zVAD-fmk prevents cisplatin-induced cleavage of autophagy proteins but impairs autophagic flux and worsens renal function

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Herzog C, Yang C, Holmes A, Kaushal GP. zVAD-fmk prevents cisplatin-induced cleavage of autophagy proteins but impairs autophagic flux and worsens renal function. Am J Physiol Renal Physiol 303: F1239–F1250, 2012. First published August 15, 2012; doi:10.1152/ajprenal.00659.2011.—Cisplatin injury to renal tubular epithelial cells (RTEC) is accompanied by autophagy and caspase activation. However, autophagy gradually decreases during the course of cisplatin injury. The role of autophagy and the mechanism of its decrease during cisplatin injury are not well understood. This study demonstrated that autophagy proteins beclin-1, Atg5, and Atg12 were cleaved and degraded during the course of cisplatin injury in RTEC and the kidney. zVAD-fmk, a widely used pancaspase inhibitor, blocked cleavage of autophagy proteins suggesting that zVAD-fmk would promote the autophagy pathway. Unexpectedly, zVAD-fmk blocked clearance of the autophagosomal cargo, indicating lysosomal dysfunction. zVAD-fmk markedly inhibited cisplatin-induced lysosomal cathepsin B and calpain activities and therefore impaired autophagic flux. In a mouse model of cisplatin nephrotoxicity, zVAD-fmk impaired autophagic flux by blocking autophagosomal clearance as revealed by accumulation of key autophagic substrates p62 and LC3-II. Furthermore, zVAD-fmk worsened cisplatin-induced renal dysfunction. Chloroquine, a lysosomotropic agent that is known to impair autophagic flux, also exacerbated cisplatin-induced decline in renal function. These findings demonstrate that impaired autophagic flux induced by zVAD-fmk or a lysosomotropic agent worsened renal function in cisplatin acute kidney injury (AKI) and support a protective role of autophagy in AKI. These studies also highlight that the widely used antiapoptotic agent zVAD-fmk may be contraindicated as a therapeutic agent for preserving renal function in AKI.

LC3; beclin-1; Atg5; p62; acute kidney injury

AUTOPHAGY IS AN EVOLUTIONARILY conserved dynamic process that involves degradation of intracellular organelles and long-lived proteins by lysosomes (57, 64). In this process, the cytoplasmic contents are first sequestered in the double-membrane vesicle called the autophagosome, which then fuses with lysosomes and the sequestered components are degraded by the lysosomal hydrolases. Free fatty acids and amino acids produced upon degradation of cellular macromolecules are recycled to synthesize new proteins and bioenergetic supplies of the cell to ensure cell survival (26, 30). The core molecular components driving autophagy are Atg (AuToPhaGy) proteins originally identified in yeast (16, 26). The formation of an autophagosome is initiated by several autophagy protein complexes including ULK1 or ULK2 complex, class III phosphatidylinositol-3'-kinase complex, Atg12-Atg5-Atg16 conjugation, and lipidation of LC3 with phosphatidylethanolamine (PE) to form LC3-II (53, 65). The LC3-II levels are generally assessed by GFP-LC3-II-positive dots that correlate with autophagosome numbers (21). Basal autophagy occurs in normal physiological process to maintain cellular homeostasis. Autophagy is upregulated under physiological stress conditions including cell starvation, hypoxia, nutrient and growth factor deprivation, and oxidant injury (16, 28). The induction of autophagy in stressed cells plays an adaptive role and promotes cell survival by providing metabolic substrates and bioenergetic needs of the cell (16, 28). However, under certain conditions, excessive or impaired autophagy may contribute to autophagic cell death (30).

Previous studies demonstrated that apoptosis is the major mechanism involved in cisplatin-induced cell death in vitro and in vivo (1). Activation of caspases was demonstrated in both in vitro (6, 24, 29, 40, 43, 54) and in vivo (12, 40, 48) models of cisplatin nephrotoxicity. However, activation of caspases was not an immediate response to cisplatin and required a preapoptotic lag-phase (6, 24) in cultured renal tubular epithelial cells (RTEC). Following this preapoptotic lag-phase, caspase activation and cell apoptosis occurred in a time- and dose-dependent manner (6, 24, 33, 44, 63). Currently, there is limited information on the specific role of autophagy in acute kidney injury (AKI). In contrast to apoptosis, autophagy was demonstrated to be induced immediately in response to cisplatin injury (44, 63) in cultured RTEC. These studies showed that cisplatin-induced autophagy preceded caspase activation, occurred during preapoptotic lag-phase, and promoted cellular survival (44, 63). Induction of autophagy during the initial period of cisplatin insult was attributed to providing an appropriate environment for maintaining cellular homeostasis before reaching the threshold for cisplatin-induced apoptosis (34, 49). Cisplatin-induced autophagy following its initial induction was markedly decreased when caspase activation or apoptosis maximally occurred (44, 63). Thus, it was of interest to investigate a cross-talk between caspase activation and autophagy induction in response to cisplatin. The present study examined the relationship between cisplatin-induced cleavage of autophagy proteins and induction of autophagy. This study involved the evaluation of the effect of the widely used antiapoptotic pan-caspase inhibitor zVAD-fmk on the cleavage of autophagy proteins and its outcome on autophagic flux in vitro and in vivo models of cisplatin nephrotoxicity. The impact of impaired autophagic flux in renal function and histology was also determined in an in vivo model of cisplatin nephrotoxicity.

MATERIALS AND METHODS

Cell culture and chemicals. LLC-PK₁ (Porcine kidney proximal tubule epithelial cells) cells obtained from ATCC were grown in M199 medium supplemented with 5% (vol/vol) heat-inactivated

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fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO₂, Caspase-3/7 substrate; Asp-Glu-Val-Asp-aminomethyl coumarin (DEVD-AMC) was purchased from Peptide International (Louisville, KY). Pancaspase inhibitor; benzylloxycarbonyl-Val-Ala-Asp-fluoro-methylketone (zVAD-fmk), cathepsin B substrate; Z-Arg-Arg-AMC, and calpain substrate; N-sucinyl-Leu-Leu-Val-Tyr-7-Amino-4-methylcoumarin were obtained from Bachem (Torrance, CA). Bafilomycin A1 and chloroquine were obtained from Sigma (St. Louis, MO). Cisplatin was purchased from Novaplus (Bedford, OH).

**Caspase activity assay.** Cells grown to ~70% confluency were treated with either vehicle or inhibitors for 30 min before addition of 50 μM cisplatin. Cells were harvested by centrifugation and cell pellets washed in cold PBS were lysed with lysis buffer (Cell Signaling) at 4°C. The supernatants were used to determine the caspase-3/7 activity as using amino-4-methylcoumarin (AMC)-tagged DEVD (DEVD-AMC) substrate as described previously (24). The amount of liberated fluorescent group, AMC, was determined using a SpectraMax M5 plate reader (Molecular Devices) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. AMC activity was used as a standard. The data for caspase activity were expressed as nanomoles of AMC liberated when 50 μg of protein extract were incubated with 50 μM AMC substrate for 60 min at 30°C.

**Lactate dehydrogenase release assay.** Cells grown in 96-well plates in quadruplicates were treated at 70% confluence with either vehicle or inhibitors 1 h before addition of cisplatin (50 μM) and the lactate dehydrogenase (LDH) release assay was performed according to the instructions in the Cytotoxicity Detection KitPlus Roche (Indianapolis, IN). Briefly, 0.1 ml of assay solution was added to cells at the indicated time points and the reaction was stopped 10 min later by mixing with 0.05 ml of stop solution. Absorbance was read at 492 and 650 nm. Readings were normalized using completely lysed cells as high control.

**Western blot analysis.** LLC-PK1 cells were treated at 70% confluence with inhibitor/s (20 μM zVAD or 20 μM chloroquine) 1 h before cisplatin (50 μM) was added. Cells were harvested at the indicated time points and washed with 1× PBS; cell pellets were stored at −20°C until used. Cell lysis was performed using RIPA buffer containing 0.5 mM PMSF and 1 μg/ml each of leupeptin and aprotinin, and acetone powder was lyophilized in the presence of 0.5 M sucrose. The aceton powders were washed in cold PBS; cell pellets were stored at −80°C until used.

**Lactate dehydrogenase release assay.** This assay was performed in a reaction mixture containing 50 mM Tris, pH 7.3, and with and without 5 mM CaCl₂ in a total volume of 200 μl. The reaction was initiated by adding 50 μg of calpain substrate (final concentration). The assay was performed in a 96-well plate in triplicate. In the buffer without CaCl₂, 1 mM EDTA was added. The reaction mixture was incubated for 15 min at 37°C. An AMC standard curve was also determined. With the use of a SpectraMax M5 plate reader (Molecular Devices), the fluorescence was measured at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Calpain activity was determined as the difference between calcium-dependent and noncalcium-dependent fluorescence and expressed as micromoles AMC released per minute of incubation time per milligram of protein.

**Cathespin B activity assay.** Cell lysates were prepared as described for the calpain assay. Cathespin B activity in the cell lysates was determined essentially as described previously (31). On a 96-well plate, in duplicate, 50 μg protein lysate were incubated with 20 μM cathepsin B fluorogenic peptide substrate (Z-Arg-Arg-AMC) in a buffer containing 340 mM sodium acetate, 60 mM acetic acid, 4 mM EDTA, pH 5.5, in total volume of 100 μl. Following incubation for 15 min, the reaction was stopped using 100 μl of stopping solution containing 100 mM trichloric acid, 30 mM sodium acetate, and 70 mM acetic acid, pH 4.3. AMC standards were prepared under the same conditions and read along with the samples using a SpectraMax M5 plate reader. At an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Cathespin B activity was expressed as nanomoles of AMC released per minute of incubation per milligram of protein.

**GFP-LC3, Atg5, and beclin-1 overexpression and autophagy detection.** Cells grown on glass coverslips in six-well plates were transfected with GFP-LC3 plasmid DNA (kindly provided by Dr. T. Yoshimori, Osaka University, Japan) or m-cherry-Atg5 plasmid DNA (Addgene, Cambridge, MA) or beclin-1 plasmid DNA (Addgene) for 36 h and then treated with 50 μM cisplatin for various time points. Transfection was carried out with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s recommendation and 2 μg/ml GFP-LC3 or m-cherry-Atg5 or beclin-1 plasmid DNA in each well was used as described previously (63). Microphotographs of GFP-LC3 fluorescence were obtained with a fluorescence microscope. The detection of punctate staining of GFP-LC3-II indicated the formation of autophagosomes.

**Induction of cisplatin-induced acute renal failure and effect of zVAD-fmk and chloroquine in renal function.** Animal protocols for these studies was approved by the Institutional Animal Care and Use Committee and Institutional Safety Committee. Ten-week-old C57BL/6 male mice were administered a single intraperitoneal injection of chloroquine (50 mg/kg body wt, n = 9) (20, 44) or zVAD-fmk (10 mg/kg body wt, n = 13) (6, 7, 14) 1 h before the injection of cisplatin (20 mg/kg body wt, n = 12) (4, 17, 54, 63). Control mice (n = 6, in each group) were administered either saline or 10% DMSO in saline. z-VAD-fmk was administered in 10% DMSO in saline. Kidneys were harvested at 1 day, 2 days, 3 days, and 4 days for histology, immunohistochemistry. Blood was collected for blood urea nitrogen (BUN) and serum creatinine level determinations. BUN and creatinine were determined using a diagnostic kit from International Bio-Analytical Industries (Boca Raton, FL).

**Histology and TUNEL assay.** Kidney tissue was fixed in phosphate-buffered 4% formalin (pH 7.4) for 24 h and then embedded in paraffin. Sections were cut into 5-μm-thick slices and used for staining with hematoxylin and eosin (H&E). Histological damage was scored essentially as described previously (on the following score: 1, <20% damage; 2, 20–40% damage; 3, 40–60% damage; 4, >60% damage). Damage was identified by TdT-mediated dUTP nick-end labeling (TUNEL) assay using a kit from Roche Applied Science (Indianapolis, IN).

**Immunofluorescence detection of LC3-II and n-p62 in kidney sections.** Ten-week-old C57BL/6 male mice were administered a single intraperitoneal injection of chloroquine 50 mg/kg body wt 1 day before zVAD-fmk (10 mg/kg body wt) 1 h before cisplatin (20 mg/kg body wt) administration or saline vehicle. Kidney tissue was fixed in phosphate-buffered 4% formalin and paraffin embedded. Deparaffinized tissue sections (5 μm) were immunostained with rabbit polyclonal anti-LC3 (Novus Biologicals, cat. no. NB-100-2220) or rabbit polyclonal anti-p62 (Sigma) overnight. After being washed with PBS, slices were incubated with...
secondary antibodies from Molecular Probes (donkey anti-rabbit Alexa Fluor 488, green). Epi-immuno-fluorescence was recorded on an Olympus BX51 microscope.

Statistical analyses. Results are reported as means ± SE. Intergroup comparisons were assessed for significance by using one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons (GraphPad Prism, version 5.04). A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Beclin 1, Atg5, and Atg12 are cleaved in response to cisplatin and zVAD-fmk blocks this cleavage. Previous studies showed that autophagy is an immediate response to cisplatin injury in cultured RTEC and cisplatin-induced autophagy is markedly decreased when caspase activation begins to elevate after cisplatin treatment (44, 63). To determine whether cisplatin-induced activation of caspases targets autophagy pro-

Fig. 1. Cleavage of beclin-1, Atg5, and Atg12 in cisplatin (CP)-treated cells and effect of pancaspase inhibitor zVAD-fmk LLC-PK1 cells were treated with 50 μM CP in the presence and absence of 20 μM zVAD-fmk for various time points as indicated. Cell lysates were subjected to Western blot analysis using antibodies to Atg5 (A), beclin-1 (B), and Atg12 (C). Actin was used as a loading control using a specific antibody to β-actin.

Fig. 2. Effect of ZVAD-fmk on CP-induced autophagic flux. A: effect of zVAD-fmk on CP-induced LC3-II formation. Fluorescence staining of GFP-LC3 and formation of GFP-LC3-II in response to zVAD-fmk in CP-treated cells. LLC-PK1 cells growing on coverslips at 70% confluency were transfected with GFP-LC3 plasmid. Following transfection as described in MATERIALS AND METHODS, cells were treated with 50 μM CP in the presence and absence of 20 μM zVAD-fmk for various times as indicated. The appearance of CP-induced punctate staining dots indicates autophagosome-associated LC3-II. B: effect of zVAD-fmk on CP-induced LC3-II formation by Western blot analysis. LLC-PK1 cells were treated with 50 μM CP in the presence and absence of 20 μM zVAD-fmk for various time periods as indicated. Conversion of LC3-I (18 kDa) to LC3-II (16 kDa) was determined in the cell lysates by Western blot using an antibody specific to LC3.
Caspase activation was initiated following cisplatin treatment (data not shown) and this led to the progressive decrease in full-length Atg proteins. Pretreatment of cells with the broad-spectrum caspase inhibitor zVAD-fmk completely prevented cisplatin-induced degradation of Atg5, beclin-1, and Atg12 proteins (Fig. 1) and caspase activation (data not shown). zVAD-fmk results in enhanced LC3-II formation but inhibits autophagic flux and decreases cell viability. Since zVAD-fmk blocked the breakdown of Atg5, beclin-1, and Atg12, we examined whether zVAD-fmk influenced the level of autophagy in response to cisplatin. Cisplatin-induced punctate staining dots for GFP-LC3-II were considerably elevated on treatment with zVAD-fmk (Fig. 2A), indicating increased accumulation of autophagosomes. Also, cisplatin-induced LC3-II protein expression was significantly increased in the presence of zVAD-fmk (Fig. 2B) further suggesting increased accumulation of autophagosomes. The accumulation of autophagosomes can result either from increased autophagic activity or impaired autophagic flux due to a block in the autophagosomal clearance. To distinguish between these two possibilities, we first employed the use of the lysomotropic agent chloroquine. Chloroquine inhibits lysosomal function (by increasing intralysosomal pH) and blocks autophagosomal clearance (8, 52) and is commonly used to distinguish between increased autophagic activity and a block in the clearance (35). Therefore, we compared cisplatin-induced LC3-II formation following treatment with zVAD-fmk in the presence and absence of chloroquine. As shown in Fig. 3, zVAD-fmk-induced increase in LC3-II was not changed significantly in the presence of chloroquine, indicating that autophagic flux is impaired in response to zVAD-fmk and occurs due to a block in the autophagosomal clearance. Another lysomotropic agent bafilomycin A (specific inhibitor of the vacuolar type H+ -ATPase that suppresses lysosomal function) also did not change zVAD-fmk-induced increase in LC3-II (data not shown), further indicating that autophagic flux is impaired in response to zVAD-fmk.

Impairment of autophagic flux can also be assessed by increased expression levels of p62 (also known as sequestosome 1 or SQSTM1 or A170) (3, 35, 36), which is one of the key autophagy substrates (25, 27, 42). During induction of autophagy, p62 directly binds to LC3-II and is selectively degraded along with the autophagosome cargo by the lysosomes (27, 42). Thus, a block in autophagosomal clearance results in increased p62 levels. As shown in Fig. 3, the expression levels of p62 were significantly increased in response to zVAD-fmk, further confirming that zVAD-fmk impaired cisplatin-induced autophagic flux. In addition, zVAD-fmk-induced increase in p62 was not changed in the presence of chloroquine, further supporting impairment of autophagic flux. Previous studies in nonrenal cells showed that zVAD-fmk can inhibit lysosomal cathepsins (46, 47, 55) and calpains (2, 34) that can suppress lysosomal function and impair autophagic flux. Thus, we examined whether zVAD-fmk inhibited cisplatin-induced activation of calpain and cathepsin B in renal cells. zVAD-fmk...

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![Fig. 3. Effect of chloroquine (CHL) on zVAD-fmk-induced accumulation of LC3-II and p62 LLC-PK1 cells were treated with 20 μM zVAD-fmk in the presence and absence of 20 μM chloroquine 1 h before the treatment with 50 μM CP for various time periods as indicated. Cell lysates were analyzed by Western blot for LC3-II and p62 using their specific antibodies. Actin was used as a loading control using a specific antibody to β-actin.](image)

![Fig. 4. Effect of zVAD-fmk on CP-induced activation of calpain and cathepsin B. A: effect of zVAD-fmk on CP-induced activation of calpain. LLC-PK1 cells were treated with 50 μM CP in the presence and absence of 20 μM zVAD-fmk for various times as indicated. Calpain activity in the cell lysates (50 μg of protein) was determined using N-succinyl-Leu-Leu-Val-Tyr-7-AMC fluorogenic substrate as described in MATERIALS AND METHODS. B: effect of zVAD-fmk on CP-induced activation of cathepsin B. LLC-PK1 cells were treated with 50 μM CP in the presence and absence of 20 μM zVAD-fmk for various times as indicated. Cathepsin B activity in the cell lysates (50 μg of protein) was determined using z-Arg-Arg-AMC fluorogenic substrate as described in MATERIALS AND METHODS.](image)

![Fig. 5. Dose-dependent effect of zVAD-fmk on CP-induced lactate dehydrogenase (LDH) release. Cells grown in 96-well plates in quadruplicates were treated at 70% confluency with either vehicle or various concentrations of zVAD-fmk 1 h before addition of CP (50 μM) and the LDH release assay was performed according to the instructions in the Cytotoxicity Detection Kit Plus Roche (Indianapolis, IN) as described in MATERIALS AND METHODS.](image)
significantly inhibited cisplatin-induced calpain and cathepsin B activities (Fig. 4), suggesting that ZVAD-fmk is capable of suppressing lysosomal function.

Since a defect in autophagosome clearance or impaired autophagic flux will be deleterious to cells (19, 27, 38), we examined whether ZVAD-fmk-mediated impaired autophagic flux decreases cell viability. ZVAD-fmk treatment increased cisplatin-induced LDH release indicating ZVAD-fmk-induced necrotic cell death (Fig. 5). Although ZVAD-fmk prevented cisplatin-induced caspase activation (data not shown), it did not suppress cisplatin-induced necrotic cell death most probably due to the impaired autophagic flux.

**Overexpression Atg5 and beclin-1 inhibits caspase activation and cell death and protects renal tubular cells from cisplatin toxicity.** We overexpressed Atg5 and beclin-1 in LLC-PK1 cells to determine whether increased expression of these Atg proteins affected cisplatin-induced apoptosis. Increased beclin-1 and Atg5 expression was shown previously to initiate autophagosome formation and improve autophagic flux (15, 37). Transient transfection with m-cherry-Atg5 vector and beclin-1 vector resulted in a significant increase in expression levels of Atg5 and beclin-1 proteins (Fig. 6A). Overexpression of Atg5 and beclin-1 proteins prevented cisplatin-induced caspase activation (Fig. 6B) and cell death (Fig. 6C).

**Induction of autophagy in kidney in response to cisplatin injury.** We first examined the formation of LC3-II protein for demonstrating autophagy in the kidney in a mouse model of cisplatin nephrotoxicity. The formation of LC3-II protein was increased in the kidney following administration of cisplatin, suggesting induction of autophagy during the progression of nephrotoxicity (Fig. 7A). Immunofluorescent staining with LC3 antibody also revealed formation of LC3-II dots, indicating formation of autophagosomes (Fig. 7B). We also examined cleavage of Atg proteins in vivo in response to cisplatin. Cleavage of beclin-1 was quite pronounced after 3 days of cisplatin administration and at least three proteolytic fragments were observed (Fig. 7C). Administration of ZVAD-fmk prevented beclin-1 cleavage. The expression level of Atg5 was reduced in response to cisplatin but the cleaved fragments were not detected (Fig. 7C).

**ZVAD-fmk and chloroquine impair autophagic flux in vivo.** We next determined the effect of ZVAD-fmk on autophagic flux in vivo in response to cisplatin. The formation of cisplatin-induced LC3-II from LC3-I was considerably elevated on administration of ZVAD-fmk as revealed by punctate staining dots for LC3-II (Fig. 8A), indicating increased accumulation of autophagosomes. Chloroquine, a lysosomotropic agent and an autophagy inhibitor that is known to impair autophagic flux (35), also elevated cisplatin-induced punctate staining for LC3-II (Fig. 8A). The protein levels of key autophagy substrates p62 and LC3-II were markedly increased on administration of cisplatin and ZVAD-fmk (Fig. 8B), indicating impairment in autophagic flux. As expected, a similar effect on the expression of p62 and LC3-II levels was also observed with

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**Fig. 6. Effect of overexpression of beclin-1 and Atg5 in CP-induced caspase activation and cell death.** A: cells were transfected with beclin-1 and Atg5 plasmids as described in MATERIALS AND METHODS and cell lysates were subjected to Western blots for beclin-1 and Atg5 protein expression. B: effect of beclin-1 or Atg5 overexpression on caspase-3/7 activation in response to CP injury. LLC-PK1 cells were transfected with Atg5 or beclin-1 plasmids and treated with and without CP (50 μM) as indicated. Cell lysates were prepared and analyzed for caspase-3/7 activation as described in MATERIALS AND METHODS. P < 0.01 compared with CP-treated cells. C: LLC-PK1 cells were transfected with Atg5 or beclin-1 plasmids and treated with and without CP (50 μM) as indicated. The cells were stained with 0.5 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) for 5 min, and the cells were washed twice in PBS. Coverslips were then mounted on slides using antifade mounting medium (Molecular Probes). Morphological changes of the nuclei were analyzed using a Zeiss deconvolution microscope.
administration of lysomotropic agent chloroquine (Fig. 8B) that is known to impair autophagic flux (35). Immunofluorescent staining of the kidney sections with p62 antibody also demonstrated that cisplatin-induced expression of p62 was enhanced further in response to zVAD-fmk and chloroquine (Fig. 8C). The TUNEL staining of kidney sections indicated that cisplatin-induced DNA fragmentation was further increased on administration of zVAD-fmk or chloroquine (Fig. 9). These studies support that zVAD-fmk and chloroquine impair autophagic flux and increase cellular injury in vivo in cisplatin nephrotoxicity.

**DISCUSSION**

In the present study, we demonstrated that the autophagy proteins beclin-1, Atg5, and Atg12 are cleaved in response to cisplatin injury in the cultured RTEC and kidney. Cisplatin-induced cleavage of Atg5, beclin-1, and Atg12 occurred con-
Fig. 8. zVAD-fmk and chloroquine impair autophagic flux in vivo. A: fluorescence staining of LC3-II in kidneys of mice treated with CP in the presence or absence of zVAD-fmk or chloroquine. C57BL/6 mice were treated with or without zVAD-fmk or chloroquine followed by CP as mentioned in MATERIALS AND METHODS. Kidneys were harvested 3 days after treatment, formalin fixed, and paraffin embedded. Deparaffinized 5-μm sections were immuno-stained with polyclonal rabbit anti-LC3 followed with anti-rabbit Alexafluor-488-labeled secondary antibody and pictures were recorded on an Olympus BX51 fluorescence microscope at ×40 magnification. Kidney sections shown are as follows: control kidney section, kidney section of mouse treated with 20 mg/kg body wt CP after 3 days (3d CP), kidney section of mouse treated with 10 mg/kg body wt zVAD-fmk followed by 20 mg/kg body wt CP for 3 days (3d CP + zVAD-fmk), and kidney section of mouse treated with 50 mg·kg body wt\(^{-1}\)·day\(^{-1}\) chloroquine + 20 mg/kg body wt CP for 3 days (3d CP + chloroquine). B: expression levels of LC3-II and p62 proteins in the presence and absence of ZVAD-fmk or chloroquine in CP nephrotoxicity. Mice were injected intraperitoneally with 20 mg/kg body wt CP in saline in presence and absence of 10 mg/kg body wt zVAD-fmk or 50 mg·kg body wt\(^{-1}\)·day\(^{-1}\) chloroquine. Control mice were administered saline. Kidneys from mice were removed at 0 day (c), 1 day, 2 days, 3 days, and 4 days. Tissue lysates from kidney cortices were prepared and 100-μg protein samples were subjected to Western blot analysis using antibodies specific to LC3, p62, and actin. Actin detection is for the loading control. C: accumulation of p62 in the presence and absence of ZVAD-fmk or chloroquine in CP nephrotoxicity. Kidney sections from 3 days and following various treatments as shown were immunostained with p62 antibody as described in MATERIALS AND METHODS. Green fluorescence shows accumulation of p62.
comitantly with caspase activation and apoptosis. zVAD-fmk treatment completely blocked the cleavage of Atg proteins. Caspase-mediated cleavage of beclin-1 has been previously reported during apoptosis induced by IL-3 depletion in the bone marrow-derived pro-B-cell line Ba/F3 (59) and death receptor TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (5) and staurosporine-induced apoptosis in HeLa cells (68). Beclin-1, also known as Atg6 in yeast, is an important component of a class III PI-3 kinase/Vps34 complex required for the formation of an autophagosome (13, 23). Beclin-1 is involved in the regulation of Vps34 activity in this complex (13, 57). Our studies demonstrated that besides beclin-1 cleavage, Atg5 and Atg12 were also cleaved during cisplatin injury. Atg5 and Atg12 are involved in the early stages of autophagosome membrane formation by assembling the protein conjugation complex Atg12-Atg5-Atg16 (39, 57). In this complex, Atg12 first conjugates with Atg5 to form the covalently linked Atg12-Atg5 complex which further associates noncovalently with Atg16. Previous studies reported calpain-mediated cleavage of Atg5 in CD95-, ceramide-, and anticancer drug (etoposide and doxorubicin)-mediated cell death (66). Calpain activity has also been shown to play an important role in regulating the levels of Atg5 in H4 cells, a human neuroblastoma cell line (62). Our studies showed that calpain activity was induced in response to cisplatin and that the pancaspase inhibitor zVAD-fmk markedly blocked this activity. Since zVAD-fmk is able to inhibit both caspases and calpain activities, future studies will identify specific caspase and calpain-specific cleaved products produced in cisplatin injury. This will involve identification of cleavage sites after obtaining amino acid sequences of the cleaved products. Nevertheless, cisplatin-induced cleavage of the Atg proteins can be attributed to diminished autophagy during the postapoptotic phase of cisplatin injury (44, 63).

The protection of Atg proteins from degradation by zVAD-fmk promoted formation of autophagosomes as revealed by increased LC3-II formation. We identified that zVAD-fmk-induced accumulation of autophagosomes was due to impairment of lysosomal function that blocks autophagosome clearance. The block in autophagosome maturation at the terminal step of the autophagic pathway results in impaired autophagic flux. Autophagic flux is an indicator of autophagic activity that involves the dynamic process of autophagosome synthesis, delivery of the autophagic substrates to the lysosome, and degradation of the sequestered substrates by the lysosomal hydrolases as illustrated in Fig. 11. Both LC3-II and p62 are integral components of the inner membrane of the autolysosome and are degraded by the lysosomal hydrolases during the completion of the autophagic process (27, 42). Accumulation of both p62 and LC3-II is known to be associated with impaired autophagic flux (25, 27, 42). Our studies demonstrated that zVAD-fmk accumulated LC3-II and p62 formation. zVAD-fmk-induced increase in LC3-II and p62 was not changed significantly in the presence of lysomotropic agent chloroquine or bafilomycin A, suggesting impairment of autophagosome clearance.

Impaired autophagic flux can result from defective lysosomal function that may retard autophagosome clearance (35). The lysosomal function is compromised when there is a block in the lysosomal hydrolases. Cathepsins are the most abundant lysosomal proteases that participate directly in the execution of autophagy (22, 56). Both calpain and cathepsins are required for macroautophagy in mammalian cells (9, 10). Our studies demonstrated that zVAD-fmk efficiently inhibited...
cathepsin B and calpain activities in RTEC at concentrations that inhibit caspases. This effect of zVAD-fmk, therefore, may be responsible for causing a defect in the autophagic flux. In nonrenal cells, zVAD-fmk was shown previously to block lysosomal enzyme cathepsin B activity, and subsequently prevented autophagosome maturation (61). In fact, zVAD-fmk has been shown to inhibit lysosomal cathepsin (45–47, 55, 58) and calpain activities (2, 34) in nonrenal cells.

Although apoptosis is generally considered to be the major mechanism involved in cisplatin cytotoxicity (40), the specific role of the widely used antiapoptotic agent zVAD-fmk in preserving renal function in cisplatin nephrotoxicity is not known. In cultured LLC-PK1 cells, zVAD-fmk provided protection from caspase-3/7 activation but not from cisplatin-induced necrosis as observed by the LDH release. In fact, zVAD-fmk significantly increased cisplatin-induced LDH release. Previous studies in renal cells showed that zVAD-fmk prevented cisplatin-induced caspase-dependent apoptosis (6) but not chemically induced necrosis (67). This is in agreement with our study that zVAD-fmk rendered the cells more vulnerable to necrotic cell death. We attribute the effect of zVAD-fmk-induced LDH release to the impaired autophagic flux. In nonrenal cells, inhibition of autophagic flux has been shown previously to induce cell death (19, 27, 38). A defect in the completion of autophagy or impaired autophagic flux would be deleterious to the cells since autophagy recycles damaged organelles and macromolecules to generate both nutrients and energy needs of the cells. Our in vitro studies indicate that indeed, zVAD-fmk increased cisplatin-induced necrotic cell death. Most importantly, in vivo studies showed that zVAD-fmk impaired autophagic flux, did not provide protection against cisplatin nephrotoxicity, and exacerbated cisplatin-induced renal dysfunction. zVAD-fmk was previously shown to provide a renoprotective role in experimental models of AKI (7, 14). On the other hand, a recent study (32) showed that zVAD-fmk is unable to protect ischemia-reperfusion-induced AKI. Our studies demonstrated that zVAD-fmk did not provide renoprotection from cisplatin-induced AKI probably because zVAD-fmk impaired autophagic flux. Thus, our studies suggest that the widely used pancaspase inhibitor for the identification of apoptosis may not work as a specific therapeutic agent for AKI.

Previous studies demonstrated that autophagy is an immediate response, preceded caspase activation, and played a survival role in cisplatin injury to cultured RTEC (44, 63). Suppression of cisplatin-induced autophagy by pharmacologic inhibitors (chloroquine and 3-methyladenine) or small interfering RNA against beclin-1 and Atg5 dramatically augmented proapoptotic activity in RTEC (44, 63). Treatment of NRK cells with cisplatin showed autophagic induction before apoptosis but autophagic inhibitors suppressed apoptosis (17). Proximal tubular cells isolated from heme oxygenase-deficient mice showed higher levels of impaired progression of autophagy and increased apoptosis after cisplatin treatment. Overexpression of heme oxygenase (HO)-1 in these cells reversed the autophagic response and inhibited apoptosis after...
that, although zVAD-fmk prevented cisplatin-induced cleavage. Our studies further demonstrated during cisplatin injury and that the pancaspase inhibitor zVAD-fmk prevents this cleavage. These studies support that autophagy is a protective agent that underlies renal function and histology. This observation is in line with the recent report that renal failure in proximal tubule-specific HO-1-deficient mice exhibited more autophagosomes in the proximal tubules of the kidney in response to cisplatin (4). Compared with wild-type mice, HO-1-deficient mice exhibited more autophagosomes in the proximal tubules of the kidney in response to cisplatin (4). These studies suggest impaired or defective autophagic flux in HO-1-deficient mice that resulted in accumulation of autophagosomes. Recent studies also demonstrated that autophagy was induced in renal ischemia-reperfusion injury (18, 20, 50, 60) and provided a renoprotective mechanism. In addition, the induction of autophagy also played a protective role in renal tubular cells against cyclosporine toxicity (41). In the present study, we demonstrated that overexpression of beclin-1 and Atg5 provided marked protection from cisplatin-induced caspase-3/7 activation and cell death. These in vitro studies suggested that autophagy would provide a protective role in vivo in cisplatin nephrotoxicity. Since chloroquine is known to inhibit autophagy by inhibiting its flux, we examined whether autophagy inhibition is maladaptive or provides protection in cisplatin nephrotoxicity. Animals treated with cisplatin and chloroquine did not improve cisplatin-induced decline in renal function and histology, but rather further displayed impaired renal dysfunction and histology. Inhibition of autophagy by chloroquine worsened cisplatin nephrotoxicity as indicated by renal function and histology. This observation is in line with the recent report that renal failure in proximal tubule-specific Atg5 knockout mice is worsened during cisplatin nephrotoxicity (51). Also, chloroquine-induced defect in autophagy was recently shown to induce cardiomyocyte necrosis in mice (49). Taken together, in vitro and in vivo studies suggest that autophagy may trigger a prosurvival signal in AKI.

Fig. 11. Scheme depicting autophagic flux and common inhibitors of the autophagic pathway. Autophagic flux involves the dynamic process of autophagosome synthesis, delivery of the autophagic substrates to the lysosome, and degradation of the sequestered substrates by the lysosomal hydrolases. The “core” autophagic machinery that utilizes Atg proteins for the phagophore formation and its elongation to mature autophagosome formation work through the following functional protein complexes: 1) the ULK1/2 kinase complex is required for the induction of autophagy and involves at the site of autophagosome formation. Ulk1/2 is negatively regulated by mTORC1. 2) A class-III phosphatidylinositol 3-kinase complex is required for nucleation of the phagophore membrane. Beclin1 (Atg6) and Atg14 are part of this complex. 3) The elongation and expansion steps in autophagosome formation involve 2 conjugation systems that require ubiquitin-like proteins, Atg12 and Atg8/LC3-II. The Atg5-Atg12 conjugate subsequently associates noncovalently with Atg16 to form an Atg12-Atg5-Atg16 multimeric complex. The second conjugation step is the formation of LC3-II (Atg8-PE) by conjugation of LC3 with phosphatidylethanolamine (PE). Once the autophagosome is formed, most of the Atg proteins are dissociated which then allows fusion with the lysosome to form autolysosome. The sequestered contents and the inner membrane of the autolysosome are degraded by the lysosomal hydrolases. The autophagic flux is an indicator that the autophagy process has undergone to completion.

In summary, our studies provided evidence that autophagy proteins were cleaved in cultured RTEC and in the kidney during cisplatin injury and that the pancaspase inhibitor zVAD-fmk prevented this cleavage. Our studies further demonstrated that, although zVAD-fmk prevented cisplatin-induced cleavage of autophagy proteins, it impaired cisplatin-induced autophagic flux both in the cultured RTEC and in the kidney. We showed that ZVAD-induced impaired autophagic flux worsened cisplatin-induced cytotoxicity in vitro and in vivo and did not provide protection from cisplasm nephrotoxicity. We further provided evidence that zVAD-fmk is capable of inhibiting lysosomal proteases and that this function of zVAD-fmk may be responsible for the impairment in clearance of the autophagic cargo during cisplatin injury. In a separate experiment, we demonstrated that impaired autophagy induced by administration of chloroquine also worsened renal function and histology. Our studies highlight that widely used zVAD-fmk as an antiapoptotic agent is unable to provide protection from cisplatin nephrotoxicity due to its ability to promote defect in autophagic flux. These studies also support that autophagy is a cytoprotective mechanism for RTEC survival.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
G.P.K. interpreted results of experiments; G.P.K. edited and revised manuscript; G.P.K. approved final version of manuscript.

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