Bid deficiency ameliorates ischemic renal failure and delays animal death in C57BL/6 mice

Qingqing Wei, Xiaoming Yin, Mong-Heng Wang, and Zheng Dong. Bid deficiency ameliorates ischemic renal failure and delays animal death in C57BL/6 mice. Am J Physiol Renal Physiol 290: F35–F42, 2006. First published August 16, 2005; doi:10.1152/ajprenal.00184.2005.—Tubular cell apoptosis is involved in ischemic renal failure, but the underlying mechanism is unclear. Bid, a proapoptotic Bcl-2 family protein, may regulate the intrinsic as well as the extrinsic pathway of apoptosis. In vivo, Bid is most abundantly expressed in the kidneys. However, the role played by Bid in renal pathophysiology is unknown. Our recent work demonstrated Bid activation during renal ischemia-reperfusion. The current study has determined the role of Bid in ischemic renal injury and renal failure using Bid-deficient mice. In wild-type C57BL/6 mice, Bid was proteolytically processed into active forms during renal ischemia-reperfusion, which subsequently targeted mitochondria. This was accompanied by the development of tissue damage and severe renal failure, showing serum creatinine of 3.0 mg/dl after 48 h of reperfusion. The same ischemic insult induced acute renal failure in Bid-deficient mice, which was nonetheless less severe than the wild-type, showing 1.3 mg/dl serum creatinine. In addition, Bid deficiency attenuated tubular disruption, tubular cell apoptosis, and caspase-3 activation during 48 h of reperfusion. Compared with wild-type, animal death following renal ischemia was delayed in Bid-deficient mice. Collectively, the results suggest a role for Bid in ischemic renal injury and renal failure.

ischemia-reperfusion; acute renal failure; apoptosis

MATERIALS AND METHODS

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BID IS A UNIQUE PROAPOPOTIC BCL-2 FAMILY PROTEIN WITH ONLY ONE BH DOMAIN, BH3 (37). THE ACTIVATION OF BID DEPENDS ON ITS PROTEOLYTIC PROCESSING INTO TRUNCATED FORMS CALLED TIBID. ON ACTIVATION, BID TARGETS MITOCHONDRIA AND, IN COLLABORATION WITH OTHER PROAPOPOTIC FACTORS, THIS LEADS TO MITOCHONDRIAL DISRUPTION AND THE RELEASE OF APOPTOTIC MOLECULES INCLUDING CYTOCHROME C (21, 24). AS A RESULT, BID MAY HAVE AN IMPORTANT ROLE IN THE DEATH RECEPTOR-INITIATED EXTRINSIC PATHWAY OF APOPTOSIS AS WELL AS THE MITOCHONDRIA-MEDIATED INTRINSIC PATHWAY OF APOPTOSIS (12, 43).

IN VIVO, BID IS MOSTLY ABUNDANTLY EXPRESSED IN THE KIDNEYS (37). HOWEVER, LITTLE IS KNOWN FOR THE INVOLVEMENT OF BID IN RENAL PATHOPHYSIOLOGY. OUR RECENT WORK DEMONSTRATED BID ACTIVATION FOLLOWING ATP DEPLETION OF RENAL TUBULAR CELLS IN VITRO AND RENAL ISCHEMIA-REPERFUSION IN VIVO (40), SUGGESTING A ROLE FOR BID IN ISCHEMIC RENAL CELL INJURY. THE CURRENT STUDY WAS DESIGNED TO DETERMINE THE INVOLVEMENT OF BID IN SEVERE ISCHEMIC RENAL INJURY AND RENAL FAILURE USING A GENE KNOCKOUT MODEL.
renal tissue collection. After clotting at room temperature, serum was purified by centrifugation. Serum samples were added to the reaction reagent, and the absorbance at 510 nm was recorded 20 and 80 s later. Creatinine standards were measured in parallel. The creatinine levels of serum samples were calculated based on the reading of the standards.

Renal histology. Kidneys were harvested and fixed with 4% paraformaldehyde for paraffin embedding. Paraffin-embedded sections were sectioned at 4 μm for hematoxylin-eosin staining. Histology was examined in a blinded fashion. Histological changes evaluated included the percentage of renal tubules that displayed cell lysis, loss of brush border, and cast formation. The development of tissue damage was scored as follows: 0: 0; 1: <10%; 2: 10–25%; 3: 26–75%; 4: >75%. Pictures of representative fields were also recorded.

Terminal transferase-mediated dUTP nick-end labeling assay. Apoptosis in renal tissues was identified by terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay as shown in our recent work (36). Briefly, renal tissues were fixed with paraformaldehyde, paraffin embedded, and cut into 4-μm sections. TUNEL assay was conducted using a in situ Cell Death Detection kit from Roche Applied Science (Indianapolis, IN), following the manufacturer’s instruction. Briefly, tissue sections were deparaffinized and permeabilized by 2 h of incubation at 65°C in 0.1 M sodium citrate, pH 6.0. The sections were then exposed to a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides including FITC-labeled dUTP. Positive staining with DNA breakage in the cell nucleus was identified by fluorescence microscopy.

In situ detection of caspase-3 activation. Caspases are synthesized as inactive preproenzymes, which are proteolytically processed into active fragments during apoptosis. In situ detection of caspase activation is based on the appearance of active caspase fragments, which are detectable by immunofluorescence using antibodies specifically reactive to these fragments. We examined in situ caspase-3 activation in renal tissues using an antibody specific for active caspase-3 fragments (32). Freshly frozen renal tissues were sectioned at 7 μm in −20°C and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed samples were permeabilized in 1% Triton X-100 for 30 min and incubated for 1 h in a blocking buffer containing 2% normal goat serum. Subsequently, the samples were incubated for 1 h with the primary antibody, a rabbit polyclonal antibody specific for the active fragments of caspase-3. Finally, the cells were exposed to a Cy3-labeled goat anti-rabbit secondary antibody. Staining was examined by fluorescence microscopy.

Cellular fractionation. To determine the subcellular localization of Bid/tBid, renal tissues were fractionated into cytosolic fraction and mitochondrial fraction. The fractionation protocol was modified from previous studies (22, 41). Briefly, kidneys were collected and immediately sliced on an ice-cold plate. The tissues were then homogenized using a Potter-Elvehjem homogenizer with a Teflon pestle in a buffer containing (in mM) 30 Tris-HCl pH 7.4, 20 EDTA, 5 EGTA, 150 NaCl, 2 DTT, and 1 PMSF as well as 1% (wt/vol) sucrose and 100 μM Z-VAD. The homogenate was centrifuged twice for 5 min at 1,000 g to remove the pellet containing nuclei and cell debris and further centrifuged at 20,000 g for 20 min to collect the cytosolic fraction in the supernatant and mitochondrial fraction in the pellet. All these steps were carried out on ice or at 4°C.

Immunoblot analysis. The NuPAGE Gel System was used for electrophoresis and protein transferring/blotting. The blots were incubated with 5% fat-free milk for blocking and then exposed to specific primary antibodies overnight at 4°C. Finally, the blots were exposed to the horseradish peroxidase-conjugated secondary antibody, and antigens on the blots were revealed using an enhanced chemiluminescence kit (Pierce, Rockford, IL).

Statistics. Data are expressed as means ± SD (n = 4). Statistical differences between two groups were determined by a Mann-Whitney test, a nonparametric method. P < 0.05 was considered to reflect significant differences between two groups of values.

RESULTS

Proteolytic activation of Bid during renal ischemia-reperfusion. An important mechanism of Bid activation involves proteolytic cleavage of Bid, leading to the removal of the COOH terminus and the exposure of the proapoptotic BH3 domain (12, 43). Thus our initial experiments examined Bid activation by immunoblot analysis of Bid cleavage. Wild-type C57BL/6 mice were subjected to sham operation or 30 min of bilateral renal ischemia followed by reperfusion. Kidneys were collected and homogenized in 2% SDS buffer to collect whole tissue lysates for immunoblotting of Bid. As shown in Fig. 1A, sham control showed intact Bid of ~22 kDa, without truncated fragments or tBid (lane 1). During renal ischemia, tBid of 15 kDa (p15) was generated, although Bid degradation or decrease was not obvious (lane 2). Subsequent reperfusion led to further generation of tBid of 13 kDa (p13), which appeared to be accompanied by progressive degradation of intact Bid (lanes 4 and 5).

Fig. 1. Proteolytic processing of Bid into truncated Bid (tBid) and tBid targeting of mitochondria during renal ischemia-reperfusion. Male C57BL/6 mice of 8–10 wk were subjected to 30 min of bilateral renal ischemia, followed by 0–48 h of reperfusion. A: whole tissue lysates were examined for Bid/tBid by immunoblot analysis. B: renal tissues were collected from ischemia-reperfused mice. The same amounts of tissue were either lysed to collect whole tissue lysate (Total) or fractionated into cytosolic (Cyto) and mitochondrial fractions (Mito) for immunoblot analysis of Bid/tBid. The blot was reprobed for mitochondria, COX IV (COX IV), an integral protein of mitochondria, to monitor fractionation. Note that the cytosolic and mitochondrial fractions were originally analyzed on the same blot; the lanes between the 2 samples were removed during graphics preparation. The blots are representatives of at least 3 experiments. The results show that Bid is proteolytically processed into tBid of 13 and 15 kDa during renal ischemia-reperfusion. Subsequently, tBid targets mitochondria.
Following the removal of the COOH fragment, tBid assumes an active conformation that targets it to mitochondria (12, 43). Thus to determine whether tBid generated during renal ischemia-reperfusion was indeed active, we analyzed its subcellular distribution. For this purpose, renal tissues of ischemia-reperfused kidneys were fractionated into cytosolic and mitochondrial fractions and analyzed for Bid by immunoblotting (Fig. 1B). Lane 1 is the results of whole tissue lysate without fractionation, showing intact Bid and p13/p15 tBid. Following fractionation, intact Bid was shown in both mitochondrial (lane 2) and cytosolic (lane 3) fractions, with the majority in the cytosolic fraction. In sharp contrast, tBid of 13 and 15 kDa was detected only in the mitochondrial fraction (lane 3). To monitor the fractionation procedure, we reprobed the blots for cytochrome oxidase subunit IV (COX IV), an integral protein of mitochondria. As shown in Fig. 1B, COX IV was detected in whole lysate, isolated mitochondrial fraction, and not in the cytosolic fraction. Using the same fractionation approach, we also detected tBid in mitochondria of ischemic renal tissues that were not reperfused (not shown). These results, together with our earlier observations (40), indicate that Bid is activated during renal ischemia-reperfusion.

Preservation of renal function following ischemia in Bid-deficient mice. To determine the involvement of Bid in ischemia renal injury and renal failure, we took advantage of theBid-deficient mouse model, which had homozygous deletion of the bid gene (44). We first confirmed that Bid was not expressed in the kidneys of these animals (Fig. 2A). Bid-deficient mice developed normally; no defects in renal histology or renal function were noticed. As shown in Fig. 2B, under control conditions, renal function measured as serum creatinine was similar in wild-type and Bid-deficient animals; both were ~0.4 mg/dl. On the contrary, their responses to renal ischemia-reperfusion were quite different. In wild-type animals, serum creatinine increased to ~3.0 mg/dl following renal ischemia and 48 h of reperfusion, whereas the creatinine increase in Bid-deficient animals was significantly lower, only 1.3 mg/dl. The results suggest a role for Bid in the development of ischemic renal failure in this experimental model.

Amelioration of tubular disruption during renal ischemia-reperfusion in Bid-deficient mice. We collected renal tissues for hematoxylin-eosin staining to examine histology. In these experiments, we particularly concentrated on the outer stripe of the outer medulla, which was the major injury site of the kidneys during renal ischemia-reperfusion (23). Representative histology is shown in Fig. 3A. Under sham-operated control conditions, the tubular systems were healthy in both wild-type and Bid-deficient animals (shown in Fig. 3A for wild-type only). After ischemia and 48 h of reperfusion, wild-type animals showed a severe disruption of renal tubules. Many tubules were lysed with sloughed materials in the lumen space; nuclei of these tubular cells were lost, suggesting degradation or autolysis. In contrast, tubular disruption in Bid-deficient animals was less severe; here, the majority of the tubules maintained their tubular structure, and the cells appeared intact with good nuclear staining, although brush borders were lost in some tubules. Representative tubules in higher magnifications are shown in the insets to Fig. 3A. We then graded the histology based on the degree of tubular disruption. As shown in Fig. 3B, wild-type animals had a pathology score of 3.2, whereas Bid-deficient animals showed a tubular disruption score of 1.4, significantly lower than the wild-type. Thus tissue damage during renal ischemia-reperfusion was ameliorated in Bid-deficient animals.

Attenuation of tubular cell apoptosis during renal ischemia-reperfusion in Bid-deficient mice. Apoptosis has been implicated in ischemic renal injury and renal failure. Bid, as a BH3-only proapoptotic protein, may regulate both the extrinsic and intrinsic pathways of apoptosis. To determine the role of Bid in tubular cell apoptosis following renal ischemia, we analyzed and compared wild-type and Bid-deficient animals by a TUNEL assay. Although the specificity of the TUNEL assay has been questioned in certain experimental models, it was shown to be reasonably specific for apoptotic cells in the settings of renal injury (17). In our experiments, TUNEL-positive cells were mainly identified in renal tubules, whereas Bid-deficient animals showed a tubular disruption score of 1.4, significantly lower than the wild-type. Thus tissue damage during renal ischemia-reperfusion was ameliorated in Bid-deficient animals.

![Fig. 2. Bid content and serum creatinine accumulation following renal ischemia in wild-type (WT) and Bid-deficient (KO) mice. A: immunoblot of Bid in renal tissues of WT and KO mice. B: serum creatinine. Male WT and KO C57BL/6 mice of 8–10 wk were subjected to sham operation or 30 min of renal ischemia followed by 48 h of reperfusion. Blood samples were collected to purify serum for measurement of creatinine. Values are means ± SD; n = 8±4. *Significantly different from WT (P < 0.05).](http://ajprenal.physiology.org/)

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In the renal medulla, much less apoptosis was shown in both wild-type and Bid-deficient kidneys (Fig. 4, A and B). To further examine the involvement of Bid in tubular cell apoptosis during renal ischemia-reperfusion, we analyzed caspase activation in wild-type and Bid-deficient mice. Caspases, a family of cysteine proteases responsible for the disassembly of apoptotic cells, are induced and activated under this pathological condition (15). We examined caspase activation in situ in renal tissue sections by immunofluorescence, using an antibody that reacted specifically with the active forms of caspase-3 (32). Compared with biochemical analysis, such as in an enzymatic assay, the in situ approach may provide information on the specific sites or cell types that activated caspases. As shown in Fig. 5, active caspase-3 staining was completely negative in the sham controls. Following ischemia-reperfusion, many cells in wild-type animals became positive for active caspase-3 in the renal cortex. Moreover, the majority of the positive cells were located in renal tubules. In sharp contrast, very few cells were positive for active caspase-3 in ischemia-reperfused kidneys of Bid-deficient animals (KO 130/R48). In the renal medulla, active caspase-3 cells were also identified, and again fewer positive cells were shown in the renal medulla of Bid-deficient animals (Fig. 5, A and B). It was noted that, following renal ischemia, there were fewer caspase-3-positive cells than TUNEL-positive cells in both wild-type and Bid-deficient animals. This was likely due to the fact that some late-stage apoptotic cells stained by TUNEL had lost or degraded caspase-3. Nevertheless, both TUNEL and active caspase-3 staining showed lower apoptosis in Bid-deficient animals, suggesting that Bid is involved in tubular cell apoptosis during renal ischemia-reperfusion.

Survival of wild-type and Bid-deficient mice during renal ischemia-reperfusion. The amelioration of tissue damage, apoptosis, and ischemic renal failure in Bid-deficient animals was associated with better survival. As shown in Fig. 6, following 30 min of bilateral renal ischemia, we started losing wild-type animals after 48 h of reperfusion. By the end of 60 h of reperfusion, one-half of the wild-type animals had expired, whereas 9 of 10 Bid-deficient mice survived. By the end of 72 h of reperfusion, all wild-type animals had expired, yet 6 Bid-deficient animals were still alive. The survival rate of Bid-deficient animals decreased during further reperfusion, and only one animal survived by the end of 120 h (Fig. 6). Thus in this experimental model of severe renal ischemia, animal death was delayed and not prevented by Bid-deficiency. The significant delay of animal death was consistent with the amelioration of renal injury and renal failure in Bid-deficient mice, supporting for a role of Bid in ischemic renal failure.
DISCUSSION

This study was designed to examine the role of Bid in renal cell injury and renal failure during ischemia-reperfusion. To extend previous work (40), Bid activation was demonstrated under pathological conditions in the mouse model of bilateral clamping. Importantly, it was shown that Bid-deficient mice were more resistant to ischemic renal tissue damage, tubular cell apoptosis, and renal failure. This was accompanied by better survival of the knockout animals. Collectively, this study has provided the first convincing evidence for the involvement of Bid in the development of ischemic renal injury and renal failure.

The Bid-deficient mouse model was originally established by homozygous deletion via gene targeting. The animals had no obvious defects in their development. Using this model, a critical role of Bid in Fas-initiated apoptosis in liver was demonstrated (44). A recent study suggested that the Bid-deficient animals might develop myeloproliferative disorder (45). Nevertheless, development of the disorder was shown to be age dependent and did not occur until 18–24 mo. In younger (<12 mo) animals, no changes were shown in hematopoietic cells, hematopoietic stem cells, myeloid cells, and myeloid progenitor cells (45). Also, neither myeloid cell nor bone marrow cell infiltration was identified at a young age (Dr. Sandra Zinkel, personal communication). In our study, young adult animals of ~2 mo were used. As expected, before ischemia, no defects in renal histology and renal function were noticed in these animals (Figs. 2 and 3). In addition, the size of the kidneys was comparable to that in wild-type animals (not shown), suggesting that Bid deficiency did not alter the overall cell turnover or tissue homeostasis. These observations further justify the use of this knockout model to study the role of Bid in ischemic renal cell injury.

In this study, Bid activation was shown during the ischemic period and continued during subsequent reperfusion. Consistently, our previous work showed early Bid activation in a rat model of renal ischemia-reperfusion (40). On the contrary, the regulation of other Bcl-2 family proteins seems to occur later. For example, no significant changes were induced in Bcl-2, Bcl-XL, and Bax during the ischemic period (4, 13). After reperfusion, these proteins in general showed increased expression, although at different time points (4, 13). These observations suggest a dynamic regulation of Bcl-2 family proteins during renal ischemia-reperfusion. Importantly, among these proteins, Bid appears to be the first to be regulated, suggesting that Bid might be an upstream signaling molecule for ischemic renal injury.
renal cell injury. These results, however, do not exclude the roles of other Bcl-2 proteins in the regulation of ischemic renal cell injury. Particularly, it is noted that Bid deficiency did not completely block tubular cell apoptosis (Fig. 4B), suggesting the involvement of Bid-independent pathways. It would be interesting to determine whether other Bcl-2 family proteins may substitute Bid to initiate the apoptotic cascade under this pathological condition.

There is a marked difference in the durations of Bid activation in the mouse model of the present study and the rat model of renal ischemia in our previous study (40). In the rat model, Bid activation indicated by Bid-to-tBid processing was shown during ischemia and early period (8 h) of reperfusion (40). After 24–48 h of reperfusion, tBid was undetectable and Bid recovered gradually to the control level (40). In the mouse model, however, Bid processing was continuous and progressive (Fig. 1, the present study). Although the exact cause of the difference remains unclear, we believe it might be related to the severity of renal injury. In the rat model, the animals were subjected to a longer period (35 min) of renal ischemia, yet the animals appeared to be less injured, and most of them recovered from the injury and survived (not shown). In contrast, all of the mice died within 3 days following 30 min of renal ischemia-reperfusion in WT and KO mice. Male WT and KO C57BL/6 mice of 8–10 wk were subjected to sham operation or 30 min of renal ischemia followed by 48 h of reperfusion. Renal tissues were fixed for immunofluorescence using an antibody specific for active caspase-3. A: representative images of active caspase-3 staining. No positive cells were shown in sham controls of WT and KO mice; shown at left is sham control of the WT only. B: quantification of active caspase-3 positive cells in reperfused renal tissues. Positive cells were counted in representative fields. Values are means ± SD (n = 4). *Significantly different from WT (P < 0.01).
ischemia (Fig. 6, wild-type). The continuous Bid activation in these terminally injured and dying animals is consistent with a role of Bid in renal injury under pathological conditions.

The mechanism underlying proteolytic activation of Bid during renal ischemia-reperfusion is unknown. Bid contains a proteolysis-sensitive domain. Thus far, several proteases have been implicated in Bid processing. In Fas-mediated apoptosis, Bid is cleaved by caspase-8 at the site of Asp59 (21, 24). During granule-mediated cytotoxic T-lymphocyte killing, granzyme B cuts Bid at Asp75 (2, 3, 34). Cell-free experiments suggest the cleavage of Bid by lysosomal proteases at Arg65 (33). More recently, calpains have been shown to process Bid at Gly70 during cisplatin-induced apoptosis (25). Interestingly, the same site was proposed for calpain cleavage following myocardial ischemia (7). In cultured renal tubular cells, we showed that Bid processing was suppressed by a peptide inhibitor of caspase-9 (40). Nevertheless, it is noteworthy that Bid processing in the in vitro model mainly occurred during the reperfusion period and not during ATP depletion (40). Thus caspase-9 might be involved in Bid activation during renal reperfusion; however, the protease(s) responsible for the initial Bid activation during renal ischemia remains to be identified.

In a mouse model of unilateral renal ischemia, Nogae et al. (27) demonstrated the activation of the Fas system during reperfusion and its involvement in subsequent tubular cell apoptosis. Whether Fas and the triggered caspase-8 activation are involved in Bid activation in the bilateral renal ischemia model remains to be determined. Apparently, mechanistic insights of Bid activation will be derived not only from the information of protease activation but more importantly from systematic analysis using pharmacological and genetic inhibitory approaches. Considering the role played by Bid in ischemic renal failure, further investigation needs to delineate the mechanism of Bid activation under pathological conditions.

On activation, Bid may regulate cell injury and death at the mitochondrial level. We demonstrated evidence for tBid targeting of mitochondria in ischemia-reperfused kidneys (Fig. 1). However, it is unclear as to how tBid leads to mitochondrial permeabilization and the release of apoptogenic molecules, including cytochrome c. Recent studies by Korsmeyer’s laboratory demonstrated that cells lacking multi-BH domain proteins (i.e., Bax and Bak) became resistant to injury induced by BH3-only proteins including Bid (39). They further showed that Bcl-2 sequestered BH3-only proteins to prevent apoptosis (8). These observations suggest that BH3-only proteins like Bid may activate multi-BH domain proteins including Bax and Bak, which subsequently permeabilize mitochondria. Death-inhibitory proteins such as Bcl-2 block cell death mainly by sequestering BH3-only proteins. This model was supported by the observation of Bax activation by Bid, resulting in supramolecular openings in the outer membrane of mitochondria (19). In HeLa cells, we demonstrated that Bid transfection activated Bax and Bak to permeabilize mitochondria, leading to cytochrome c release and cell death. Bcl-2 attenuated Bid-induced apoptosis by suppressing tBid insertion and Bax/Bak activation in mitochondria (42). With these results taken together, it is suggested that Bid/tBid may collaborate with other Bcl-2 family proteins to permeabilize mitochondria and induce tubular cell apoptosis during renal ischemia-reperfusion.

The amelioration of overall tubular disruption in Bid-deficient mice was impressive (Fig. 3). Apparently, in addition to apoptosis, tubular necrosis was suppressed in these animals as well. The mechanism of tubular cell necrosis during renal ischemia-reperfusion remains elusive; however, it may involve an interplay of cell injury, inflammation, oxidative stress, and other processes (6, 26, 30). In the experimental model of this study, necrosis became obvious after 24 h of reperfusion, whereas apoptosis was not fully developed until 48 h (not shown), suggesting that the initial wave of necrosis was not secondary to apoptosis. Because necrotic damage was also ameliorated in Bid knockout animals (Fig. 3), Bid seemed to contribute to necrosis in an apoptosis-independent manner. A role of Bid in necrosis has been suggested by the results from a hyperoxia injury model (38), although it remains unclear how Bid directly contributes to necrosis.

In conclusion, this study has extended previous observations of Bid activation during renal ischemia-reperfusion. Importantly, using Bid-deficient mice, the results have suggested a role of Bid in the development of ischemic renal cell injury and renal failure.

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GRANTS

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