Prevention of apoptosis averts glomerular tubular disconnection and podocyte loss in proteinuric kidney disease

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There is a great need for treatment that arrests progression of chronic kidney disease. Increased albumin in urine leads to apoptosis and fibrosis of podocytes and tubular cells and is a major cause of functional deterioration. There have been many attempts to target fibrosis, but because of the lack of appropriate agents, few have targeted apoptosis. Our group has described an ouabain-activated Na,K-ATPase/IP3R signalosome, which protects from apoptosis. Here we show that albumin uptake in primary rat renal epithelial cells is accompanied by a time- and dose-dependent mitochondrial accumulation of the apoptotic factor Bax, down-regulation of the antiapoptotic factor Bcl-xL and mitochondrial membrane depolarization. Ouabain opposes these effects and protects from apoptosis in albumin-exposed proximal tubule cells and podocytes. The efficacy of ouabain as an antiapoptotic and kidney-protective therapeutic tool was then tested in rats with passive Heymann nephritis, a model of proteinuric chronic kidney disease. Chronic ouabain treatment preserved renal function, protected from renal cortical apoptosis, up-regulated Bax, down-regulated Bcl-xL, and rescued from glomerular tubular disconnection and podocyte loss. Thus we have identified a novel clinically feasible therapeutic tool, which has the potential to protect from apoptosis and rescue from loss of functional tissue in chronic proteinuric kidney disease.

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Chronic kidney disease (CKD) is a rapidly increasing world-wide public health problem.[1] CKD results from many causes, including diabetes, glomerulonephritis, hypoxia, hypertension, infections, and polycystic kidney disease. Most forms of CKD are progressive[1–3] and characterized by disrupted glomerular perm-selectivity, albuminuria, loss of podocytes, interstitial fibrosis, and glomerular-tubular disconnection.[4–9] Albuminuria, a well-documented predictor of progressive loss of kidney function, is considered a cause of kidney damage and loss of function.[10–13] The nature of albumin toxicity has been extensively studied in the past decade, and it is well recognized that prolonged exposure of renal tubular cells to albumin results in apoptosis and fibrosis,[14–18] but their interrelationship is not yet fully understood. There are several ongoing trials aimed at halting progression of CKD using drugs targeted to inhibit profibrotic and/or stimulate antifibrotic molecular pathways,[19–22] but there are few attempts to target the apoptotic process, mainly because of lack of nontoxic agents.

Apoptosis is triggered either via an extrinsic pathway stimulated by activation of plasma membrane death receptors or via an intrinsic mitochondrial pathway. The intrinsic apoptotic pathway is controlled by the family of B-cell lymphoma (Bcl)-2 proteins. The mitochondrial apoptotic pathway is initiated by activation of Bax, a prominent pro-apoptotic member of the Bcl-2 family, to the outer mitochondrial membrane, where it oligomerizes and penetrates the inner mitochondrial membrane. This results in release of cytochrome C and caspase activation, the apoptotic executors. B-cell lymphoma–extra large (Bcl-xL), a prominent anti-apoptotic member of the Bcl-2 family, counteracts Bax accumulation on the mitochondria and Bax-induced permeabilization of the mitochondrial membranes.[23,24]

Our group has identified an antiapoptotic signal activated by the cardiotonic steroid ouabain, which involves interaction between sodium-potassium adenosine triphosphatase (Na,K-ATPase) and the inositol 1,4,5-triphosphate receptor (IP3R) and triggering of slow intracellular calcium oscillations. We have shown that the ouabain signal may interfere with the apoptotic process by down-regulation of the apoptotic factor Bax and up-regulation of the antiapoptotic factor Bcl-xL.[25–30] Studies from us and other investigators

have provided evidence for a tissue protective effect of ouabain.[30–36]

The aim of this study has been to test the hypothesis that activation of the ouabain signal can, via down-regulation of Bax and up-regulation of Bcl-xL, rescue from the onset of albumin-triggered apoptotic process and thereby halt the progression of CKD. If this would be the case, ouabain may be a good candidate for a clinically feasible antiapoptotic drug. Proximal tubular cells (PTCs) are the main target for the toxic effects of albumin overload,[17] and loss of early PTCs will result in glomerular-tubular disconnection and irreversible renal damage.[5,7]

To assess at which stage ouabain interferes with the apoptotic process, Bax recruitment to the mitochondria, changes of the mitochondrial membrane potential, and cellular abundance and localization of Bcl-xL were sequentially studied in a homogenous preparation of primary rat PTCs (RPTCs). Podocytes, which constitute a well-recognized locus minoris resistentiae in CKD,[37,38] were also examined with regard to their apoptotic response to albumin and the rescuing effect of ouabain. To obtain the first proof of principle that ouabain may protect from apoptosis and progressive renal damage in proteinuric CKD, we used a well-established rat model of human proteinuric kidney disease, passive Heymann nephritis (PHN).[39,40]

RESULTS

Albumin uptake into primary renal cells triggers apoptosis followed by increased expression of TGF-β 1: protective effect of ouabain

It is well documented that excessive uptake of albumin into RPTCs triggers apoptosis[30,41–43] and generation of profibrotic factors, such as transforming growth factor (TGF)-β.[44] To determine the order by which these processes are initiated, we incubated RPTCs with fatty acid and endotoxin-free albumin (10 mg/ml) for 2, 4, 8, and 18 hours. The level of apoptosis triggered by albumin was determined with terminal deoxynucleotidyltransferase–mediated dUTP nick end-labeling (TUNEL) staining[45] and expression of the profibrotic TGF-β1 precursor with immunoblotting (Figure 1a–d). Apoptotic index (AI) was significantly increased after 2 hours of albumin incubation and continued to increase during the following 16 hours. In contrast, expression of the TGF-β1 precursor was not increased until after 8 hours of albumin incubation.

The apoptotic effect of albumin was dose-dependent and coincubation with ouabain (5 nM) resulted in a robust reduction of AI with all tested albumin concentrations (5, 10, or 20 mg/ml for 8 or 18 hours) (Figure 1e and f). The expression of TGF-β precursor in RPTCs exposed to albumin for 8 or 18 hours was also attenuated by ouabain (5 nM) (Supplementary Figure S1B). Ouabain did not interfere with albumin uptake in RPTCs (Supplementary Figure S1C).

To test whether albumin might trigger apoptosis of primary rat podocytes, isolated glomeruli were plated, cultured for 3 days, and stained with the podocyte-specific transcriptional factor WT1.[46] AI was determined in cells that had migrated out from the glomerulus and were WT1-positive (Figure 1g). Incubation with albumin for 18 hours triggered apoptosis in a dose-dependent manner. Coincubation with ouabain resulted in significant reduction of AI (Figure 1h).

Ouabain interferes with the albumin triggered intrinsic apoptotic pathway

In RPTCs exposed to albumin (10 mg/ml) for 8 hours, Bcl-xL abundance was decreased and the Bax abundance was increased as measured by immunoblotting (Figure 2b and c). The abundance of cleaved caspase-3 was increased, indicating that the apoptotic process was reaching the point of no return. Ouabain (5 nM) partially rescued from Bcl-xL down-regulation, Bax up-regulation, and increase of cleaved caspase-3.

The ouabain signaling pathway includes activation of the IP3R, release of calcium from endoplasmic reticulum via the IP3R and activation of the prosurvival nuclear factor κB (nuclear factor kappa-light-chain-enhancer of activated B cells) p65 subunit (Figure 2a).[25,27,47] When cells coincubated with albumin and ouabain were depleted of calcium from endoplasmic reticulum stores by sarco/endoplasmic reticulum Ca2+−ATPase pump inhibition with cyclopiazonic acid or coincubated with helenalin, a specific nuclear factor κB p65 subunit inhibitor, the rescuing effects of ouabain were abolished (Figure 2b and c). Ouabain had little effect on Bax and Bcl-xL expression in control cells. The extrinsic apoptotic pathway can be triggered by incubation with lipopolysaccharide. Ouabain (5 nM) did not protect against lipopolysaccharide-triggered cytokine release (Figure 2d, Supplementary Figure S2).

Ouabain protects from albumin-triggered mitochondrial dysfunction

To further characterize the involvement of mitochondria in albumin toxicity, time-sequence studies were performed with regard to mitochondrial membrane potential, Bax colocalization with mitochondria and Bcl-xL abundance (Figure 3a–c). Albumin (2.5 mg/ml) caused time-dependent depolarization of the mitochondrial membrane (Figure 3g). Colocalization between Bax and mitochondria in albumin-exposed cells increased in a time-dependent manner and the increase was significant after 2 hours (Figure 3g). The intensity of the fluorescent signal from Bcl-xL in albumin-incubated RPTCs decreased in a time-dependent manner. Changes were significant after 2 hours of incubation with albumin (Figure 3g). Albumin had no effect on colocalization between Bcl-xL and mitochondria (Supplementary Figure S3A). In RPTCs coincubated with albumin (2.5 mg/ml) and ouabain (5 nM) for 8 hours, the effects on mitochondrial accumulation of Bax, mitochondrial membrane potential, and Bcl-xL abundance were greatly attenuated (Figure 3d–f). Incubation of RPTCs with 10 mg/ml albumin resulted in a more pronounced effect (Supplementary Figure S3B–E) and was seen after 30 minutes (Supplementary Figure S4A–C).