

Role of Annexin A5 in Cisplatin-induced Toxicity in Renal Cells

MOLECULAR MECHANISM OF APOPTOSIS*

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Background: There is increasing evidence that annexin A5 is related to cytotoxicity, but the precise function has yet to be elucidated.

Results: Cisplatin induces mitochondrial translocation of annexin A5, and annexin A5 mediates VDAC oligomerization.

Conclusion: Annexin A5 may play a role as a mediator of cisplatin-induced apoptosis in renal epithelial cells.

Significance: Learning how annexin A5 is involved in the apoptotic pathway is crucial for understanding cisplatin-induced toxicity.

Annexin A5 belongs to a large family of calcium-binding and phospholipid-binding proteins and may act as an endogenous regulator of various pathophysiological processes. There is increasing evidence that annexin A5 is related to cytotoxicity, but the precise function of this protein has yet to be elucidated. In this study, we aimed to verify the function of annexin A5 in the apoptosis of renal epithelial cells. Real-time PCR and Western blot analysis, together with immunofluorescence analysis, showed that the expression of annexin A5 significantly increased in the presence of cisplatin in both human and rat renal epithelial cells. With regard to the mechanism of cisplatin-induced apoptosis, apoptosis-inducing factor (AIF) release into the cytosol was observed, and the underlying mechanism was identified as voltage-dependent anion channel (VDAC) oligomerization. Mitochondrial membrane potential ($\Delta\psi_m$) was found to be greatly disrupted in cisplatin-treated cells. Moreover, cisplatin strongly induced translocation of annexin A5 into mitochondria. To understand the functional significance of annexin A5 in renal cell death, we used a siRNA-mediated approach to knock down annexin A5. Annexin A5 depletion by siRNA led to decreased annexin A5 translocation into mitochondria and significantly reduced VDAC oligomerization and AIF release. Annexin A5 siRNA also increased cell viability compared with the control. Moreover, expression of annexin A5 was induced by other nephrotoxicants such as CdCl₂ and bacitracin. Taken together, our data suggest that annexin A5 may play a crucial role in cisplatin-induced toxicity by mediating the mitochondrial apoptotic pathway via the induction and oligomerization of VDAC.

Cisplatin is one of the most widely used chemotherapeutic agents for the treatment of various types of cancers, including some types of carcinomas, sarcomas, and lymphomas (1), but

the risk of nephrotoxicity frequently limits the use of higher doses to maximize its antineoplastic effects. Nephrotoxicity after cisplatin treatment is common and can manifest as either acute renal failure or a chronic syndrome characterized by electrolyte wasting. The cytotoxic effects of cisplatin have been thought to occur via a number of diverse mechanisms, including inhibition of protein synthesis, mitochondrial injury, and DNA damage (2). However, the underlying molecular mechanisms of cisplatin-induced apoptosis of kidney cells are not yet fully understood. The lack of early biomarkers is another issue that makes it difficult to initiate potential therapeutic or preventive interventions for cisplatin-induced nephrotoxicity in a timely manner (3, 4).

After conducting two-dimensional gel electrophoresis followed by MALDI-TOF-MS analysis of cisplatin-treated cells to determine which proteins are differentially expressed (5), we selected annexin A5 as a marker candidate based on GEO microarray data showing increased expression of annexin A5 mRNA in rats injected with various nephrotoxicants (6). Annexin A5 is a calcium-regulated, phospholipid-binding protein that belongs to the annexin family and has a molecular mass of 32–35 kDa. This protein is present in many human tissues and exists mostly in the endothelium and parietal epithelium of glomeruli, macula densa, and renal cortical tubular epithelium (7, 8). Annexin A5 has been reported to have anticoagulant activity and to be involved in the inhibition of phospholipase A₂ and the regulation of membrane transport. It has also been implicated in many cellular functions, including cellular proliferation and signal transduction, although the precise role of annexin A5 has not yet been identified (7). Recently, annexin A5 has emerged as a promising biomarker of cytotoxicity in patients with Alzheimer disease, heart injury (9), and renal diseases such as nephritic syndrome and systemic lupus erythematosus nephritis (7, 10).

The voltage-dependent anion channel (VDAC)³ could have an important role in apoptotic mechanisms. It is known that

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³ The abbreviations used are: VDAC, voltage-dependent anion channel; TMRM, tetramethylrhodamine methyl ester; AIF, apoptosis-inducing factor.

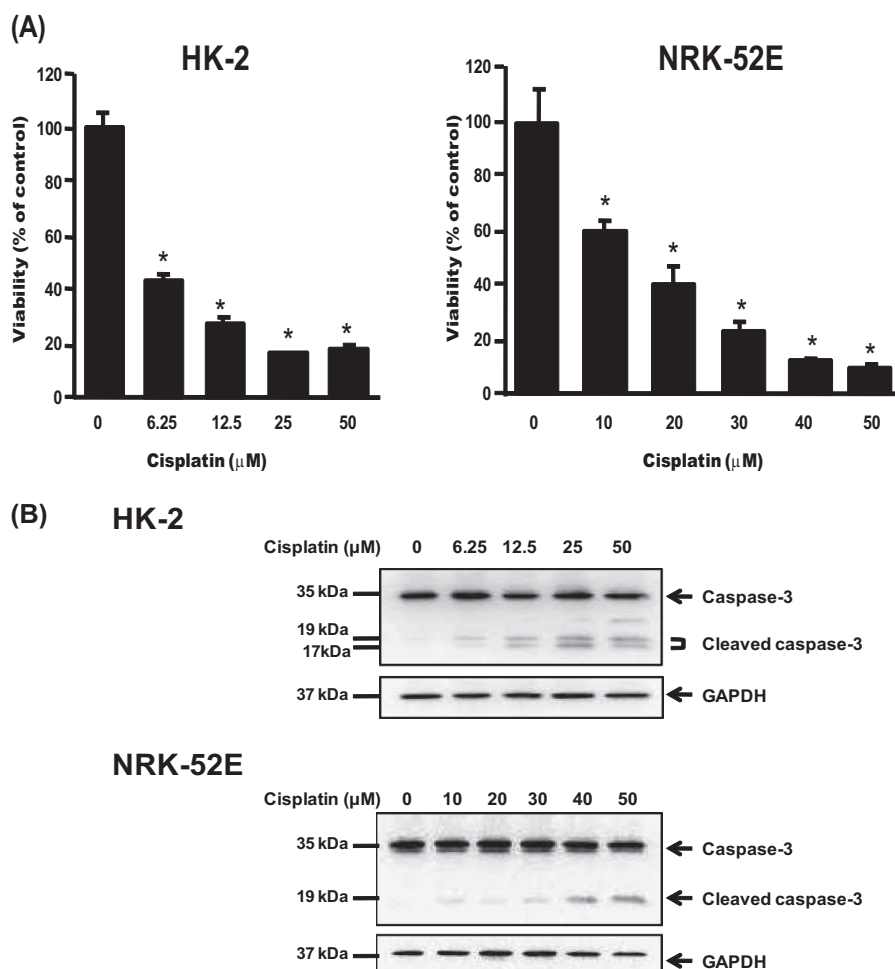


FIGURE 1. Cisplatin induces apoptotic cell death in renal epithelial cells. A, cell cytotoxicity. HK-2 or NRK-52E cells were treated with cisplatin at various concentrations for 48 h. Cell viability was detected with CCK. Formazan formation was quantified by spectrophotometry at 450 nm. The percentage of cells surviving in each group relative to the control was calculated. B, caspase-3 activity. Cells were treated with cisplatin at the indicated concentrations for 24 h. Whole cell lysates from HK-2 or NRK-52E cells were prepared. Total caspase-3 and cleaved caspase-3 were measured by Western blot analysis. *, significantly different from untreated cells ($p < 0.05$).

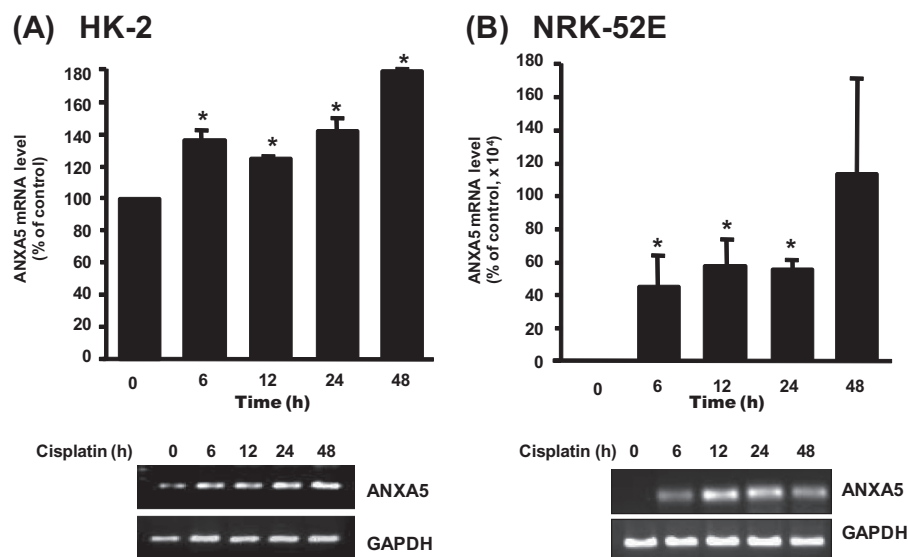
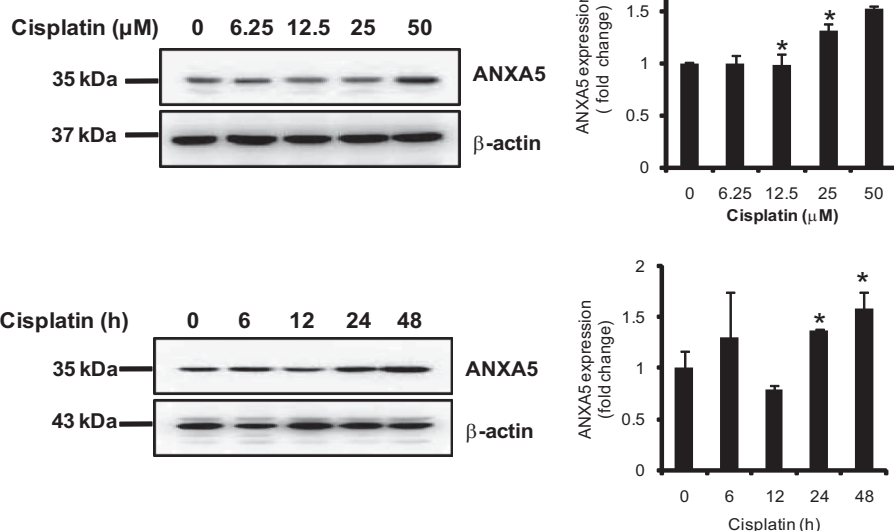


FIGURE 2. Cisplatin induces annexin A5 mRNA expression. Cells were treated with cisplatin (20 μ M for HK-2 (A) and 24 μ M for NRK-52E (B) cells) for 0, 6, 12, 24, or 48 h. Expression of annexin A5 (ANXA5) mRNA was determined by real-time quantitative PCR. HK-2 cells. *, significantly different from untreated cells ($p < 0.05$).

(A) HK-2



(B) NRK-52E

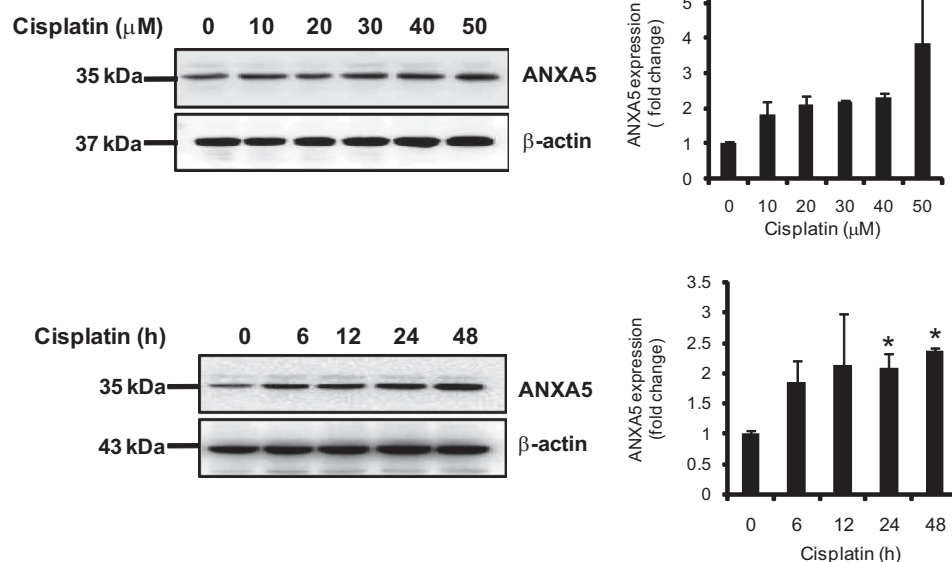


FIGURE 3. Cisplatin induces annexin A5 protein expression. Cells were treated with cisplatin (0, 10, 20, 30, 40, or 50 μ M) for 24 h or were treated with cisplatin (20 μ M for HK-2 (A) and 24 μ M for NRK-52E (B) cells) for the designated time periods (0, 6, 12, 24, or 48 h). Total cell lysates were prepared and used for Western blot analyses with anti-annexin A5 (ANXA5) antibody. β -Actin was used as a loading control. The intensities of the protein bands were quantified using Quantity One software and are plotted as the ratio of annexin A5 to β -actin intensity. Data are the mean $\pm$ S.D. ($n = 3$). *, significantly different from untreated cells ($p < 0.05$).

VDAC functions in mitochondria-mediated apoptosis through the release of apoptogenic proteins (11). VDAC is a 30-kDa mitochondrial protein located in the mitochondrial outer membrane that controls cross-talk between the mitochondria and the rest of the cell by functioning as a gatekeeper for the entry and exit of mitochondrial metabolites (12). VDAC associates with higher order structures to allow folded proteins to pass the mitochondrial membrane to achieve apoptosis (13, 14). Various apoptosis inducers, including staurosporine, curcumin, selenite, and arsenic oxide, induce formation of the VDAC dimer and oligomers (15). Cisplatin also induces VDAC oligomerization in HEK-293 cells at relatively high concentra-

tions (40–50 μ M), which indicates that cisplatin may possibly cause apoptosis via VDAC oligomerization (16, 17).

In this study, we determined that annexin A5 is up-regulated in both human and rat renal epithelial cells treated with cisplatin and explored the mechanism of toxicity. In cross-linking experiments, VDAC appeared to form a homodimer and oligomers after cisplatin treatment in a concentration- and time-dependent manner. Because there has been no evidence to suggest that annexin A5 plays a role in apoptosis despite annexin A5 up-regulation being implicated in apoptosis, we carried out knockdown experiments using annexin A5 siRNA to explore the function of annexin A5. Our results suggest that

Role of Annexin A5 in Cisplatin-induced Toxicity

annexin A5 can act as an apoptotic mediator via the modulation of mitochondrial membrane potential and apoptogenic factors.

EXPERIMENTAL PROCEDURES

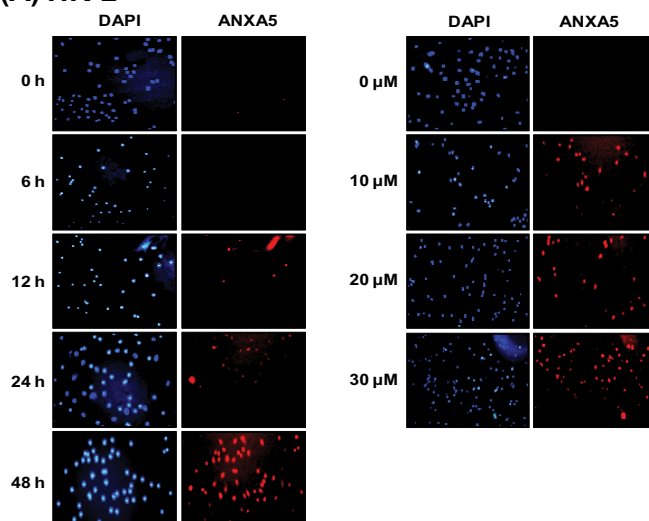
Chemicals—Cisplatin, CdCl₂, and bacitracin were purchased from Sigma-Aldrich. FBS and RPMI 1640 medium were purchased from HyClone (Logan, UT). Anti-annexin A5 antibody was purchased from Abcam (Cambridge, United Kingdom). The transfection reagent LipofectamineTM, Alexa Fluor[®] 594-conjugated donkey anti-goat IgG, Texas Red-conjugated goat anti-rabbit IgG, and MitoTracker[®] probes were supplied by Invitrogen. Tetramethylrhodamine methyl ester (TMRM) was from Molecular Probes (Eugene, OR). Antibodies for VDAC (N-18) and apoptosis-inducing factor (AIF) were purchased from Millipore (Bedford, MA). Anti-caspase-3 antibody was from Cell Signaling Technology (Beverly, MA). BCA protein assay kits and ECL kits were obtained from Pierce. Moloney murine leukemia virus reverse transcriptase and RNase inhibitor (RNasin) were purchased from Promega (Madison, WI). *Ex Taq*TM DNA polymerase was obtained from TaKaRa Bio (Shiga, Japan).

Cell Culture and Treatment—Immortalized human renal proximal epithelial cells (HK-2) and rat renal proximal tubular epithelial cells (NRK-52E) were obtained from American Type Culture Collection (Manassas, VA) and were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. Cisplatin, CdCl₂, or bacitracin was dissolved in sterile 0.9% NaCl solution (3 mM, 2 M, or 2 mM, respectively). Each stock solution was used within 3 days.

Cell Viability Assay—Cells (1 × 10⁴ cells/well) were plated onto 96-well round-bottom plates and treated with cisplatin at various concentrations. After 48 h, 10 µl of EZ-CyTox (Daeil Lab Service, Seoul, Korea) was added to each well and incubated for 2 h. The absorbance at 450 nm was measured using a GENios Pro microplate reader (Tecan, Männedorf, Switzerland). For flow cytometry analysis, cells (1 × 10⁶ cells/ml) were collected, and the resulting pellets were resuspended in 100 µl of MuseTM annexin V and dead cell reagent (Millipore) and 100 µl of culture medium. After resuspended cells were completely mixed and stained for 20 min in the dark, viable cells were analyzed using a MuseTM cell analyzer (Millipore).

RT-PCR—Total RNA was extracted using an RNeasy mini kit (Qiagen). Total RNA (500 ng) was transcribed at 37 °C for 1 h in a volume of 20 µl containing 5× RT buffer, 10 mM dNTPs, 40 units of RNase inhibitor, 200 units of Moloney murine leukemia virus reverse transcriptase, and 100 pmol of oligo(dT) primer. Subsequently, 0.8 µl of the reaction mixture from each sample was amplified with 10 pmol of each oligonucleotide primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, and 1.25 units of *Taq* DNA polymerase in a final volume of 25 µl. PCR was performed as follows: one cycle at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 63 °C for 15 s, and extension at 72 °C for 15 s. Human annexin A5 cDNA was amplified using sense primer 5'-CAGTCTAGGTGCAGCTGCCG-3' and antisense primer 5'-GGTGAAGCAGGACCAGACTGT-3'. For

(A) HK-2



(B) NRK-52E

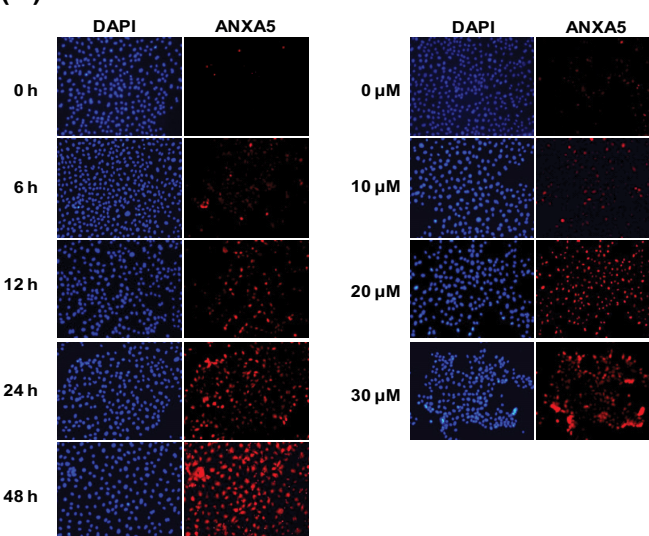


FIGURE 4. Immunofluorescence analysis of annexin A5 expression by cisplatin in renal epithelial cells. HK-2 (A) and NRK-52E (B) cells were cultured on microscope slides and treated with cisplatin (20 µM) for 0, 6, 12, 24, or 48 h in a concentration-dependent manner. After treatment, cells were fixed, permeabilized, and subsequently stained using anti-annexin A5 (ANXA5) antibody. Cells were then stained with the Alexa Fluor 594-labeled secondary antibody. DAPI was used for nuclear staining. Fluorescence was determined using a fluorescence microscope.

amplification of rat annexin A5 cDNA, sense primer 5'-GGC-CCTGCTGCTCCTCTG-3' and antisense primer 5'-GTA-AGGCAGCGTGGCAGGC-3' were used. Human GAPDH cDNA was amplified using sense primer 5'-TGAACGGGAA-GCTCACTGG-3' and antisense primer 5'-TCCACCACCCT-GTTGCTGTA-3'. The number of amplification cycles was optimized in preliminary experiments to ensure that the PCR did not reach a plateau. PCR products were analyzed by 2% (w/v) agarose gel electrophoresis using a ChemiDoc XRS system (Bio-Rad).

Quantitative PCR—To quantitatively determine the concentration of expressed mRNA, quantitative PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad) with a SYBR Green I PCR kit (TaKaRa Bio) as

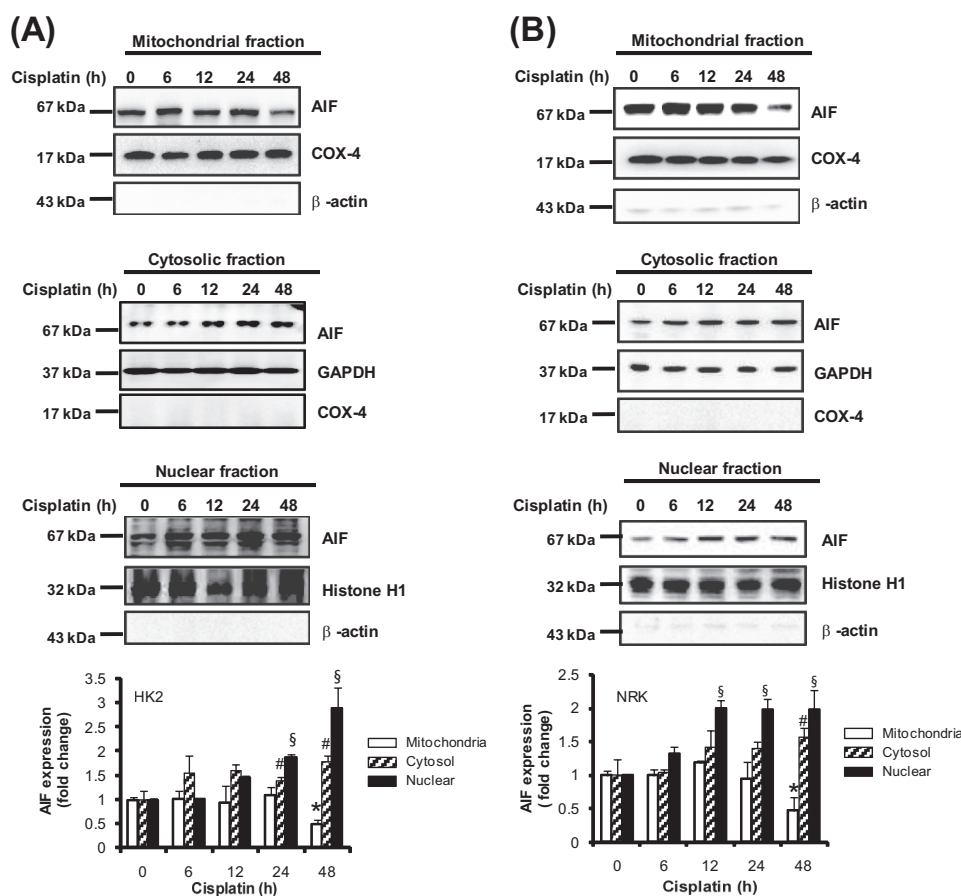


FIGURE 5. Cisplatin induces translocation of AIF from mitochondria to the cytosol and nucleus. HK-2 (A) and NRK-52E (B) cells were treated with cisplatin for 0, 6, 12, 24, or 48 h. Mitochondrial, cytosolic, and nuclear fractions were prepared and used for Western blot analyses with antibody against AIF. COX-4, GAPDH, and histone H1 were used as loading controls for the mitochondrial, cytosolic, and nuclear fractions, respectively. The intensities of the protein bands were quantified using Quantity One software and are plotted as the AIF/COX-4, AIF/GAPDH, and AIF/histone H1 intensity ratios. Data are the mean $\pm$ S.D. ($n = 3$). *, significantly different from the mitochondrial fraction of untreated cells ($p < 0.05$); #, significantly different from the cytosolic fraction of untreated cells ($p < 0.05$); \$, significantly different from the nuclear fraction of untreated cells ($p < 0.05$).

recommended by the manufacturers. Each reaction contained 10 μ l of 2 $\times$ SYBR Green Premix *Ex Taq*, 15 pmol of each oligonucleotide primer, and 2 μ l of cDNA in a final volume of 20 μ l. Quantitative PCR was performed as follows: one cycle at 95 $^{\circ}$ C for 10 min, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 10 s, annealing at 63 $^{\circ}$ C for 15 s, and extension at 72 $^{\circ}$ C for 15 s. Melting curve analysis was performed to verify amplification specificity. For data analysis, the comparative C_T (threshold cycle) method (18) was used to calculate the relative changes in gene expression.

Cross-linking of VDAC—Following treatment with cisplatin, cells were washed twice with PBS. Sulfo-EGS (Pierce) in Me₂SO was added to a final concentration of 250 μ M. After a 20-min incubation at 30 $^{\circ}$ C, the cross-linker was quenched by the addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 20 mM. Samples were then solubilized in 1% Nonidet P-40 and sonicated five times for 7 s with a 30% pulse using a Vibra-Cell sonicator (Sonics & Materials, Inc., Newtown, CT). VDAC was detected by Western blotting using anti-VDAC1 monoclonal antibody.

Western Blot Assay—Cells were solubilized with ice-cold lysis buffer (pH 7.4) containing 25 mM HEPES, 1% Triton X-100, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 1 μ g/ml leupeptin. Extracted proteins (30 μ g) were sepa-

rated by SDS-PAGE on 10% polyacrylamide gels and were electrophoretically transferred onto a PVDF membrane. Membranes were blocked in 5% (w/v) nonfat dried milk in Tris-buffered saline for 2 h at 4 $^{\circ}$ C. Membranes were then incubated overnight with several primary antibodies at a 1:500 dilution in 5% (w/v) nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were incubated with HRP-conjugated secondary antibodies. Proteins were visualized by an enhanced chemiluminescence method, and the band intensity was analyzed using a ChemiDoc XRS densitometer and quantified using the Quantity One software (Bio-Rad). Protein concentrations were estimated using the BCA method according to the supplier's recommendations, and bovine serum albumin was used as the standard. Subcellular localization of AIF was studied using a mitochondria isolation kit for cultured cells and NE-PER nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific) according to the manufacturer's protocols.

siRNA Transfection—HK-2 cells were plated 24 h prior to transfection. At 50–70% confluence, cells were transfected using DharmaFECT 2 (Thermo Fisher Scientific) with siRNA specific for human annexin A5 or with control scrambled siRNA. The target sequence of annexin A5 siRNA is GUAAUG-GGAUCUAUAAAGG.

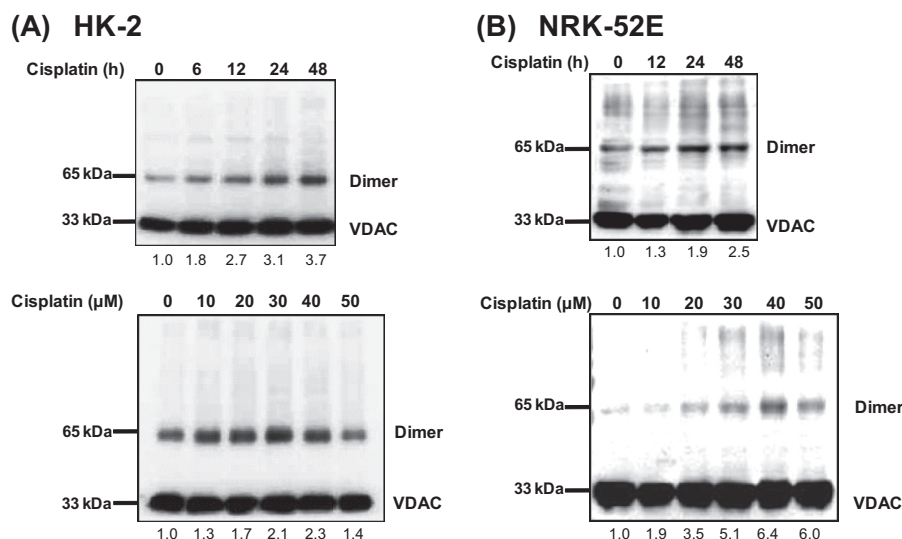


FIGURE 6. Cisplatin induces VDAC expression and dimerization. HK-2 (A) and NRK-52E (B) cells were treated with cisplatin (0, 10, 20, 30, 40, or 50 μM) for 24 h or with 20 μM cisplatin for the designated time periods. Cells were then harvested and incubated with sulfo-EGS (250 μM) for 20 min at 30 $^{\circ}\text{C}$. After proteins were resolved by 8% SDS-PAGE, VDAC proteins were measured by Western blot analysis. A 33-kDa band represents VDAC monomers, whereas a band at 65 kDa represents the VDAC dimer.

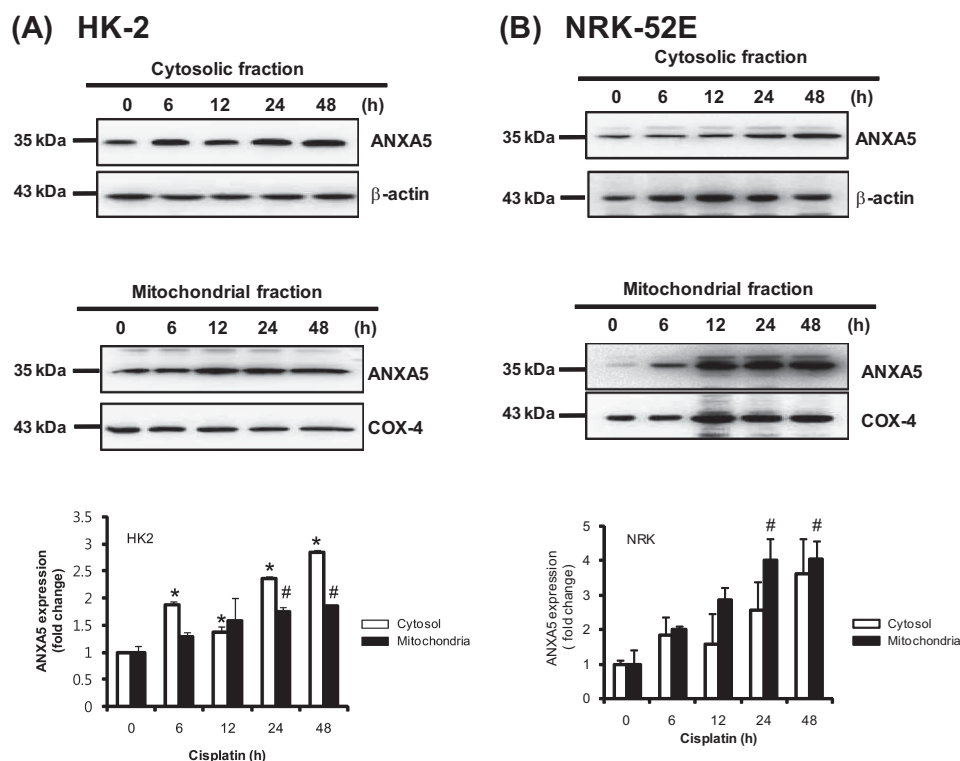


FIGURE 7. Induction of annexin A5 translocation from the cytosol to mitochondria by cisplatin. Cells were treated with cisplatin (20 μM for HK-2 (A) and 24 μM for NRK-52E (B) cells) for 0, 6, 12, 24, or 48 h. After incubation, cells were harvested, and the cytosolic and mitochondrial fractions were isolated. Extracted proteins were resolved by 10% SDS-PAGE, and Western blot analysis was conducted using anti-annexin A5 (ANXA5) antibody. β -Actin and COX-4 were used as loading controls for cytosolic and mitochondrial fractions, respectively. The intensities of the protein bands were quantified using Quantity One software and are plotted as the annexin A5/ β -actin or annexin A5/COX-4 intensity ratio. Data are the mean $\pm$ S.D. ($n = 3$). *, significantly different from the cytosolic fraction of untreated cells ($p < 0.05$); #, significantly different from the mitochondrial fraction of untreated cells ($p < 0.05$).

Immunofluorescence—Cells grown on coverslips were treated with cisplatin for 24 h in growth medium and then washed with PBS and treated with growth medium containing 100 nM Mito-Tracker[®] probes. After 1 h, the cells were fixed with 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. After washing with PBS, cells were blocked for 15 min in PBS containing 5% goat serum and 0.2% Triton X-100. The cells were

then incubated with anti-annexin A5 antibody (1:500) for 1 h, washed extensively, and stained for 1 h with either Alexa Fluor 594- or Texas Red-conjugated goat anti-rabbit IgG (1:1000). After washing, the coverslips were mounted on glass slides using Ultra-Cruz[™] mounting medium (Santa Cruz Biotechnology). Fluorescence signals were analyzed using a Zeiss LSM 510 META confocal laser scanning microscope.

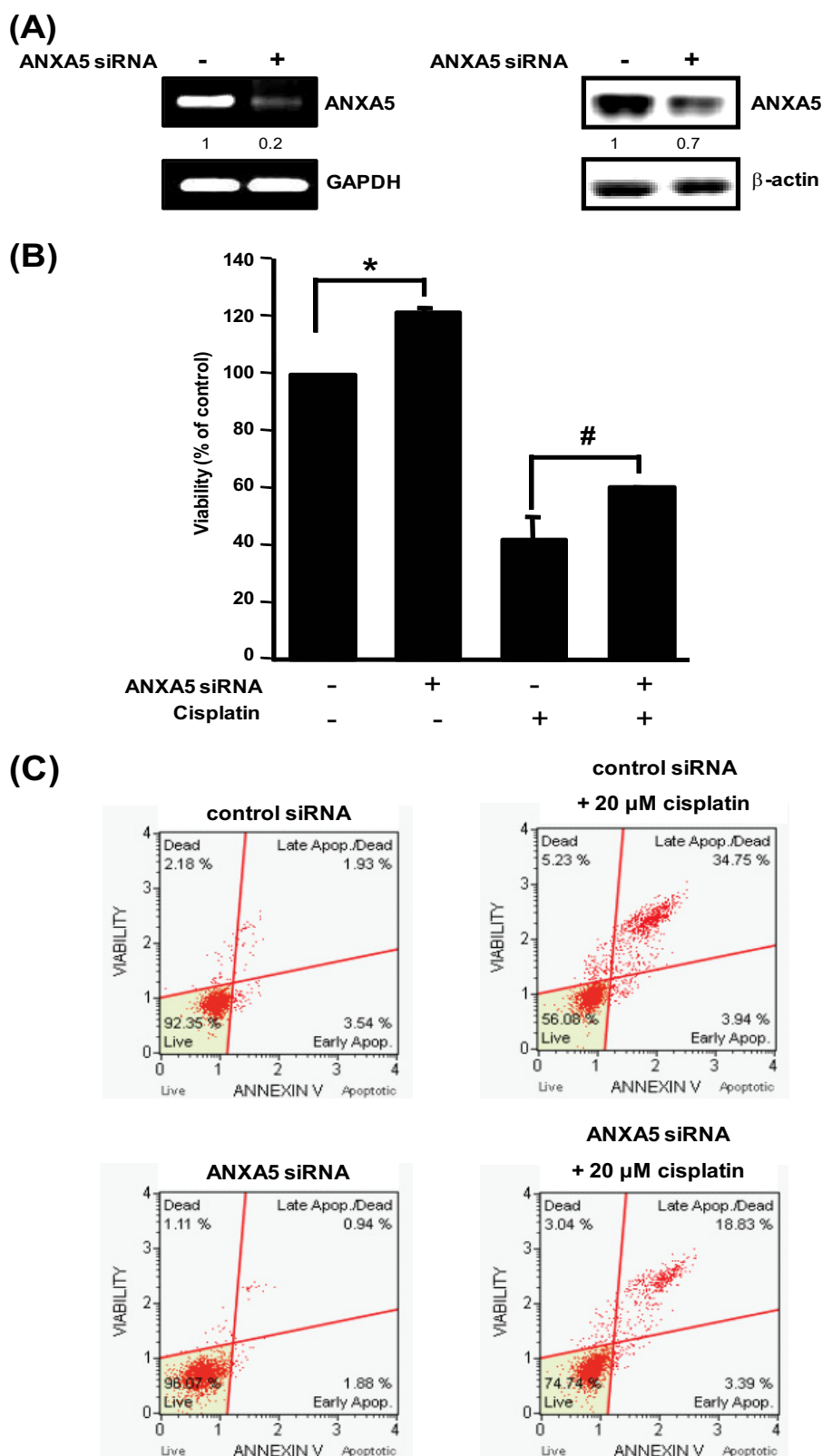


FIGURE 8. Effect of annexin A5 knockdown on cell viability in HK-2 cells following cisplatin treatment. A, HK-2 cells were treated with annexin A5 (ANXA5) siRNA (38 nM) for 48 h and then treated with cisplatin (20 μ M) for 24 h. Annexin A5 mRNA and protein were determined by RT-PCR and Western blot analysis, respectively. The numbers between the blots are the ratios of the intensity of bands after normalization to the control. B, the CCK assay was carried out to determine cell viability. The percentage of cells surviving in each group relative to the control was calculated. *, significantly different from untreated cells ($p < 0.05$); #, significantly different from cisplatin-treated cells ($p < 0.05$). C, apoptosis assay using flow cytometry. The cells were stained with MuseTM annexin V and dead cell reagent. Viability and cell density were assessed using a MuseTM cell analyzer.

Role of Annexin A5 in Cisplatin-induced Toxicity

Mitochondrial Membrane Potential—Cells (1×10^6 cells) were labeled with $2 \mu\text{M}$ JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) for 30 min at 37°C in a 5% CO_2 incubator. After washing, cells were resuspended in PBS and analyzed immediately using a BD FACScan flow cytometer (BD Biosciences). Cells were methodically gated to exclude debris, and JC-1 was monitored by log FL1 (x axis, 530 nm) versus log FL2 (y axis, 582 nm). The ratio of JC-1 aggregate (FL2, red) to monomer (FL1, green) intensity was calculated. Mitochondrial membrane potential ($\Delta\psi_m$) was also measured in cells with TMRM using a flow cytometer. Cells were loaded with 100 nM TMRM for 20 min in culture medium at 37°C . After washing with PBS, cells were resuspended in PBS and analyzed immediately using the BD FACScan flow cytometer. Red fluorescence was measured in the FL2 mode. For each sample, 10,000 events were acquired.

Statistical Analysis—Statistical analysis was performed by one-way analysis of variance and Dunnett's pairwise multiple comparison *t* test using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) when appropriate. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Annexin A5 Is Up-regulated by Cisplatin Treatment—Exposure of renal epithelial cells to various concentrations of cisplatin for 48 h in serum-free medium led to a concentration-dependent decrease in cell viability, as assessed by cell counting kit (CCK) assay. Cisplatin IC_{50} values were $10 \mu\text{M}$ for HK-2 cells (human renal epithelial cell line) and $12 \mu\text{M}$ for NRK-52E cells (rat renal epithelial cell line) (Fig. 1A). We also found that cisplatin induced activation of caspase-3, which is an indicator of apoptosis in both cell lines. Cleaved fragments (17/19 kDa) of activated caspase-3 were significantly increased by cisplatin treatment in a concentration-dependent manner (Fig. 1B). Although total caspase-3 was found at comparatively high levels, cleaved caspase-3 was not detected in the control. In subsequent experiments, a 2-fold concentration of IC_{50} was used for each cell line ($20 \mu\text{M}$ for HK-2 cells and $24 \mu\text{M}$ for NRK-52E cells). Because annexin A5 was strongly induced upon cisplatin treatment upon two-dimensional gel electrophoresis/MS analysis (data not shown), we continued to study the function of annexin A5 in cisplatin-mediated cytotoxicity. To ensure that annexin A5 expression is induced by cisplatin, the expression level of annexin A5 mRNA after cisplatin treatment was measured. Annexin A5 mRNA levels increased by up to 80% in cisplatin-treated HK-2 cells compared with control cells, whereas cisplatin-treated NRK-52E cells showed a $>10,000$ -fold increase in the expression of annexin A5 mRNA compared with control cells (Fig. 2). As expected, annexin A5 protein levels were also significantly increased with cisplatin in a concentration- and time-dependent manner in human and rat renal epithelial cells. In NRK-52E cells, annexin A5 increased by ~ 1.8 -fold compared with control cells 6 h after treatment with $24 \mu\text{M}$ cisplatin, and its expression was prolonged for 48 h (Fig. 3). Although the degree of increase was limited, cisplatin also increased annexin A5 expression in HK-2 cells, with a similar pattern in NRK-52E cells (Fig. 3). Immunofluorescence analysis using anti-annexin A5 antibody was used to confirm annexin

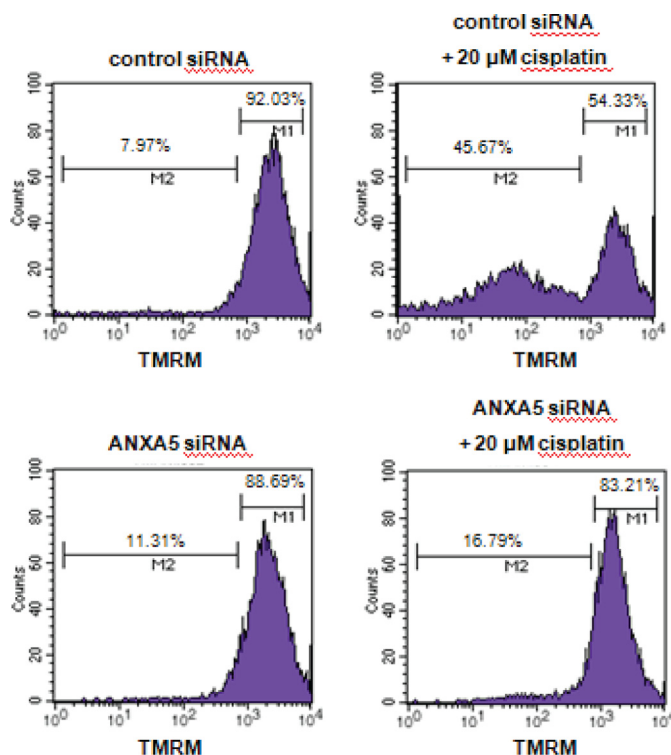


FIGURE 9. Effect of annexin A5 knockdown on mitochondrial membrane potential ($\Delta\psi_m$) in HK-2 cells following cisplatin treatment. HK-2 cells were treated with annexin A5 siRNA (38 nM) for 48 h and then treated with cisplatin ($20 \mu\text{M}$) for 24 h. Cells were labeled with 100 nM TMRM for 20 min. After washing, cells were analyzed by flow cytometry. TMRM was monitored as log FL2-H (x axis, 574 nm) versus relative cell number (y axis) in the histogram.

A5 induction by cisplatin in both cells (Fig. 4). Annexin A5 expression was shown to increase after cisplatin treatment in a concentration-dependent ($0, 10, 20$, and $30 \mu\text{M}$) and time-dependent ($0, 6, 12, 24$, and 48 h) manner.

VDAC Oligomerization May Be Critical for Mediating Cisplatin-induced Apoptosis—In an effort to investigate the mechanism of cisplatin-induced apoptosis, we isolated mitochondria and nuclei from cell lysates and detected apoptogenic factors such as AIF and VDAC after cisplatin treatment. AIF, a protein known to be released from mitochondria into the cytosol upon exposure to apoptotic stimuli (19), was verified to be increased in the cytosolic fraction after cisplatin treatment, and nuclear translocation was also found to be enhanced, which may possibly mediate DNA fragmentation (Fig. 5). To elucidate the mechanism responsible for AIF release, we measured VDAC protein by Western blot analysis (Fig. 6). Because VDAC, which is an abundant mitochondrial outer membrane protein, is reported to form a higher order complex that participates in gating the efflux of cytochrome *c* and AIF (15, 20), we suspected that AIF release would be accompanied by oligomerization of VDAC proteins. Interestingly, we found that cisplatin enhanced expression of VDAC itself (Fig. 6). Moreover, in the detection of VDAC oligomers using sulfo-EGS, we found that cisplatin induced the homodimerization of VDAC in a concentration- and time-dependent manner (Fig. 6). These results suggest that VDAC could be a mediator of cisplatin-induced apoptosis and that VDAC homodimerization induced by cisplatin could potentially determine its gating capacity to efflux AIF.

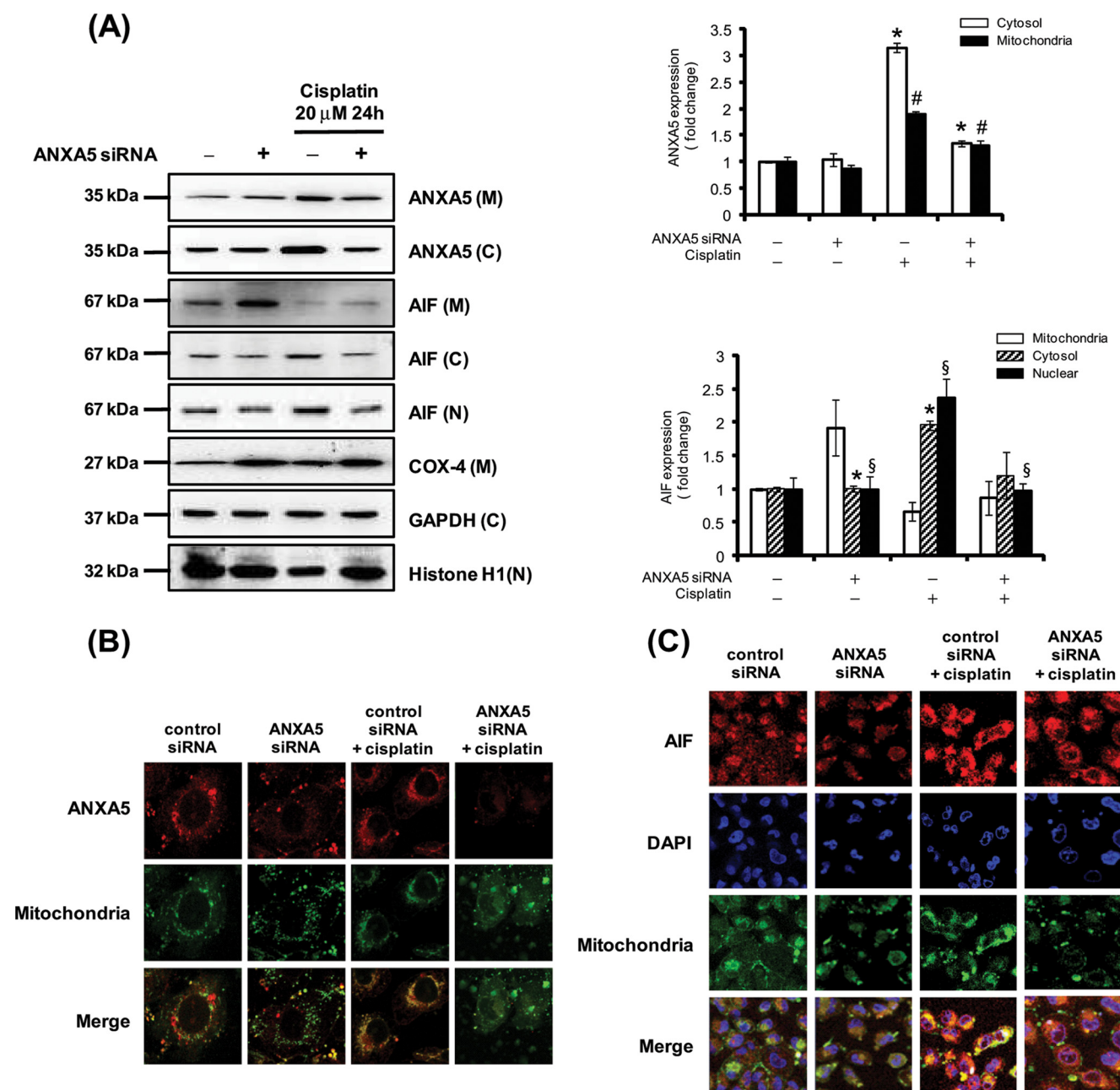


FIGURE 10. Blockade of cisplatin-mediated annexin A5 translocation by siRNA. *A*, HK-2 cells were treated with annexin A5 (ANXA5) siRNA (38 nm) for 48 h and then treated with cisplatin (20 μ M) for 24 h. After incubation, cells were harvested, and the mitochondrial fractions were isolated. Extracted proteins were resolved by 10% SDS-PAGE, and Western blot analysis was conducted using either anti-annexin A5 or anti-AIF antibody. COX-4, GAPDH, and histone H1 were used as loading controls for the mitochondrial (M), cytosolic (C), and nuclear (N) fractions, respectively. The intensities of the protein bands were quantified using Quantity One software and are plotted as the annexin A5/COX-4 and annexin A5/GAPDH (upper) and AIF/COX-4, AIF/GAPDH, and AIF/histone H1 (lower) intensity ratios. Data are the mean $\pm$ S.D. ($n = 3$). *, significantly different from the cytosolic fraction of untreated cells ($p < 0.05$); #, significantly different from the mitochondrial fraction of untreated cells ($p < 0.05$); §, significantly different from the nuclear fraction of untreated cells ($p < 0.05$). *B* and *C*, cells were cultured on microscope slides, treated with annexin A5 siRNA (38 nm) for 48 h, and then treated with cisplatin (20 μ M) for 24 h. After treatment, cells were permeabilized and subsequently stained using anti-human annexin A5 (*B*) or anti-AIF (*C*) antibody. After staining with the Texas Red-conjugated secondary antibody, fluorescence was determined by confocal microscopy.

Role of Annexin A5 in Cisplatin-induced Toxicity in Renal Cells—To determine whether cisplatin is able to induce the level of annexin A5, we determined the amount of annexin A5 in mitochondria. The data shown in Fig. 7 show that cisplatin strongly induced the translocation of annexin A5 from the cytosol into mitochondria in a time-dependent manner. Translocation of annexin A5 into mitochondria by cisplatin was significant in HK-2 and NRK-52E cells. Although annexin A5 is

thought to be related to cisplatin-induced toxicity, the function of annexin A5 as a mediator of apoptosis is not yet known. To further investigate the role of annexin A5, both annexin A5 mRNA and protein levels were reduced using specific siRNA. Treatment with annexin A5 siRNA (38 nm) for 48 h strongly suppressed annexin A5 mRNA and protein expression (Fig. 8A). Knockdown of annexin A5 by siRNA caused a significant increase in HK-2 cell viability in the presence or absence of

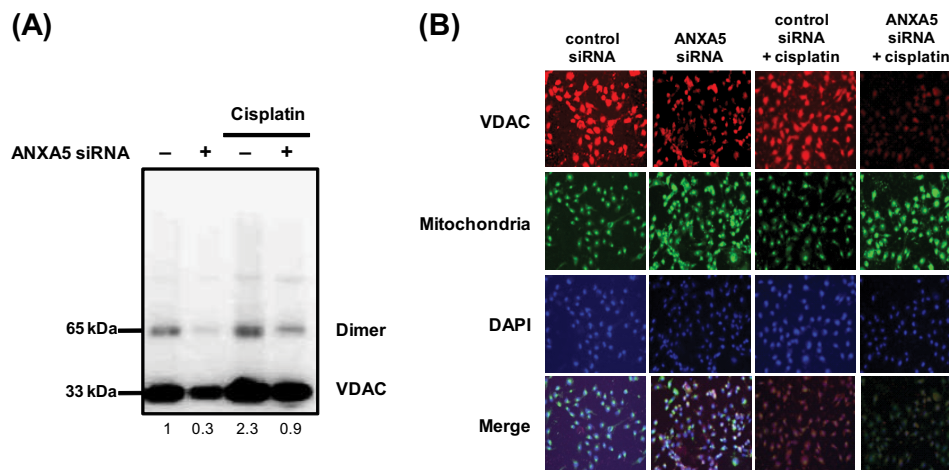


FIGURE 11. Effect of annexin A5 knockdown on VDAC levels following cisplatin treatment. A, effect on VDAC oligomerization. HK-2 cells were treated with annexin A5 (ANXA5) siRNA (38 nM) for 48 h and then treated with cisplatin (20 μ M) for 24 h. After incubation, cells were harvested and incubated with sulfo-EGS (250 μ M) for 20 min at 30 $^{\circ}$ C. Cells were then lysed, and Western blot analysis was conducted using anti-VDAC antibody. B, cells were cultured on microscope slides and treated with annexin A5 siRNA and 20 μ M cisplatin. After treatment, cells were permeabilized and subsequently stained using anti-VDAC antibody. Cells were then stained with the Texas Red-conjugated secondary antibody. DAPI was used for nuclear staining. Fluorescence was determined using a fluorescence microscope. Mitochondrial morphology was examined by MitoTracker staining.

cisplatin (Fig. 8B), indicating that annexin A5 may play an important role in mediating apoptosis. Flow cytometry analysis also showed that the decreased cell viability observed in the presence of 20 μ M cisplatin was recovered by annexin A5 siRNA treatment (Fig. 8C).

Cisplatin is known to induce mitochondrial permeability transition, leading to apoptotic changes such as cytochrome *c* release and Bax translocation (2). To identify the effect of annexin A5 on mitochondrial depolarization, HK-2 cells were treated with annexin A5 siRNA and subsequently with cisplatin and then stained with TMRM to measure mitochondrial membrane potential ($\Delta\psi_m$). TMRM allows for sensitive measurements of the changes in $\Delta\psi_m$ at the level of individual cells (21). In the case of healthy cells, TMRM enters the mitochondria, where it accumulates in an innermembrane potential-dependent manner, but in the case of apoptotic cells, TMRM no longer accumulates inside the mitochondria and become more evenly distributed throughout the cytosol (22, 23). As shown in Fig. 9, a 24-h exposure to cisplatin (20 μ M) caused a marked decrease in FL2-H fluorescence in HK-2 cells. In contrast, cells treated with annexin A5 siRNA maintained high FL2-H levels under the same conditions, indicating that annexin A5 may be involved in controlling $\Delta\psi_m$ (Fig. 9).

The translocation of annexin A5 into mitochondria by cisplatin was examined to identify whether annexin A5 plays a role in cisplatin-induced apoptosis in mitochondria. Our results indicated that cisplatin increased the level of mitochondrial annexin A5, whereas cytoplasmic annexin A5 remained unchanged as the net annexin A5 level increased (Fig. 10A). The result that annexin A5 translocates into mitochondria in cisplatin-treated cells suggests that annexin A5 might function in mitochondria as a regulator of cisplatin-induced apoptosis. To further understand the functional aspects of annexin A5 in mediating cisplatin-induced apoptosis, we examined the expression levels of VDAC and AIF following annexin A5 knockdown and cisplatin treatment. Interestingly, VDAC, which may partially mediate cisplatin-induced apoptosis, has

shown to be reduced by annexin A5 siRNA in its monomeric form and as a homodimer generated by apoptotic stimuli (Fig. 11A). AIF release into the cytosol was also decreased by annexin A5 siRNA (Fig. 10A). As shown in Fig. 10B, the data from confocal microscopy analyses also showed that cisplatin induced translocation of annexin A5 into mitochondria and that annexin A5 siRNA prevented annexin A5 expression and mitochondrial translocation. Fig. 10C also shows that cisplatin treatment induced AIF levels in the cytosol and that annexin A5 siRNA was able to block AIF release into the cytosol. VDAC expression was strongly decreased by annexin A5 siRNA (Fig. 11B). Thus, it could be inferred that annexin A5 may be necessary for maintaining VDAC protein levels after translocation into the mitochondria by cisplatin.

Effect of Other Nephrotoxicants on Annexin A5 Expression—To determine whether annexin A5 is induced by other nephrotoxicants such as CdCl₂ and bacitracin, the cytotoxicity of CdCl₂ or bacitracin was measured in NRK-52E cells. Based on a cytotoxicity assay (data not shown), cells were treated with either 30 μ M CdCl₂ or 80 μ M bacitracin. When the cells were treated with CdCl₂, annexin A5 expression was increased in a concentration- and time-dependent manner, similar to cisplatin (Fig. 12A). Bacitracin was also able to increase annexin A5 expression, although the level was relatively lower compared with CdCl₂ (Fig. 12B). From these results, it can be inferred that annexin A5 is up-regulated by a spectrum of nephrotoxicants such as cisplatin, cadmium, and bacitracin (24). The possibility that reactive oxygen species produced by nephrotoxicants like cisplatin are able to induce annexin A5 expression remains to be determined.

DISCUSSION

Annexin A5 is a 35-kDa plasma protein that has a high affinity for phosphatidylserine in the nanomolar range. The biological function of this protein is unclear, although a previous report suggests that annexin A5 may play an important role in cytotoxicity (25). Here, we identified annexin A5 as an impor-

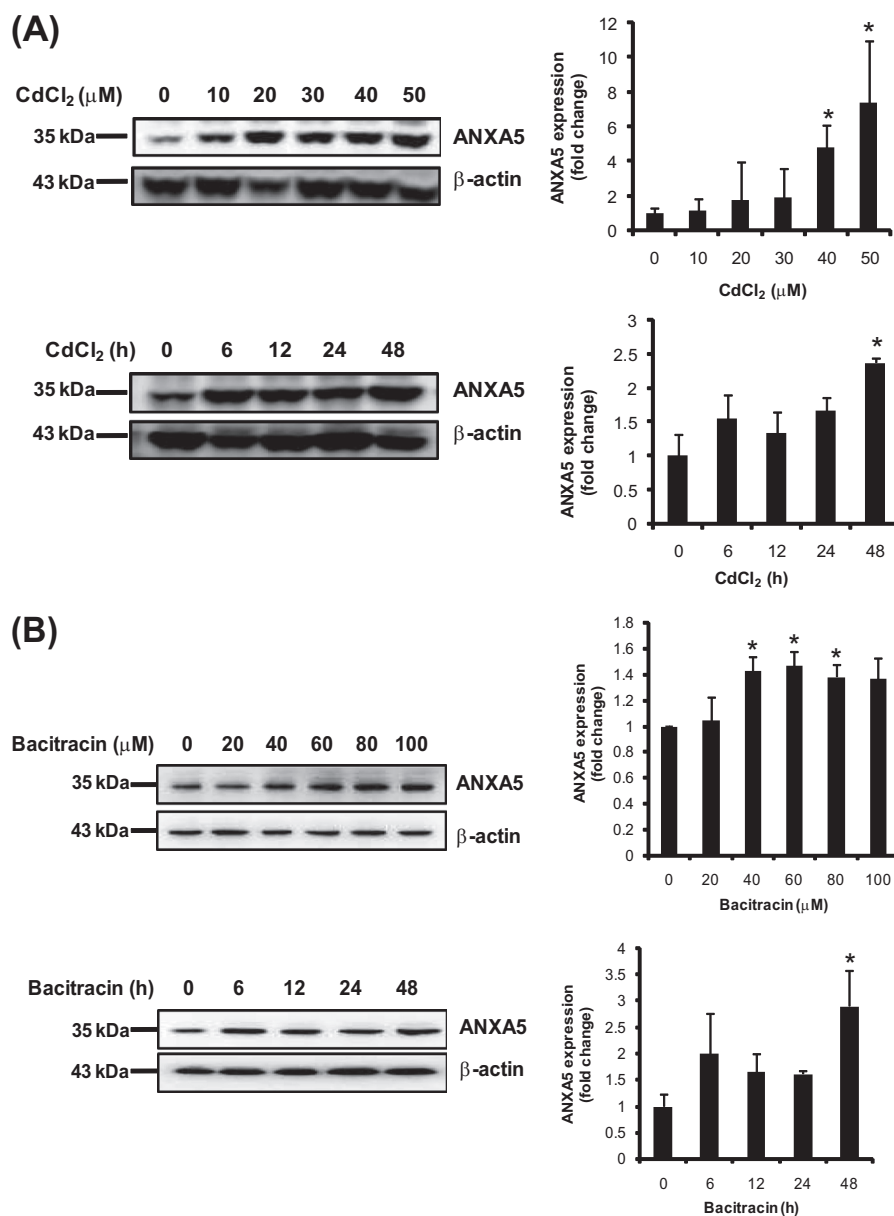


FIGURE 12. Effect of CdCl₂ and bacitracin on annexin A5 expression in rat kidney cells. A, CdCl₂ treatment. NRK-52E cells were treated with CdCl₂ (0, 10, 20, 30, 40, or 50 μM) for 24 h or with 30 μM CdCl₂ for 0, 6, 12, 24, or 48 h. After incubation, the cell lysates were isolated. Extracted proteins were resolved by 10% SDS-PAGE. Western blot analysis was conducted using anti-annexin A5 (ANXA5) antibody. β-Actin was used as a loading control. B, bacitracin treatment. Cells were treated with bacitracin (0, 20, 40, 60, 80, or 100 μM) for 24 h or with 80 μM bacitracin for 0, 6, 12, 24, or 48 h. Western blot analysis was conducted using anti-annexin A5 antibody. β-Actin was used as a loading control. The intensities of the protein bands were quantified using Quantity One software and are plotted as the annexin A5/β-actin intensity ratio. Data are the mean ± S.D. (n = 3). *, significantly different from untreated cells (p < 0.05).

tant mediator protein of cisplatin-induced toxicity and suggested the possibility of its function as a VDAC controller. Our data show that annexin A5 expression increased significantly 6 h after cisplatin treatment. Annexin A5 mRNA levels were also elevated, suggesting that cisplatin induced transcription of annexin A5 mRNA, which was further supported by the finding that protein expression increased as well. It has been reported that the annexin A5 gene promoter has consensus sequences for MTF-1 (metal regulatory transcription factor-1) (26). MTF-1 binds to a *cis*-acting DNA element, termed a metal response element, and activates the transcription of metallothionein genes and other target genes in response to potentially toxic concentrations of zinc and cadmium and to strong oxidiz-

ing agents, including hydrogen peroxide (27). The annexin A5 up-regulation in cisplatin-treated cells may be related to the activation of MTF-1. If annexin A5 up-regulation is related to cytotoxicity, then annexin A5 could possibly have a role in either mediation of apoptosis or compensation for cytotoxicity. In experiments using annexin A5 siRNA, cell viability was found to be significantly increased in siRNA-treated cells compared with control cells, suggesting that annexin A5 may have a crucial function in cell death. In previous studies, annexin A5 overexpression was found to result in increased cell death. A progressive depolarization of the mitochondrial membrane in association with an increase in human annexin A5 was observed in annexin A5-transfected HL-1 cells along with increased cyto-

chrome *c* release and caspase-3 activation (28). Pro-apoptotic characteristics were also reported in cells overexpressing annexin A5. In chondrocytes, annexin A5 overexpression increased caspase-3 activity induced by calcium phosphate crystals or TNF- α (29). These previous reports strongly support our results showing increased cell viability when annexin A5 is knocked down.

In contrast, VDAC was shown to be critical in cisplatin-induced renal cell death. As reported, under apoptotic conditions induced by toxic agents such as arsenic trioxide and staurosporine, VDAC forms a protein complex composed of two or more VDAC proteins, through which apoptogenic proteins such as cytochrome *c* and AIF are released into the cytosol (30, 31). Cisplatin has also been reported to induce VDAC oligomerization at a concentration of 40 μM in HEK-293 cells (15, 16). In both HK-2 and NRK-52E cells, the formation of the VDAC dimer as well as other homo-oligomers was increased by cisplatin in a time- and concentration-dependent manner. The level of VDAC protein itself also increased slightly, a finding that was reported in a previous publication examining the toxic mechanism of arsenic trioxide (14). These results demonstrate that cisplatin-induced apoptosis is partly dependent on VDAC oligomerization, which, in turn, enables AIF release.

To identify the role of annexin A5 in apoptosis, we knocked down annexin A5 and observed the resulting changes in mitochondrial function after cisplatin treatment. As it has been previously reported that cisplatin induces a duration-dependent transition in mitochondrial permeability (24), we measured mitochondrial membrane potential ($\Delta\psi_m$) after annexin A5 knock-down. $\Delta\psi_m$ was greatly disrupted upon cisplatin treatment, as indicated by a marked decrease in FL2 fluorescence and a slight increase in FL1 fluorescence in control siRNA-treated cells. In contrast, in cells treated with annexin A5 siRNA, FL2 was maintained at a high level under the same conditions. These results indicate that annexin A5 has a key role in cisplatin-induced disruption of $\Delta\psi_m$.

It is known that VDAC is regulated by changes in $\Delta\psi_m$, and we further observed the effect of annexin A5 silencing on VDAC and related proteins such as AIF. Surprisingly, both the VDAC dimer, which had increased upon cisplatin treatment, and VDAC protein levels were markedly decreased when annexin A5 was knocked down. AIF release into the cytosol was also shown to be decreased in annexin A5 siRNA-treated cells, confirming the effect of annexin A5 on VDAC. Our results collectively suggest that annexin A5 plays a crucial role in mediating cisplatin-induced apoptosis via action on mitochondria. This includes disruption of $\Delta\psi_m$ and positive regulation of VDAC protein levels and VDAC homodimerization, which, in turn, mediate AIF release into the cytosol, facilitating the function of the caspase-independent apoptotic pathway. Our result that cisplatin induces annexin A5 translocation to mitochondria also supports this explanation.

To explore the effects of other nephrotoxicants on annexin A5 induction, NRK-52E cells were treated with cadmium or bacitracin. Cadmium, a heavy metal known to be nephrotoxic, increased annexin A5 expression in a manner similar to that of

cisplatin. Bacitracin is also able to increase annexin A5 expression in NRK-52E cells. These results suggest the possibility that up-regulation of annexin A5 can be an indicator of chemical-mediated nephrotoxicity, although *in vivo* experiments need to be performed to verify the usefulness of annexin A5 as a biomarker of nephrotoxicity.

Annexin A5 has been used as a tool for the detection of apoptosis for decades (32). Unfortunately, the biochemical role of this protein is not yet fully understood. Our current data suggest the use of annexin A5 as a marker candidate for cisplatin-induced toxicity in renal cells and indicates its function as a mediator of the mitochondrial apoptotic pathway. How this protein regulates mitochondrial function is still not clear. Previously, annexin A5 has been regarded as an endogenous regulator of PKC (33). It has also been reported that annexin A5 is necessary for PKC δ translocation and activation (34). PKC δ translocation and subsequent activation are implicated in apoptosis induced by phorbol esters or H₂O₂ (35), and recently, it has been suggested that PKC δ inhibition is an effective strategy to reduce cisplatin-induced nephrotoxicity (36). There is a possibility that annexin A5 acts through PKC δ in mediating cisplatin-induced apoptosis. To verify this relationship, further studies are needed.

In conclusion, our results clearly show that annexin A5 plays an important role as a mediator of cisplatin-induced renal cell apoptosis by regulating mitochondrial functions, and we suggest that annexin A5 may be a crucial marker for cisplatin-induced nephrotoxicity that could be applied as a valuable target for therapy or preventive intervention.

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Role of Annexin A5 in Cisplatin-induced Toxicity in Renal Cells: MOLECULAR MECHANISM OF APOPTOSIS

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