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Loss of α(E)-Catenin Potentiates Cisplatin-Induced Nephrotoxicity via Increasing Apoptosis in Renal Tubular Epithelial Cells

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ABSTRACT

Cisplatin is one of the most potent and widely used antitumor drugs. However, the use of cisplatin is limited by its side effect, nephrotoxicity. Evidence has shown an increased incidence and severity of acute kidney injury (AKI) in the elderly. Previous studies from our laboratory demonstrate a decrease in α(E)-catenin expression in aged kidney. In this study, we investigated whether the loss of α(E)-catenin may increase cisplatin nephrotoxicity. To study the effects of reduced α(E)-catenin, a cell line with stable knockdown of α(E)-catenin (C2 cells) was used; NT3 is nontargeted control. C2 cells exhibited a significant loss of viability as determined by MTT assay compared with NT3 cells after cisplatin challenge, but showed no difference in lactate dehydrogenase (LDH) leakage. Increased caspase 3/7 activation and PARP cleavage was observed in C2 cells after cisplatin treatment. Z-VAD, a pan-caspase inhibitor, abolished the difference in susceptibility between NT3 and C2 cells. Interestingly, the expression of α(E)-catenin was further decreased after cisplatin treatment. Furthermore, *in vivo* data demonstrated a significant increase in serum creatinine at 72 h after a single dose of cisplatin in 24-month-old rats, but not in 4-month-old rats. Increased expression of KIM-1 and *in situ* apoptosis were also detected in aged kidney after cisplatin challenge. Taken together, these data suggest that loss of α(E)-catenin increases apoptosis of tubular epithelial cells which may contribute to the increased nephrotoxicity induced by cisplatin in aged kidney.

Key words: aging; cisplatin; α-catenin; apoptosis; AKI

Acute kidney injury (AKI), a common clinical complication, is characterized by a rapid and progressive loss of renal function (Bellomo *et al.*, 2012; de Almeida *et al.*, 2013). Evidence has shown that there is an increased incidence and severity of AKI in the elderly (Ning *et al.*, 2013). Approximately 20% of AKI cases are induced by nephrotoxic drugs and the incidence of drug-induced nephrotoxicity among elderly who developed AKI in the hospital can be as high as 66% (Peres and da Cunha, 2013).

cis-Diamminedichloroplatinum II or cisplatin is a widely used and highly effective anticancer drug for the treatment of various solid tumors in the head, neck, breast, lung, ovary, testis, and uterus (Oh *et al.*, 2014). However, the use of cisplatin is dramatically limited by its side effects including nephrotoxicity, gastrotoxicity, ototoxicity, and myelosuppression (Hartmann and Lipp, 2003). Approximately 28–36% of patients experience AKI following the treatment of cisplatin (Madias and Harrington, 1978) which makes nephrotoxicity a dose-limiting side effect of cisplatin (Yao *et al.*, 2007). Like most alkylating antineoplastic agents, cisplatin causes crosslinking of DNA, triggering apoptosis in tumor cells, and other proliferating cells (Jamieson and Lippard, 1999). However, cisplatin can also cause considerable injury to proximal tubular epithelial cells (McDuffie *et al.*, 2013). The mechanism of cisplatin-induced nephrotoxicity has been studied for >30 years and recent studies suggest that local accumulation of cisplatin, intracellular conversion to nephrotoxins, inflammation response, oxidative stress, DNA damage, and activation of apoptotic pathways, can partially explain this injury (dos Santos *et al.*, 2012; Pabla and Dong, 2008; Peres and da Cunha, 2013).

α-Catenin, which works at the interface between the cadherin-β-catenin complex and F-actin, is very important in the relationship between the adherens junction (AJ) and cytoskeleton which is essential for cell adhesion (Desai *et al.*, 2013). There are three forms of α-catenin: α(E)-catenin is mainly expressed in epithelial tissues, α(N)-catenin is most prevalent in neural tissues, and α(T)-catenin is restricted to the heart and testes (Kobielak and Fuchs, 2004). Recent studies indicate that besides a simple structural function, α-catenin is involved in multiple pathways controlling membrane and actin dynamics, cell proliferation, migration, and apoptosis (Benjamin and Nelson, 2008). Our laboratory has reported a dramatic decrease of α-catenin expression in proximal tubular epithelium in aged male Fisher 344 rats (Jung *et al.*, 2004). Decreased expression of α-catenin was coupled with an increase in p53 expression in both lung and gastric cancer (Nozawa *et al.*, 2006). In addition, an increase in apoptosis was observed in α-catenin deleted mouse mammary glands (Nemade *et al.*, 2004). In this study, the role of α(E)-catenin in cisplatin-induced apoptosis was determined in a cell line (C2 cells) that has stable knockdown of α(E)-catenin. C2 cells are characterized by decrease cell-cell aggregation, increased monolayer permeability, and decreased repair in a wound healing assay due to migration deficits (Nichols *et al.*, 2014a,b). The hypothesis that loss of α(E)-catenin increases susceptibility to cisplatin was examined in series of experiments.

MATERIALS AND METHODS

Materials. *cis*-Diamineplatinum(II) dichloride (Sigma-Aldrich, Cat. no. P4394), N-cadherin antagonist peptide (GenScript, SWELYYPLRANL-NH_2), N-cadherin antagonist control peptide (GenScript, SRELYYPLRANL-NH2), Caspase Inhibitor I (Z-VAD (OMe)-FMK) (CalBiochem, Cat. no. 627610), mercury(II) chloride (Sigma-Aldrich, Cat. no. 203777), Staurosporine (CalBiochem, Cat. no. 569396), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Cat. no. M2128), neutral red (Sigma, Cat. no. N4638).

Animals. Male Fisher 344 rats (4-, 20-, and 24-month-old) were obtained from the NIA colony. Animals were randomly assigned to the cisplatin treated group and saline control group with $n = 5$ each. Animals received a single intraperitoneal (IP) injection of 2.75 mg/kg cisplatin, or 2 mg/kg mercuric chloride, or an equal volume of saline as control. Animals were placed in metabolic cages overnight before harvesting. On the day of the experiment (72 h after cisplatin injection; 48 h after mercury injection), rats were anesthetized with ketamine (80–120 mg/kg)/xylazine (5–10 mg/kg) via IP injection. Urine was collected and a cardiac puncture was performed to obtain blood. Kidney tissue was fixed in 4% paraformaldehyde overnight and stored in 70% ethanol. All animal experiments and care were approved by the Animal Care and Use Committee in accordance with the National Institutes of Health (NIH).

Cell culture. Cells were plated at a density of 5×10^4 cells/cm^2 and cultured in Dulbecco's modified eagle medium/F12 (DMEM/F12) (1:1) with L-glutamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco, Cat. no. 11039-021) supplemented with 10% fetal bovine serum (FBS) (Altanta Biologicals, Cat. no. S11150), 5 μg/ml puromycin dihydrochloride (Sigma, Cat. no. P9620) and incubated at 37°C in 5% CO_2. Cells were harvested with TrypLE Express (Gibco, Cat. no. 12604-021) and pelleted at 1250 rpm for 5 min at room temperature (RT). Single cell colonies were grown to confluence and passaged to larger plates. The cell lines (NT3 and C2) were used within 20 passages of establishing a clonal cell line, as described by our laboratory (Nichols *et al.*, 2014a,b).

Cell viability. Cell viability was assessed by the MTT assay based on mitochondrial's ability to convert soluble MTT to its insoluble purple formazan. Cells were seeded in 96-well flat bottom tissue culture plates (Sigma, Cat. no. Z707910) at a density of 5×10^4 cells/cm^2. After 24 h, culture media was replaced by serum-free (SF) media supplemented with desired treatments. Three hours before harvest, 10 μl of 5 mg/ml MTT (Sigma, Cat. no. M2128), dissolved in Dulbecco's phosphate buffered saline (DPBS) (Gibco, Cat. no. 14190-144), was added to each well. Upon harvesting, cells were washed with cold DPBS and dissolved by adding 50 μl solubilization solution (10% Triton X-100, 0.1N HCl in isopropanol). The plates were read at 570/690 nm on the Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT). The results are expressed by percentage viability as: $Abs_{570-690}$ treated/$Abs_{570-690}$ control × 100.

Cell viability was also estimated by neutral red assay which stains lysosomes red, in live cells. Briefly, confluent cultures of NT3 and C2 cells, in 96-well plates, were challenged with cisplatin in SF media for 24 h. Three hours before harvest, 10 μl of 500 μg/ml neutral red (Sigma, Cat. no. N4638), dissolved in DPBS, was added to each well. After incubation, the media was aspirated and cells were fixed with 50 μl fixative solution (1% formaldehyde, 1% $CaCl_2$) for 5 min. Then the fixative solution was aspirated, the plates were dried in RT and cells were dissolved by adding 100 μl solubilization solution (1% acetic acid, 50% ethanol) for 15 min. The plates were read at 540 nm on the Synergy HT Multi-Detection Microplate Reader. The results are expressed by percentage viability as: Abs_{540} treated/Abs_{540} control × 100.

LDH assay. Confluent cultures of NT3 and C2 cells in 96-well plates, were challenged with cisplatin in SF media for 24 h. Cytotoxicity was determined by lactate dehydrogenase (LDH) assay. The LDH assay was performed using Pierce LDH Cytotoxicity Assay Kit (Pierce, Cat. no. 88953) according to the manufacturer's instructions.

Caspase 3/7 activity assay. Confluent cultures of NT3 and C2 cells in 96-well plates were challenged with cisplatin in SF media for 24 h. Caspase 3/7 activity was determined by Caspase-Glo 3/7 Assay Kit (Promega, Cat. no. G8091) according to the manufacturer's instructions.

Western blot. Subconfluent cells were washed twice with ice-cold DPBS and lysed with lysis buffer (10mM Tris-HCl, 1% SDS) containing Halt Protease/Phosphatase inhibitors (ThermoScientific, Cat. no. 78444). Cells were scraped and incubated on a rocker for 15 min at 4°C. Cells were further disrupted by pipette 15 times and spun at 12,000 × g for 15 min at 4°C. Protein concentration was determined by NanoDrop 2000c Spectrophotometer (ThermoScientific, Waltham, MA) at 280 nm.

The following antibodies were used: anti-caspase 3 (Cell Signaling, Cat. no. 9662), anti-caspase 7 (Cell Signaling, Cat. no. 9492), anti-PARP (Cleaved-Asp214) (Sigma, Cat. no. SAB4500487), anti-α-catenin (BD Transduction Laboratories, Cat. no. 610194), anti-N-cadherin (BD Transduction Laboratories, Cat. no. 610920), and anti-β-actin (Sigma, Cat. no. A2228). Goat-antimouse horseradish peroxidase (HRP) conjugate and Goat-antirabbit HRP conjugate (Jackson ImmunoResearch Laboratories, Cat. nos. 115035003 and 305035003) were used at 1:20,000 dilutions. Blots

were developed using SuperSignal West Femto Chemiluminescent Substrate (Pierce, Cat. no. 34095), imaged using the Chemi-Doc imaging system (Bio-Rad, Hercules, CA), and quantitation performed using the ImageLab 3.0 software (Bio-Rad).

Real-time PCR. From 5×10^6 to 1×10^7 cells were harvested and suspended in 1 ml PBS. RNA was isolated using the RNeasy mini kit (Qiagen, Cat. no. 74104) with on-column DNase digestion. RNA concentration and quality was determined by NanoDrop 2000c Spectrophotometer. cDNA was generated from 2 μg RNA using the High Capacity cDNA Synthesis Kit (Life Technologies, Cat. no. 4368814) following the kit protocol. Quantitative PCR was performed in duplicate using 50 ng cDNA/reaction via Taqman assays with SsoFast Probes Supermix with ROX (Bio-Rad, Cat. no. 172-5251), and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The following cycling conditions were used: 95°C for 20 s, then 95°C for 1 s and 60°C for 20 s repeated 40 times.

Commercially available TaqMan primer sets were used to assess α(E)-catenin (Rn01406769_mH) (Life Technologies, Cat. no. 4331182). Relative quantitation was performed using the Pfaffl method normalized to glyeraldehyde-3-phosphate dehydrogenase (Gapdh).

In situ *apoptosis.* Paraffin-embedded kidney sections were used to detect the *in situ* apoptosis. The *in situ* apoptosis assay was performed using. *In situ* Apoptosis Detection Kit (Genway, Cat. no. 40-831-160019) according to the manufacturer's instructions. To quantify the *in situ* apoptosis, the positively stained area was measured using the point tool of CellSense.

Statistics. Results are expressed as mean ± SE. A two-way analysis of variance (ANOVA) was performed with the exception of Figures 1A 1E and 4A in which a one-way ANOVA was performed, followed by Student's *t*-test using the statistical software GraphPad Prism 6 (GraphPad Software, La Jolla, CA). The differences were considered statistically significant when $p < 0.05$.

RESULTS

Initial experiments demonstrated that decreased cell density increased the sensitivity to cisplatin challenge, indicating that cell-cell adhesion increases cell resistance to cisplatin injury (Fig. 1A). Because α-catenin is well known to play important role in cell adhesion (Kobielak and Fuchs, 2004), a clonal α(E)-catenin knockdown cell line (C2) was generated in NRK-52E cells using lentiviral shRNA; the NT3 cell line is the nontargeted control. Confluent cultures of NT3 and C2 cells were challenged with cisplatin for 24 h. C2 cells exhibited a significant loss of viability at all concentrations of cisplatin as compared with NT3 cells (Figs. 1B and 1C). A time course assay was also performed using 150μM cisplatin. C2 cells have significantly lower cell viability than NT3 cells at early (6–12 h) and late (21–48 h) time points following cisplatin treatment (Fig. 1D). The increased cell viability in the early time points (6–12 h) can be caused by the protective cellular stress response (Fulda *et al.*, 2010). As previously demonstrated by our laboratory, the expression of N-cadherin is also decreased in C2 cells (Nichols *et al.*, 2014b). In order to exclude the potential effect of decreased N-cadherin expression in the increased sensitivity of C2 cells, NRK-52E cells were treated with an N-cadherin antagonist peptide (H-SWELYYPLRANL-NH2) alone or in the presence of 150μM cisplatin. Another peptide (H-SRELYYPLRANL-NH2) was used as a control (Devemy and Blaschuk, 2009). The N-cadherin antagonist peptide did not increase the sensitivity of NRK-52E cells to cisplatin (Fig. 1E). These results indicate that loss of α(E)-catenin, rather than loss of N-cadherin, increases cell susceptibility to cisplatin injury.

Apoptosis and necrosis are two major mechanisms of cell death (Edinger and Thompson, 2004). LDH leakage, a marker of necrosis, was not different in C2 cells as compared with NT3 after 24 h of cisplatin challenge (Fig. 2A). This suggests that the increased susceptibility of C2 cells to cisplatin is not due to necrosis, an interpretation that is supported by the finding that C2 cells are not more sensitive to inorganic mercury, which induces necrosis in proximal tubular epithelial cells (Stacchiotti *et al.*, 2011) (Fig. 2B). However, staurosposine, a commonly used drug to induce apoptosis in cell culture (Mannherz *et al.*, 2006), decreased viability in C2 cells to a greater extent than NT3 cells (Fig. 2C). Taken together, these observations suggest that the increased susceptibility of C2 cells to cisplatin is more likely to be caused by apoptosis rather than necrosis.

In order to study the role of apoptosis in the increased susceptibility of C2 cells to cisplatin, a Caspase-Glo 3/7 Assay was performed to detect the activity of caspase 3/7, which plays key effector roles in apoptosis. The basal caspase activity in C2 cells is not significantly different from that in NT3 cells. However, after cisplatin treatment, increased caspase 3/7 activity was observed in C2 cells as compared with NT3 cells (Fig. 3A). This finding was confirmed by Western blot (Fig. 3B) which revealed increased caspase 3/7 activation (Figs. 3C and 3D) and PARP cleavage (Fig. 3E) in C2 cells at 18 h of cisplatin treatment. Importantly, the susceptibility difference between NT3 and C2 cells to cisplatin was abolished by Z-VAD, a pan-caspase inhibitor (Fig. 3F). Together, these data identify apoptosis as the underlying mechanism of the increased susceptibility of C2 cells to cisplatin injury.

Another interesting finding is that cisplatin significantly suppressed mRNA (Fig. 4A) and protein (Figs. 4B and 4D) expression of α-catenin. After challenge with 100μM cisplatin for 24 h, α(E)-catenin levels were at 20% in the NT3 cells. The expression of α(E)-catenin in C2 cells also decreased even further after cisplatin treatment. Similar reduction of α-catenin protein expression was observed by Western blot. However, the expression of N-cadherin did not change after the cisplatin challenge (Figs. 4B and 4C). These results demonstrate that α-catenin is also a target of cisplatin-induced cell injury.

Consistent with previous studies (Jung *et al.*, 2004), no significant difference was observed between 4- and 24-month-old male Fisher 344 rats in either serum creatinine (Fig. 5A) or blood urea nitrogen (BUN) levels (data not shown). However, a significant increase in serum creatinine was seen at 72 h after a single dose of cisplatin in aged rats, but not in young rats. No significant difference was seen in BUN levels (data not shown), this is probably because BUN is less sensitive than serum creatinine as a biomarker of AKI (Edelstein, 2008). Interestingly, when aged (20-month-old) and young rats were challenged with mercuric chloride, no significant difference was seen (Fig. 5B). The aged rat expressed higher level of KIM-1 compared with young animal in control groups which is consistent with previous reports from our laboratory (Gardiner *et al.*, 2012). A significant increase in urine KIM-1 was detected after the cisplatin challenge in both young and aged rats (Fig. 5C). Interestingly, the expression of α(E)-catenin in the tubules of young rats was not influenced by cisplatin. However, α(E)-catenin expression was further decreased in aged kidney after cisplatin injury (Figs. 5D and 5F). Conversely, the expression of N-cadherin was not affected by cisplatin in neither young nor aged kidney (Figs. 5D and 5E). *In*

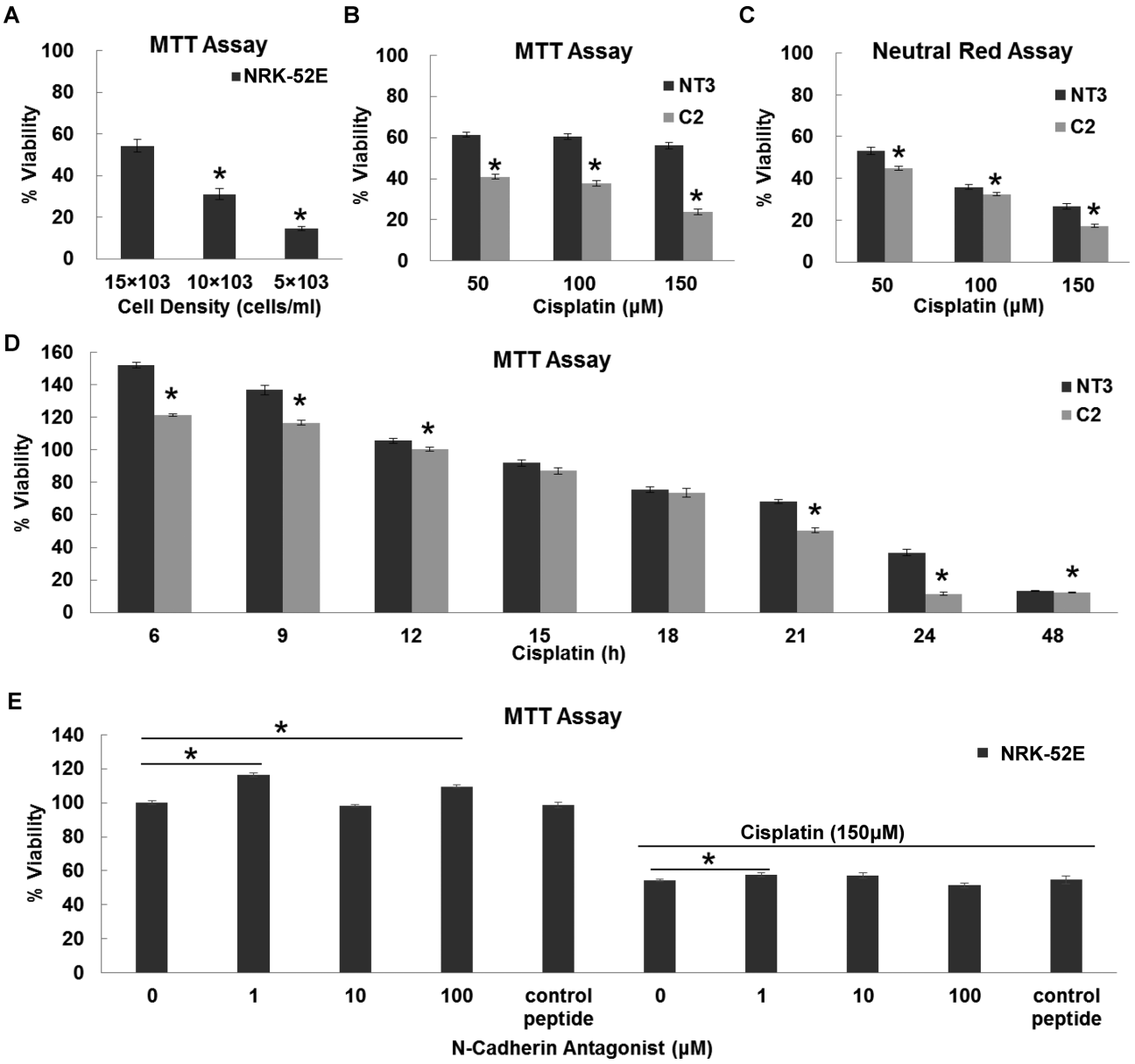

FIG. 1. Effect of decreased α(E)-catenin on susceptibility to cisplatin injury. (A) Cell viability was determined by MTT assay in cultures of NRK-52E cells seeded with the indicated densities and challenged with 150μM cisplatin in SF media for 24 h. The densities correspond with 100, 75, and 50% confluence. The results are presented as percentage viability of untreated control with corresponding cell density in SF media. The asterisks indicate significant differences from the 15×10^3 group ($n = 16$). Cell viability determined by MTT (B) and neutral red (C) assays in confluent cultures of NT3 and C2 cells treated with the indicated concentrations of cisplatin in SF media for 24 h. (D) Cell viability determined by MTT assay upon treatment for the indicated time periods with 150μM cisplatin. The asterisks in (B), (C), and (D) indicate significant differences between NT3 and C2 cells for each treatment ($n = 24$). (E) Cell viability determined by MTT assay in NRK-52E cells treated with the indicated concentrations of cadherin antagonist ($IC_{50} = 0.09\mu M$) alone or in the presence of 150μM cisplatin. Control peptide was used at a concentration of 1mM ($n = 16$). The results in (B), (C), (D), and (E) are presented as percentage viability of untreated control in SF media.

situ apoptosis was detected by TUNEL assay (Fig. 6A). The aged kidney exhibited higher level of *in situ* apoptosis compared with the young kidney. The *in situ* apoptosis was increased to a larger extent by cisplatin in aged group than young group (Fig. 6B). These data demonstrate that aged kidney, which is marked by loss of α-catenin, is more susceptible to cisplatin injury, but not necrosis.

DISCUSSION

α-Catenin has been viewed as a simple linkage molecule between cadherin-β-catenin complex and actin cytoskeleton mediating cell-adhesion in a cadherin-dependent manner for decades (Benjamin and Nelson, 2008). Recent studies, however, have revealed cadherin-independent functions of α-catenin (Scott and Yap, 2006). Mis-localization or loss of α-catenin has been reported to be a more severe prognosis of cancer progression than loss of E-cadherin in several tumors (Gofuku *et al.*, 1999). Moreover, α-catenin is also involved in cell proliferation, apoptosis, and actin cytoskeleton dynamics in a cadherin-

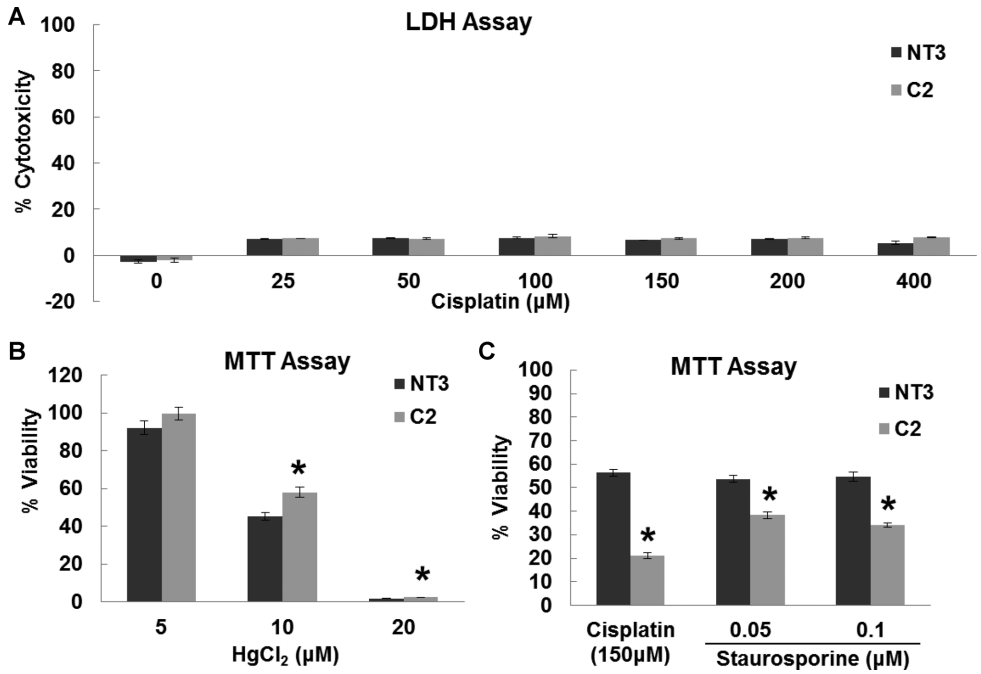

FIG. 2. Susceptibility of NT3 and C2 cells to injuries induced by different chemicals. (A) Cytotoxicity was determined by LDH assay in confluent cultures of NT3 and C2 cells treated with the indicated concentrations of cisplatin in SF media for 24 h. The results are presented as the percentage cytotoxicity of the maximum LDH release control ($n = 6$). Cell viability was determined by MTT assay in cells treated with the indicated concentrations of mercury (B), cisplatin, and staurosporine (C). The results are presented as percentage viability of untreated control in SF media (B and C). The asterisks indicate significant differences between NT3 and C2 cells of each treatment ($n = 24$).

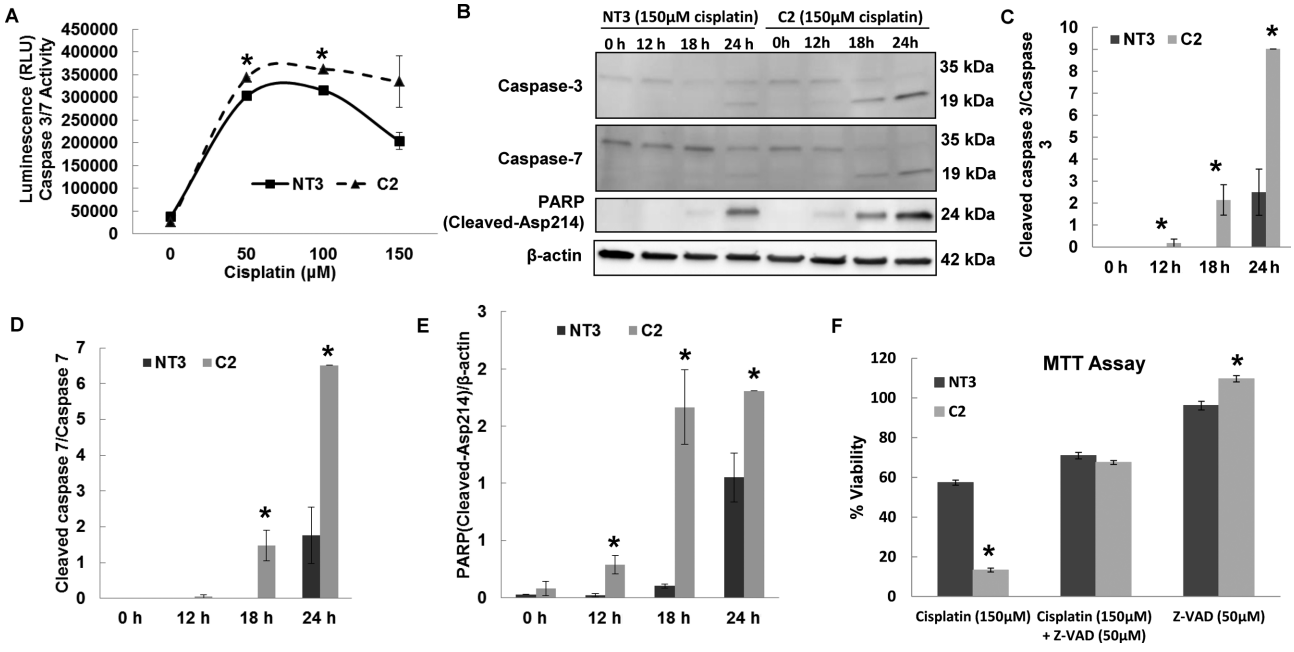

FIG. 3. Apoptosis induced by cisplatin in NT3 and C2 cells. (A) Caspase 3/7 activity in confluent cultures of cells treated with the indicated concentrations of cisplatin in SF media for 24 h, was determined by luminescent assay. The asterisks indicate the significant differences between NT3 and C2 cells of each treatment ($n = 8$). Immunoblot analysis (B) and quantification of cleaved caspase 3/caspase 3 (C), cleaved caspase 7/caspase 7 (D) and PARP (cleaved-Asp214)/ β-actin (E) in whole cell lysates (70 μg protein) obtained from cells treated with 150μM cisplatin for the indicated time periods. The β-actin blot serves as the loading control. The significant difference between C2 and NT3 at each time point is indicated by asterisk ($n = 2$). (F) Cell viability determined by MTT assay in cells treated with 150μM cisplatin alone, in the presence of 50μM Z-VAD or 50μM Z-VAD alone in SF media for 24 h. The results are presented as percentage viability of untreated control in SF media. The asterisks indicate significant differences between NT3 and C2 cells of each treatment ($n = 24$).

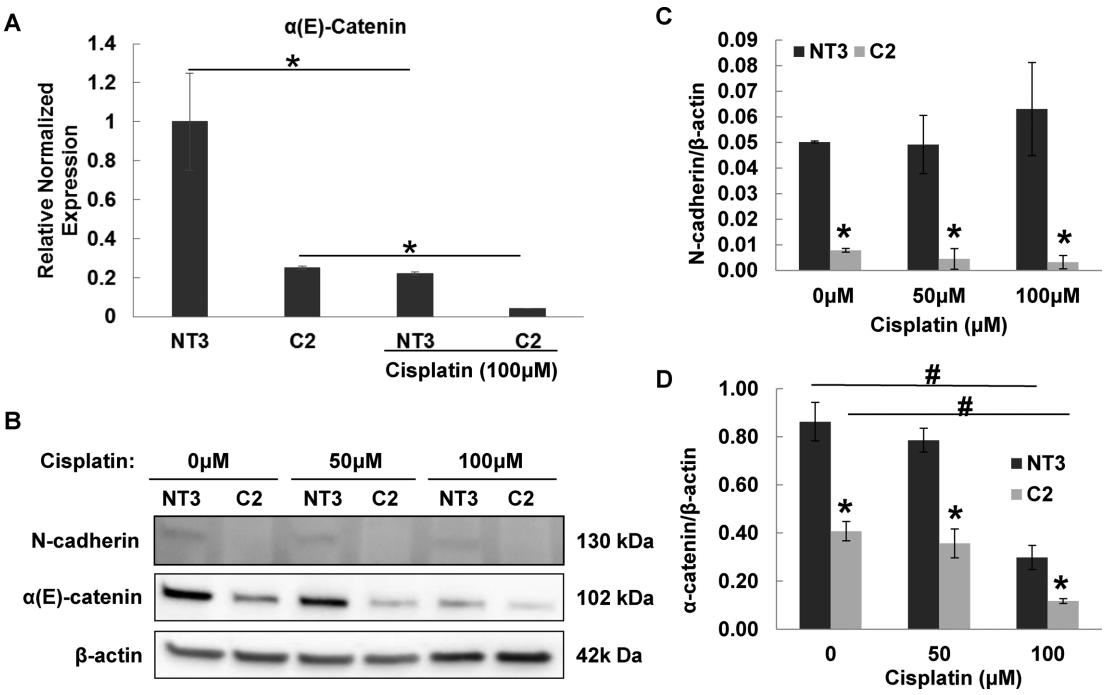

FIG. 4. The effect of cisplatin on α-catenin and N-cadherin expression. (A) qPCR for α(E)-catenin in NT3 and C2 cells with or without 100μM cisplatin treatment for 24 h. The result is normalized to untreated NT3 cells. The significant differences are indicated by asterisks ($n = 3$). Immunoblot analysis (B) and quantification of N-cadherin/β-actin (C) and α-catenin/β-actin (D) in whole cell lysates (40 μg protein) obtained from cells treated with the indicated concentrations of cisplatin in SF media for 24 h. The β-actin blot serves as loading control. The significant differences between C2 and NT3 are indicated by asterisks ($n = 2$). The hash symbol indicates significant difference from the untreated control group ($n = 2$).

independent manner (Benjamin and Nelson, 2008; Scott and Yap, 2006). The role of α-catenin in apoptosis is contentious. On one hand, deletion of α-catenin in the central nervous system or epidermis was reported to cause a decrease in apoptosis due to up-regulation of NF-κB (Kobielak and Fuchs, 2006). Reintroducing α-catenin into a myeloid leukemia cell line resulted in a decrease in proliferation and increase in apoptosis (Benjamin and Nelson, 2008). On the other hand, evidence has shown a decreased expression of α-catenin coupled with an increase in p53 expression in both lung and gastric cancer (Nozawa *et al.*, 2006). In addition, an increase in apoptosis was observed in α-catenin deleted mouse mammary gland (Nemade *et al.*, 2004). Hence, whether loss of α-catenin increases or decreases apoptosis may depend on the cellular context. In our study, C2 cells exhibited a significant loss of viability as compared with NT3 cells after the cisplatin treatment (Figs. 1B, 1C, and 1D), but showed no cytotoxicity (LDH leakage) difference (Fig. 2A). These results indicate that the increased susceptibility of C2 cells to cisplatin is not due to necrosis. Furthermore, increased caspase 3/7 activation and PARP cleavage was observed in C2 cells after cisplatin treatment. Z-VAD, a pan-caspase inhibitor, abolished the susceptibility difference between NT3 and C2 cells to cisplatin (Fig. 3). Taken together, our study is the first to provide evidence that loss of α(E)-catenin increases cisplatin-induced apoptosis of renal tubular epithelial cells.

Interestingly, the mRNA and protein expression of α-catenin was further decreased in both NT3 and C2 cells after the cisplatin challenge (Fig. 4). Correspondingly, our *in vivo* study showed a further decrease of α(E)-catenin expression at 72 h after a single dose of cisplatin in aged kidney, but α(E)-catenin expression was not altered in young rats (Figs. 5D and 5F). This results indicate that loss of α(E)-catenin during aging is exacerbated by further loss following cisplatin injury, which initiates further apoptosis of renal tubular epithelial cells, leading to increased injury.

The aging population is a critical issue for the 21st century. There will be >70 million Americans over the age of 65 by 2030 according to the United States Census Bureau (Ricketts, 2011). Aging can change the structure and function of several organs, which increases the incidence of many diseases. With the exception of lung, the change in kidney with normal aging, is most dramatic among all human organ systems (Long *et al.*, 2005). Aging related changes in the kidney may not be obvious under normal conditions. However, these changes may contribute to renal dysfunction under stress or chemical insults, including drug-induced AKI (Ning *et al.*, 2013). This is consistent with our *in vivo* study which shows no significant difference between 4- and 24-month-old male Fisher 344 rats in basal serum creatinine level. However, after 72 h of an IP dose of cisplatin treatment, increased serum creatinine level were observed in aged rats but not young ones (Fig. 5A).

Cisplatin is a widely used chemotherapeutic agent with broad-spectrum against a variety of tumors. However, its clinical usage is dramatically limited by a dose-limiting side effect, nephrotoxicity (Miller *et al.*, 2010). The mechanism underlying cisplatin-induced nephrotoxicity involves several factors, including DNA damage, oxidative stress, inflammatory response, and apoptosis (Yao *et al.*, 2007). Two major pathways of apoptosis have been studied in cisplatin-induced nephrotoxicity: the intrinsic and extrinsic pathways (Peres and da Cunha, 2013). Both pathways will ultimately activate caspase 3/7 leading to the morphological changes of apoptosis including cell shrinkage, membrane blebbing, and DNA fragmentation (Pabla and Dong, 2008). The intrinsic pathway, involving the mitochondria,

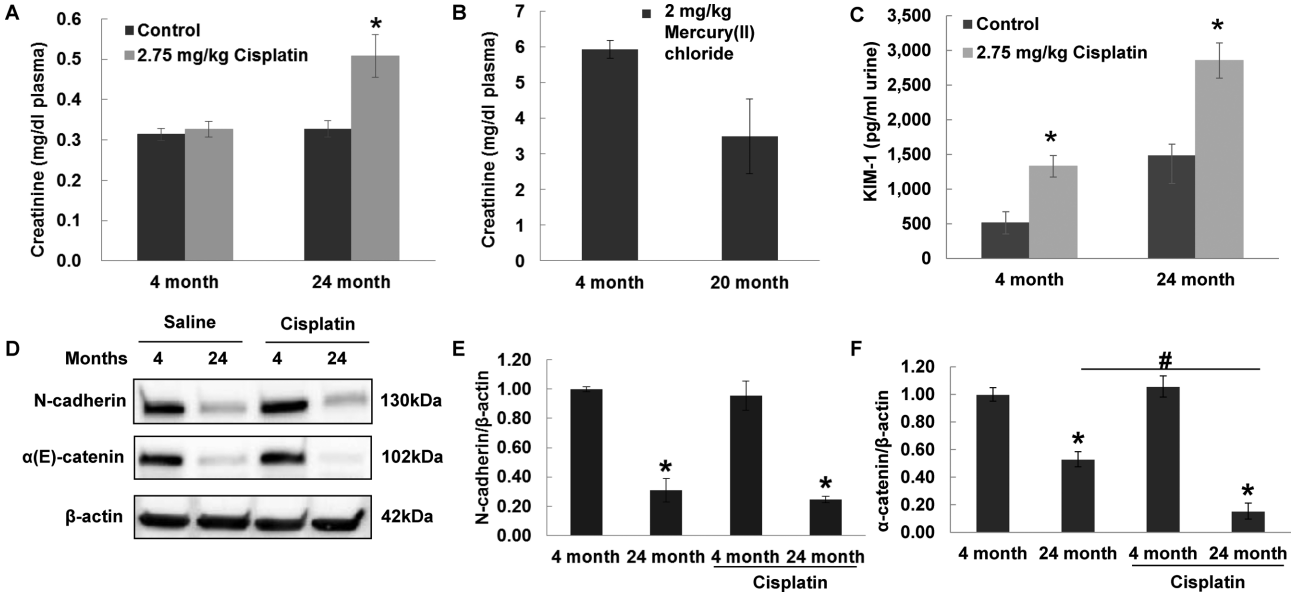

FIG. 5. Cisplatin or mercury(II) chloride-induced changes in young and aged kidney. Serum creatinine levels were measured after 72 h of cisplatin treatment (A) or 48 h of mercury(II) chloride treatment (B). Urine KIM-1 levels were measured after 72 h of cisplatin treatment (C). Equal volume of saline is used as control. The asterisk indicates significant differences from the control group ($n = 5$). Immunoblot (D) and quantification of N-cadherin/ β-actin (E) and α-catenin/β-actin (F) in kidney tissue lysates (100 μg protein) obtained from young and aged rats after 72 h of cisplatin treatment. The asterisk indicates significant differences between young and aged group of each treatment ($n = 5$). The pound indicates significant difference from the untreated group ($n = 5$).

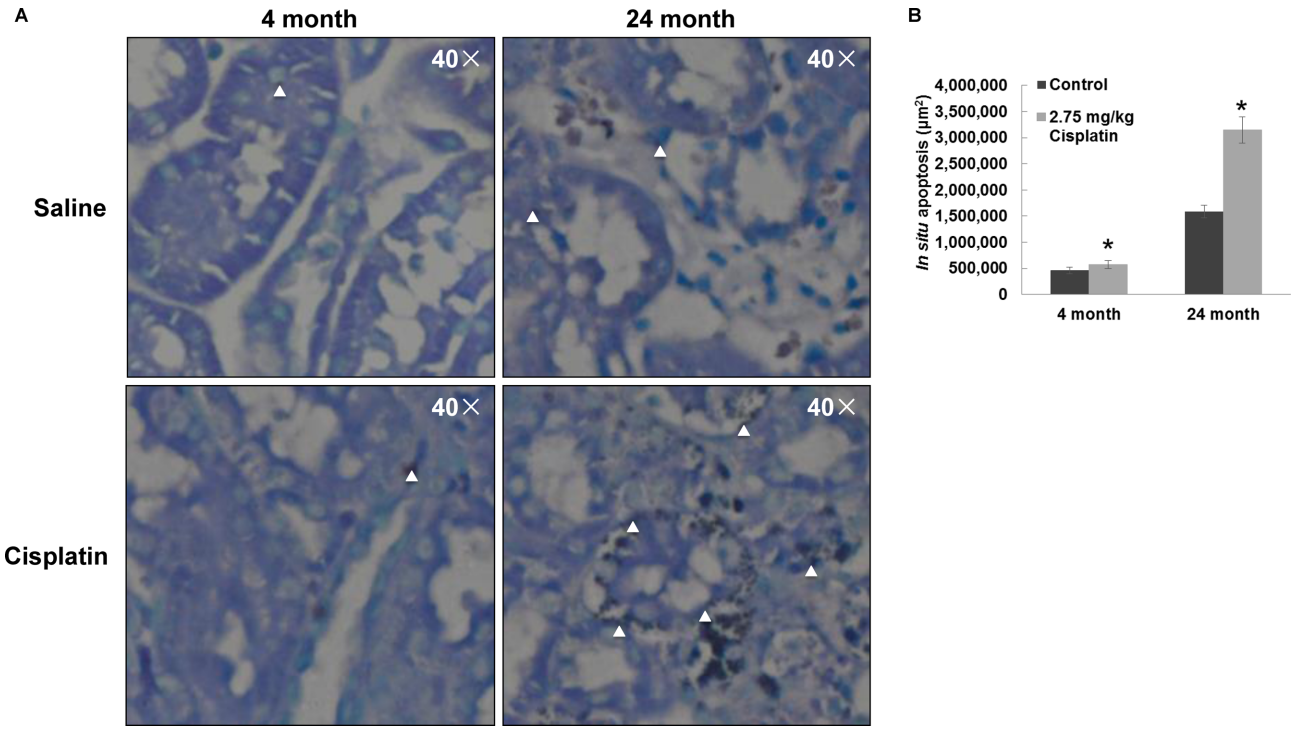

FIG. 6. *In situ* apoptosis induced by cisplatin in aged kidney. *In situ* apoptosis was detected (A) and quantified (B) via TUNEL assay. The white arrow heads point the apoptotic cells. The asterisks indicate significant difference from the untreated group ($n = 5$).

has been reported to play a key role in cisplatin-induced renal tubular cell death. After exposure to cisplatin, proapoptotic proteins BAX, and BAK are activated, altering the integrity of mitochondrial membrane. As a result, cytochrome C and apoptosis-inducing factor (AIF) will be released from the mitochondria, which leads to the caspase-dependent or -independent apoptosis pathway correspondently (Rodriguez-Garcia *et al.*, 2009). Because our study showed that pan-caspase inhibitor completely abolished the susceptibility difference between NT3 and C2 cells to cisplatin (Fig. 3C), loss of α-catenin may only influence the caspase-dependent apoptosis pathway. In addition, apoptosis mediated by endoplasmic reticulum (ER) stress involving

caspase 12, phosphorylated extracellular signal regulated kinase (p-ERK) and Ca^{2+}-independent phospholipase A2 may also play an important role in cisplatin-induced nephrotoxicity (Gao *et al.*, 2014). Furthermore, evidence has shown that the inhibition of p38-MAPK can attenuate cisplatin-induced nephrotoxicity in mice (Ramesh and Reeves, 2005). The extrinsic pathway is mainly initiated by activating cell death receptors by binding TNF-α or Fas, which cause the recruitment and activation of caspase 8/10 leading to the activation of caspase 3 or the mitochondrial apoptosis pathway (Pabla and Dong, 2008; Peres and da Cunha, 2013). Our future studies will be focused on which specific pathway is influenced by the loss of α(E)-catenin causing the increased susceptibility of the aged kidney to apoptosis.

In conclusion, this study showed that loss of α(E)-catenin increases cisplatin-induced apoptosis of renal tubular epithelial cells. Considering the loss of α(E)-catenin in aged kidney, this result could partially explain the increased nephrotoxicity induced by cisplatin in the aged kidney.

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