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APELA/ELA32 Reduces Iodixanol-induced Apoptosis, Inflammatory Response and Mitochondrial and DNA Damage in Renal Tubular Epithelial Cells

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Abstract. *Background/Aim: Contrast-induced AKI (CI-AKI) is an important clinical complication of intravascular use of iodinated contrast agents. The aim of the present study was to investigate the renoprotective effect of Apela on contrast-induced acute kidney injury. Materials and Methods: Blood samples from patients exposed to iodinated contrast agent were collected to assay for Apela and creatinine levels. The effects of ELA32 (Apela 32) on iodixanol-induced apoptosis, inflammation response, mitochondrial ROS production and DNA damage were examined in NRK-52E renal tubular epithelial cells. Results: Plasma Apela levels were decreased in patients exposed to the contrast agent. Iodixanol-induced apoptosis was reduced in ELA32 treated NRK-52E cells ($p<0.05$). ELA32 treatment inhibited iodixanol-induced mitochondrial ROS generation ($p<0.01$). Iodixanol-induced inflammatory cytokines TNFa and IL-6 and inflammatory genes Nrf2 and ICAM-1 were reduced by ELA32 treatment ($p<0.01$). Reduced Apela expression in iodixanol-treated cells was partially restored by ELA32 treatment ($p<0.05$). ELA32 treatment suppressed iodixanol-induced up-regulation of DNA damage-associated gene P-ATR and p-CHK1 as well as apoptosis-associated gene C-caspase 3 ($p<0.05$). Conclusion: Administration of iodinated contrast agent reduces Apela expression. ELA32 treatment reduces iodixanol-induced apoptosis, inflammatory response and mitochondrial and DNA damage in renal tubular epithelial cells.*

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Key Words: Apela, iodinated contrast agent, acute kidney injury.

Acute kidney injury (AKI) is a clinical syndrome characterized by a deteriorating kidney function (1). Ischemic or toxic injury to renal tubular cells is the most common cause for AKI (2). With the increasing use of contrast agents in the angiography, contrast-induced acute kidney injury (CI-AKI) has been reported to be a serious clinical problem related with increased mortality and high incidence of complications (3-5).

The exact mechanisms underlying the development of CI-AKI have not yet been fully elucidated. Accumulating evidence has demonstrated that the toxic effect of iodinated contrast agent on renal tubules might lead to DNA damage and apoptosis (6, 7). Mitochondrial damage and excess of reactive oxygen species (ROS) generation are involved in CI-AKI (8). At present, hydration is the only effective and standard preventive measure for the patients with CI-AKI (9). Therefore, there is an urgent need to identify the molecular mechanism and effective prevention and therapy for CI-AKI.

Apela is an endogenous ligand for apelin receptor and is also known as ELABELA (ELA). Apela 32 (ELA32) is a 32-residue peptide that is specifically expressed in the kidney in adult mice and humans (10-12). ZHANG *et al*. have shown that the level of Apela (ELA) in diabetic nephropathy gradually decreased with an increase in albuminuria and serum creatinine (13). In the renal ischemia-reperfusion mice model, both endogenous and exogenous Apela significantly inhibited DNA damage, apoptosis and inflammatory response of renal tubular cells (14). Therefore, we examined whether ELA has also renoprotective role in CI-AKI. We examined Apela expression in patients undergoing coronary angiography and the renoprotective effect of Apela (ELA32) on the iodixanol-induced AKI using renal tubular epithelial cells.

Materials and Methods

Patient's plasma samples. Blood samples were obtained from 22 patients who undergone coronary angiography at the Department of Cardiology of Nanjing Chest Hospital (Nanjing, PR China) in March 2019. Blood samples were drawn before coronary angiography, and 12 h, 24 h and 48 h after administration of iodixanol (GE Healthcare, Shanghai, PR China). A total of 5 ml blood was collected in EDTA tube and centrifuged (1,000 × *g*) at 4˚C for 10 min to isolate the plasma. The study was approved by the Institutional Medical Ethics Committee of Nanjing Chest Hospital and written informed consent was obtained from all patients.

Cell culture. NRK-52E cells (a rat renal tubular epithelial cell line) were obtained from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. They were cultured in DMEM (Gibco, NY, USA) plus 5% fetal bovine serum (FBS, Wisent, Montreal, Canada) at 37˚C in a 5% CO_2 atmosphere.

Cell proliferation assay. Cell proliferation was measured using the CCK8 assay. NRK52E cells in the logarithmic growth phase were harvested and plated into a 96-well plate at a density of 5×10^3 cells/well. After 24 h culture, the culture medium was replaced with the serum-free medium plus 1% BSA. Different concentrations of iodixanol (IOD) or ELA32 (R&D) were added into the cultured cells and incubated for different periods of time. Then 10 μl CCK8 reagent was added to each well and incubated for 4 h. The absorbance at 450 nm was measured and the inhibition rate was calculated.

Annexin V detection of apoptotic cells. NRK-52E cells were plated into a 96-well plate and cultured at 37˚C in a 5% CO_2 atmosphere for 24 h. Then the cells were grouped and treated with iodixanol (IDO), iodixanol plus 3 nM ELA32 (IDO+ELA32-30 nM) or iodixanol plus 3 μM ELA32 (IDO+ELA32-3 μM). Untreated cells served as a control group. After 2 h incubation, the cells were digested with EDTA-free trypsin and collected in separate tubes. The cells were resuspended in 500ul Binding Buffer after PBS wash. Then, 5 μl Annexin V-FITC reagent (KGA106, KeyGEN BioTECH, Nanjing, PR China) was added and mixed with the cells. Five μl Propidium iodide (PI) dye was added into the tube and incubated at room temperature in the dark for 20 min. Apoptotic cells were detected with flow cytometry.

Mitochondrial ROS measurement. NRK-52E cells were plated into 48-well plates and cultured at 37˚C in a 5% CO_2 atmosphere for 24 h. Then the cells were grouped and treated with iodixanol or iodixanol plus ELA32 for 2 h. After PBS wash, a 500 nm working solution of mitochondrial Mito Tracker Red CMXRos probe (Thermoscientific, Shanghai, PR China) were added and incubated with the cells for 45 min at 37˚C. The cells were then fixed with 1 ml of precooled 4% paraformaldehyde at room temperature for 15 min. One ml of 0.3% Triton X100 was added and incubated at room temperature for 30 min, followed by the addition of 50 μl DAPI for 30 min. The cells were observed for the distribution of mitochondrial ROS with a fluorescence microscope at 561 nm excitation.

Enzyme-linked immunosorbent assay. The TNFa and IL-6 levels were assayed in the supernatants from NRK-52E cells treated with iodixanol or iodixanol plus ELA32 using a Rat TNF-alpha ELISA Kit (KF20001, Proteintech, Shanghai, PR China) and a Rat Interleukin 6 (IL-6) ELISA Kit (CSB-E04640r, Cusabio, Wuhan, PR China), respectively. The detection was performed according to manufacturer's instructions. Optical density (OD) of supernatant samples and standard was measured at 450 nm to determine TNFa and IL-6 concentrations.

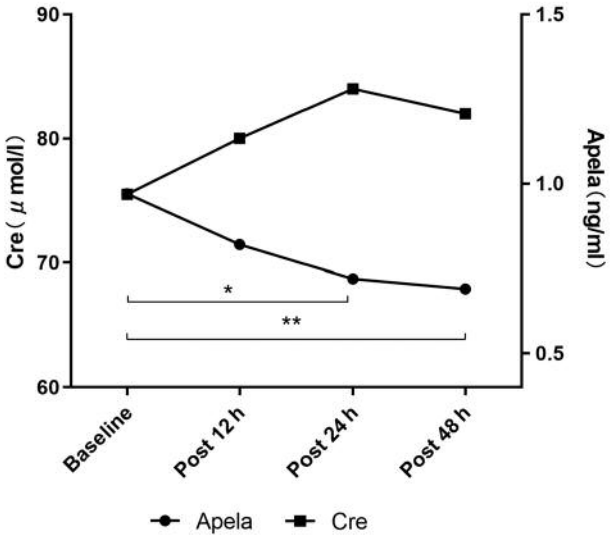

Figure 1. *Effect of iodinated contrast agent on patient's Apela expression and kidney function. Plasma Apela and creatinine (Cre) levels were examined in 22 patients exposed to iodinated contrast agent during coronary angiography. Iodixanol administration significantly reduced patient's plasma Apela levels. *p=0.016 and **p=0.019, compared to baseline.*

Immunocytofluorescense. NRK-52E cells were plated into 48-well plates and cultured at 37˚C in a 5% CO_2 atmosphere for 24 h. Then the cells were grouped and treated with iodixanol or iodixanol plus ELA32 for 2 h. After PBS wash, the cells were fixed with 1 ml of precooled 4% paraformaldehyde at room temperature (18-25˚C) for 15 min. One ml of 0.3% Triton X100 was added and incubated at room temperature (18-25˚C) for 30 min. One ml of 5% BSA blocking solution was added and incubated at room temperature (18-25˚C) for 30 min. Then Rabbit anti-human Apela antibody (1: 500) (Baode Bio) was added and incubated at 4˚C overnight. FITC conjugated sheep anti-rabbit secondary antibody (1: 1000) was added and incubated at 37˚C for 1 h, followed by the addition of 50 μl DAPI for 30 min. The cells were observed with a fluorescence microscope at 488 nm excitation.

Reverse transcription-polymer chain reaction (RT-PCR). Total Trizol (Invitrogen, Carlsbad, CA, USA) reagent was used to isolate RNA from NRK-52E cells according to the manufacturer's protocol. VazymE reverse transcription kit (R123-01, Vazyme Biotech, Nanjing, Jiangsu, PR China) was used to reversely transcribe RNA into cDNA. The following specific primers of each gene were used for PCR amplification: Nrf2 (sense, 5'-GAGAAAACGACAGAAACCTCCAT-3', and antisense, 5'-TGTATCTGGCTTCTTGCTCTTGG-3'), ICAM-1 (sense, 5'-CTGGAGAGCACAAACAGCAGAGAT-3', and antisense, 5'-ATGGGAGCTGAAAAGTTGTAGATTC-3') and GAPDH (sense, 5'-TATGTCGTGGAGTCTACTGGCGTCT-3' and antisense, 5'-AAG CAGTTGGTGGTGCAGGATG-3').

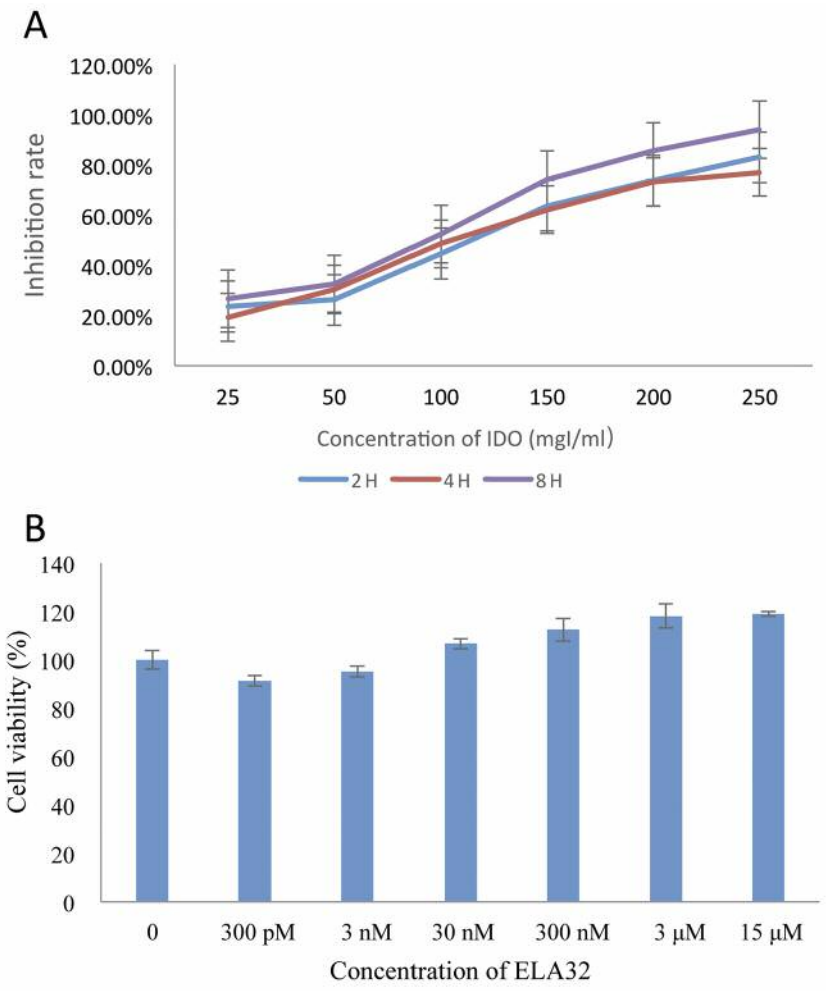

Figure 2. *Effects of iodixanol and ElA32 on NRK-52E cell proliferation. Cell proliferation was measured using the CCK8 assay. A. NRK-52E cell proliferation was significantly inhibited by iodixanol treatment. Iodixanol* IC_{50} *value for 2 h incubation time was 93.6 mg I/ml. B. ELA32 treatment did not significantly inhibit proliferation of NRK-52E cells.*

Quantitative RT-PCR was carried out by using the Step One Plus™ real-time PCR system (Applied Biosystems, Waltham, MA, USA). The relative RNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

Western blotting. NRK-52E cells were harvested and lysed in 100 μl RIPA lysis buffer. After determination of protein concentration, an equal amount of protein from each lysate from was resolved by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies to caspase 3, APELA, P-ATR, p-CHK1 and β-actin (Cell Signaling Technology, Boston, MA, USA). The membranes were then incubated with an appropriate horseradish peroxidase-linked secondary antibody. Analysis of electrochemiluminescence was performed according to the manufacturer's instructions (WBKLS0050, Millipore).

Statistical analysis. The SPSS 24.0 software was used for the statistical analysis. Data were expressed as mean values±standard deviation (mean±SD). The significance of the differences was examined by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the LSD test for post-hoc comparisons. One-way analysis of variance followed by a Dunnett's *post-hoc t*-test was performed to evaluate statistical differences for serum creatinine concentration and plasma Apela. $p<0.05$ was considered to indicate statistically significant differences.

Results

Plasma Apela levels are decreased in patients exposed to contrast agent. To investigate Apela expression in human contrast induced-acute kidney injury (CI-AKI), plasma creatinine and Apela levels were examined in the patients

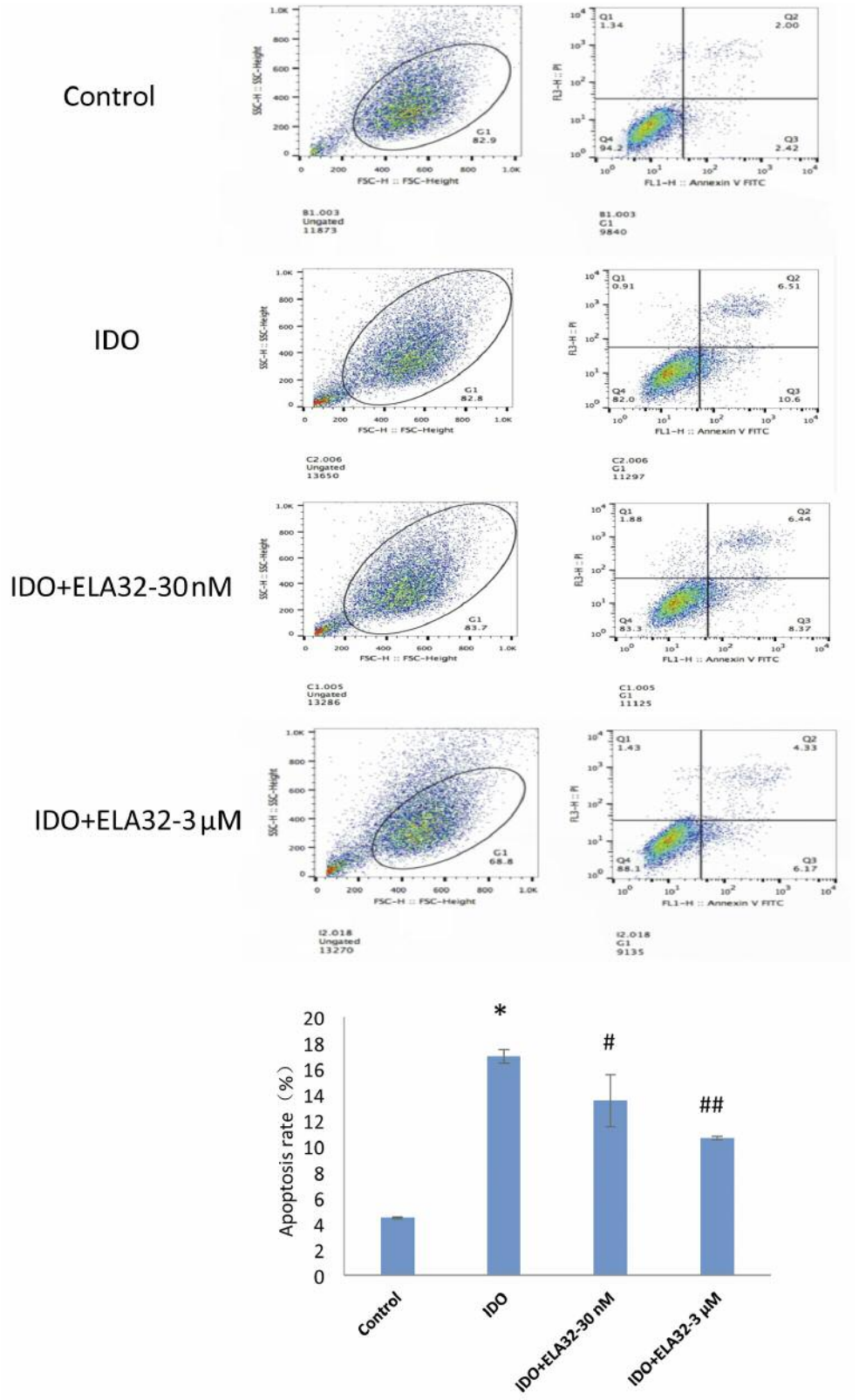

Figure 3. *ELA32 reduces iodixanol-induced apoptosis of renal tubular epithelial cells. Effect of ELA32 on iodixanol-induced apoptosis in NRK-52E cells was examined using the Annexin V-PE/7-AAD method. Iodixanol-induced cell apoptosis was significantly reduced in the NRK-52E cells treated with ELA32. *p<0.01, compared to control cells. #p<0.05, ##p<0.01, compared to iodixanol treated cells.*

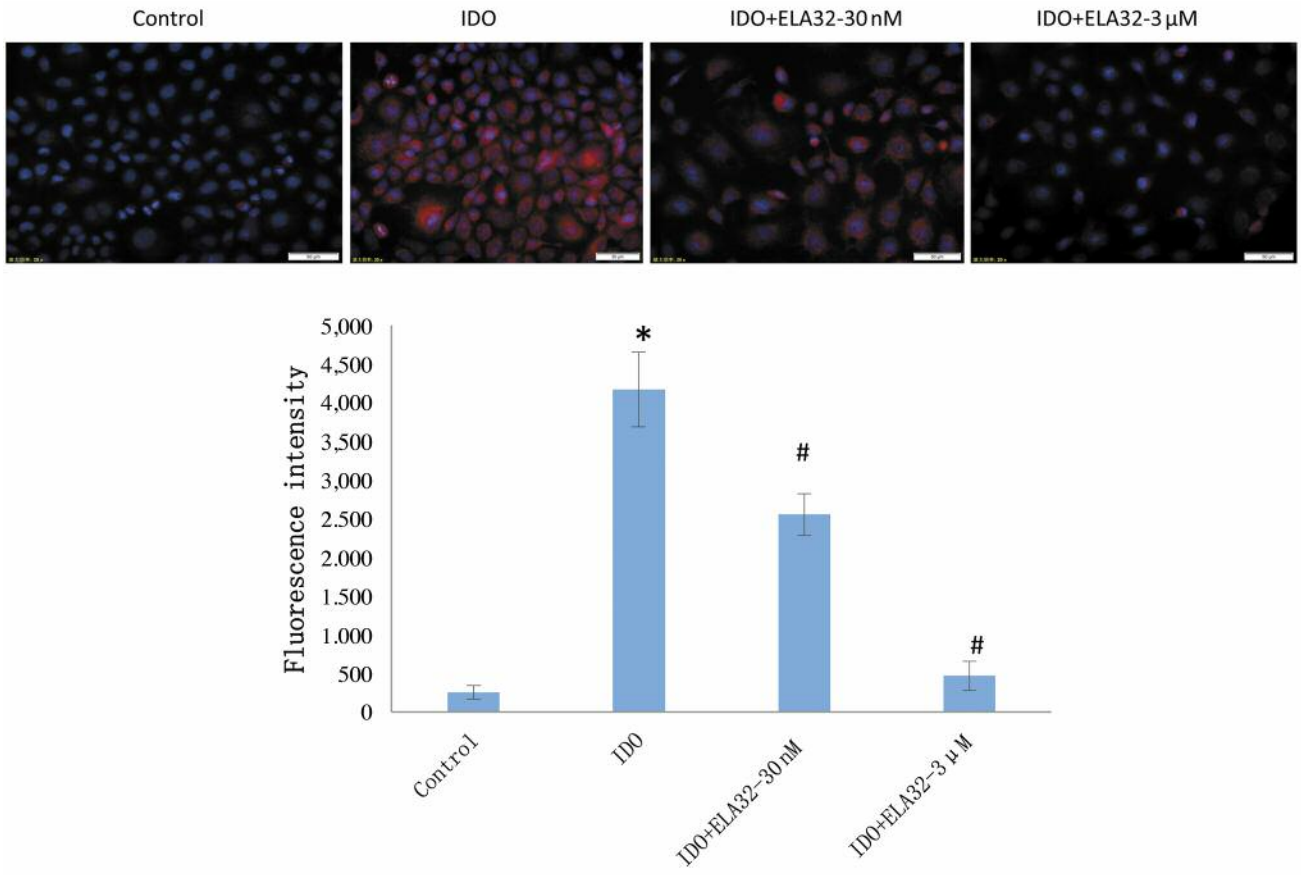

Figure 4. *ELA32 reduces iodixanol-induced mitochondrial ROS generation in renal tubular epithelial cells. A. Representative images of mitochondrial ROS generation in each group. Mitochondrial ROS were labeled by a mitochondrial probe (Mito Tracker Red CMXRos) (Red). Nuclei were stained by DAPI (blue). B. Quantification of intracellular ROS generation in each group. Iodixanol-induced cell mitochondrial ROS generation was significantly reduced in the NRK-52E cells treated with ELA32. *p<0.01, when compared to control cells. #p<0.01, compared to iodixanol treated cells.*

exposed to iodinated contrast agent during coronary angiography. As shown in Figure 1, there was no significant increase of creatinine levels in the patients with administration of iodixanol although creatinine levels were increased after the administration and peaked in 24 h (p>0.05). However, plasma Apela levels were significantly reduced in the patients at 24 h (0.72±0.44 ng/ml, p=0.016) and 48 h (0.69±0.43 ng/ml, p=0.019) after administration of iodixanol compared to baseline level (0.97±0.48 ng/ml). The result indicated that Apela is reduced in patients exposed to iodinated contrast agent although there was not a significant kidney function damage.

Iodixanol induces cytotoxicity in renal tubular epithelial cells. Cytotoxicity of iodixanol and ElA32 on NRK-52E cells was evaluated using the cell proliferation assay. As shown in Figure 2, after treatment with different concentrations (25 mg I/ml, 50 mg I/ml, 100 mg I/ml, 150 mg I/ml, 200 mg I/ml, 250 mg I/ml) of iodixanol for 2 h, 4 h or 8 h, NRK-52E cell proliferation was significantly inhibited in a concentration-dependent manner as compared to the untreated control cells (p<0.05). Iodixanol IC_{50} values at 2 h and 4 h incubation time were 93.6 and 89.32 mg I/ml, respectively, indicating that proliferation inhibition was not time-dependent. However, treatment of treated with 300 pmol/l, 3 nmol/l, 30 nmol/l, 300 nmol/l, 3 μmol/l, or 15 μmol/l ELA32 for 2 h did not significantly inhibit proliferation compared to the untreated control cells (p>0.05). Based on these results, half of the iodixanol IC_{50} value (46 mg I/ml) and two doses of ELA32 (3 μM and 30 μM) were selected for the subsequent experiments.

ELA32 reduces iodixanol-induced apoptosis in renal tubular epithelial cells. The renoprotective effect of ELA32 on iodixanol-induced apoptosis in NRK-52E cells was examined using the Annexin V-PE/7-AAD method. As shown in Figure 3, the apoptosis rate was significantly increased in the NRK-52E cells treated with iodixanol compared to the untreated control cells (p<0.01). Iodixanol-induced cell apoptosis was significantly reduced in the NRK-

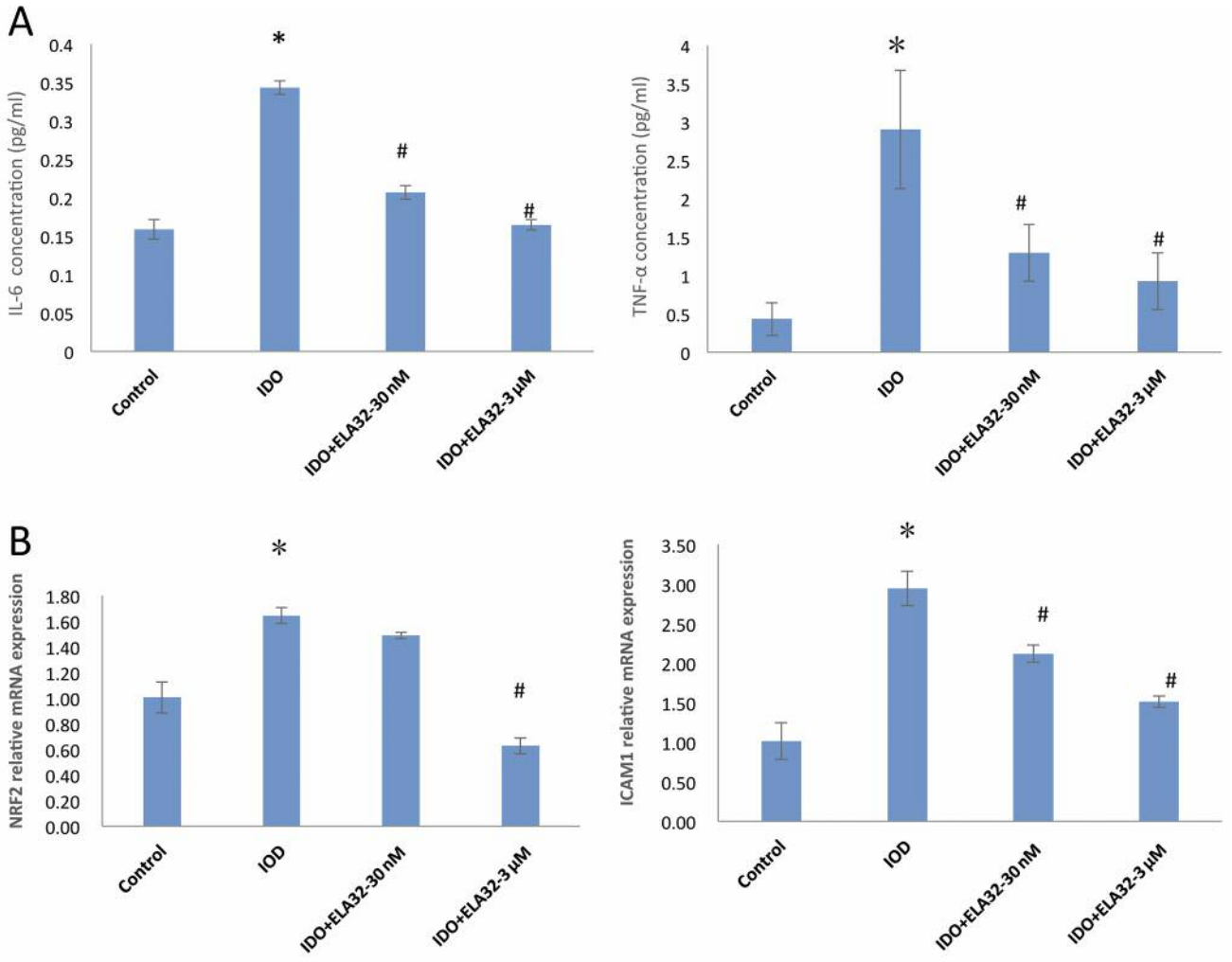

Figure 5. *ELA32 inhibits iodixanol-induced inflammation in renal tubular epithelial cells. The effect of ELA32 on iodixanol-induced inflammation in NRK-52E cells was assessed by examining the production of TNFa and IL-6 and the expression of inflammatory genes Nrf2 and ICAM-1. A. ELA32 treatment significantly reduced iodixanol-induced TNFa and IL-6 levels. B. ELA32 treatment significantly reduced iodixanol-induced Nrf2 and ICAM-1 expression. *$p<0.01$, compared to control cells. #$p<0.01$, compared to iodixanol-treated cells.*

52E cells treated with ELA32 in a dose-dependent manner ($p<0.05$).

ELA32 reduces iodixanol-induced mitochondrial ROS generation in renal tubular epithelial cells. As shown in Figure 4, NRK-52E cells treated with iodixanol showed increased signals of fluorescent MitoSOX compared to the untreated control cells ($p<0.01$), indicating that iodixanol exposure induced mitochondrial damage and ROS generation. However, ELA32 treatment significantly reduced the signals of fluorescent MitoSOX in NRK-52E cells compared to iodixanol treated cells in a dose dependent manner ($p<0.01$), suggesting a renoprotective effect of ELA32 on iodixanol-induced mitochondrial injury.

ELA32 inhibits iodixanol-induced inflammation in renal tubular epithelial cells. The effect of ELA32 on iodixanol-induced inflammation in NRK-52E cells was assessed by examining the levels of TNFa and IL-6. As shown in Figure 5A, production of TNFa and IL-6 was significantly increased in NRK-52E cells treated with iodixanol compared to the untreated control cells ($p<0.01$). ELA32 treatment resulted in significantly reduced production of TNFa and IL-6 in NRK-52E cells compared to iodixanol treated cells ($p<0.01$).

Expression of inflammatory genes *Nrf2* and *ICAM-1* was also analysed for the effect of ELA32 on iodixanol-induced inflammation in NRK-52E cells. As shown in Figure 5B, expression of *Nrf2* and *ICAM-1* was significantly increased in the NRK-52E cells treated with iodixanol compared to the

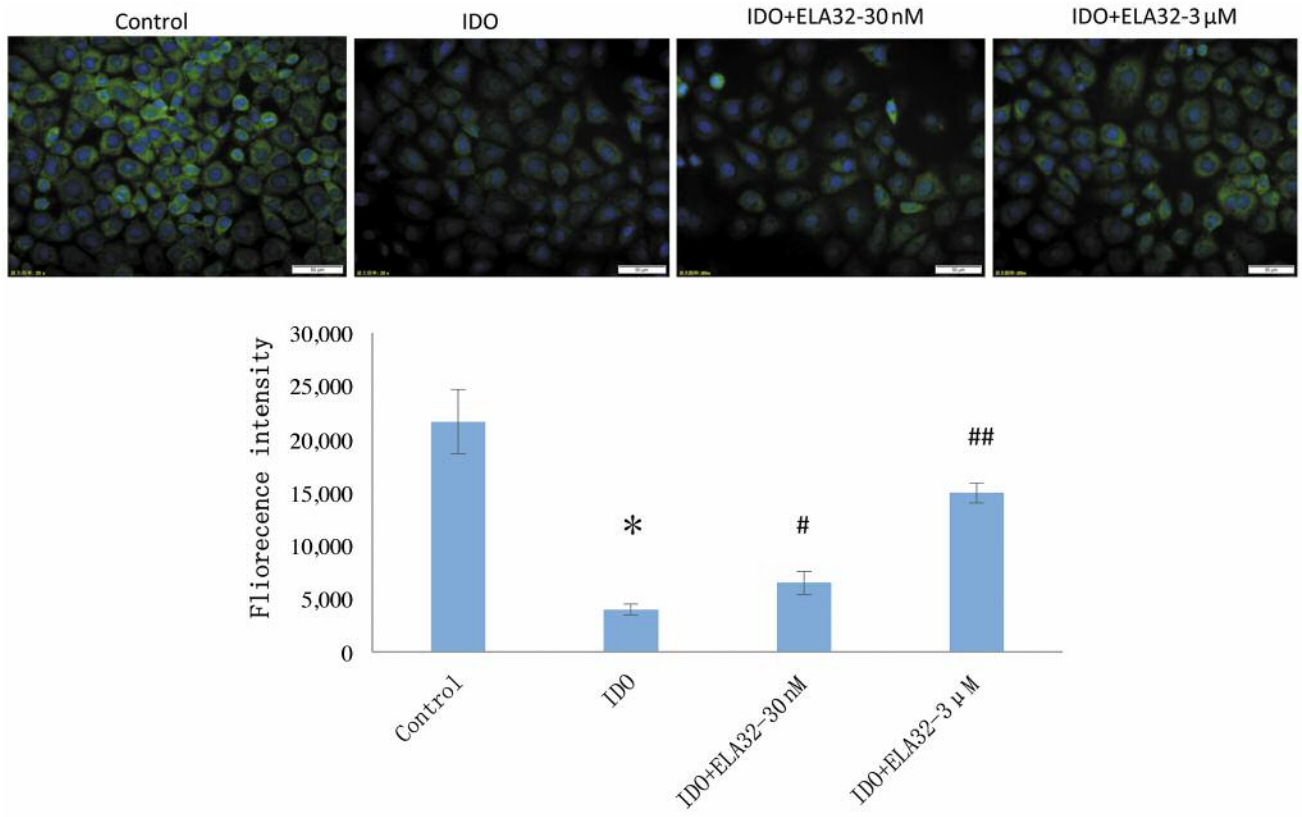

Figure 6. *ELA32 partially restores the levels of Apela in iodixanol-treated renal tubular epithelial cells. A. Representative images of Apela expression in each group. Apela expression was detected by immunocytofluorescense (green). Nuclei were stained by DAPI (blue). B. Quantification of Apela expression in each group. Iodixanol induced a reduction in Apela expression, which was significantly restored by ELA32 treatment. *p<0.01, when compared to control cells. #p<0.05, ##p<0.01, compared to iodixanol treated cells.*

untreated control cells ($p<0.01$). ELA32 treatment significantly reduced expression of *Nrf2* and *ICAM-1* compared to iodixanol treated cells ($p<0.01$). These results suggested that ELA32 inhibits iodixanol-induced inflammation in NRK-52E cells

ELA32 partially restores the levels of Apela in iodixanol-treated renal tubular epithelial cells. Apela expression was examined in the NRK-52E cells treated with iodixanol and ELA32. Apela expression was significantly inhibited in cells treated with iodixanol compared to the untreated control cells ($p<0.01$). However, the levels of Apela expression were partially but significantly restored following co-treatment with ELA32 ($p<0.05$) (Figure 6).

ELA32 partially restores expression of DNA damage and apoptosis-associated genes in renal tubular epithelial cells co-treated with iodixanol. As shown in Figure 7, the expression of DNA damage-associated genes p-ATR and p-CHK1 as well as apoptosis-associated gene C-caspase 3 were significantly increased in the NRK-52E cells treated with iodixanol compared to the untreated control cells ($p<0.05$), whereas ELA32 treatment significantly suppressed *iodixanol-induced* gene up-regulation ($p<0.05$), suggesting that ELA32 protects renal tubular epithelial cells against iodixanol-induced DNA damage and apoptosis.

Discussion

Contrast-induced AKI (CI-AKI) is an important clinical complication of the intravascular use of iodinated contrast agents. However, effective prevention and therapy for CI-AKI are still lacking. Apela has been found to be involved in renal ischemia-reperfusion (I/R) injury and have renoprotective effect on renal I/R injury (14). In the present study, we report for the first time that Apela levels decreased in the patients following administration of iodixanol during coronary angiography, suggesting that Apela also plays a critical role in CI-AKI.

Renal I/R injury triggers inflammatory responses in the tubulointerstitium, leading to kidney damage and development of fibrosis (15, 16). The crucial roles of inflammatory genes

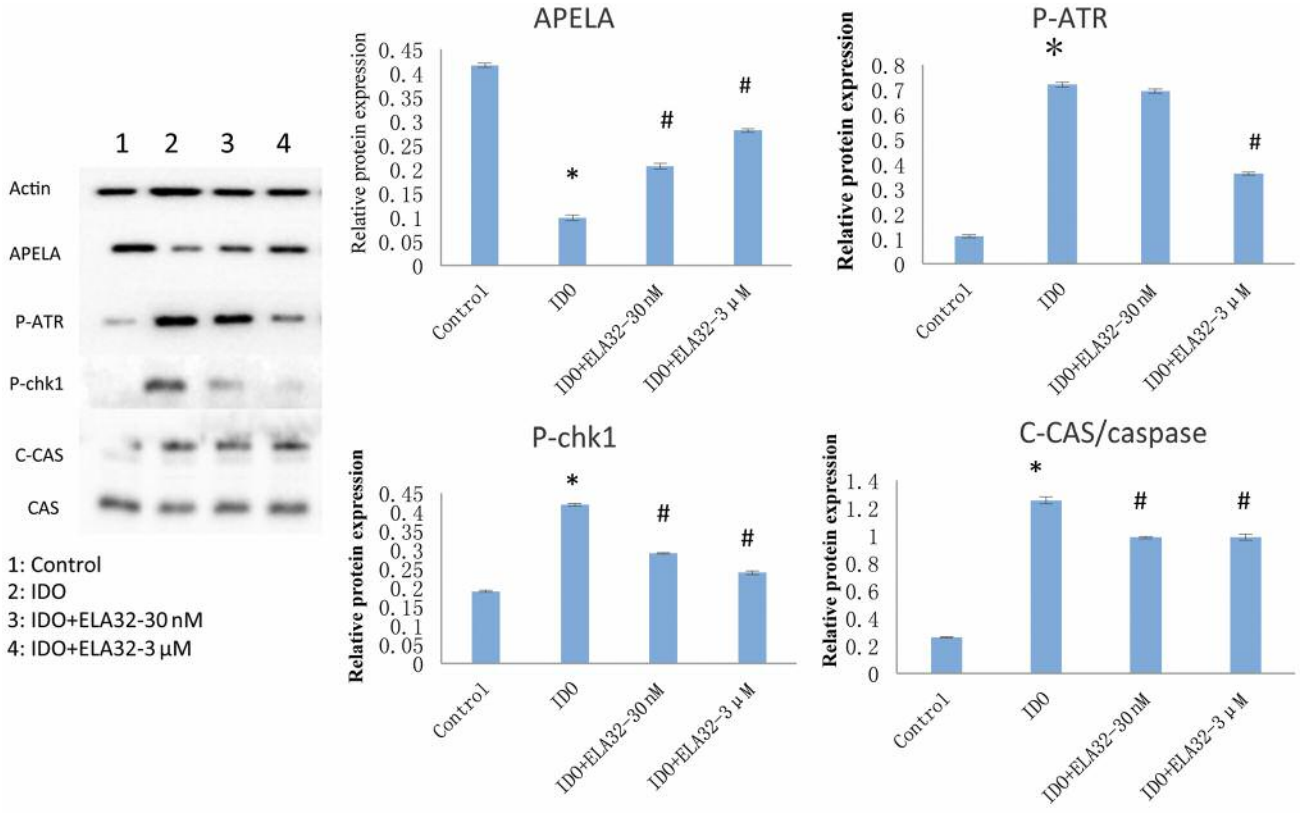

Figure 7. *ELA32 reduces iodixanol-induced DNA damage and apoptosis-associated genes in renal tubular epithelial cells. Expression of Apela, p-ATR, p-chkl, C-caspase and caspase proteins were analyzed by western blotting. ELA32 treatment significantly reduced iodixanol-induced p-ATR, p-chkl and C-caspase expression. Iodixanol induced an increase in protein expression, was significantly restored in NRK-52E cells treated with ELA32. *p<0.01, when compared to control cells. #p<0.01, when compared to iodixanol treated cells.*

in renal I/R injury have been suggested (16, 17). Apela has been found to reduce renal inflammation and fibrosis markers and lead to improvement in renal pathology and kidney dysfunction in an animal model of Type 1 diabetes mellitus (13). Here, we found that iodixanol induced cytotoxicity and apoptosis in renal tubular epithelial cells. ELA32 not only significantly suppressed production of inflammatory molecules TNFa and IL-6, but also inhibited expression of inflammatory genes Nrf2 and ICAM-1 in iodixanol treated cells, indicating that it protects renal cells against iodixanol induced inflammatory response.

It has been reported that DNA damage, such as accumulation of apoptosis-associated DNA cleavage in renal tubular cells, occurs in the I/R-injured kidney (18, 19). Apela32 or Apela11 (the 11-residue furin-cleaved fragment) significantly suppressed I/R injury-induced renal fibrosis, inflammation, apoptosis, and the DNA damage response and markedly reduced the renal tubular lesions and renal dysfunction (14). Our results demonstrated that ELA32 reduces iodixanol-induced DNA damage and apoptosis-associated genes in renal tubular epithelial cells, suggesting its renoprotective effect on iodixanol-induced DNA damage and apoptosis.

Excess generation of mitochondrial ROS has been found in AKI and has been considered to play an important role in the pathogenesis of AKI (20, 21). Mitochondrial damage is recognized as a leading cause to many acute renal diseases (22). Mitochondrial damage and ROS generation in CI-AKI have been reported (8). However, the effect of Apela on mitochondrial ROS generation remained unclear. Our study demonstrated that *ELA32* reduces iodixanol-induced mitochondrial damage and ROS generation in renal tubular epithelial cells.

In conclusion, administration of iodinated contrast agent reduces Apela expression. ELA32 treatment reduces iodixanol-induced apoptosis, mitochondrial ROS generation, inflammatory response and DNA damage in renal tubular epithelial cells.

Conflicts of Interest

None of the Authors have any conflicts of interest with regard to this study.

Authors' Contributions

Yimin Li and Qinghua Wu designed the study and wrote draft manuscript; Jin Huang, Shenghu He, Zhiping Lu, Jing Zhang and

Xiangyu Li performed experiments and analyzed the data; Zhijian Yang and Robert M. Hoffman revised the manuscript.

Acknowledgements

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