The pathological role of Bax in cisplatin nephrotoxicity

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Nephrotoxicity induced by cisplatin involves tubular cell necrosis and apoptosis; the latter of which may be initiated by multiple mechanisms including activation of the intrinsic mitochondrial pathway. In cultured tubular epithelial cells, cisplatin can activate the proapoptotic protein Bax resulting in cytochrome *c* release, caspase activation, and apoptosis. Definitive evidence for the involvement of Bax in cisplatin nephrotoxicity *in vivo*, however, is lacking. We analyzed Bax regulation during cisplatin nephrotoxicity in wild-type mice and determined the pathological role of Bax using mice in which this gene was knocked out. In wild-type mice, cisplatin induced Bax in renal tubular cells which became active, accumulated in the mitochondria, and was accompanied by acute kidney injury. Compared with the wild-type mice renal function, as measured by blood urea nitrogen and serum creatinine, was partially but significantly preserved in Bax knockout mice. The number of apoptotic cells was decreased as was general tissue damage. Additionally, cisplatin-induced cytochrome *c* release was attenuated in the Bax-deficient mice. This significant decrease in apoptosis and in cytochrome *c* release was also mirrored in primary cultures of proximal tubular cells prepared from Bax knockout animals. Collectively, our results provide compelling evidence for a role of Bax and its related apoptotic pathway in cisplatin nephrotoxicity.

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Cisplatin, a widely used chemotherapy drug, induces renal injury, which limits its therapeutic efficacy.[1,2] Nephrotoxicity by cisplatin involves necrosis as well as apoptosis of renal tubular cells. Mechanistically, tubular cell death under the pathological condition may be initiated by multiple mechanisms.[3–16] For apoptosis, both the intrinsic mitochondrial pathway and the extrinsic death–receptor pathway have been implicated.[6,12,17–20] In addition, *in vitro* studies have further suggested the involvement of ER stress related mechanisms.[21]

Using cultured renal cells, Lee *et al*. and Park *et al*. showed Bax activation during cisplatin treatment, accompanied by mitochondrial release of cytochrome *c* and apoptosis, demonstrating the first evidence for the involvement of the intrinsic pathway of apoptosis in cisplatin nephrotoxicity. The observations were confirmed and further extended by recent work.[6,17,22] Despite the findings, these studies were all conducted in *in vitro* systems of cell cultures. Whether and to what extent Bax and related apoptotic pathway contribute to *in vivo* cisplatin nephrotoxicity is unclear. Moreover, the evidence collected from the earlier studies was correlative, and definitive evidence for a role of Bax in tubular cell apoptosis and renal injury during cisplatin nephrotoxicity remains to be established.

In this study, we have examined and compared cisplatin nephrotoxicity in wild-type and Bax-deficient mice and tubular cells. We have demonstrated Bax induction and activation in kidneys following cisplatin treatment of wild-type animals. We have further shown that Bax-deficient mice are more resistant to cisplatin injury than wild-type animals. In primary cultures of proximal tubular cells, Bax deficiency attenuates cytochrome *c* release and apoptosis during cisplatin incubation. Together, this study has demonstrated compelling evidence for a role of Bax and related apoptotic pathway in cisplatin nephrotoxicity.

RESULTS

Bax expression and activation during cisplatin nephrotoxicity in C57BL/6 mice

A single dose of cisplatin (30 mg/kg, intraperitoneally) induced acute kidney injury in C57BL/6 mice, leading to the loss of renal function. Three days after cisplatin injection, the serum level of creatinine increased from 0.3 mg/dl to 3.1 mg/dl, and blood urea nitrogen (BUN) increased from

~50 mg/dl to over 180 mg/dl. To examine changes in Bax, we initially analyzed Bax expression under the pathological condition. Immunoblotting of whole kidney lysates detected an increase of Bax expression during cisplatin treatment (Figure 1a). Bax was induced at day 1 (lane 2) and intensified by day 3 (lane 4).

In normal healthy cells, Bax mainly resides in the cytosol. Following activation, Bax translocates to mitochondria to induce membrane permeabilization. Thus to detect Bax activation, we analyzed Bax translocation or accumulation in mitochondria. To this end, cytosolic and mitochondrial fractions were isolated from kidney tissues for immunoblot analysis of Bax. As shown in Figure 1b, in control tissues, Bax was mainly detected in the cytosolic fraction (lane 1). Following cisplatin treatment, cytosolic Bax increased to some extent, whereas mitochondrial Bax had a marked increase (lane 2), suggesting Bax accumulation and activation in mitochondria under the pathological condition. We further determined Bax activation by immunofluorescence using an antibody that specifically recognized active Bax. As expected, following the staining control renal cortical tissues had a weak fluorescence background (Figure 1c: control). After cisplatin treatment, active Bax immunofluorescence was clearly shown in specific cells of cortical tissues. Noticeably, Bax staining in these cells was in punctiform, suggesting organellar or mitochondrial accumulation of Bax (Figure 1c: cisplatin). Together these results demonstrate Bax induction and activation during cisplatin nephrotoxicity *in vivo*.

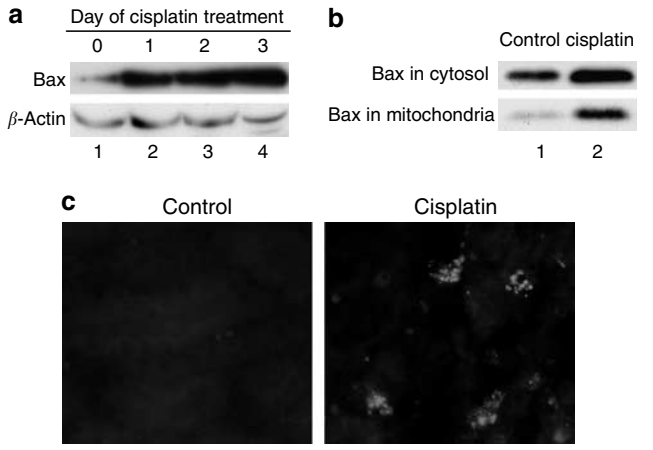

Figure 1 | Bax expression and activation during cisplatin nephrotoxicity. Wild-type C57BL/6 mice were injected with cisplatin or saline as control. (**a**) Immunoblot of Bax and β-actin in whole tissue extracts. Renal tissues were collected 0, 1, 2, or 3 days after cisplatin injection for immunoblot analysis using an antibody for total Bax. The blot was reprobed for β-actin to monitor protein loading and transferring. (**b**) Immunoblot analysis of Bax in mitochondrial and cytosolic fractions of renal tissues. Renal tissues were collected at day 3 of cisplatin treatment and fractionated into mitochondrial and cytosolic fractions for immunoblot analysis using an antibody for total Bax. (**c**) Immunofluorescence of active Bax in renal cortex. Renal cortical tissues of control and 3 day cisplatin-treated mice were processed for immunofluorescence using an antibody specific for active Bax. The blots and immunofluorescence are representatives of at least two separate experiments. The results show Bax expression, mitochondrial accumulation, and activation during cisplatin nephrotoxicity.

Localization of Bax activation in renal tubular cells

To identify the cell type(s) that activated Bax, we co-stained the kidney tissues for active Bax and tubular cell markers. For this purpose, we used two fluorescein isothiocyanate (FITC)-labeled lectins, Phaseolus Vulgaris Agglutinin (PHA) and Peanut Agglutinin (PNA), which bind and stain proximal and distal tubules respectively.[23,24] PHA binds to proximal tubular cells mainly at the brush border, whereas PNA binds to the apical membrane of distal tubular cells. In renal cortex, many cells were labeled by FITC-PHA (Figure 2a), whereas fewer cells were labeled by FITC-PNA (Figure 2b). This was expected, because the majority of renal tubules in the cortex are proximal tubules. When the lectin staining images were superimposed with Bax immunofluorescence, most of Bax

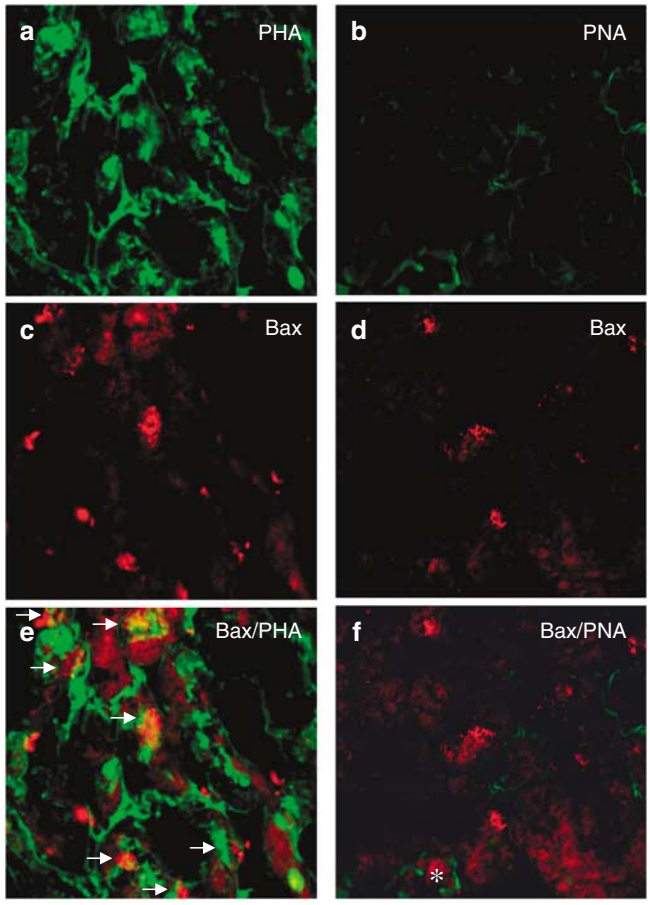

Figure 2 | Bax activation in renal tubular cells during cisplatin nephrotoxicity. Wild-type C57BL/6 mice were injected with cisplatin to collect renal cortical tissues 3 days later. (**c**, **d**) The tissues were processed for immunofluorescence of active Bax, followed by staining with FITC-labeled (**a**) PHA or (**b**) PNA. (**e**, **f**) The images of Bax immunofluorescence were superimposed with PHA or PNA staining of the same tissues. Arrow: colocalization of active Bax and PHA staining. Asterisk: colocalization of active Bax with PNA staining. The results suggest that the majority of Bax active cells are in proximal tubules.

active cells overlapped with PHA staining (Figure 2e, arrowed). Occasionally, we detected PNA-positive cells that had active Bax, but the number was very limited (Figure 2f, asterisk). The results suggest that cisplatin mainly induces and activates Bax in cells of the proximal tubules.

Preservation of renal function in Bax-deficient mice during cisplatin injury

To identify the role of Bax in cisplatin nephrotoxicity, we compared wild-type ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) mice for renal function during cisplatin treatment. We routinely conducted genotyping to select $Bax^{-/-}$ homozygote mice for experiment. At the end of experiment, we confirmed Bax deficiency in renal tissues of these animals by immunoblot analysis (Figure 3a). Without cisplatin treatment, $Bax^{+/+}$ and $Bax^{-/-}$ mice showed similar basal levels of BUN ($\sim$50 mg/dl) and serum creatinine (0.3–0.4 mg/dl) (Figure 3b and c). Cisplatin treatment for 3 days induced obvious increases in BUN (184 mg/dl) and serum creatinine (3.1 mg/dl) in wild-type $Bax^{+/+}$ mice (Figure 3b and c: open columns). The increases were significantly ameliorated in $Bax^{-/-}$ mice, which had a BUN value of 113 mg/dl and serum creatinine of 1.4 mg/dl, suggesting a partial preservation of renal function in the gene knockout animals.

Cisplatin-induced renal tissue damage in wild-type and Bax-deficient mice

We examined renal histology following hematoxylin and eosion staining (Figure 4a). Without cisplatin treatment, $Bax^{+/+}$ and $Bax^{-/-}$ mice showed similar normal renal histology. Following cisplatin injection, $Bax^{+/+}$ mice developed renal tissue damage, which started from day 2 and became obvious at day 3 (Figure 4a). The damage was characterized by tubular cell lysis, complete loss of brush border, and sloughed debris in tubular lumen space. During the observation period, the damage was mainly concentrated in the outer stripe of outer medulla and renal cortex. Compared with $Bax^{+/+}$ animals, $Bax^{-/-}$ mice showed less tubular damage and the majority of the proximal tubules preserved their brush border, despite signs of stress and injury including tubular dilation and distortion (Figure 4a). We further determined the pathological scores of the collected tissues. As shown in Figure 4B, the $Bax^{+/+}$ animals had an injury score of 3.5, whereas $Bax^{-/-}$ mice had a score of 1.8.

Cisplatin-induced renal cell apoptosis in wild-type and Bax-deficient mice

Bax is a critical pro-apoptotic molecule, which upon activation accumulates in mitochondria to permeabilize the outer membrane, leading to the release of apoptogenic

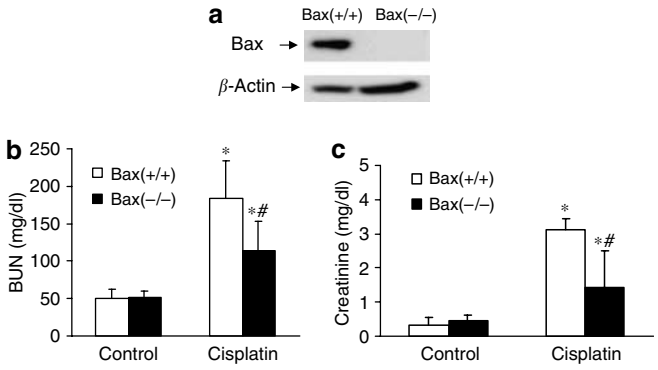

Figure 3 | Loss of renal function during cisplatin treatment of Bax-deficient and wild-type mice. Wild-type ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) mice were injected with cisplatin or saline (control) for 3 days. (**a**) Immunoblot analysis of Bax and β-actin in whole kidney lysate to confirm Bax deficiency in $Bax^{-/-}$ mice. (**b**) BUN. Data are presented as mean $\pm$ s.d., ($n \geqslant 8$). (**c**) Serum creatinine concentration. Data are presented as mean $\pm$ s.d., ($n \geqslant 3$). *Statistically significant difference compared with control. #statistically significant difference compared with cisplatin-treated wild-type group. The results show an amelioration of cisplatin-induced renal failure in Bax-deficient mice.

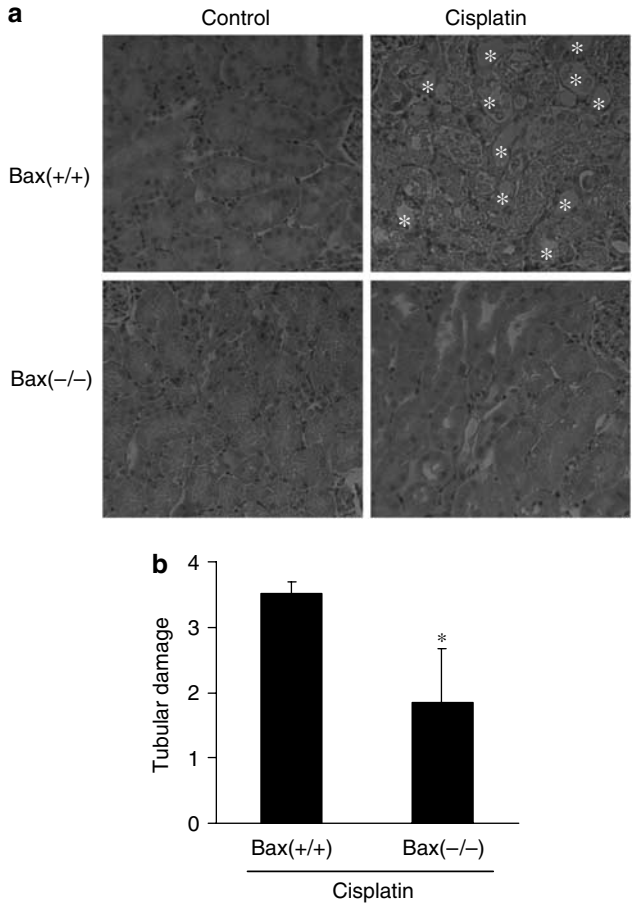

Figure 4 | Cisplatin-induced tissue damage in Bax-deficient and wild-type mice. Wild-type ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) mice were injected with cisplatin or saline (control) to collect renal cortical tissues 3 days later. The tissues were fixed and paraffin-embedded for hematoxylin and eosin (H&E) staining. (**a**) Representative images of hematoxylin and eosin staining. Asterisks: lysed tubules. (**b**) Pathological scores of tubular damage. Data are presented as mean $\pm$ s.d. ($n = 5$). *Statistically significant difference compared with the wild-type ($Bax^{+/+}$) group. The results show lower tubular damage in renal cortex of Bax-deficient mice.

factors such as cytochrome *c*. To determine the effects of Bax-deficiency on renal apoptosis during cisplatin nephrotoxicity, we analyzed kidney tissues by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, which detects DNA cleavage in apoptotic cells (as shown in Figure 5a). Regardless the Bax status, no TUNEL-positive cells were shown in control renal tissues. Cisplatin treatment led to significant amounts of TUNEL-positive cells in $Bax^{+/+}$ kidneys of wild-type animals (Figure 5a, arrowed). These cells were mainly detected in renal cortex and outer medulla, and not in inner medulla (not shown). Importantly, $Bax^{-/-}$ mice had much fewer TUNEL-positive cells in kidney tissues (Figure 5a). By counting the numbers of TUNEL-positive cells, we further confirmed the significant difference between wild-type ($Bax^{+/+}$: 37 TUNEL-positive cells/mm^2) and Bax-deficient ($Bax^{-/-}$: 15 TUNEL-positive cells/mm^2) tissues (Figure 5b).

To identify the origin(s) of the apoptotic cells, we co-stained the wild-type renal tissues for TUNEL and PHA or PNA. As shown in Figure 6a, the majority of TUNEL-positive cells were shown in PHA-stained tubules (arrows). We also detected some cells that had co-staining of TUNEL and PNA (arrowheads), but the number of this type of cells was relatively small. The results suggest the occurrence of apoptosis in both proximal and distal tubules in this experimental model. We further examined the colocalization of TUNEL-positive cells with active Bax (Figure 6b). For this purpose, active Bax was revealed by immunofluorescence using an antibody specific for active Bax. As shown in Figure 6b, some cells were co-stained by both TUNEL assay and active Bax immunofluorescence (arrowheads). Nevertheless, we also detected cells that were TUNEL-positive but showed very weak or no active Bax (Box). There were also cells that had active Bax but did not show clear TUNEL

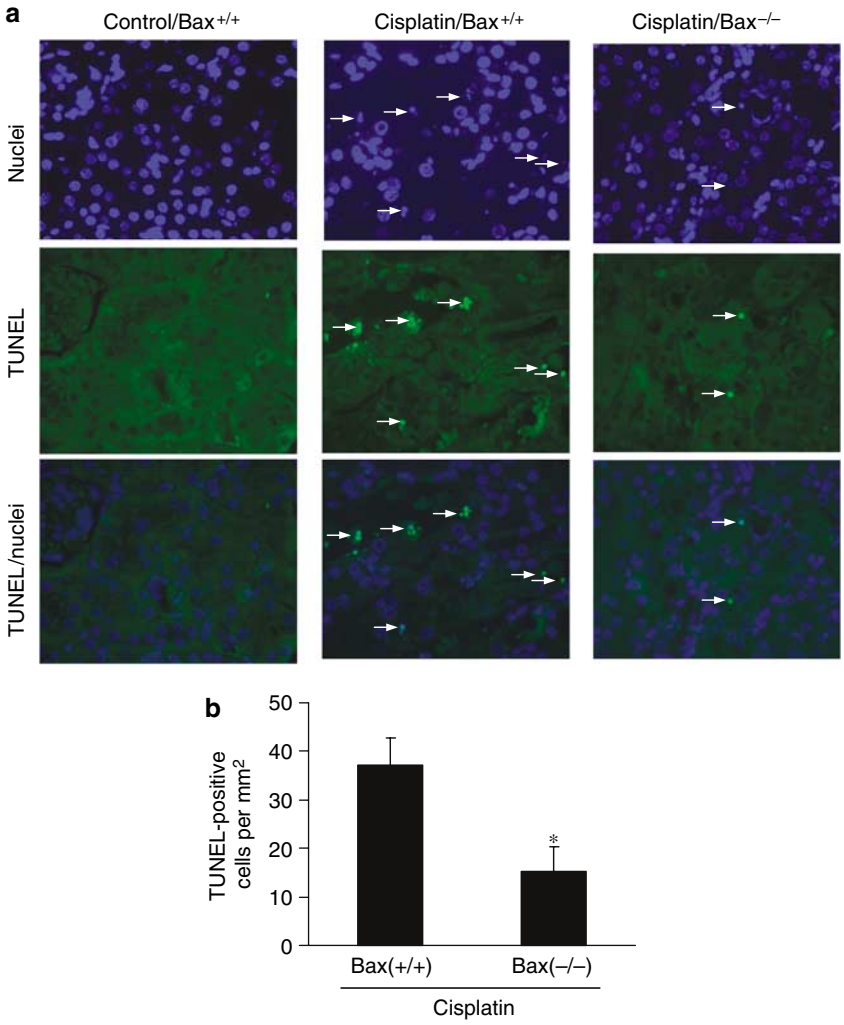

Figure 5 | Cisplatin-induced renal apoptosis in Bax-deficient and wild-type mice. Wild-type ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) mice were injected with cisplatin or saline (control) to collect renal cortical tissues 3 days later. The tissues were fixed and paraffin-embedded for TUNEL assay. (**a**) Representative images of TUNEL assay. Arrows: TUNEL-positive cells with condensed or fragmented nuclei. (**b**) Counting of TUNEL-positive cells in renal cortical tissues. *Statistically significant difference compared with the wild-type group. Data are expressed as mean $\pm$ s.d. ($n \geqslant 10$).

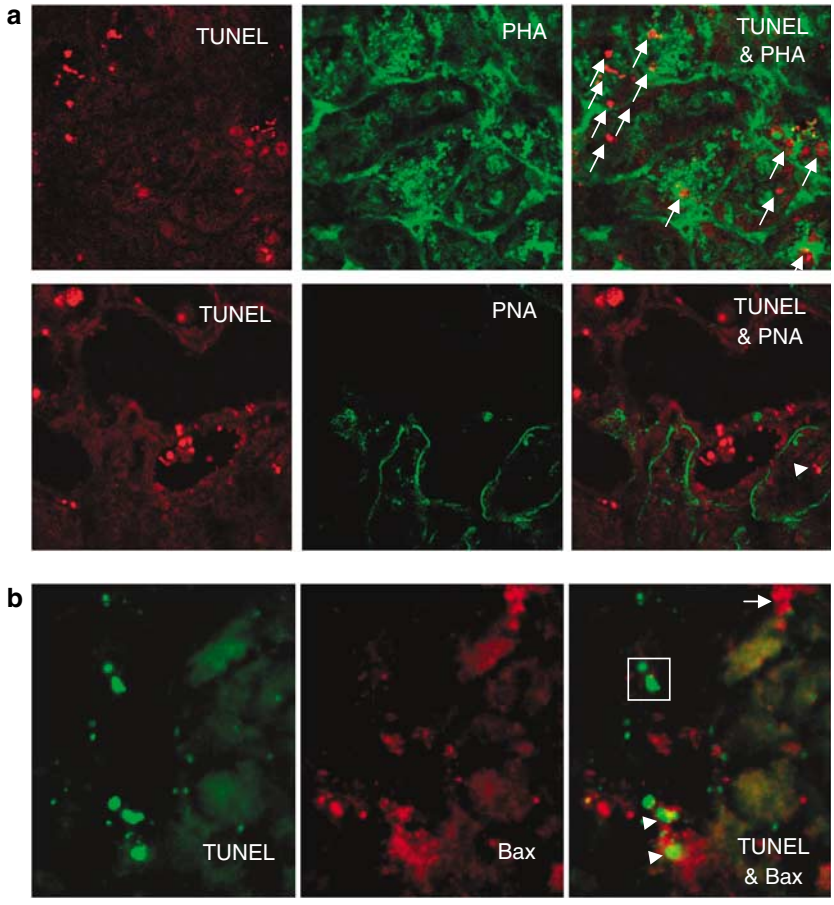

Figure 6 | Localization of Bax activation and apoptosis in cisplatin-injured renal tissues. Wild-type C57BL/6 mice were injected with cisplatin to collect renal cortical tissues at day 3. **(a)** The tissues were processed for TUNEL assay, followed by staining with FITC-labeled PHA or PNA. The TUNEL images were superimposed with PHA or PNA staining of the same tissues to show the possible colocalization. Arrows: localization of TUNEL-positive cells in PHA-stained tubules. Arrowheads: localization of TUNEL-positive cells in PNA-stained tubules. **(b)** The tissues were processed for TUNEL assay and immunofluorescence of active Bax. The TUNEL image was superimposed with active Bax staining to show colocalization. Arrowheads: colocalization of active Bax and TUNEL staining in the same cell. Box: TUNEL-positive cells with very weak or no active Bax. Arrow: cell with active Bax but no TUNEL staining.

staining (arrow). Analysis of the co-staining images indicated that about 30–40% TUNEL-positive cells had active Bax.

Cisplatin-induced cytochrome *c* release in renal tissues of wild-type and Bax-deficient mice

We further examined cytochrome *c* release in renal tissues during cisplatin treatment of $Bax^{+/+}$ and $Bax^{-/-}$ animals. To this end, renal cortical tissues were collected to isolate cytosolic and mitochondrial fractions for immunoblot analysis. In wild-type $Bax^{+/+}$ mice, there was a small yet consistently detectable cytochrome *c* release after 2 days of cisplatin treatment (Figure 7a: lane 2). At day 3, cytochrome *c* released into the cytosolic fraction became obvious (Figure 7a: lane 3). Nevertheless, the released portion was $<20\%$ of total cytochrome *c* and, as a result, mitochondrial cytochrome *c* did not show an obvious decrease. Importantly, cytochrome *c* release during cisplatin treatment was ameliorated in kidney tissues of $Bax^{-/-}$ animals (Figure 7b).

Effects of Bax deficiency on cisplatin-induced apoptosis in primary cultures of proximal tubular cells

We hypothesized that the observed resistance of Bax-deficient animals to cisplatin nephrotoxicity was largely due to Bax deficiency in proximal tubular cells. To test directly this possibility, we isolated proximal tubular cells from wild-type ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) mice. Primary cultures of the cells were then treated with 50 μM cisplatin. Representative cell morphology was shown in Figure 8a–d. Apoptosis was minimal under control incubation, regardless the presence or absence of Bax (not shown). Following cisplatin treatment, some primary proximal tubular cells developed typical apoptotic morphology, showing cellular shrinkage, apoptotic body formation, and nuclear condensation and fragmentation (Figure 8a–d, arrows). Apoptosis was obviously lower in $Bax^{-/-}$ cells (Figure 8a–d). We further quantified apoptosis by cell counting. As shown in Figure 8e, under control conditions, the primary tubular cells had very low apoptosis. Cisplatin treatment for 16 and 20 h induced

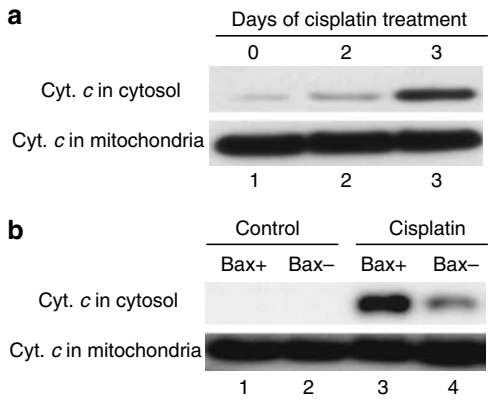

Figure 7 | Cytochrome *c* release during cisplatin nephrotoxicity in Bax-deficient and wild-type mice. (**a**) Cisplatin-induced cytochrome *c* release in renal tissues of wild-type mice. After 0–3 days of cisplatin treatment, renal cortical tissues were collected to isolate the mitochondrial and cytosolic fractions for immunoblot analysis of cytochrome *c*. (**b**) Cytochrome *c* release in renal cortical tissues during cisplatin treatment of wild-type and Bax-deficient mice. Three days after cisplatin or saline control injection, renal cortical tissues were collected to isolate mitochondrial and cytosol fractions for immunoblot analysis of cytochrome *c*. The blots are representatives of three separate experiments. The results show cytochrome *c* release during cisplatin nephrotoxicity, which is partially attenuated in Bax-deficient mice.

about 20 and 30% apoptosis in $Bax^{+/+}$ tubular cells, but only 5 and 10% in $Bax^{-/-}$ cells (Figure 8e). Under the experimental conditions, cisplatin did not induce significant necrosis as shown by propidium iodide staining (not shown).

Cisplatin-induced cytochrome *c* release in wild-type and Bax-deficient primary cultures of proximal tubular cells

We further determined cytochrome *c* release during cisplatin treatment of primary cultures of $Bax^{+/+}$ and $Bax^{-/-}$ tubular cells. To this end, cells were fractionated into cytosolic and organellar (mitochondrial) fractions for immunoblot analysis. As shown in Figure 9a, without cisplatin treatment, both $Bax^{+/+}$ and $Bax^{-/-}$ cells had cytochrome *c* in mitochondria, with very little cytochrome *c* in the cytosolic fraction (lanes 1, 2, 5, and 6). Following cisplatin incubation, significant amounts of cytochrome *c* were detected in the cytosolic fraction of $Bax^{+/+}$ cells, accompanied by obvious loss of cytochrome *c* from the mitochondria fraction (lanes 3 and 4). In $Bax^{-/-}$ cells, cisplatin induced cytochrome *c* release (lanes 7, 8), but at a much lower level than $Bax^{+/+}$ cells. The immunoblot results were confirmed by immunofluorescence staining of cytochrome *c* (Figure 9b–m). In control cells, cytochrome *c* immunofluorescence was exclusively mitochondrial, showing a perinuclear organellar staining (Figure 9b and d). Following cisplatin treatment, many $Bax^{+/+}$ cells lost mitochondrial cytochrome *c*, resulting in a faint staining in the cytosol (Figure 9c). The same cells showed apoptotic morphology including nuclear fragmentation and condensation (Figure 9g and k, arrowed). Cytochrome *c* release was also noticed in some cisplatin-treated $Bax^{-/-}$ cells (Figure 9e and m, arrowed), but the percentage was significantly lower.

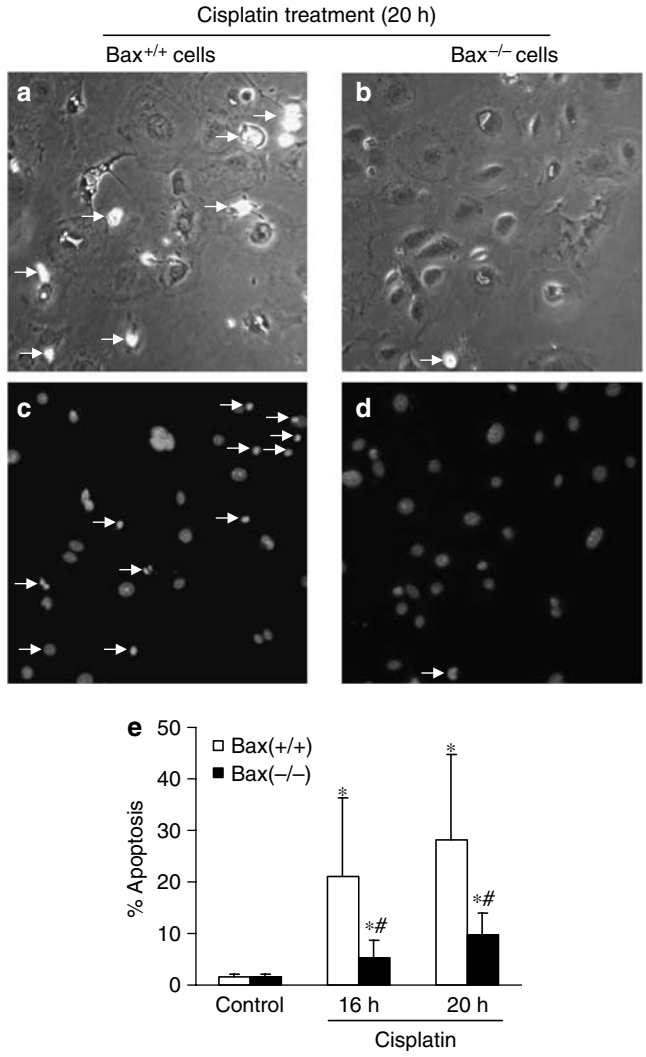

Figure 8 | Cisplatin-induced apoptosis in primary proximal tubular cells isolated from wild-type and Bax-deficient mice. Proximal tubular cells were isolated from wild-type ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) mice and cultured for experiment. The cells were incubated with or without 50 μM cisplatin. (**a–d**) Representative images of cell morphology after 20 h of cisplatin incubation. Cells after cisplatin incubation were stained with Hoechst 33342. (**a**, **b**) Cell and (**c**, **d**) nuclear morphology of the same fields were recorded by phase-contrast and fluorescence microscopy, respectively. Arrows: apoptotic cells showing cellular and nuclear condensation and fragmentation. (**e**) Percentage of apoptosis evaluated by cell counting. The cells with typical apoptotic morphology were counted after control or cisplatin incubation for 16 or 20 h. Data are expressed as mean $\pm$ s.d. ($n \geqslant 6$). *Statistically significant difference compared with control. #Statistically significant difference compared with cisplatin-treated wild-type group.

Together the results support an important role of Bax in mitochondrial membrane permeabilization, cytochrome *c* release, and apoptosis during cisplatin nephrotoxicity.

DISCUSSION

Bax was implicated in cisplatin nephrotoxicity by previous studies.[6,17,18,22] However, those experiments were mainly conducted in cultured cells and whether Bax has a role in

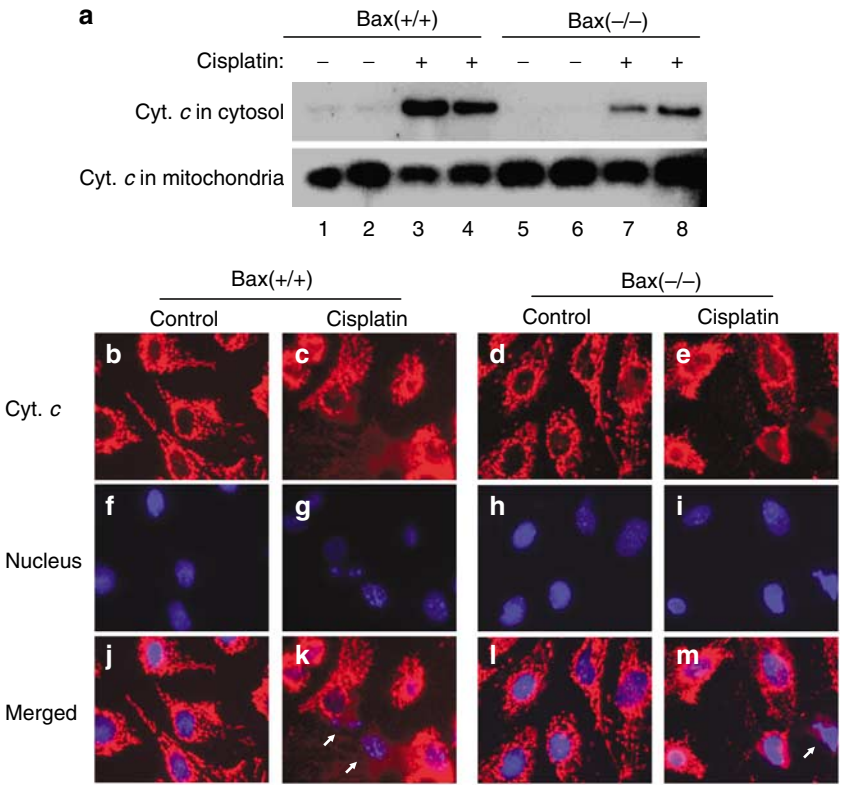

Figure 9 | Cisplatin-induced cytochrome *c* release in primary cultures of proximal tubular cells isolated from wild-type and Bax-deficient mice. Proximal tubular cells were isolated from wild-type ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) mice and cultured for experiment. The cells were incubated with or without 50 μM cisplatin for 20 h. (**a**) Immunoblot analysis of cytochrome *c* release. After control or cisplatin incubation, the cells were fractionated into cytosolic and organellar (mitochondrial) fractions for immunoblot analysis of cytochrome *c*. Samples from two separate experiments were analyzed on the same blot. (**b–m**) Immunofluorescence of cytochrome *c*. (**b–e**) Control and cisplatin-treated cells were processed for immunofluorescence of cytochrome *c* and (**f–i**) then stained for nucleus with Hoechst 33342. (**j–m**) The immunofluorescence and nuclear staining of the same cells were superimposed. Arrows: cells with cytochrome *c* in cytosol and apoptotic nuclear morphology.

cisplatin nephrotoxicity *in vivo* is unclear. Importantly, the earlier evidence was correlative and circumstantial, and did not establish the role of Bax by inhibitory approaches. Using gene knockout mouse and primary culture models, the current study has demonstrated the first compelling evidence for the involvement of Bax in cisplatin nephrotoxicity.

The Bax-deficient mouse model was originally established by Knudson *et al.*[25] via targeted gene disruption.$Bax^{-/-}$ homozygotes are viable with cell lineage-specific aberrations in apoptosis, which are detected mainly in thymocytes, B cells and male reproductive systems.[25] The knockout model has been used to identify a role for Bax in cell death during tumorigenesis,[26] spinal cord injury,[27] and more recently during myocardial ischemia–reperfusion.[28] In our study, $Bax^{-/-}$ mice were used at the young adult age of 8–10 weeks. The shape and size of the kidneys of these animals were similar to those of the wild type. There were no noticeable defects in renal function and structure in these animals either. Unchallenged wild-type and Bax-deficient mice showed similar basal levels of BUN and serum creatinine. The lack of detectable abnormalities in kidneys of the $Bax^{-/-}$ mice justifies the feasibility of using this gene knockout model to determine the involvement of Bax in the development of renal pathology.

In wild-type C57BL/6 mice, cisplatin induced an early Bax expression, starting from day 1 of treatment. At this time point, renal injury shown by tissue damage, apoptosis, and loss of renal function was not obvious (data not shown).[29] Consistently, cytochrome *c* release was not detected (Figure 7) until day 2 and 3, time points of Bax activation (Figure 1c). The results suggest that the expressed Bax needs to be activated to induce apoptosis. The temporal analysis supports a role of Bax in tubular cell injury in this experimental model.

Active Bax was shown mainly in proximal tubules with occasional occurrence in distal tubules (Figure 2). During cisplatin nephrotoxicity, both proximal and distal tubules are injured. Although work suggested distal tubules as the primary site of apoptosis,[30] more recent studies also showed apoptosis in proximal tubules.[9,20] As a result, primary and transformed proximal tubular cells are commonly used to study cisplatin-induced apoptosis as a critical pathogenic event. Nevertheless these studies did not clearly identify the origin(s) of apoptotic cells by using specific markers. We stained the tissues with the lectins that are specific for

proximal or distal tubules. The results show that many apoptotic cells were stained with PHA, a proximal tubule-binding lectin (Figure 6a). We also detected positive staining of some apoptotic cells by PNA, a distal tubule-binding lectin. It is suggested that apoptosis occurs in both proximal and distal tubules in this experimental model. These observations support the significance of Bax expression and activation in proximal tubular cells during cisplatin nephrotoxicity.

Co-staining of active Bax and apoptosis (TUNEL) indicated that 30–40% of apoptotic cells had Bax activation (Figure 6b). Some TUNEL-positive cells did not show clear active Bax staining. The straightforward explanation is that Bax may be involved in apoptosis of the 30–40% cells but not others. However, some of the apoptotic cells might have entered late stage of apoptosis and thus have lost Bax due to extensive proteolytic degradation. This possibility is supported by the quantitative comparison of apoptosis in wild-type and Bax-deficient mice. As shown in Figure 5b, apoptosis during cisplatin treatment was reduced from 37 in wild-type mice to 15 (TUNEL-positive cells/mm^2) in Bax-deficient mice, suggesting that Bax may be involved in 60% (22/37) apoptosis in this experimental model. It is not surprising that Bax deficiency did not completely block apoptosis, because multiple pathways of apoptosis can be activated by cisplatin in renal tubular cells.

In addition of apoptosis, necrotic tissue damage during cisplatin nephrotoxicity was also suppressed in Bax-deficient mice (Figure 4). This is an interesting observation because Bax and related molecules are generally considered to be apoptotic. However, it is noteworthy that these proteins, although originally identified in apoptosis, may promote necrosis under certain pathological conditions. Despite the distinct morphological differences, recent studies suggest that apoptosis and necrosis may share some of the critical signaling events including mitochondrial damage.[31] It is speculated that by damaging mitochondria, Bax and related molecules may also be involved in necrotic cell death. Alternatively, some of the necrotic cells may result from the lysis of apoptotic cells; by reducing apoptosis, Bax deficiency may decrease this type of 'secondary necrosis'. Our recent work showed that both necrosis and apoptosis of tubular cells during renal ischemia–reperfusion were ameliorated in mice that were deficient of Bid, another proapoptotic Bcl-2 family protein.[32]

The mechanisms of Bax expression and activation during cisplatin nephrotoxicity are currently unclear. Nevertheless, cisplatin activates p53 in renal tubular cells,[5–7,13,17] which reportedly is a transcriptional regulator of Bax expression.[33] In p53-deficient mice, Bax expression during cisplatin nephrotoxicity is ameliorated (Wei and Dong, unpublished data). For Bax activation, our recent work suggests that p53-upregulated modulator of apoptosis (PUMA-α) is induced in a p53-dependent manner during cisplatin nephrotoxicity. Following induction, PUMA-α translocates to mitochondria to bind and neutralize Bcl-XL, freeing Bax for activation.[6] This scenario emphasizes a role of PUMA-α in Bax activation. However, it is noteworthy that, in addition to protein interaction, Bax can also be regulated by phosphorylation. In this direction, recent work by Kim *et al.*[34] has suggested the involvement of ERK in Bax regulation during cisplatin incubation of opossum kidney cells. Further investigations are needed to clarify the signaling pathways that lead to Bax induction and activation during cisplatin nephrotoxicity.

The inhibitory effects of Bax deficiency on cisplatin-induced renal injury are substantial but not complete. Apoptosis shown by TUNEL assay was suppressed to $\sim$40% and tissue damage reduced to $\sim$50%. Consistently, renal function measured as BUN and serum creatinine was partially protected in these animals. These results suggest that Bax is important to the development of cisplatin nephrotoxicity, but it is not the single mediating factor. This conclusion is consistent with previous studies, which suggest the involvement of other apoptotic pathways in cisplatin nephrotoxicity, including the death receptor-mediated extrinsic pathway.[19,20] In addition, other proapoptotic Bcl-2 proteins may have a redundant role of Bax. For example, upon apoptosis induction, Bak may also oligomerize in mitochondria to permeabilize the outer membrane, leading to cytochrome *c* release and apoptosis.[35]

In conclusion, this study has demonstrated compelling evidence for the involvement of Bax in cisplatin-induced tubular cell apoptosis, acute kidney injury and renal failure. Targeting Bax by pharmacological and genetic approaches may offer a new therapeutic strategy for cisplatin nephrotoxicity.

MATERIALS AND METHODS

Animals

Breeders of Bax-deficient and wild-type mice with C57BL/6 background were originally purchased from Jackson Laboratory (Bar Harbor, ME, USA). Male Bax$^{-/-}$ homogyzotes were infertile; thus colonies of heterozygous Bax$^{-/+}$ were maintained. Bax$^{-/+}$ animals were interbred to produce homozygous Bax$^{-/-}$ mice for experiments. The genotypes of the animals were determined by polymerase chain reaction-based genotyping according to a published protocol.[36] In this study, male animals of 8–10 weeks were used. As wild-type controls, both littermates and age/sex-matched nonlittermate C57BL/6 mice were used.

Cisplatin treatment of animals

The animals were housed with a 12/12 h light–dark cycle, food and water available *ad libitum*. Cisplatin was freshly prepared at 1 mg/ml in saline and was administered by a single intraperitoneal injection at a dose of 30 mg/kg as described previously.[29] As control, a group of animals was injected with a comparable volume of saline.

Analysis of renal function

Renal function was monitored by measuring serum creatinine and BUN as described previously.[29,32] Briefly, blood samples were collected from the tail of the animals or at the time of killing. After clotting at room temperature, serum was collected by centrifugation. Creatinine was measured with a commercial kit from Stanbio Laboratory (Boerne, TX, USA). BUN was measured with a kit from Biotron Diagnostics Inc. (Hemet, CA, USA).

Renal histology

Kidneys were harvested and fixed with 4% paraformaldehyde for paraffin embedding. Paraffin-embedded tissues were sectioned at 4 μm for hematoxylin and eosin staining. Renal histology was examined in a blinded fashion by an experimental pathologist. 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, no damage; 1, $<$25%; 2, 25–50%; 3, 50–75%; 4, $>$75%. Pictures of representative fields were also recorded.

TUNEL assay

Apoptosis in renal tissues was identified by TUNEL assay as shown in our recent work.[29,32] Renal tissues were fixed with 4% paraformaldehyde, paraffin-embedded, and cut into 4-μm sections. TUNEL assay was conducted using the *in situ* Cell Death Detection kit from Roche Applied Science (Indianapolis, IN, USA), 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 was identified in cell nucleus with DNA breakage by fluorescence microscopy.

Immunofluorescence

For staining of renal tissues, kidneys were collected and immediately frozen in liquid nitrogen. Freshly frozen tissues were cryosectioned at 7 μm and fixed with 4% paraformaldehyde for 20 min at room temperature. The fixed samples were permeabilized with 1% Triton X-100 and incubated for 1 h in a blocking buffer containing 2% BSA, 0.2% nonfat milk, 0.8% Triton X-100, and 2% normal goat serum. The samples were further incubated for 1 h with the primary anti-active Bax antibody (Upstate Biotechnology, Lake Placid, NY, USA). Finally, the slides were exposed to a Cy3-labelled goat anti-rabbit secondary antibody (Chemicon, Temecula, CA, USA) for examination by fluorescence microscopy. For immunofluorescence analysis of cytochrome *c* in proximal tubular cells, the cells were grown on collagen-coated coverslips and fixed with a modified Zamboni's fixative containing picric acid and 4% paraformaldehyde. After blocking in 2% normal goat serum, the cells were incubated with a mouse monoclonal anti-cytochrome *c* (BD Pharmingen, San Jose, CA, USA), followed by exposure to Cy3-labelled goat anti-mouse secondary antibody (Chemicon). The cells were finally stained with 5 μg/ml Hoechst 33342 to examine nuclear morphology by fluorescence microscopy.

Tissue staining with tubular cell markers

After immunofluorescence staining of active Bax, kidney tissues were incubated again with the blocking buffer (see the above section), followed by exposure to a FITC-labeled lectin. Two kinds of lectins, PHA and PNA (Vector Laboratories, Burlingame, CA, USA), were used in this study, which stain proximal tubules and distal tubules, respectively.[23,24] The double-stained tissues were examined by confocal microscopy using a LSM 510 Zeiss microscope.

Preparation of cell or tissue lysate and fractions

To obtain whole kidney lysate, renal tissues were ground in liquid N_2 and then immediately dissolved in 2% sodium dodecyl sulfate buffer. Benzonase (VWR, West Chester, PA, USA) was added to digest the genomic DNA. The insoluble part was further removed by 10 min of centrifugation at 15 000 *g*.

The mitochondrial and cytosolic fractions of kidney tissues were collected as described by Weinberg *et al.*[37] with some modifications. Briefly, fresh or frozen kidney tissues were homogenized in a buffer containing 0.27 M sucrose, 1mM EGTA, and 5 mM Tris–HCl (pH 7.4). The homogenates were centrifuged at 600 *g* for 10 min to remove cell debris and nuclei. The supernatants were further centrifuged at 10 000 *g* for 10 min to collect the mitochondrial fraction in the pellets. The supernatant fractions were centrifuged again at 100 000 *g* for 60 min to collect the cytosolic. The whole isolation procedure was conducted at 4°C.

Primary cultures of proximal tubular cells were fractionated into cytosolic fraction and the membrane-bound organellar fraction by using low concentrations of digitonin, which specifically permeabilized the plasma membrane to release cell cytosol.[6,17,38] Briefly, cells were incubated with 0.05% digitonin in isotonic sucrose buffer (in mM: 250 sucrose, 10 Hepes, 10 KCl, 1.5 $MgCl_2$, 1 EDTA, and 1 EGTA; pH 7.1) for 5 min at room temperature. The portion released by digitonin was collected as the cytosolic fraction. Digitonin insoluble part was further extracted with 2% sodium dodecyl sulfate to collect the membrane bound fraction that contained mitochondria.

Primary proximal tubular cell culture

Proximal tubular cells were isolated for primary culture as described by Nowak *et al.*[39] and Zager *et al.*[40] with minor modifications. Briefly, immediately after the harvest of the kidneys, the renal cortex part was collected and minced on an ice-cold plate. The minced tissues were digested for 15 min with 0.75 mg/ml collagenase 4 at 37°C in Hank's solution containing 0.75 mg/ml trypsin inhibitor. The digestion was stopped by mixing with ice-cold 10% horse serum. Subsequently, the cells were washed with ice-cold Hanks' solution and serum-free Dulbecco's modified Eagle's medium/F-12 medium. The proximal tubular cells were purified by 10 min of centrifugation at 2000 *g* in 32% Percoll, 4°C. The pellet was collected and washed twice with ice-cold serum-free Dulbecco's modified Eagle's medium/F-12 medium. Finally, the cells were plated into collagen-coated dishes and cultured in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 5 μg/ml transferrin, 5 μg/ml insulin, 0.05 μM hydrocortisone, and 50 μM vitamin c.

Cisplatin treatment of primary proximal tubular cells

The isolated cells were grown for 6–7 days and then plated at 0.3×10^6/35 mm dish for experiments. The next day, the cells were incubated with 50 μM cisplatin in fresh culture medium. Cell morphology was monitored by phase-contrast microscopy. Nuclear morphology was examined by fluorescence microscopy following Hoechst 33342 staining. Apoptosis was assessed by morphological criteria including cellular shrinkage, formation of apoptotic bodies, and condensation and fragmentation of nucleus. Cells with typical apoptotic morphology were counted to quantify apoptosis.[6,17]

Immunoblot analysis

Immunoblotting was performed by standard procedures after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Briefly, the blots were blocked with 5% fat-free milk and exposed to a specific primary antibody. The blots were then exposed to the horseradish-peroxidase-conjugated secondary antibody, and antigens on the blots were revealed by using the enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

Statistics

Data were expressed as mean $\pm$ s.d. Statistical differences between two groups were determined by Student's *t*-test with Microsoft Excel 2000. $P < 0.05$ was considered significantly different.

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