed using a chronic allograft damage index, fibrosis was present in 70%–85% of grafts at 1 yr, making it the most common feature of nephropathy (16, 17). A histological analysis of kidneys from extrarenal transplant recipients demonstrated that many areas of the kidneys were irreversibly affected by CNI treatment, including the tubulointerstitium (interstitial fibrosis) and glomeruli (fibrosis of Bowman's capsule and focal segmental/global glomerulosclerosis) (18). These fibrotic lesions, at least partially attributable to CsA nephrotoxicity, were seen in virtually all histological sections 10 yr after transplantation (19). These structural and functional changes were not only exclusive to long-term use of CsA but also were seen with tacrolimus treatment. CNI-induced nephropathy was later characterized as progressive and irreversible deterioration of renal function associated with fibrosis and tubular atrophy (13, 18).

A MOLECULAR CULPRIT: TRANSFORMING GROWTH FACTOR- β

Upregulation of the proinflammatory cytokine and potent stimulator of fibrosis transforming growth factor- β (TGF- β) is known to be a major contributor to this fibroproliferative disease (20, 21). Anti-TGF β antibody staining of renal biopsy samples revealed higher levels of active TGF- β in patients on CsA therapy than in patients receiving tacrolimus (17, 22). Khanna et al. (23) found that renal biopsy samples from patients diagnosed with tacrolimus nephrotoxicity also had increased mRNA expression of TGF- β , fibronectin, and collagen, additional contributors to fibrosis. CNIs have also been

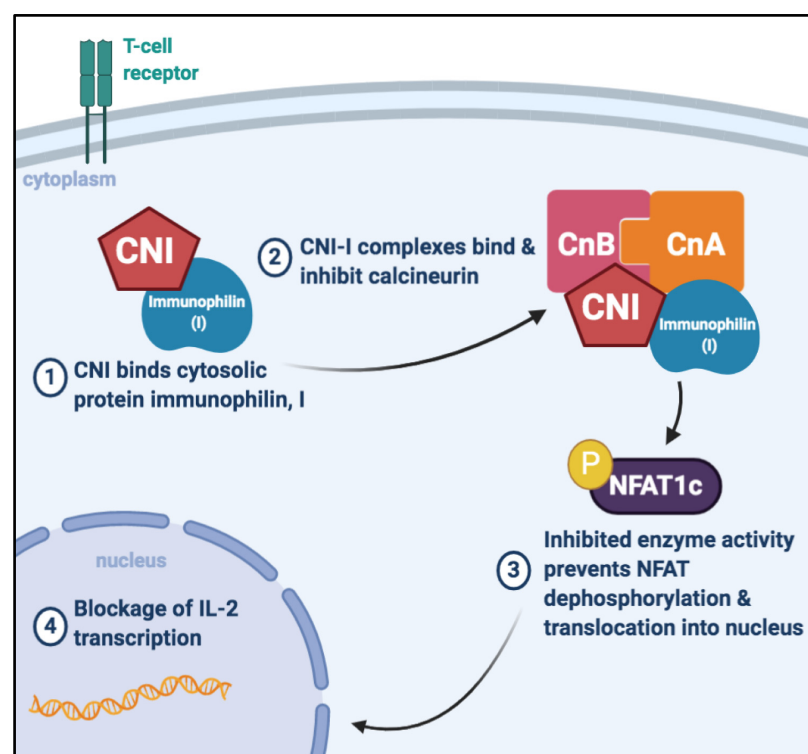


Figure 1. Calcineurin inhibitors (CNIs) blunt the immune response by inactivating calcineurin phosphatase activity. CNIs bind to cytosolic proteins called immunophilins (I) (step 1) enabling the CNI-I complex to bind calcineurin active site, thereby inhibiting phosphatase activity (step 2). Upon inhibition, transcription factors such as nuclear factor of activated T cells (NFAT) are unable to become activated by dephosphorylation (step 3). This prevents NFAT translocation into the nucleus to increase IL-2 transcription (step 4), thereby blunting immune activation. [Image created with BioRender.com.]

reported to increase in vitro and in vivo TGF- β expression and receptor activity in experimental models (24, 25). Molecular mechanisms of TGF- β regulation have been proposed, with microRNA expression emerging as suspected culprits. MicroRNAs, short noncoding RNAs that regulate protein-coding RNA expression, have been previously linked to renal fibrosis (26). Differential regulation of microRNAs regulating TGF- β signaling genes have also been identified by Gooch et al. (27). Among 76 differentially expressed microRNAs, 16 microRNA/mRNA clusters were identified that regulate genes involved in the TGF- β signaling pathway in CsA-treated mice (27). This study identified microRNAs previously linked to renal fibrosis that includes let-7d, miR-21, miR-29, miR-30, miR-130, miR-192, and miR-200, as well as microRNAs that have not been reported to be related to nephropathy or immune suppression. Pathway analysis of microRNA/mRNA changes highlights the TGF- β , Wnt, mTOR, and VEGF signaling pathways (27). It is unclear if tacrolimus alters microRNA levels in a similar manner as CsA. Additional mechanisms of TGF- β regulation, extracellular matrix accumulation and fibrosis following calcineurin inhibition remain better to be understood.

A TALE OF TWO MAIN ISOFORMS: CnA α AND CnA β

Investigation of the distinct calcineurin isoforms that signal independently of one another will offer additional

insight into CNI nephrotoxicity. Full catalytic activation of calcineurin requires formation of a Ca²⁺/calmodulin-dependent subunit heterodimer consisting of catalytic subunit A (CnA) and regulatory subunit B (CnB) (28). In 1989, Kuno et al and Kincaid (11, 31) published reports discerning the existence of three major calcineurin A isoforms expressed in humans: CnA α , CnA β , and CnA γ (29, 30). CnA γ is mainly enriched in the testis and brain, whereas CnA α and CnA β exist in most, if not all, organs and tissues (29, 30).

Although separate genes control the expression of these isoforms, PPP3CA (CnA α), PPP3CB (CnA β), and PPP3CC (CnA γ), it is unknown whether these genes arose from duplication. Amino acid sequences of CnA α and CnA β are 81% identical, with these sequences being highly conserved throughout evolution (28). The most striking difference between the catalytic subunit CnA α and CnA β isoforms is the amino acid proline-rich region located in the N-terminus of CnA β . It was later revealed that the proline-rich N-terminal sequence of CnA β promotes substrate binding (31).

Although these isoforms share a high degree of sequence homology, they contribute to a more precise regulation of diverse calcineurin functions in different tissues (26). To distinguish roles of CnA α and CnA β in T cell activation, Zhang (32) utilized CnA α ^{-/-} mice lacking a functional CnA α gene. When this isoform was ablated in mice, no substantial deficiency in T cell receptor (TCR)-mediated IL-2 production was observed, raising the suspicion that CnA β mediates TCR signaling (32). CnA β was later confirmed to play a critical

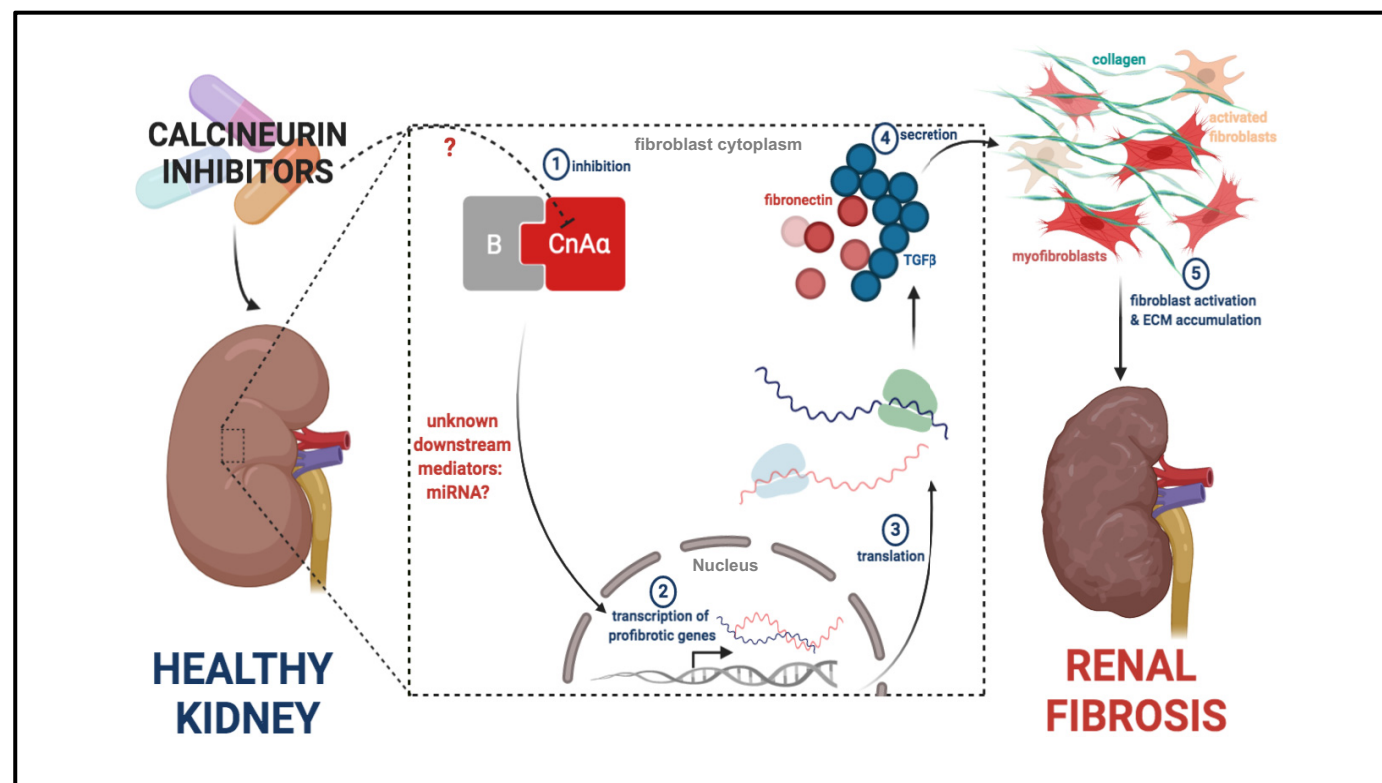


Figure 2. Calcineurin inhibitor (CNI)-induced nephropathy may be isoform-specific. Loss of the CnA α isoform reproduces features of cyclosporine nephrotoxicity, causing histopathological changes including matrix expansion. Further studies are necessary to confirm whether CNIs specifically inhibit the CnA α isoform (step 1), altering expression of downstream mediators that promote profibrotic gene expression and secretion (steps 2–4). CNI-induced secretion of profibrotic proteins (such as TGF β and fibronectin) activates resident fibroblasts to differentiate into contractile myofibroblasts capable of secreting additional extracellular matrix proteins that accumulate (step 5), ultimately causing renal fibrosis. [Image created with BioRender.com.]

role in T cell development, whereas CnA α was shown to mediate the antigen-specific T cell response (32, 33).

Data have shown CnA α to be the predominant active isoform expressed in the kidneys (34). In primary CnA $\alpha^{-/-}$ renal fibroblasts, nuclear translocation of the calcineurin substrate NFAT ϵ is unaffected, but it fails to occur in CnA $\beta^{-/-}$ fibroblasts (35). In addition, Gooch et al. (34) found CnA α to be essential in renal development, as CnA $\alpha^{-/-}$ mice on a mixed genetic background experienced kidney failure, with death occurring within weeks after birth. In addition to the kidney, there are isoform-specific differences in several other systems including the skin, where loss of CnA α results in increased apoptosis of squamous epithelial cells and salivary glands, where CnA α is required for vesicle transport (5, 36).

Upregulation of the CnA β isoform was shown to promote kidney hypertrophy, a feature of diabetic nephropathy (37, 38). This evidence was provided when Reddy et al. (37) induced type I diabetes in both wild-type and CnA $\beta^{-/-}$ mice. They found that there was a significant increase in kidney hypertrophy in wild-type mice as early as 1 wk after the induction of diabetes. However, there was no increase in whole kidney hypertrophy in diabetic CnA $\beta^{-/-}$ mice. After several weeks, both wild-type and CnA $\beta^{-/-}$ mice demonstrated significant whole kidney hypertrophy. However, the degree of hypertrophy was significantly less in diabetic CnA $\beta^{-/-}$ mice compared with diabetic wild-type. Using kidney fibroblasts lacking CnA α or CnA β , in vitro findings confirmed that the CnA β isoform is required for high glucose-induced cellular hypertrophy (38). Taken together, findings indicate that CnA isoforms possess distinct functions.

In addition to studies demonstrating these isoforms having distinct tissue-specific roles, Kilka et al. provided evidence that CnA α , CnA β , and CnA γ each confer substrate specificities (31). Utilizing a comparative kinetic analysis of the dephosphorylation of five specific calcineurin substrates: NFAT, DARPP-32, Elk-1, Tau, and RII peptide, their results revealed that the substrate preferences of the isoforms may contribute to distinct physiological functions. Additional supporting data demonstrate that CnA β utilizes its downstream target NFAT in kidney hypertrophy signaling in response to hyperglycemia (38).

Gooch et al. (39) identified CnA α as a potential key player in CNI-induced renal fibrosis. They found that loss of this isoform reproduces features of CsA nephrotoxicity in vivo and in vitro. Particularly, loss of CnA α in vivo results in histopathological changes including matrix expansion, whereas loss of the β isoform does not (39). Consistent with their in vivo findings, CnA $\alpha^{-/-}$ renal fibroblasts exhibited increased fibronectin and TGF- β protein expression also similar to what is seen in CsA nephrotoxicity (39). They found that CnA $\alpha^{-/-}$ cells had higher basal levels of fibronectin transcription activity compared with wild type cells and that administration of neutralizing TGF- β antibodies did not reduce fibronectin protein levels in CnA $\alpha^{-/-}$ renal fibroblasts. Another study demonstrated that both CsA treatment and loss of CnA α are accompanied by a significant increase in metalloproteinase-9 (MMP-9) expression and activity in renal fibroblasts (40). MMP-9 works in conjunction with TGF- β to promote extracellular matrix remodeling, which also contributes to fibrosis. The additional molecules acting

upstream of TGF- β and MMP-9 to induce renal fibrosis currently remain a mystery. Although these pivotal studies paved the way into a better understanding of calcineurin signaling and fibrosis, unexpected findings leave important questions to be answered.

FUTURE DIRECTIONS

CNI-induced hypertension is a well-known adverse effect of CNI therapy that indirectly contributes to renal damage. Vasoconstriction, sympathetic excitation, and sodium retention by the kidney have all been proposed in contributing to this form of hypertension (41). Hoorn et al. (42) have shown that CNIs increase the activity of the thiazide-sensitive sodium chloride cotransporter through an effect on the kinases WNK and SPAK. Future studies are needed to conclusively determine which calcineurin isoform contributes to this phenomenon. Recently, Borschewski et al. (43) demonstrated that loss of CnA β activity increases the abundance and activity of the Na $^{+}$ -K $^{+}$ -2Cl $^{-}$ cotransporter (NKCC2) in rats. NKCC2 overactivity is known to promote electrolyte (and water) retention, leading to hypertension (43). Further, CNIs downregulate the sodium-bicarbonate cotransporter-1 (NBCn1) expression in the epithelial cells of the medullary thick ascending limb, thereby causing acid-base disturbances that also promote renal dysfunction (44). It is currently unknown which calcineurin isoform is involved in regulating NBCn1 expression or activity, underscoring the need for further investigation.

There has been no published information regarding sex differences in outcomes using CNIs, which currently remains a gap in the field. In addition, it remains to be known whether there exist any single-nucleotide polymorphisms in any of the CnA isoforms that correlate with patient outcomes. Taken together, these data generate a compelling need to investigate the distinct functional roles of CnA α and CnA β , despite their structural similarities. Although more studies point to loss of CnA α activity in directly reproducing features of CNI-nephrotoxicity, more studies are necessary to rule out any indirect contribution from CnA β to this phenomenon. Strategies and therapies to preserve CnA α function could, therefore, reduce side effects and prevent subsequent kidney transplants because of CNI toxicity. The key to taking advantage of the established experimental findings is designing studies that investigate how CNI impacts regulation of these isoforms, particularly CnA α . Moreover, it will be quite interesting to learn additional molecular signals altered by the loss of CnA α , ultimately providing clues into potential downstream mediators promoting renal fibrosis. The emergence of systemic CsA analogues, such as voclosporin in long-term treatment of autoimmune diseases such as lupus nephritis, highlights the real need to investigate this phenomenon further (1, 45–48). Clinical trials are currently underway to assess its efficacy and safety compared with CsA and tacrolimus (48).

As strides have been made in untangling the complexities of calcineurin signaling, researchers have provided clues as a road map for future work to elucidate exact mechanisms of chronic CNI-induced nephropathy. This matter is quite complex, as both direct and indirect mechanisms contribute to CNI nephropathy. It is crucial that researchers continue in

the pursuit to understand molecular mechanisms underlying CNI-induced renal fibrosis. Until then, managing optimum immune suppression while minimizing renal side effects will be an ongoing challenge.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

A.C.U. prepared figures; A.C.U. drafted manuscript; A.C.U., T.-Y.W., and C.R.W. edited and revised manuscript; T.-Y.W. and C.R.W. approved final version of manuscript.

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