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Calcineurin inhibitors recruit protein kinases JAK2 and JNK, TLR signaling and the UPR to activate NF-κB-mediated inflammatory responses in kidney tubular cells

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ABSTRACT

The calcineurin inhibitors (CNIs) cyclosporine (CsA) and tacrolimus are key drugs in current immunosuppressive regimes for solid organ transplantation. However, they are nephrotoxic and promote death and profibrotic responses in tubular cells. Moreover, renal inflammation is observed in CNI nephrotoxicity but the mechanisms are poorly understood. We have now studied molecular pathways leading to inflammation elicited by the CNIs in cultured and kidney tubular cells.

Both CsA and tacrolimus elicited a proinflammatory response in tubular cells as evidenced by a transcriptomics approach. Transcriptomics also suggested several potential pathways leading to expression of proinflammatory genes. Validation and functional studies disclosed that in tubular cells, CNIs activated protein kinases such as the JAK2/STAT3 and TAK1/JNK/AP-1 pathways, TLR4/Myd88/IRAK signaling and the Unfolded Protein Response (UPR) to promote NF-κB activation and proinflammatory gene expression. CNIs also activated an Nrf2/HO-1-dependent compensatory response and the Nrf2 activator sulforaphane inhibited JAK2 and JNK activation and inflammation. A murine model of CsA nephrotoxicity corroborated activation of the proinflammatory pathways identified in cell cultures. Human CNIs nephrotoxicity was also associated with NF-κB, STAT3 and IRE1α activation.

In conclusion, CNIs recruit several intracellular pathways leading to previously non-described proinflammatory actions in renal tubular cells. Identification of these pathways provides novel clues for therapeutic intervention to limit CNIs nephrotoxicity.

Introduction

Calcineurin inhibitors (CNIs) are key drugs in current immunosuppressive regimes for solid organ transplantation. However, both cyclosporine A (CsA) and tacrolimus (formerly known as FK506) are toxic drugs that may cause acute and chronic nephrotoxicity (Naesens et al., 2009). The three key biological processes underlying acute and chronic CNIs nephrotoxicity are kidney cell death, inflammation and residual fibrosis. In acute kidney injury, fibrosis is only evident in later stages when kidney regeneration fails to fully restore kidney structure and function. A body of evidence has shown that CsA and tacrolimus promote tubular cell death and pro-fibrotic changes in tubular cells, including epithelial-to-mesenchymal transition (EMT) (Berzal et al., 2012; Justo et al., 2003; Neria et al., 2009). However, there is very little information on the effects of CNIs on kidney inflammation, despite the fact that inflammation plays a key role in nephrotoxic kidney injury induced by some drugs and has been observed in CNIs nephrotoxicity. Specifically, the cisplatin-elicited inflammatory response in tubular cells plays a key amplification role in cisplatin nephrotoxicity (Pabla and Dong, 2008).

Inflammation is an essential event in the progression of renal disease. The transcription factor NF-κB is a key promoter of renal inflammation that integrates intracellular signals from many stimuli and drives the expression of cytokines and chemokines (Sanz et al., 2008). NF-κB contributes to lymphocyte activation following recruitment by the calcineurin/NFAT signaling pathway. CsA and tacrolimus inhibit

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calcineurin in lymphocytes, thus hindering the formation of cooperative NFAT, NF-κB and AP-1 complexes and hence, NF-κB-dependent IL-2 and IL-8 production and clonal T cell expansion (Granelli-Piperno et al., 1990; Nishiyama et al., 2005). This antiinflammatory effect of CNIs in lymphocytes contrasts to tubulointerstitial inflammation observed in animals chronically treated with CsA (Ling et al., 2003; Mizui et al., 2004) or in those with deletion of the α isoform of calcineurin A (Gooch et al., 2007). However, the molecular mechanisms of kidney inflammation in response to CNIs have not been studied and it is unknown whether CNIs have direct pro-inflammatory actions on renal cells. The response of tubular cells and leukocytes to CNIs may differ. Thus, CsA promotes cell death in tubular cells but protects from cell death in macrophages (Hortelano et al., 1999, 2000).

Numerous molecules and signaling pathways contribute to CNIs toxicity. Thus, CNIs elicit mitochondrial injury, endoplasmic reticulum (ER) stress and activate protein kinases (including MAPK and JAK/STAT) and caspase cascades to produce apoptosis (Justo et al., 2003; Neria et al., 2009; Xiao et al., 2013). However, whereas some of these pathways are also able to recruit NF-κB and elicit inflammation in other cellular systems or in tubular cells exposed to other stressors, their role in the generation of inflammation in CNIs nephrotoxicity has not been addressed.

The Unfolded Protein Response (UPR) is an ancient physiological adaptive mechanism to cope with potential deleterious protein misfolding in the ER. CsA- and tacrolimus-induced synthesis of the UPR protein GADD153/CHOP restrained TNFα-elicited inflammation in tubular cells (Du et al., 2009). This antiinflammatory action of CNIs was proposed to potentially protect renal tissue after transplantation. However, the fact remains that CNIs contribute to chronic allograft nephropathy, a process characterized by tubulointerstitial inflammation. Innate immunity responses involving toll like receptors (TLR) may also lead to NF-κB activation during infection as well as during sterile inflammation (Gonçalves et al., 2011; Loiarro et al., 2010). In this regard, increased TLR2 and TLR4 levels have also been observed in chronic human CsA nephrotoxicity (Lim et al., 2005, 2009). In resting macrophages, calcineurin may restrain TLR basal activity since CNIs favored TLR downstream signaling (Kang et al., 2007; Loiarro et al., 2010), but the interaction of CNIs with TLRs in kidney cells has not been explored.

Renal tubular cells compose most of the mass of the functioning kidney and they are thought to be a central cell type in renal inflammation and CNIs nephrotoxicity (Daha and van Kooten, 2000). We have now explored the hypothesis that CNIs trigger early proinflammatory signaling directly on tubular cells. We show that CNIs engage several protein kinase-dependent pathways that converge at and result in NF-κB activation and hence in inflammation. Both the UPR and TLR natural immunity also contributed to NF-κB activation.

Materials and methods

Cells and reagents. MCT cells are a cultured line of murine proximal tubular epithelial cells originally obtained from Eric Neilson (Vanderbildt University, Nashville, TN) that have been extensively characterized and used as model to study renal inflammation (Haverty et al., 1988; Sanz et al., 2008; Ucero et al., 2013). MCT proximal tubular epithelial cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% decomplemented fetal bovine serum (DFBS), 2 mM glutamine, 100 U/mL penicillin and 10 mg/mL streptomycin, in 5% CO_2 at 37 °C (Berzal et al., 2012). Human kidney 2 (HK2) proximal tubular epithelial cell line was cultured in the same RPMI medium supplemented with Insulin–Transferrin–Sodium Selenite (100 μg/ml) and hydrocortisone (5 ng/ml). Cells were depleted of DFBS and stimulated when 70–80% confluent. Endotoxin free CsA (Calbiochem, Merck Chemicals, Darmstadt, Germany) and tacrolimus (USBiological, Salem, MA) stock solutions (both 10 mg/ml) were dissolved in ethanol. Human Embryonic Kidney 293 (HEK293) cells were grown in high glucose (4.5 g/l) DMEM with the same additives as RPMI. The following inhibitors (specificity indicated between brackets) were used at concentration derived from prior dose–response studies in our lab or from the literature: AG490 (JAK2), SP600025 (JNK), (5Z)-7-Oxozeaenol (TAK1), IRAK1/4 inhibitor, 4μ8C (IRE1α) and salubrinal (eIF-2α) from Calbiochem; Pepinh-Myd88 (Myd88) and CLI095 (TLR4) from Invivogen (San Diego, CA); IL-1Rn (IL-1R) from Abnova (Taipei, Taiwan); Parthenolide (Iκ-Bα), PBA (UPR) and 3-Ethoxy-5,6-dibromosalicylaldehyde (IRE1α) from Sigma-Aldrich (Spain). Sulforaphane (SFN, Nrf2 activator) was obtained from Calbiochem.

Transcriptomics arrays. Transcriptomics arrays of MCT renal tubular cells were performed at Unidad Genómica Moncloa, Fundación Parque Científico de Madrid, Madrid, Spain. Affymetrix microarray analysis was performed following the manufacturer's protocol. Image files were initially obtained through Affymetrix GeneChip Command Console Software. Subsequently, Robust Multichip Analysis was performed using the Affymetrix Expression Console Software. Starting from the normalized Robust Multichip Analysis, the Significance Analysis of Microarrays was performed using the limma package (Babelomics, http://www.babelomics.org), using a false discovery rate of 5% for values increased for at least 1.5 times over the control to identify genes that were significantly differentially regulated between the analyzed groups. The analysis of concurrent annotations was performed with the web-based tool GENECODIS (Carmona-Saez et al., 2007).

Gene expression studies. One μg RNA isolated by Tripure (Roche, Spain) was reverse transcribed with High Capacity cDNA Archive Kit and real-time PCR was performed on a ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA) using the DeltaDelta Ct method. Expression levels are given as ratios to GAPDH. Pre-developed primer and probe assays were all from Applied.

Elisa. Cells were stimulated with 10 μg/ml CsA or 20 μg/ml tacrolimus, and murine MCP-1 was determined in the supernatants by ELISA (BD, Franklin Lakes, NJ) according to manufacturer's intructions. Briefly, 96 well plates were covered with anti MCP-1 capture antibody overnight at 4 °C, then incubated at RT with the samples properly diluted to fit the measuring linear range and finally with an HRP-conjugated anti MCP-1 secondary antibody. Signal was developed with the colorimetric TMB substrate reagent set (BD), stopped with 2 N sulfuric acid and read it at a wavelength of 450 nm. A wavelength of 570 nm was used to correct experimental intra-assay variations.

Western blot. Protein content from cell extracts homogenized in lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.1 mmol/L PMSF, 25 mmol/L NaF) was determined by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). Proteins were separated by 10% SDS-PAGE under reducing conditions and then blotted onto nitrocellulose membranes. Membrane blockade was accomplished with 5% defatted milk in TBS-T (0.05 mol/L Tris, 0.15 mol/L NaCl, 0.05% Tween 20, pH 7.8). Thereafter, membranes were overnight probed at 4 °C with specific primary antibodies made in the same blocking solution or 5% BSA in TBS-T and then incubated with secondary HRP-conjugated antibodies for 1 h at room temperature. Primary antibodies to p65, JAK2, CHOP, XBP1 and Nrf2 were from Santa Cruz Biotechnology (Santa Cruz, CA); to p-p65 (Ser536), p-IkBα, IkBα, p-STAT3 (Ser727), STAT3, p-JNK, JNK, p-TAK1, TAK1, p-IRAK1 from Cell Signaling Technology (Danvers, MA); to p-JAK2 (pYpY1007-1008) from Invitrogen (Camarillo, CA); to p-IRE1α (Abcam, Cambridge, UK); to IRE1α from Lifespan Biosciences (Seattle, WA) and to HO-1 from Enzo Life Sciences (Farmingdale, NY). Antibodies to α-Tubulin (Sigma-Aldrich) and mouse polyclonal anti-GAPDH (Merck-Millipore, Darmstadt, Germany) were used to correct minor differences in protein loading.

Electrophoretic mobility shift assay (EMSA). Nuclei and cytosolic fractions from cell pellets were separated by using NE-PER® extraction