Role and regulation of activation of caspases in cisplatin-induced injury to renal tubular epithelial cells

GUR P. KAUSHAL, VARSHA KAUSHAL, XIAOMAN HONG, and SUDHIR V. SHAH

Departments of Medicine and Biochemistry, University of Arkansas for Medical Sciences, and Central Arkansas Veterans Healthcare System, Little Rock, Arkansas, USA

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Background. Cellular and molecular mechanisms responsible for cisplatin-induced nephrotoxicity to renal tubular epithelial cells are not well understood. Although caspases play a critical role in the execution of the cell death pathway, their specific role in toxic injury to renal tubular epithelial cells has not been elucidated previously.

Methods. The role of caspases in cisplatin-induced injury was determined using caspase inhibitors and p35 transfected LLC-PK1 cells. The Akt/PKB phosphorylation pathway was studied for the regulation of caspase activation in these cells.

Results. The activation of initiator caspases-8, -9 and -2, and executioner caspase-3 began after eight hours of cisplatin treatment, thereafter markedly increased in a time (8 to 24 hours) and dose-dependent manner (0 to 200 μmol/L). Proinflammatory caspase-1 did not show cisplatin-induced activation. Inhibition of caspase-3 by over expressing cowpox virus p35 protein or alternatively by the peptide inhibitor DEVD-CHO provided marked protection against cell death and partial protection against DNA damage. We then examined the role of the Akt/PKB phosphorylation pathway in regulation of cisplatin-induced caspase activation. There was a marked induction of Akt/PKB phosphorylation in a time (0 to 8 hours) and dose-dependent (0 to 200 μmol/L) manner during the course of cisplatin injury. Cisplatin-induced Akt/PKB activation was associated with Bad phosphorylation, suggesting induction of a cell survival signal mediated by the Bcl-2 family member, Bad. Wortmannin or LY294002, two structurally dissimilar inhibitors of phosphatidylinositol 3'-kinase (PI-3 kinase), abolished both cisplatin-induced Akt phosphorylation and Bad phosphorylation, and promoted cisplatin-induced early and accelerated activation of caspase-3 and caspase-9, but not of caspase-8 and caspase-1, indicating that inhibition of the Akt/PKB phosphorylation pathway enhances the mitochondrial-dependent activation of caspases. The impact of enhanced activation of caspases by wortmannin or LY294002 was reflected on accelerated cisplatin-induced cell death.

Conclusions. These studies demonstrate differential activation and role of caspases in cisplatin injury, and provide the first evidence of cisplatin-induced induction of the Akt/PKB phosphorylation pathway, inhibition of which enhances activation of caspase-3 and caspase-9.

The chemotherapeutic drug cisplatin, commonly used for the treatment of solid tumors of a wide range of tissues [1–3], is generally associated with nephrotoxicity as one of its major side effects. The primary targets of cisplatin in kidney are the proximal tubular epithelial cells where it accumulates and promotes the damage of these cells [3, 4]. The cellular and molecular mechanisms responsible for drug-induced nephrotoxicity to renal tubular epithelial cells are not well understood. Although caspases play a critical role in the execution of the cell death pathway, their specific role in toxic injury to renal tubular epithelial cells has not been elucidated previously.

Caspases (originally known as interleukin 1β-converting enzyme and its family of proteases) are a family of cell death proteases [5] that plays an essential role in the execution phase of apoptosis [6–11]. Thus far, 14 members of the caspase family have been identified from mammalian cells [11, 12]. Caspase-8, -9, -10, and -2 have large prodomains and initiate the activation of downstream caspases, and caspase-3, -6, and -7 with smaller domains are identified as executioner caspases [11]. The downstream executioner caspase-3 is responsible for the cleavage of a wide variety of physiological substrates including DNA repair enzymes [13, 14], ICAD (inhibitor of caspase-activated DNase) or DNA fragmentation factor [15, 16], nuclear structural proteins [11, 17], and cytoskeleton proteins [11, 18, 19]. At present, there are two relatively well-characterized cell death pathways that result in the activation of downstream or executioner caspases. One is receptor-mediated and the other is mitochondrial-dependent. The receptor-dependent pathway is initiated by activation of cell death receptors (Fas and tumor necrosis factor) that activates procaspase-8, which in turn cleaves and activates downstream caspases-3 [20–22]. The mitochondrial-dependent pathway is triggered by cytochrome c release from the mitochondria.
that promotes the activation of procaspase-9 through Apaf-1 and dATP. Activated caspase-9 then cleaves and activates the downstream pro-caspase-3 [23–25]. However, whether cisplatin-induced activation of caspases are involved with these signaling pathways is not known.

Currently, there is limited information on the role of caspases in cisplatin-induced renal tubular epithelial (RTE) cell injury. Cisplatin has been shown to induce cell death in renal tubular epithelial cells [26–30]. Caspase-3 is activated in renal proximal tubular cells [31, 32] by cisplatin treatment, suggesting that cisplatin-induced cell death is mediated by caspases. We have previously demonstrated that renal tubular epithelial cells transcribe and express multiple caspases, including the initiator and executioner caspases [33]. However, there is no information on the specific role and regulation of the underlying mechanism(s) of signaling pathways responsible for the activation of caspases in cisplatin-induced cellular injury.

Cells have inherited information to initiate the apoptotic pathway in response to a death stimulus unless they are challenged by cell survival signals that maintain cell viability. On receiving a death stimulus, both cell death and cell survival pathways can be induced during the course of cellular injury. Therefore, the extent of cellular injury caused by a toxic agent will depend on the balance between activation of caspases triggered by toxic injury and on the induction of survival factors capable of blocking the activation of caspases. At present, there is no information on the induction of survival signals that regulate caspase activation during cisplatin-induced cellular injury. The present study provides evidence that cisplatin not only triggers selective and differential activation of caspases but also causes induction of the PI3/Akt pathway of cell survival, a signaling pathway previously unrecognized in cisplatin-induced cellular injury. The studies also demonstrate the specific role of executioner caspase-3 and enhanced activation of this caspase and the initiator caspase, caspase-9, by inhibition of the PI-3 kinase/Akt phosphorylation pathway during cisplatin-induced injury to renal tubular epithelial cells.

**METHODS**

**Cell culture and reagents**

LLC-PK1 cells obtained from American Type Culture Collection (Rockville, MD, USA) were cultured as in our previous studies [34]. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 20 mmol/L glycine, 50 mmol/L sodium azide (HEPES), and 2 mmol/L nonessential amino acids. Cultures were maintained in a humidified incubator gassed with 5% CO2 and 95% air at 37°C and fed with fresh medium at intervals of 48 to 72 hours. Experiments were performed with cells grown to 80% confluence. Caspase inhibitors, DEVD-CHO, IETD-CHO, and LEHD-CHO were purchased from Bachem Inc. (Torrance, CA, USA) and ZVAD-FMK was from Enzyme Systems products (Dublin, CA, USA). Caspase substrates were purchased from Peptide International (Louisville, KY, USA) and antibodies to caspases were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Akt, anti-ser-473-phosphorylated Akt, anti-Bad, anti-ser-136 phosphorylated Bad, anti-ser-112 phosphorylated Bad antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Expression plasmid for p35 and antibody to p35 were kindly provided by Dr. Anu Srini-vasan, Idun Pharmaceuticals (La Jolla, CA, USA).

**Treatment with cisplatin**

Cells were replaced with fresh DMEM containing serum, glutamine, pyruvate, nonessential amino acids, glucose, 3.7 g/L NaHCO3 and 20 mmol/L HEPES at pH 7.4 and incubated either with or without cisplatin of various concentrations for the period of time indicated. In initial studies, we determined the optimum exposure time and the suitable concentration of cisplatin. To determine a role of caspases in cisplatin-induced cell death, cells were preincubated with caspase inhibitors, DEVD-CHO for caspase-3 and LEHD-CHO for caspase-9 for 30 minutes, washed, and exposed to cisplatin (50 μmol/L).

**Determination of caspase activity**

Cells were harvested by centrifugation and the pellets were washed in cold phosphate-buffered saline (PBS) twice. The washed cell pellets were lysed with 20 mmol/L HEPES, pH 7.5, containing 10% sucrose, 0.1% CHAPS-3-[(3-cholamidopropyl)dimethylamino-1]propane-sulfonate), 2 mmol/L dithiothreitol (DTT), 0.1% NP40, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, and 1 μg/mL pepstatin A at 4°C. The supernatants obtained after centrifugation were used to determine the enzyme activity. The activities of caspase-1, -2, -3, -8, and -9 were determined by fluorometric assay using the substrates; YVAD-AMC for caspase-1, VDVAD-AMC for caspase-2, DEVD-AMC for caspase-3, LEHD-AMC for caspase-9, and IETD-AMC for caspase-9, which are specifically cleaved by the respective enzymes at the Asp residue to release the fluorescent leaving group, amino-4-methyl coumarin (AMC) [35, 36]. The enzyme extracts containing 50 μg protein were incubated with 100 mmol/L HEPES, pH 7.4, containing 10% sucrose, 0.1% CHAPS, 10 mmol/L DTT, and 50 μmol/L of caspase substrate in a total reaction volume of 0.25 mL. The reaction mixture was incubated for 60 minutes at 30°C. At the end of incubation, the liberated fluorescent group AMC was determined using a fluorescent spectrophotometer (Perkin Elmer) with an excitation wavelength of 380 nm and an emission wavelength.
of 460 nm [36]. AMC was used as a standard. Based on the standard curve made with fluorescence reading with free AMC, the data for caspase activity are expressed as nmol of AMC liberated when 50 μg of protein extract was incubated with 50 μmol/L of substrate for 60 minutes at 30°C.

**Determination of DNA damage**

The residual double-stranded DNA was measured by the alkaline unwinding assay and determination of ethidium bromide fluorescence according to the method of Birnboim and Jevcak [37], as utilized in our previous studies [38, 39]. Ethidium bromide fluorescence was measured at 520 nm excitation and 590 nm emission using a fluorescence spectrophotometer. Under the conditions employed, ethidium bromide binds preferentially to double-stranded DNA. Percent double-strand DNA (D) was determined by the equation: 

\[ \%D = 100 \times \frac{F(T) - F(B)}{F(T) - F(P)} \]

where F(P) is the sample fluorescence, F(T) is the total fluorescence prior to alkaline treatment, and F(B) is the background fluorescence that was obtained from sonicated, alkali-treated DNA, a condition under which the DNA is completely unwound and represents the fluorescence due to all components other than double-stranded DNA.

**Detection of DNA fragmentation by agarose gel electrophoresis**

To assess DNA fragmentation, fragmented DNA was isolated from LLC-PK1 cells as previously described [38, 39]. Cells were collected by centrifugation at 200 × g for 10 minutes, and the pellets were lysed with 1.0 mL of lysis buffer containing 0.5% Triton X-100, 10 mM of substrate for 60 minutes. The cell lysates were prepared as described previously in this article. [38, 39]. Ethidium bromide fluorescence was measured at 520 nm excitation and 590 nm emission using a fluorescence spectrophotometer. Under the conditions employed, ethidium bromide binds preferentially to double-stranded DNA. Percent double-strand DNA (D) was determined by the equation:

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where F(P) is the sample fluorescence, F(T) is the total fluorescence prior to alkaline treatment, and F(B) is the background fluorescence that was obtained from sonicated, alkali-treated DNA, a condition under which the DNA is completely unwound and represents the fluorescence due to all components other than double-stranded DNA.

**Western blot analysis**

The cell lysates were prepared as described previously in this article for caspase assay and 100 μg protein samples were subjected to reducing SDS-gel electrophoresis. The resolved proteins were electrophoretically transferred to Immobilon polyvinylidene difluoride (Millipore, Bedford, MA, USA) membrane and processed further for antibody staining as described by Towbin, Staehelin and Gordon [40]. Following this transfer, the membranes were washed in a buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for five minutes, and then in the same buffer containing 5% non-fat dry milk for an hour at room temperature. The membranes were then incubated for five hours with antibodies to caspases (usually 1:1000 dilution) in the same buffer with 5% dry milk as described previously in this article. The incubations were done at room temperature on a platform shaker to ensure thorough mixing. At the end of this time, the membranes were thoroughly washed (at least 4 times) with 50 mM Tris-Cl, pH 7.5, containing 0.05% Tween 20. The membrane filters were then incubated for two hours with horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1:3000 in Tris buffer containing 5% dry milk. After incubation, membrane filters were washed in the same buffer (50 mM Tris-Cl and 150 mM NaCl only) containing 0.05% Tween 20 and developed by exposure to chemiluminescent substrates (Pierce, Rockford, IL, USA).

**Overexpression of p35**

LLC-PK1 cells were transfected with expression plasmid pcDNA-3 containing the p35 full-length cDNA. p35 viral protein has been reported to be an effective inhibitor of caspase-3 [41–43]. Transfection was performed using Lipofectamine (Life Technologies, Grand Island, NY, USA) as per the manufacturer’s recommendation. In brief, cells were plated at a density of 2 × 10^5 in six-well plates. After 24 hours, cells were washed thrice with serum-free media and incubated for five hours with a mixture of Lipofectamine and plasmids containing the inserts (1.5 μL of Lipofectamine, 50 μL of serum-free medium and 1 μg of DNA in 50 μL of serum-free medium) in serum-free media at 37°C with 10% CO₂. Cells were then grown in the presence of G418 and selected for G418-resistant clones. After cell recovery, cells were examined for the expression of p35 A by Western blot using p35 antibody. The transfected cells were also examined for the suppression of caspase-3 activity using the fluorogenic substrate DEVD-AMC. These cells were then used to examine the protection against cisplatin-induced injury. After variable periods of cisplatin treatment, DNA damage and cell death were determined in the over-expressing cells as described...
Fig. 1. Time course effect of cisplatin on activation of caspases. LLC-PK1 cells were exposed to 50 μmol/L of cisplatin at various times as shown. To determine the caspase activity, cell lysates (50 μg protein) were incubated with fluorogenic peptide substrates at 37°C for 60 minutes as described in the Methods section. The production of liberated fluorescent amino-4-methyl coumarin (AMC) was determined with an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a spectrofluorometer (Perkin Elmer). AMC was used as a standard. The data for caspase activity are expressed as nmol of AMC liberated when 50 μg of protein extract was incubated with 50 μmol/L of substrate for 60 minutes at 30°C. Values are means ± SE (N = 5). Symbols from top to bottom curves are: (●) DEVD-MCA, caspase-3; (▲) VDVAD-MCA, caspase-2; (■) LEHD-MCA, caspase-9; (▲) IETD-MCA, caspase-8; (●) YVAD, caspase-1.

in our studies [34] and compared to control cells to examine the effect of the inhibitor in this model of injury.

Statistical analyses
Results are mean ± SE. Comparison between values was determined by the Student t test. A P value of less than 0.05 was considered significant.

RESULTS
Role of caspases in cisplatin-induced injury
To elucidate the role of caspases in cisplatin-induced injury to LLC-PK1 cells, we first examined the activity of the initiator caspases, caspase-9, -8, -2, and the executioner caspase, caspase-3 in response to cisplatin injury to LLC-PK1 cells. The activity of caspases was determined using fluorometric peptide substrates specific for each of the caspases. As shown in Figure 1, the activity of DEVD-AMC cleavage began at approximately eight hours of cisplatin treatment and then significantly increased with increasing periods of cisplatin (50 μmol/L) treatment, suggesting the activation of caspase-3. The maximal activation occurred at 24 hours of cisplatin treatment. The activities of cleavage of IETD-AMC, LEHD-AMC, and VDVAD-AMC substrates for caspase-8, caspase-9, and caspase-2, respectively, also were increased but to lesser degree than caspase-3 (Fig. 1). However, the activity of proinflammatory caspase-1 [11] was not detected in cisplatin-treated cells, indicating that cisplatin does not activate proinflammatory caspase-1. The substrate specificity of the caspases may be promiscuous, but was overcome by identifying the activation by Western blot analysis (discussed later in this article) using specific antibodies to each caspase. The cisplatin-induced activation of caspases was suppressed by peptide inhibitors of caspases (Fig. 2).

Caspases are synthesized as inactive proenzymes and in response to an apoptotic stimulus are activated by proteolytic processing of the proenzyme. In the process of activation the inactive proenzyme is proteolytically cleaved to the smaller subunits of about 20 and 10 kD in size [11]. In response to an injury, the identity of the active form of the caspase produced can be revealed by Western blot analysis using a specific antibody to the caspase. Thus, to examine the exact identity and activation of initiator and executioner caspases during cisplatin-induced injury to LLC-PK1 cells, we performed Western blot analysis using specific antibodies to caspase-3, -8, and -9. As shown in Figure 3, proteolytic processing of procaspase-3 results in the formation of a 17 kD subunit of the active caspase-3. This activation began at eight hours of cisplatin (50 μmol/L) treatment and thereafter progressively increased up to 24 hours of the treatment. Caspase-8 was also proteolytically processed as shown by the gradual decrease in the band of its proform (the antibody to caspase-8 used was specific...
to the proform only and did not recognize the processed subunits; Fig. 3). Similarly, caspase-9 was proteolytically processed to result in a 37 kD (prodomain + large fragment) and a 17 kD subunit of the active form. Moreover, caspase-9 protein expression apparently was increased upon cisplatin treatment. The proteolytic processing of caspase-3, -8, and -9 to their active forms was in agreement with the increased activities of these caspases, as shown in Figure 1. The activation of caspase-8 and -9 should precede caspase-3 according to the known pathways of receptor-mediated or mitochondrial-dependent activation of caspase-8 and -9, respectively. The data presented in Figures 1 and 3 show that activation of caspase-3 may precede that of caspase-8 and -9, suggesting that this activation of caspase-3 may be contributed by an unknown pathway or other caspases.

Since cowpox viral protein is a potent inhibitor of caspase-3 [41–43], we developed a stably transfected LLC-PK1 cell line that overexpressed the p35 caspase inhibitor. The transfected cells provided marked protection against cisplatin-induced cell death (Fig. 4A). Peptide inhibitors of caspase-3 and caspase-9 also provided significant protection against cisplatin-induced cell death in LLC-PK1 cells (Fig. 4B). However, inhibition of caspases provided partial protection against cisplatin-induced DNA damage as measured by DNA unwinding assay (residual double-stranded DNA in control cells, 95 ± 3%; 50 μmol/L cisplatin, 46 ± 2%; 50 μmol/L cisplatin + 25 μmol/L DEVD-CHO, 72 ± 3%; p35, 77 ± 2%; N = 3, P < 0.002) and as determined by DNA fragmentation pattern on agarose–gel electrophoresis (Fig. 5).

**Induction of cisplatin-induced PI-3 kinase/Akt phosphorylation and its effect on caspase activation and cell death**

The serine/threonine kinase Akt is one of the downstream targets of phosphatidylinositol 3'-kinase (PI-3 kinase) [44, 45] and PI-3 kinase-dependent Akt phosphorylation provides an important mechanism for resistance to cell death in response to injury [44, 46]. Phosphorylation of Akt leads to full activation of Akt that can result in phosphorylation of its direct downstream target, Bad, a member of the Bcl-2 family [47–49]. When phosphorylated, Bad dissociates from Bcl-XL or Bcl-2 [50] and re-
not changed during the course of cisplatin injury. Since activated Akt can phosphorylate Bad, we examined the time course of Bad phosphorylation in cisplatin-induced injury. As shown in Figure 7, Bad phosphorylation was markedly increased at least up to eight hours of cisplatin treatment and then declined in longer periods. These studies indicate that Bad phosphorylation is associated with Akt phosphorylation during cisplatin-induced injury and may be involved in the suppression of caspase activation in early time periods (at least up to 8 hours) of cisplatin treatment. Indeed, phosphorylation of both Akt and Bad were abolished in a dose-dependent manner by LY294002 (0 to 10 μmol/L; Fig. 8) or by wortmannin (0 to 0.25 μmol/L; data not shown), inhibitors of PI-3 kinase. Since Akt phosphorylation is known to block apoptosis and promote cell survival [44, 46], the inhibition of Akt phosphorylation should potentiate enhanced caspase activation during the course of cisplatin injury.

As shown in Figure 9, wortmannin (0.25 μmol/L) resulted in an early and enhanced increase in cisplatin-induced caspase-3 and caspase-9 activation compared with the activation by cisplatin alone in a time- (0 to 24 hours; Fig. 9A) and dose-dependent (0 to 100 μmol/L of cisplatin for 12 hours) manner (Fig. 9B). Wortmannin (0.25 μmol/L) alone (as shown in Fig. 9B at zero cisplatin concentration) slightly increased caspase-3 and caspase-9 activation, but this activation was far less compared to the activation by cisplatin alone or by wortmannin and cisplatin together. Thus, these studies indicate that inhibition of Akt phosphorylation enhance mitochondrial-dependent caspase-9 and caspase-3 activation in cisplatin-induced injury. In marked contrast, inhibition of Akt phosphorylation did not affect the cisplatin-induced caspase-8 and proinflammatory caspase-1 activities (Fig. 10). Thus, the receptor-mediated pathway that activates procaspase-8 and proinflammatory caspase-1 are not affected by inhibition of Akt phosphorylation in cisplatin-induced injury. These studies indicate that Akt phosphorylation regulates the mitochondrial-dependent caspase-9 and caspase-3 activation in cisplatin-induced injury. We next examined the effect of LY294002 on the cisplatin-induced proteolytic processing of caspase-3 and caspase-9 from their proforms to the active forms. This was done by Western blot analysis using specific antibodies to caspase-3 and caspase-9. As shown in Figure 11, LY294002 (5 μmol/L) resulted in the appearance of active caspase-3 and caspase-9 as early as two hours of cisplatin treatment and enhanced activation of these caspases in later time points. These data suggest that inhibition of the PI-3 kinase/Akt pathway not only results in earlier activation of caspase-3 and -9 but also at higher levels in cisplatin-induced injury to renal tubular epithelial cells. The inhibition of cisplatin-induced Akt phosphorylation was also reflected in early and enhanced cell death (Fig. 12). Figure 12 shows that cisplatin-induced cell death is markedly enhanced by inhibi-

Fig. 5. Effect of caspase inhibition on cisplatin–induced DNA fragmentation. Lanes are: C; control, CP (50 μmol/L); cells were exposed to 50 μmol/L cisplatin for 20 hours. CP (50 μmol/L)+1 (25 μmol/L); cells were exposed to 50 μmol/L cisplatin and 25 μmol/L of DEVD-CHO for 20 hours. CP (50 μmol/L) + p35; p35 transfected cells exposed to 50 μmol/L cisplatin for 20 hours. The fragmented DNA was isolated from each sample as described in the Methods section and subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide.
Fig. 6. Time course and dose-dependent activation of Akt in response to cisplatin. (A) Cells were treated with 50 μmol/L cisplatin for various time periods as indicated. Cell lysates (100 μg protein) were analyzed for Akt activation by Western blot analyses using specific antibodies to phosphorylated Akt (ser-473) and Akt, respectively. (B) Cells were treated with various concentrations of cisplatin for 20 hours as indicated. Cell lysates (100 μg protein) were analyzed for Akt activation by Western blot analyses using specific antibodies to phosphorylated Akt and Akt, respectively.

Fig. 7. Time course of Bad phosphorylation in response to cisplatin. Cells were treated with 50 μmol/L cisplatin for various time periods as indicated. Cell lysates (100 μg protein) were analyzed for Bad phosphorylation by Western blot analyses using specific antibodies to phosphorylated Bad (ser-136) and Bad, respectively.

Fig. 8. (A) Dose-dependent inhibition of Akt phosphorylation by PI-3 kinase inhibitor, LY294002. Cells were treated with 50 μmol/L cisplatin in the presence or absence of various concentrations (0 to 10 μmol/L) of LY294002 (LY) for four hours as indicated. Cell lysates were analyzed for Akt activation by Western blot analysis using antibodies to phosphorylated Akt (ser-473) and Akt. (B) Dose-dependent inhibition of Bad phosphorylation by PI-3 kinase inhibitor, LY294002. Cells were treated with 50 μmol/L cisplatin in the presence or absence of various concentrations (0 to 10 μmol/L) of LY294002 for four hours. Cell lysates were analyzed for Bad phosphorylation by Western blot analysis using specific antibodies to phosphorylated Bad (ser-136) and Bad alone.

These studies indicate that enhanced activation of caspase-3 and caspase-9 by inhibition of Akt phosphorylation also has an impact on cisplatin-induced cell death.

DISCUSSION

The executioner caspase-3 recently has been shown to be activated by cisplatin treatment in renal proximal tubular cells [30, 31]. However, there is no information on the specific role and underlying mechanism(s) of signaling pathways responsible for the regulation of downstream caspase-3 and other upstream caspases that may be activated during cisplatin-induced injury to renal tubular epithelial cells. Our data indicate that cisplatin induces selective and differential activation of caspases including executioner caspase-3 and initiator caspase-8 and -9, but not proinflammatory caspase-1. The selective activation of these caspases was markedly inhibited by their respective peptide inhibitors, suggesting that these caspases may play an important role in cisplatin-induced injury to renal tubular epithelial cells. DEVD-CHO or
LEHD-CHO, inhibitors of caspase-3 and caspase-9, respectively, provided marked protection against cisplatin-induced cell death and partial protection against DNA damage in LLC-PK1 cells as revealed by an alkaline unwinding assay and by agarose gel electrophoresis. The specific role of caspase-3 and its more direct involvement in cisplatin-induced injury came from studies utilizing the baculovirus protein p35, which is a potent inhibitor of caspase-3 [42–44]. Over-expression of p35 blocks the induction of apoptosis in insect and mammalian cells [54–56]. Thus, a stably transfected LLC-PK1 cell line developed to over-express p35 was capable of providing protection against cisplatin-induced injury indicating that cisplatin injury involves the participation of caspases.

Our studies also show that cisplatin induces the phosphorylation of the antiapoptotic serine/threonine kinase Akt and that this phosphorylation is inhibited by PI-3 kinase inhibitors LY294002 and wortmannin [57], indicating that Akt phosphorylation is dependent on the activation of PI-3 kinase. Akt (also known as protein kinase B) was originally identified as the cellular homolog of the transforming oncogene of the AKT8 retrovirus [58, 59]. Recent studies from several laboratories have recognized PI-3 kinase/Akt phosphorylation as one of the signalingspecific role of caspase-3 and its more direct involvement in cisplatin-induced injury came from studies utilizing the baculovirus protein p35, which is a potent inhibitor of caspase-3 [42–44]. Over-expression of p35 blocks the induction of apoptosis in insect and mammalian cells [54–56]. Thus, a stably transfected LLC-PK1 cell line developed to over-express p35 was capable of providing protection against cisplatin-induced injury indicating that cisplatin injury involves the participation of caspases.

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in vitro and in vivo [47, 48, 61], and may render Bad incapable of binding to Bcl-XL and restore the antiapoptotic function of Bcl-2 [44, 62]. Sequestering phosphorylated Bad by 14-3-3 proteins [50, 61] also may participate in the Akt survival pathway by making it unavailable to bind to Bcl-2 or prevent it from damaging the mitochondria. Many studies have provided evidence that Bcl-2 family members regulate activation of caspases through control of cytochrome c release from the mitochondria [51, 62] and by directly binding to Apaf-1, thus preventing the activation of procaspase-9 and subsequently caspase-3 [53]. Our study presents evidence that PI-3 kinase-mediated Akt phosphorylation is associated with Bad phosphorylation and suppression of caspase-9 and caspase-3 activation. Indeed, our studies show that wortmannin and LY294002, inhibitors of PI-3 kinase block cisplatin-induced phosphorylation of both Akt and Bad and enhance activation of caspase-9 and caspase-3 by cisplatin. On the other hand, these inhibitors had no effect on the activation of proinflammatory caspase-1 and receptor-dependent initiator caspase-8. A recent study has shown that Akt also can phosphorylate human caspase-9, resulting in a reduction of caspase-9 activity [63]. Based on these studies, the inhibition of Akt phosphorylation as well as Bad phosphorylation by wortmannin and LY294002 in cisplatin-induced injury may contribute to enhanced activation of mitochondrial dependent caspase-3 and caspase-9, but not receptor-mediated activation of caspase-8 or proinflammatory caspase-1. In addition, our studies show that inhibition of PI-3 kinase/Akt phosphorylation triggers early activation of caspase-3 and caspase-9 and enhanced cell death, indicating the possibility that activation of Akt modulates the response to cisplatin injury.

The apoptotic pathway is a highly regulated process and cells have inherited information to initiate this pathway in response to a death stimulus. Once initiated by an apoptotic stimulus, the cell death pathway can be challenged by cell survival signals to overcome injury and maintain cell viability. Thus, the extent of cell injury caused by a toxic agent will depend on the balance between activation of caspases triggered by a toxic agent and on the induction of survival signals capable of blocking the activation of caspases. The data presented in this article clearly demonstrate that cisplatin not only triggers caspase activation, but also causes induction of the PI-3 kinase/Akt pathway and impacts on cell survival. These observations have not been previously recognized in renal tubular epithelial cell injury. Our studies thus provide new targets to modulate cisplatin-induced injury to renal tubular epithelial cells. It is possible that enhancing the renal-specific cell survival signals and inhibiting death signals will lead to preservation of renal function in toxic injury.

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Reprint requests to Gur P. Kaushal, Ph.D., Department of Medicine, Slot 501, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, Arkansas 72205, USA.
E-mail: kaushalgurp@uams.edu
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