Vitamin E (α-tocopherol) ameliorates aristolochic acid-induced renal tubular epithelial cell death by attenuating oxidative stress and caspase-3 activation

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Abstract. Aristolochic acid (AA) is a component identified in traditional Chinese remedies for the treatment of arthritic pain, coughs and gastrointestinal symptoms. However, previous studies have indicated that AA can induce oxidative stress in renal cells leading to nephropathy. α-tocopherol exists in numerous types of food, such as nuts, and belongs to the vitamin E isoform family. It possesses antioxidant activities and has been used previously for clinical applications. Therefore, the aim of the present study was to determine whether α-tocopherol could reduce AA-induced oxidative stress and renal cell cytotoxicity, determined by cell survival rate, reactive oxygen species detection and apoptotic features. The results indicated that AA markedly induced H_2O_2 levels and caspase-3 activity in renal tubular epithelial cells. Notably, the presence of α-tocopherol inhibited AA-induced H_2O_2 and caspase-3 activity. The present study demonstrated that antioxidant mechanisms of α-tocopherol may be involved in the increased survival rates from AA-induced cell injury.

Introduction

Aristolochic acid (AA) is a component present in Chinese herbs (for example *Asarum* and *Aristolochia*) from remedies for the treatment of arthritis pain, coughs and gastrointestinal symptoms (1-4). Previous studies have indicated that AA can lead to renal injury (5,6) and this finding has led to further studies (7,8). Previous studies have indicated that renal damage from renal cell death and renal fibrosis is associated with AA treatment (9,10).

AA-induced oxidative stress may serve an important role in the development of renal injury (11-13). Previous studies have demonstrated that oxidative stress causes lipid peroxidation, DNA damage and protein peroxidation, and results in cell damage (14-16). O_2^- and H_2O_2 are key reactive oxygen species (ROS) identified in cells (17,18). Normally, O_2^- and H_2O_2 are produced in the mitochondria via electron transport chain (19,20) and these ROS are removed by cellular superoxide dismutase (SOD), glutathione peroxidase (Gpx) and catalase (CAT) (21-23). However, various toxins also induce O_2^- and H_2O_2 production (24-26). The excessive O_2^- and H_2O_2 lead to cell injury (27,28) and it has additionally been reported that AA-induced H_2O_2 leads to renal damage (29).

Various studies have demonstrated that oxidative stress can induce cell apoptosis or cell necrosis (30-32), and consequently AA-induced oxidative stress can cause apoptosis or necrosis of renal cells (29,33-35). Concerning apoptosis, caspase-dependent and caspase-independent pathways have been reported previously (36,37). Although certain mechanisms of AA-induced cell death remain unclear, the caspase activation may be associated with AA-induced apoptosis (38,39). Previous studies indicated that AA can activate caspase-9 and caspase-3 leading to cell apoptosis (40-42).

The isoforms of vitamin E consist of α-tocopherol, β-tocopherol, δ-tocotrienol and γ-tocotrienol (43). Among them, α-tocopherol possesses anti-oxidative activities and has been used in a clinical setting (44,45). In addition, previous studies have suggested that α-tocopherol can inhibit renal fibrosis (46,47). Due to the fact that AA-induced renal injury was associated with oxidative damage and fibrotic renal injury (9,11-13), the effects of α-tocopherol on AA-induced renal cell cytotoxicity were studied. The results of the present study demonstrated that α-tocopherol can inhibit the H_2O_2

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level and caspase-3 activities to attenuate renal tubular epithelial cell death under AA treatment.

Materials and methods

Materials. The MTT assay kit was obtained from Bio Basic Canada, Inc. (Markham, OT, Canada). Vitamin E (α-tocopherol), luminol, lucigenin, tubulin polyclonal antibody and Hoechst 33342 were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Caspase-3 and cleaved caspase-3 polyclonal antibodies were obtained from Cell Signaling Technology, Inc. (9662; 1:1,000; Danvers, MA, USA). Fetal bovine serum, DMEM, non-essential amino acid, L-glutamine, and penicillin/streptomycin were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell culture. Rat renal tubular epithelial cells (NRK-52E) were obtained from the Bioresource Collection and Research Center (Shin Chu, Taiwan). NRK-52E cells were cultured with DMEM medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 0.1 mM non-essential amino acids. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C.

ROS detection. H_2O_2 and O_2^- levels were measured by using the lucigenin-amplified chemiluminescence method (48,49). The culture supernatant (200 μl) were added with 0.2 mmol/l of luminol solution (100 μl) and measured subsequently by using a chemiluminescence analyzing system (CLA-FSI; Tohoko Electronic Industrial Co., Ltd., Sendai, Japan) for the determination of H_2O_2 levels. The samples (200 μl) were treated with 0.1 mmol/l lucigenin solution (200 μl) and then O_2^- levels were measured using the CLA-FSI chemiluminescence analyzing system.

Cell survival rates determination. The cell survival rates were determined using the MTT assay kit according to the manufacturer's instructions. In brief, NRK-52E cells were cultured into 96-well plates at a density of $8x10^3$ cells/well and incubated for 24 h in 100 μl DMEM medium. The suitable concentration and optimum exposure time of AAI were determined as 5, 10, 20 and 100 μM at 6 h time intervals. Cells were treated with MTT assay kit for 3 h at 37˚C and were measured at 570 nm absorbance using a Multiskan™ FC microplate photometer (Molecular Devices, Inc., Sunnyvale, CA, USA). The cell survival rate was calculated as the following formula: Optical density (OD) 570 experimental group/OD 570 control group x100%.

Observation of apoptotic features. Apoptotic features containing DNA fragmentation and nuclear condensation were observed by using Hoechst 33342 (23491-52-3; Sigma-Aldrich; Merck KGaA) nuclear staining (49,50). Control and experimental cells were treated with Hoechst 33342 (10 μg/ml) at 37˚C for 10 min. DNA fragmentation and nuclear condensation were observed under an Olympus DP71 fluorescence microscope (excitation, 352 nm; emission, 450 nm; Olympus Corporation, Tokyo, Japan).

Western blotting. Cells were treated with radioimmunoprecipitation assay buffer (20-188; EMD Millipore, Billerica, MA, USA). Following 10 min centrifugation (16,000 x g) at 4˚C, proteins were obtained from the supernatant layer and the concentration was determined by using the protein assay kit (23200; Thermo Fischer Scientific, Inc.). Equal quantities of samples were separated on a 13.3% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and then transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with 5% milk for 2 h at room temperature. Next, the membranes were washed with phosphate-buffered saline (PBS) then incubated with the primary antibodies for 4 h. Following that, membranes were washed with PBS and treated with anti-rabbit-horseradish peroxidase secondary antibodies (NA934; 1:1,000 Amersham; GE Healthcare Life Sciences, Chalfont, UK) for 1 h at room temperature. Finally, proteins were observed by using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Inc., Waltham, MA, USA).

Statistical analysis. Student's t-test and two-way analysis of variance were utilized for the analysis of the data using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean ± standard error. $P<0.05$ was considered statistically significant different between values.

Results

Increases of H_2O_2 and O_2^- levels by different concentrations of AA treatment. Previous studies have demonstrated that AA induced ROS generation in renal tubular cells (13,51). H_2O_2 and O_2^-, two major types of ROS, were measured in AA-treated renal tubular cells. Experimental results indicated that H_2O_2 levels were increased dose-dependently in the AA-treated cells (Fig. 1A). Compared with H_2O_2 levels, O_2^- levels were increased only at the 100 μM AA treatment however not at 20-50 μM AA concentrations (Fig. 1B). The data suggested low-dose AA (5-20 μM) can induce increases in H_2O_2 levels, but not O_2^- levels. Additionally, high-dose AA (100 μM) can induce increases of H_2O_2 and O_2^- levels.

AA decreased cell survival rates in dose- and time- dependent manners. In order to determine the cytotoxic effects on AA-treated renal tubular cells, various concentrations (5-100 μM) of AA were studied. As presented in Fig. 2, the cell survival rates were below 50% at 100 μM AA (6 h), 20 μM AA (12 h), 10 μM AA (24 h) and 5 μM AA (48 h) treatment. These results demonstrated AA-induced cell cytotoxicity was dose- and time-dependent.

Apoptotic characteristics in AA-treated renal tubular cells. Cell death can be described as apoptosis or necrosis (52,53). Apoptotic cells can be removed by macrophages in order to prevent serious inflammatory responses (54,55), and previous studies have indicated that nuclear condensation and DNA fragmentation are key apoptotic characteristics (49,56). In the present study, the cell nuclei were observed by Hoechst 33342 staining (49,50). As presented in Fig. 3, compared with control cell, the nuclear condensation and DNA fragmentation were identified in AA-treated renal tubular cells. The results indicated that AA-induced cell death was associated with the apoptotic death pathway.

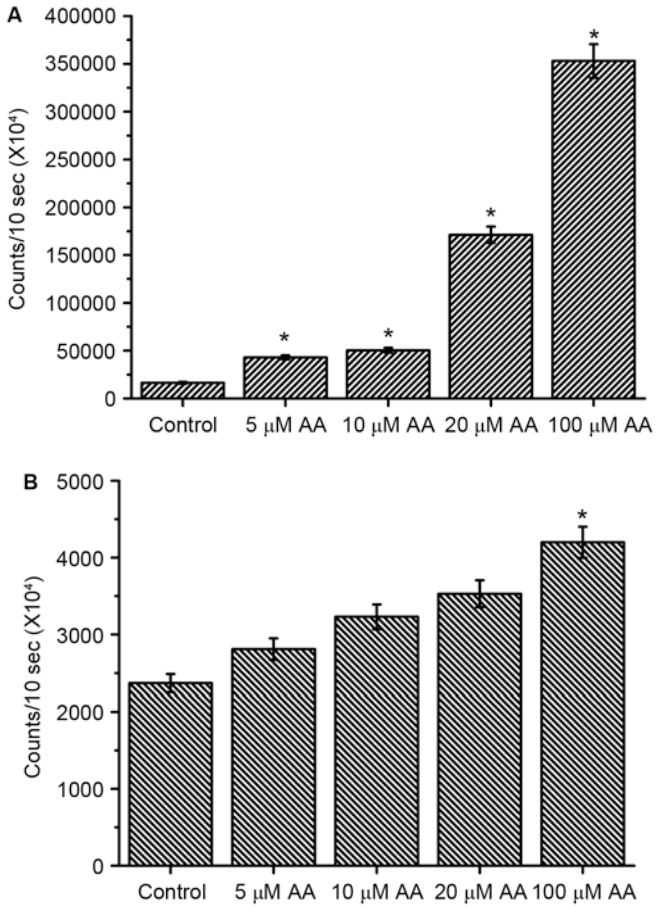

Figure 1. H_2O_2 and O_2^- levels. (A) H_2O_2 levels and (B) O_2^- levels were measured in control group (0 μM AA) and AA-treated groups (5-100 μM AA). The levels were measured following 2 h treatment. Data from four independent experiments were presented as the mean ± standard deviation. *P<0.05, vs. the control group (0 μM AA). AA, aristolochic acid.

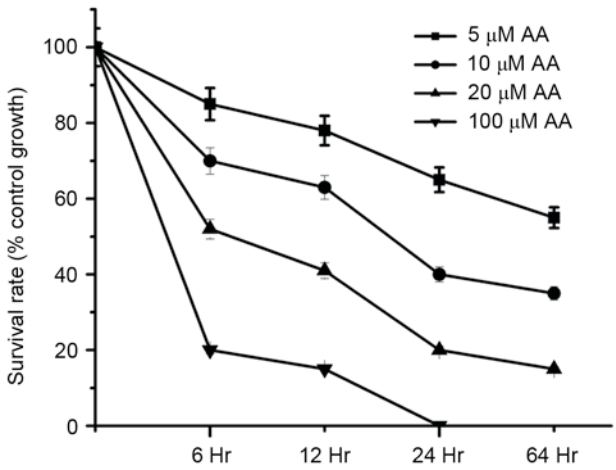

Figure 2. Cell survival rates. The renal tubular cells were treated with 5-100 μM AA for 48 h. Data from four independent experiments are presented as the mean ± standard deviation. AA, aristolochic acid.

α-tocopherol attenuated H_2O_2 levels and increased cell survival in AA-treated cells. The antioxidant stress activities of vitamin E (α-tocopherol) has been demonstrated in clinical cases (44,45). Due to the fact that AA elevated H_2O_2 levels (Fig. 1A), it was investigated whether α-tocopherol could inhibit H_2O_2 in AA-treated cells with various concentrations of α-tocopherol (5, 10, 20 and 100 μM). The data indicated that α-tocopherol attenuated AA-induced H_2O_2 levels (Fig. 4). Notably, it was observed that 10 μM α-tocopherol appeared to have a more marked effect on AA-induced H_2O_2 compared with other concentrations. Subsequently, the effects of α-tocopherol on AA-induced renal cell death were investigated. As presented in Fig. 5, the cell survival rates were increased in AA-treated renal tubular cells undergoing 10 or 20 μM α-tocopherol treatment. These results first demonstrated that α-tocopherol attenuated AA-induced H_2O_2 levels and increased cell survival of AA-treated cells.

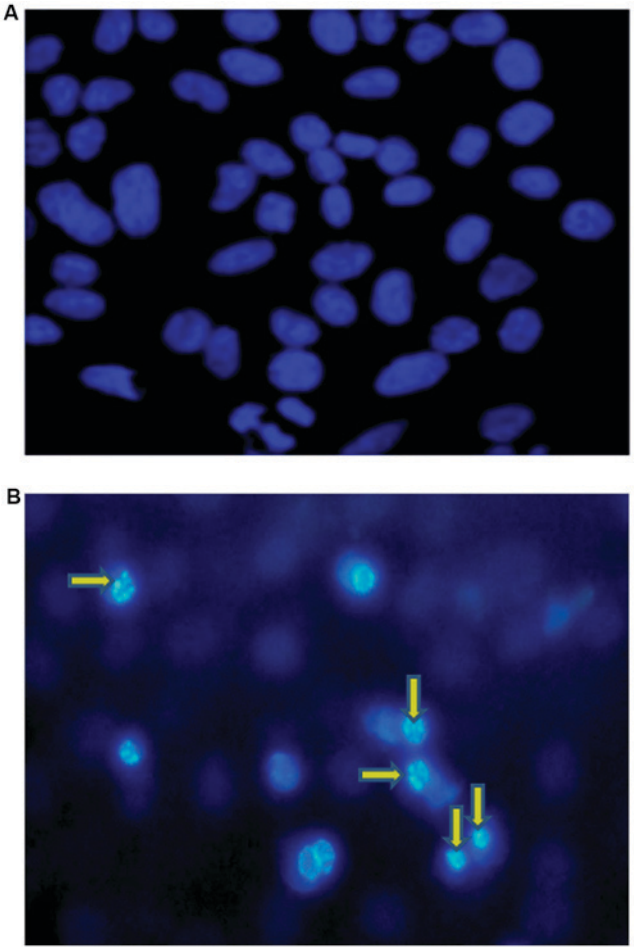

Figure 3. Nuclear condensation and DNA fragmentation. (A) Control group. (B) AA-treated group. Renal tubular cells were treated with 10 μM AA for 24 h, nuclear condensation and DNA fragmentation (yellow arrows) were observed in the AA-treated cells under a phase-contrast microscope at magnifications of x400. AA, aristolochic acid.

α-tocopherol reduced AA-activated caspase-3. Caspase-3 activation is associated with the apoptotic death pathway (40-42). Due to the fact that apoptotic characteristics were predominantly identified in AA-treated renal tubular cells (Fig. 3B), whether AA could activate caspase-3 was investigated. As presented in Fig. 6, compared with the control group (lane 1), the level of cleaved caspase-3 was markedly increased in the AA-treated group (lane 2). AA was identified to induce caspase-3 activity and the effect of α-tocopherol on AA-induced caspase-3 was

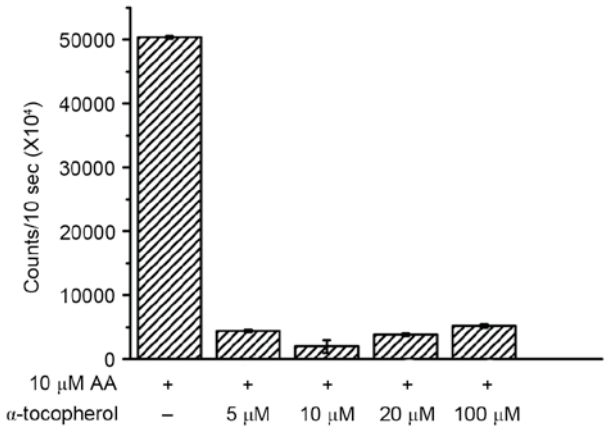

Figure 4. H_2O_2 levels. H_2O_2 levels were measured in 10 μM AA-treated groups (0 μM α-tocopherol vehicle) and 10 μM AA + 5-100 μM α-tocopherol treated group. The levels were measured following 2 h treatment. Data from four independent experiments were presented as the mean ± standard deviation. *P<0.05, vs. the AA alone-treated group (0 μM α-tocopherol cotreatment). AA, aristolochic acid.

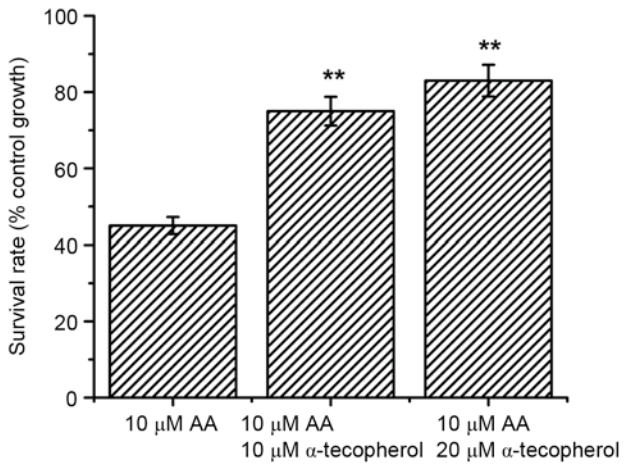

Figure 5. Cell survival rates. The renal tubular cells were treated with 10 μM AA + 0, 10 and 20 μM α-tocopherol treatment, respectively. At 24 h the survival rates were calculated. Data from four independent experiments are presented as the mean ± standard deviation. **P<0.001, vs. the 10 μM AA alone-treated group. AA, aristolochic acid.

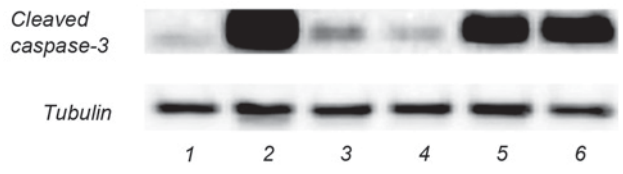

Figure 6. Caspase-3 activation by western blotting. The caspase-3 activity was analyzed at 24 h in the control (lane 1), 10 μM AA (lane 2), 10 μM α-tocopherol (lane 3), 20 μM α-tocopherol (lane 4), 10 μM AA + 10 μM α-tocopherol (lane 5), and 10 μM AA + 20 μM α-tocopherol (lane 6) cells. AA, aristolochic acid.

further determined. AA was reported to decrease mitochondrial membrane depolarization and to lead to an increase of caspase-3 (42). The results demonstrated that cleaved caspase-3 levels were reduced in the AA plus α-tocopherol group (lane 5 and 6) compared with the AA treatment group (lane 2). Due to the fact that AA-induced H_2O_2 levels are also reduced in the AA + α-tocopherol group (Fig. 4), this suggested that α-tocopherol attenuation of AA-induced cell cytotoxicity may be associated with caspase-3 activity from the reduce of H_2O_2 levels.

Discussion

The results of the current study indicated that AA causes increases in H_2O_2 levels and a reduction in cell survival rates in renal tubular cells and α-tocopherol (10 and 20 μM) attenuated AA-induced H_2O_2 levels and inhibited AA-induced cytotoxicity in these cells. These data suggested that AA-induced cell cytotoxicity may be associated with H_2O_2 levels. By contrast, α-tocopherol could not inhibit AA-induced cytotoxicity under high-dose (100 μM) AA treatment (data not shown), however it effectively ameliorated AA-induced cytotoxicity under low-dose (5-20 μM) AA treatment. This suggested that α-tocopherol ameliorated AA-induced renal cell damage was dependent on AA dosage. Furthermore, high-dose AA alone elevated both H_2O_2 and O_2^- levels. Therefore, this may partially explain why α-tocopherol could not inhibit high-dose AA-induced cell cytotoxicity.

CAT, Gpx and SOD are major cellular antioxidant enzyme systems (57,58). CAT is a tetrameric iron-porphyrin protein in peroxisomes that converts H_2O_2 to H_2O and O_2. CAT and Cu/Zn-SOD are expressed constitutively, whereas Mn-SOD expression within the mitochondria is induced by oxidative stress. GSH is a sulfhydryl peptide that may directly react with O_2^- or N_2^- containing free radicals, or is able to donate electrons in the enzymatic dismutation of H_2O_2 to H_2O and O_2 by GPx (59,60). Cellular CAT and Gpx can remove H_2O_2, whereas SOD removes O_2^- (61). In the present study, the data indicated that AA induced increases in H_2O_2 in a dose-dependent manner, however the 100 μM AA alone was capable of increasing O_2^- levels. This result suggested that low-dose AA may influence the activity of CAT and Gpx while high-dose AA may influence CAT, Gpx and SOD activities. However, further studies are required to confirm this hypothesis.

Studies have indicated that antioxidant can attenuate AA-induced renal damage (11,29,59) and it has been additionally demonstrated that vitamin C attenuated AA-induced renal damage (29). However, the effect of antioxidant α-tocopherol on AA-induced renal damage remains to be reported. The current study demonstrated that α-tocopherol ameliorated AA-induced renal cell cytotoxicity.

Vitamin C and α-tocopherol are common antioxidants and have been used in clinical cases (44,45,62,63). Vitamin C and α-tocopherol both have antioxidant activities, however their antioxidant mechanisms differ (64-66). These studies indicated that α-tocopherol (lipid-soluble material) is able to pass through the cell membrane and catch the free radicals to protect the cell from oxidative damage. However, vitamin C (water-soluble material) cannot pass through cell membrane to remove free radicals directly. Based on the results of a previous study (29) and the current study, it is suggested that both vitamin C and α-tocopherol scavenge H_2O_2 produced by AA-treated renal cells, leading to an increase of survival rate. It was suggested that AA-induced H_2O_2 existed not only in the cytosol however additionally in the cell membrane. In addition,

another key antioxidant function of vitamin C is converting the oxidized α-tocopherol radical back to α-tocopherol (62). It was suggested that the combination of vitamin C and α-tocopherol may be more powerful for protection of AA-induced renal damage. In patients with coronary artery bypass surgery, vitamin cocktail (ascorbic acid and α-tocopherol) effectively attenuated oxidative stress than control (44). In summary, the results demonstrated that α-tocopherol attenuates AA-induced H_2O_2 and caspase-3 and ameliorated AA-induced renal cell cytotoxicity.

Acknowledgements

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