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Effect of aristolochic acid on intracellular calcium concentration and its links with apoptosis in renal tubular cells

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Abstract Aristolochic acid (AA) has been demonstrated to play a causal role in Chinese herbs nephropathy. However, the detailed mechanism for AA to induce apoptosis of renal tubular cells remains obscure. In this study, we show that AA evokes a rapid rise in the intracellular Ca^{2+} concentration of renal tubular cells through release of intracellular endoplasmic reticulum Ca^{2+} stores and influx of extracellular Ca^{2+}, which in turn causes endoplasmic reticulum stress and mitochondria stress, resulting in activation of caspases and finally apoptosis. Ca^{2+} antagonists, including calbindin-D_{28k} (an intracellular Ca^{2+} buffering protein) and BAPTA-AM (a cell-permeable Ca^{2+} chelator), are capable of ameliorating endoplasmic reticulum stress and mitochondria stress, and thereby enhance the resistance of the cells to AA. Moreover, we show that overexpression of the anti-apoptotic protein Bcl-2 in combination with BAPTA-AM treatment can provide renal tubular cells with almost full protection against AA-induced cytotoxicity. In conclusion, our results demonstrate an impact of AA to intracellular Ca^{2+} concentration and its link with AA-induced cytotoxicity.

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Abbreviations

ER	endoplasmic reticulum
GRP	glucose-regulated protein
AA	aristolochic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid
ZVAD-fmk	benzyloxycarbonyl-Phe-Val-Ala-Asp (Ome)-fluoromethyl-ketone
MDCK	Madin-Darby canine kidney
COX4	cytochrome oxidase subunit IV
PBS	phosphate-buffered saline
$[Ca^{2+}]_i$	intracellular calcium concentration
PLA_2	phospholipase A_2
CsA	cyclosporine A

Introduction

Early and pivotal events in apoptosis are now known to occur in the endoplasmic reticulum (ER) and mitochondria [1, 2]. The release of Ca^{2+} (from the ER) and cytochrome *c* (from mitochondria) into the cytosol are requisites for apoptosis in many cases [3]. The ER is a cellular organelle where secretory and membrane proteins are synthesized and modified and is also a major intracellular Ca^{2+} storage compartment inside the cell. Disturbances in any of these ER functions can lead to so-called ER stress [4, 5]. Both of Ca^{2+} overload and depletion of the ER Ca^{2+} storage can results in changes in

protein folding and in ER stress [6–8]. The mammalian ER stress response can be separated into two phases, adaptation and apoptosis. Cells initially adapt to the accumulation of unfolded proteins by inducing ER-resident chaperones such as glucose-regulated protein (GRP) 78 [9] and GRP94 [10]. The induction of GRP78, also referred as immunoglobulin-binding protein (BiP), has been used widely as an indicator of ER stress and the onset of the unfolded protein response [11]. However, if this adaptation does not prove sufficient, the apoptotic response is initiated, as exemplified by induction of the gene encoding C/EBP homologous transcriptional factor (CHOP/GADD153) [12, 13] and activation of ER-associated caspases [14, 15].

Recent studies indicate that ER stress can finally trigger the mitochondrial permeability transition [3]. A significant fraction of the cytosolic Ca^{2+} released from the ER is captured by mitochondria, presumably because mitochondrial Ca^{2+} uptake sites are concentrated in regions of the membrane located near Ca^{2+}-release channels of the ER [16]. This ER-mitochondria connection enables Ca^{2+} signals not only to fine-tune cellular metabolism but also to modulate the ability of mitochondria to undergo apoptosis [17]. Several treatments, such as Ca^{2+} ionophores and thapsigargin, have been found to trigger apoptosis through Ca^{2+}-mediated mitochondrial permeability transition in various cell types [3, 18].

Aristolochic acid (AA), a component present in at least six types of Chinese herbs including *Aristolochia fangchi* and *Aristolochia manshuriensis* Kom [19], has been demonstrated to play an unequivocal role in so-called Chinese herbs nephropathy, characterized by extensively interstitial fibrosis without glomerular abnormalities [19–21]. AA was shown to be toxic to renal tubular epithelium and carcinogenic to urethral epithelium [22, 23], and it formed DNA adducts in renal tissues of patients with Chinese herbs nephropathy [24]. Chinese herbs nephropathy-like disease has been reproduced in rabbits and rats treated with AA [25, 26]. *In vitro*, AA was shown to induce apoptosis in cultured proximal tubular cells [27–29]. However, the mechanism for AA to trigger apoptosis of renal tubular cells remains unclear. Intriguingly, AA has also been reported to ameliorate apoptosis of some non-renal cells under certain stressful circumstances [30–32]. In this study, we attempt to examine the mechanism for AA to induce apoptosis of renal tubular cells and the role of the Ca^{2+} signals in this event.

Materials and methods

Chemicals and reagents

The rabbit polyclonal anti-cleaved caspase-3 (Asp175), anti-phospho-ERK (Thr202/Tyr204), anti-phospho-p38 (Thr180/Tyr182), and anti-phospho-JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The rabbit polyclonal anti-GRP78 (H-129), anti-heat shock protein 60 (H-300), anti-heat shock protein 90 (H-114), anti-ERK (C-17), and anti-JNK (C-16) and mouse monoclonal anti-cytochrome *c* (A-8) and anti-actin (C-2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse monoclonal anti-cytochrome oxidase subunit IV (COX4) was purchased from Invitrogen Life Technologies, Inc. The mouse monoclonal anti-calbindin-D_{28K}, aristolochic acid, propidium iodide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. The rabbit polyclonal anti-heat shock protein-70, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA-AM), G418 sulfate and caspase inhibitor ZVAD-fmk were purchased from Calbiochem, Inc. (San Diego, CA, USA). The mouse monoclonal anti-Bcl-2 was purchased from Cashmere Biotech, Inc. (Houston, TX, USA). Fura-2 acetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes, Inc. (Eugene, OR, USA).

Cell culture and transfections

LLC-PK1 cells and Madin-Darby canine kidney (MDCK) were maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% and 95% air atmosphere. To generate cells stably expressing calbindin D_{28K}, MDCK cells were transfected with pCMV-calbindin-D_{28K} [33] using LipofectAMINE (Invitrogen Life Technologies). 48 h after transfection, the cells were harvested and seeded on 100-mm dishes at an appropriate density in the medium containing 0.5 mg/ml G418. Two weeks later, G418-resistant cell clones were picked using cloning cylinders and screened for calbindin-D_{28K} expression by immunoblotting. Multiple positive clones were obtained for further analysis. MDCK cells stably overexpressing Bcl-2 were established in our laboratory and described previously [34].

Measurement of cell viability

Cells were seeded in 96-well plates (10^4/well) for 12 h in the medium with 10% serum before pharmacological treatments. Cells were treated with AA (in most cases at 100 μM) in the medium supplemented with 5% serum. After the corresponding treatments, cell viability was evaluated by MTT assay. For MTT assay, the cells were incubated with 5 mg/ml MTT solution (50 μl/well) for 4 h at 37°C and lyzed in 20% SDS. The absorbance of the dissolved formazan grains within the cells was measured at 570 nm by a microplate reader.

Detection of DNA fragmentation

Cells were harvested, washed in phosphate-buffered saline (PBS), and lysed in the buffer containing 0.5% Triton X-100, 50 mM Tris, pH 8.0, 10 mM EDTA, and 0.5 mg/ml proteinase K at 56°C for 16 h. RNase A (0.5 μg/ml) was added to the mixture and incubated at 56°C for another 3 h. DNA was extracted by phenol/chloroform and analyzed by 2% agarose gel electrophoresis at constant 50 V for 2 h. DNA was stained with ethidium bromide and visualized by Kodak EDAS 290 UV light system with Kodak 1D version 3.6 image software.

Flow cytometry

Various times after AA treatment, MDCK cells were washed in PBS and fixed in 70% ethanol at −20°C for at least 8 h. The cells were collected by centrifugation and suspended (5×10^6/ml) in PBS supplemented with 0.1% Triton X-100, 0.5 μg/ml RNase A, and 20 μg/ml propidium iodide. 30 min later, fluorescence emitted from the propidium iodide-DNA complex was quantified by Beckman Coulter FC500 flow cytometer and the DNA contents in the sub-G1 phase were analyzed by Beckman Coulter CXP software.

Measurement of $[Ca^{2+}]_i$

Cells were grown on coverslips and loaded with 2 μM fura-2/AM in Dulbecco's modified Eagle's medium for 30 min at 37°C. After loading, the cells were washed twice with Ca^{2+} medium (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4) or Ca^{2+}-free medium (with 1 mM EGTA). Fura-2-loaded cells were then incubated in Ca^{2+} medium or Ca^{2+}-free medium for 30 min at 37°C and transferred to the recording chamber (approximately 0.5 ml in volume) mounted on the stage of an inverted Olympus microscope IX-70 linked to a Delta Scan System (Photon Technology International, Princeton, NJ). Fluorescence was monitored by alternately recording excitation signals at 340 and 380 nm and emission signal at 510 nm. Paired images were acquired every 2–10 s. 7–15 cells were monitored for each experiments. Maximum and minimum fluorescence value were obtained by adding Triton X-100 (0.1%) and EGTA (20 mM) sequentially at the end of an experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously [35].

Detection of cytochrome *c* in cytosolic and mitochondrial fractions

For isolation of cytosol and mitochondrial fractions, cells were lysed by Dounce homogenizer in the buffer containing 20 mM HEPES, pH 7.5, 250 mM sucrose, 20 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitory units/ml aprotinin, and 20 μg/ml leupeptin at 4°C. The cell lysates were centrifuged at $1500 \times g$ for 3 min to remove unbroken cells and nuclei. The supernatants were centrifuged at $10000 \times g$ for 15 min at 4°C and the resulting pellet was collected as the mitochondrial fraction. The supernatant from $10000 \times g$ spin was centrifuged at $100000 \times g$ for 1 h at 4°C for preparation of cytosol. For detection of cytochrome *c*, equal amount of proteins from mitochondrial and cytosolic fractions were separated by 12% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with monoclonal anti-cytochrome *c*.

Immunoblotting

Cells were lyzed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, and 1 mM Na_3VO_4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitory units/ml aprotinin, and 20 μg/ml leupeptin). The lysates were centrifuged for 10 min at 4°C to remove debris, and the protein concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA). Equal amount of cell lysates was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH). Immunoblotting was performed with appropriate antibodies using the Amersham Pharmacia Biotech enhanced chemiluminescence system for detection. Chemiluminescent signals were detected by Fuji LAS-3000 luminescence image system. The result for each immunoblotting is a representative of at least three separate experiments.

Statistics

Student's *t* test was one-tailed and used to determine whether there was a significant difference between two means ($P<0.05$); statistical differences are indicated with an asterisk.

Results

AA induces apoptosis of renal tubular MDCK cells

Although AA is known to induce apoptosis of renal proximal tubular cells [27–29], its effect on renal distal tubular cells has never been reported. Our results clearly show that AA was cytotoxic not only to proximal tubular LLC-PK1 cells but also to distal tubular MDCK cells, which caused their death in a dose- and time-dependent manner (Fig. 1A, B). However, MDCK cells seemed to be more resistant to AA-induced cytotoxicity than LLC-PK1 cells. 24 h after

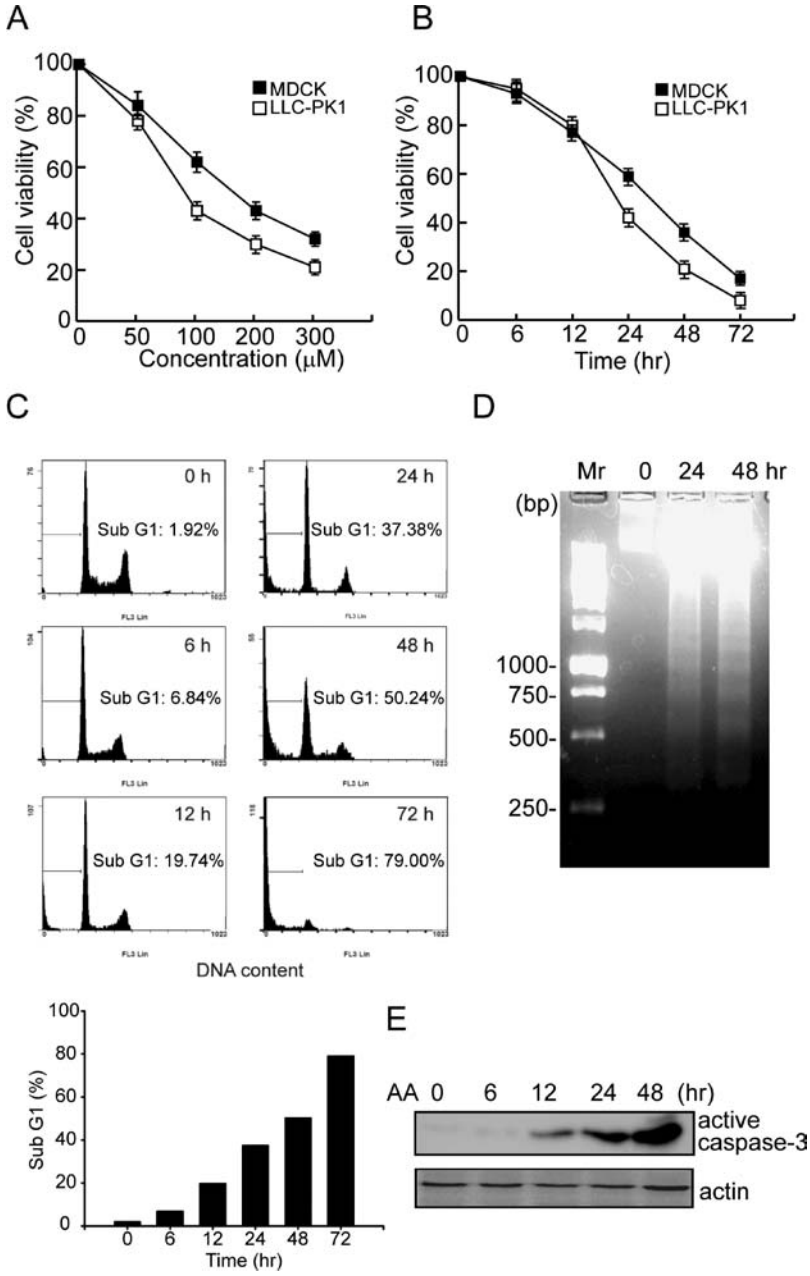

Fig. 1 AA induces apoptosis of MDCK cells. (**A**) MDCK cells and LLC-PK1 cells were treated with AA at various concentrations. 24 h later, the viability of the cells was measured by the MTT assay, as described under "Experimental Procedures." The viability of the cells without AA treatment is defined as 100%. Values (means ± S.E.) are from three independent experiments. (**B**) MDCK cells were treated with 100 μM AA for various times and their viability was measured. (**C**) MDCK cells were treated with 100 μM AA treatment for various times and subjected to a flow cytometer for analyzing their DNA contents. The percentage of the cells whose DNA contents in the sub-G1 phase was measured. Data is the average of two experiments. (**D**) MDCK cells were treated with 100 μM AA. 24 or 48 h later, the chromosomal DNA of the cells was extracted and analyzed by agarose gel electrophoresis. The DNA marker (*Mr*) is shown on the left as base-pair (*bp*). (**E**) MDCK cells were treated with 100 μM AA for various times. The activation of caspase-3 was analyzed by immunoblotting with an antibody specific to active (cleaved) form of caspase-3

100 μM AA treatment, at least 60% of MDCK cells remained alive, which was approximately 20% more than LLC-PK1 cells (Fig. 1A). The decreased viability of MDCK cells was closely correlated with increased DNA content in the sub-G1 phase (Fig. 1C). Moreover, the AA-induced MDCK cell death was associated with characteristics of apoptosis, such as DNA fragmentation (Fig. 1D) and caspase-3 activation (Fig. 1E).

AA induces both ER stress and mitochondria stress

The mechanism for AA to induce apoptosis of renal tubular cells was investigated. We first examined whether mitochondria is involved in the AA-induced apoptosis. It is known that induction of the mitochondrial permeability transition is accompanied by the release of cytochrome *c* from the mitochondria to the cytosol. Figure 2A shows that AA produced a decrease in the mitochondrial content of cytochrome *c* that was evident after 3 h and more prominent at 6 h. This depletion of cytochrome *c* in the mitochondrial fraction was mirrored by a concomitant increase of the protein in the cytosolic fraction. The cytochrome oxidase subunit IV (COX4) and actin were respectively used as loading controls for mitochondrial proteins and cytosolic proteins. Next, the possibility for AA to induce ER stress was examined. The induction of the ER-resident chaperone GRP78 has been widely used

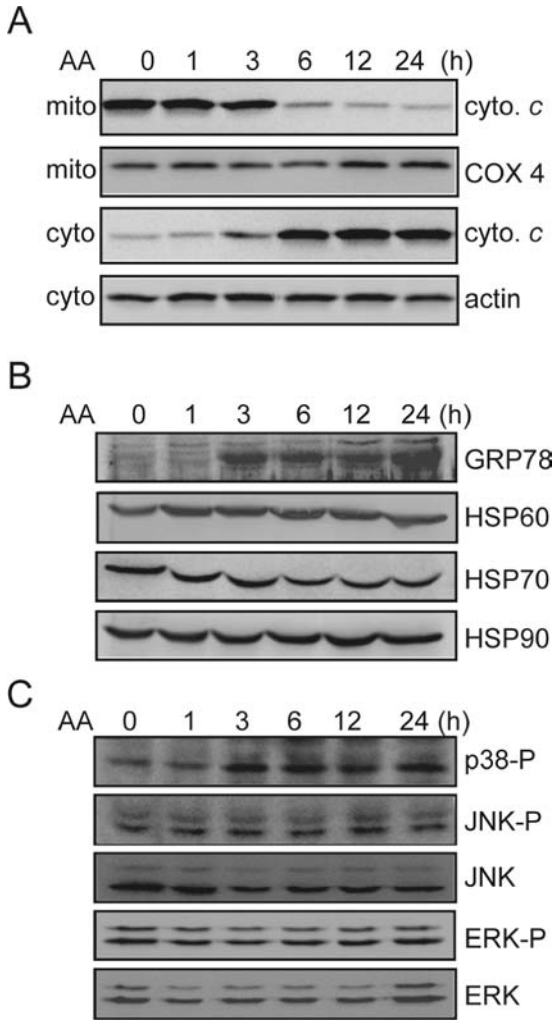

Fig. 2 AA induces both mitochondria stress and ER stress. (**A**) MDCK cells were treated with 100 μM AA for various times and then subjected to fractionation. The presence of cytochrome *c* in the mitochondrial fraction (*mito*) or the cytosol fraction (*cyto*) was detected by immunoblotting. Note that the presence of cytochrome c (*cyto. c*) in the cytosol fraction is used as a marker for mitochondria stress. COX4 and actin are respectively used as loading controls for mitochondrial proteins and cytosolic proteins. (**B**) MDCK cells were treated with 100 μM AA for various times and lyzed. Equal amount of whole cell lysates was analyzed by immunoblotting with antibodies as indicated. Note that the expression of GRP78 is used as a marker for ER stress. (**C**) Equal amount of whole cell lysates from MDCK cells as described in the panel B was analyzed for activation of the mitogen-activated protein kinases, p38, JNK, and ERK

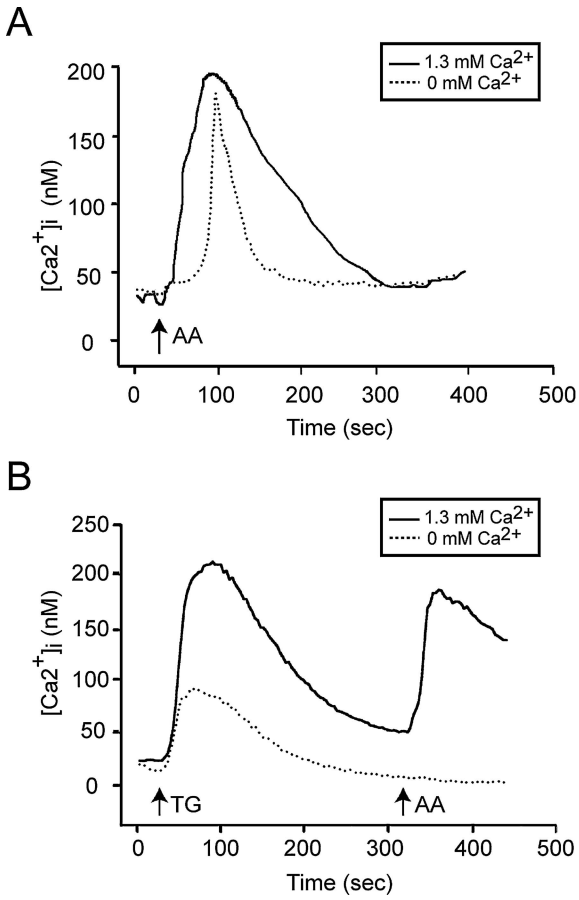

Fig. 3 AA induces a rapid rise in $[Ca^{2+}]_i$ through both release of ER stores and influx of extracellular Ca^{2+}. (**A**) MDCK cells were loaded with the Ca^{2+} indicator dye Fura-2 (2 μM) for 30 min, and then exposed to 100 μM AA in the buffer with or without Ca^{2+}. The changes in $[Ca^{2+}]_i$ were monitored as described under "Experimental Proceudres." (**B**) MDCK cells loaded with Fura-2 were pretreated with 1 μM thapsigargin (*TG*) for 300 sec and then exposed to 100 μM AA. The changes in $[Ca^{2+}]_i$ were monitored. Note that thapsigargin is used to induce the release of intracellular ER Ca^{2+} stores and prevent refilling by inhibition of the ER Ca^{2+}—ATPase

as an indicator of ER stress and the onset of the unfolded protein response [11]. Figure 2B shows that the expression of GRP78, but not other heat shock chaperones, was selectively induced by AA, which could be detected as early as 1 hour after AA treatment and became more evident after 3 h (Fig. 2B). Moreover, AA induced activation of the p38 mitogen-activated protein kinase (Fig. 2C) that is known to be activated upon ER stress and involved in the induction of GRP78 [36]. These results together indicate that AA causes both ER stress and mitochondria stress in renal tubular cells.

AA induces a rapid rise in $[Ca^{2+}]_i$ through both release of ER stores and influx of extracellular Ca^{2+}

Since disturbance of the $[Ca^{2+}]_i$ homeostasis has been reported to cause ER stress and mitochondria stress [1, 2], we speculated that AA might have some impact to the $[Ca^{2+}]_i$ To examine this possibility, MDCK cells were loaded with the Ca^{2+} indicator dye Fura-2 and the $[Ca^{2+}]_i$ was monitored upon exposure to 100 μM AA. In the presence of extercellular Ca^{2+}, AA rapidly (within seconds) increased the $[Ca^{2+}]_i$, which reached the maximum within 1.5 min and declined to the baseline by 5 min (Fig. 3A). In the absence of extracellular Ca^{2+}, AA could still evoke an increase in

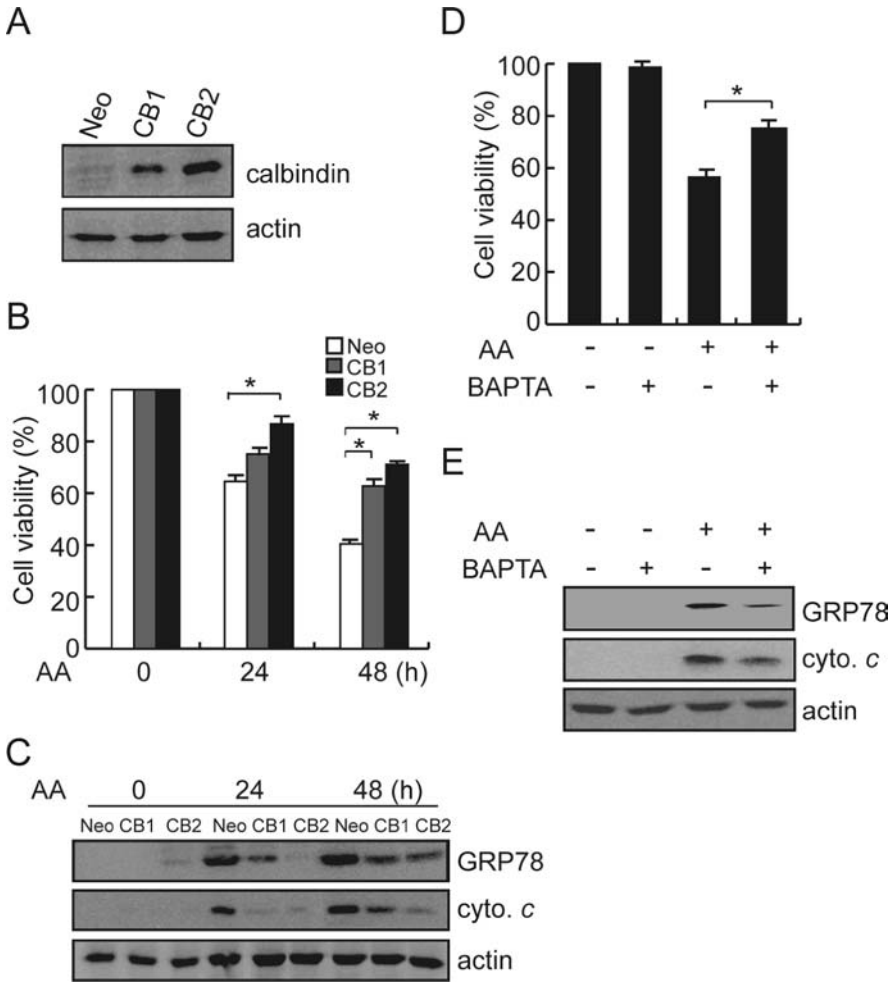

Fig. 4 Calcium antagonists ameliorate AA-induced cell death. (**A**) Calbindin-D_{28k}, an intracellular Ca^{2+} buffering protein, was stably expressed in MDCK cells and its expression was analyzed by immunoblotting. (**B**) Two MDCK cell clones (*CB-1 and CB-2*) stably expressing calbindin-D_{28k} and a neomycin-resistant control clone (*Neo*) were treated with 100 μM AA. 24 or 48 h later, the cell viability was measured by the MTT assay. Values (means ± S.E.) are from three independent experiments. $^{*}P<0.05$. (**C**) The cells as described in the panel *B* were treated with 100 μM AA for 24 or 48 h and subjected to analysis for the expression of GRP78 in whole cell lysates and the presence of cytochrome *c* in the cytosolic fraction. (**D**) MDCK cells were pretreated with 10 μM BAPTA-AM, a cell-permeable cytosolic Ca^{2+} chelator, for 1 h and treated with (+) or without (−) 100 μM AA in the presence or absence of 10 μM BAPTA-AM for 24 h. The viability of the cells was measured. Values (means ± S.E.) are from three independent experiments. $^{*}P<0.05$. (**E**) MDCK cells were treated as described in the panel *D*, and GRP78 in whole cell lysates and cytochrome *c* in the cytosol fraction were analyzed

the $[Ca^{2+}]_{i,}$ although to a lesser extent (Fig. 3A). These results suggest that AA may cause an increase in the $[Ca^{2+}]_i$ through release of intracellular ER Ca^{2+} stores and influx of extracellular Ca^{2+}. To further confirm this, MDCK cells were pretreated with thapsigargin before exposure to AA. Thapsigargin is known to induce the release of intracellular ER Ca^{2+} stores and prevent refilling by inhibition of the ER Ca^{2+}—ATPase [37]. In the presence of extracellular Ca^{2+}, AA increased the $[Ca^{2+}]_i$ of the thapsigargin-treated cells, but on the other hand, in the absence of extracellular Ca^{2+}, it failed to increase the $[Ca^{2+}]_i$ of those cells (Fig. 3B). These results together support our notion that the AA-induced increase in the $[Ca^{2+}]_i$ is through both release of ER stores and influx of extracellular Ca^{2+}.

Calcium antagonists ameliorate AA-induced cell death

To examine the significance of the increased $[Ca^{2+}]_i$ in AA-induced cytotoxicity, calbindin-D_{28k}, a cytosolic Ca^{2+}-binding protein, was stably expressed in MDCK cells (Fig. 4A). Calbindin-D_{28k} has been used as an intracellular Ca^{2+} buffer to reduce Ca^{2+}-mediated cytotoxicity in renal cells [33] and non-renal cells [38]. The expression of calbindin-D_{28k} in MDCK cells partially (20–30%) suppressed AA-induced cell death (Fig. 4B), accompanied by inhibition in the GRP78 expression and the release of cytochrome c from mitochondria (Fig. 4C). To further confirm the role of increased $[Ca^{2+}]_i$ in the AA-induced cytotoxicity, BAPTA-AM, a cell-permeable Ca^{2+} chelator, was applied

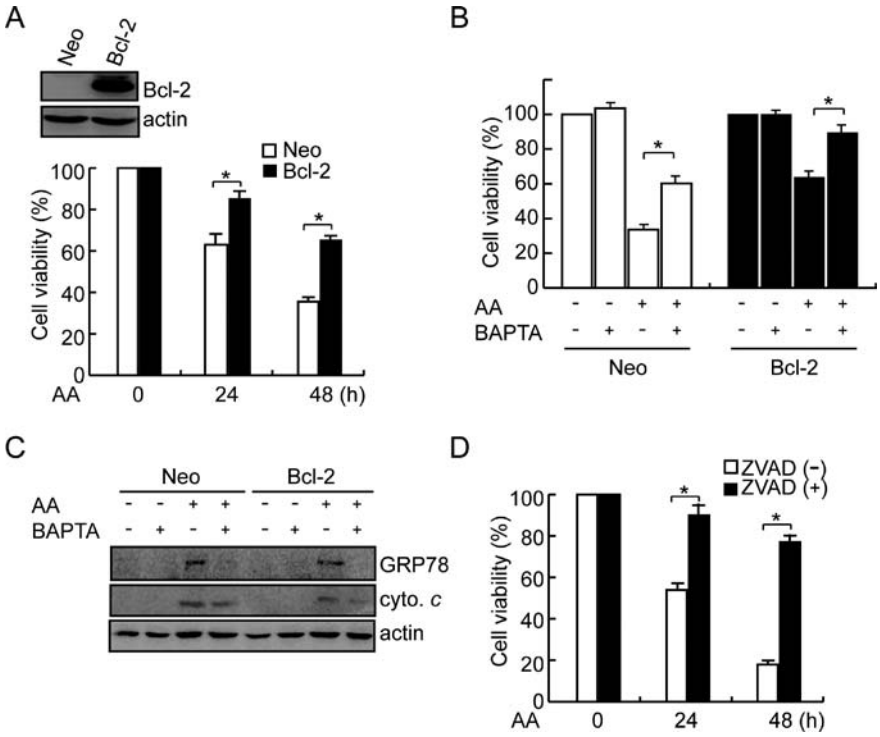

Fig. 5 Bcl-2 overexpression and BAPTA treatment synergistically protect MDCK cells against AA-induced cytotoxicity. (**A**) MDCK cells stably overexpressing Bcl-2 (*MDCK/Bcl-2*) and their neomycin-resistant control cells (*MDCK/Neo*) were treated with 100 μM AA. 24 or 48 h later, the viability of the cells were measured by the MTT assay. Values (means ± S.E.) are from three independent experiments. $^{*}P<0.05$. (**B**) MDCK/Bcl-2 cells and MDCK/Neo cells were pretreated with 10 μM BAPTA-AM for 1 h and challenged with (+) or without (−) 100 μM AA in the presence or absence of 10 μM BAPTA-AM for 48 h. The viability of the cells was measured. Values (means ± S.E.) are from three independent experiments. $^{*}P<0.05$. (**C**) MDCK/Bcl2 cells and MDCK/Neo cells were treated as described in the panel *B*. GRP78 in whole cell lysates and cytochrome *c* in the cytosolic fraction were analyzed. (**D**) Parental MDCK cells were treated with or without 100 μM AA in the presence (+) or absence (−) of 50 μM ZVAD-fmk (ZVAD), a broad range caspase inhibitor. 24 or 48 h later, the cell viability was measured. Values (means ± S.E.) are from three independent experiments. $^{*}P<0.05$

in our experiments. Similar to calbindin-D_{28k} expression, BAPTA-AM partially (~20%) rescued MDCK cells from AA-induced cytotoxicity (Fig. 4D). The protective effect of BAPTA-AM against AA cytotoxicity was correlated with its inhibition in the GRP78 expression and the release of cytochrome *c* from mitochondria (Fig. 4E).

Bcl-2 overexpression and BAPTA-AM treatment synergistically rescue cells from AA-induced cell death

The anti-apoptotic protein Bcl-2 is known to prevent mitochondrial permeability transition and cytochrome *c* release [39], and increase the overall mitochondrial Ca^{2+}-uptake capacity [40]. To examine the significance of the mitochondria stress in AA-induced cytotoxicity, Bcl-2 was stably overexpressed in MDCK cells (Fig. 5A). Overexpression of Bcl-2 partially (20–30%) suppressed AA-induced cell death (Fig. 5A). In the presence of BAPTA-AM, the Bcl2-overexpressed cells retained more than 90% viability even 48 h after AA treatment (Fig. 5B). The enhanced resistance of MDCK cells to AA by the combination of Bcl-2 overexpression and BAPTA-AM treatment was correlated with more evident inhibition in the GRP78 expression and the release of cytochrome *c* from mitochondria (Fig. 5C). These results suggest that the synergistic effect of Bcl-2 overexpression and BAPTA-AM treatment on protecting cells against AA-induced cytotoxicity may be through suppression on both ER stress and mitochondria stress. Since ER stress and mitochondria stress lead to activation of caspases, it may be possible to rescue cells from AA-induced cytotoxicity by a broad range caspase inhibitor. Indeed, the broad range caspase inhibitor ZVAD-fmk effectively suppressed AA-induced cell death to an extent similar to that inhibited by the combination of Bcl-2 overexpression and BAPTA-AM treatment (Fig. 5D).

Discussion

In this study, we demonstrate that AA is cytotoxic to renal distal tubular MDCK cells. The concentration of AA used in the most of our experiments is 100 μM. Compared to other *in vitro* studies [27–29] where AA was used to treat different types of cells at a range between 18 μM and 117 μM, we thus

consider 100 μM of AA is within an acceptable range for an *in vitro* study. However, it is not clear whether 100 μM of AA is a physiologically relevant concentration. To address this question, we reviewed previous reports describing the "Chinese herbs nephropathy" [20, 21, 41, 42] and found no clinical data for the answer. Next, we turned to the animal models of AA-induced nephropathy for the answer [25, 26, 28]. Unfortunately, in those studies the authors did not measure the concentration of AA or its metabolites in the serum of those animals. Therefore, the concentration of AA in patients or animals with Chinese herbs nephropathy-like disease remains obscure.

We (this study) and others [27–29] demonstrate that AA induces apoptosis of renal tubule cells *in vitro*. It is important to known whether this is the case *in vivo*. Indeed, apoptotic cells were detected within renal tubules of AA-treated mice [28]. The apoptosis of tubular cells induced by AA may account for tubular atrophy found in Chinese herbs nephropathy. However, on histological examination there is widespread remarkably acellular or hypocellular interstitial fibrosis and tubular atrophy associated with only focal mononuclear infiltration and either normal or globally sclerosed glomeruli. It should be noted that interlobular fibroelastosis, which is absent to moderate in early stages of Chinese herbs nephropathy, becomes severe in later stages of the disease, suggesting that it is secondary to the progressive kidney destruction [41, 42]. The mechanism for AA to cause interstitial fibrosis is not clear yet. It could be because of increased proliferation of mesenchymal cells and/or induction of epithelial-mesenchymal transition. The possibility of AA to induce transdifferentiation of renal tubular epithelial cells into myofibroblastic phenotype is currently under investigation.

In contrast to its cytotoxicity to renal epithelial cells, AA has been shown to be cytoprotective to some non-renal cells under certain stressful conditions. For instances, AA at the concentration of 50 μM, which is cytotoxic to cultured renal tubular cells [27–29], attenuates Bax overexpression-induced apoptosis in Jurkat T cells [30] and tumor necrosis factor-induced apoptosis in mouse L929 fibroblasts [31] and rat MH1C1 hepatocytes [32]. The cytoprotective effect of AA shown in those studies was attributed to its inhibition in the activity of phospholipase A_2 (PLA_2) [43, 44], an enzyme produces lysophosphatidic acid and arachidonic acid by catalyzing the release of *sn*-2 fatty acid from membrane phospholipids [45]. Since arachidonic acid is a powerful inducer of the mitochondrial permeability transition [46], suppression of the PLA_2 activity by AA may reduce arachidonic acid production and in turn prevent the mitochondrial permeability transition. In those studies, PLA_2 seems to play a pro-apoptotic role. Intriguingly, PLA_2 can also play an anti-apoptotic role. Zhang et al. [47] showed that overexpression of PLA_2 in baby hamster kidney cells significantly increased

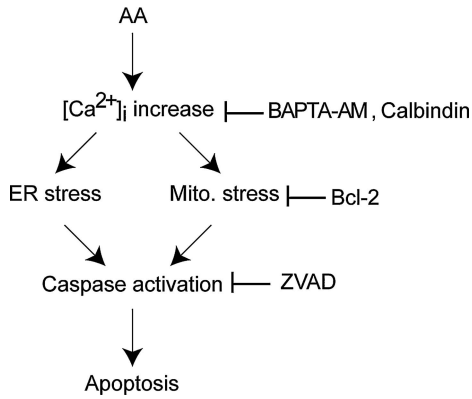

Fig. 6 Role of increased $[Ca^{2+}]_i$ in AA-induced apoptosis. AA induces a rapid increase in $[Ca^{2+}]_i$, which could cause both ER stress and mitochondria stress, leading to activation of caspases and finally apoptosis. The works in this study show that Ca^{2+} antagonists, including calbindin-D_{28k} and BAPTA-AM, partially rescue cells from AA-induced cytotoxicity through ameliorating ER stress and mitochondria stress. Bcl-2 overexpression also partially prevents cell from AA-induced cell death. However, Bcl-2 overexpression in combination with BAPTA-AM treatment renders renal tubular cells maximal resistance to AA

their resistance to growth factor withdrawal-induced apoptosis. This PLA_2 promoted-cell survival was abrogated by AA. Therefore, the fate of cells upon AA treatment may depend on cellular context, in which the interplay between the PLA_2 signaling pathway and other intracellular signals may be crucial for cell's commitment to live or to die.

Gao et al. [27] reported that AA induced apoptosis of LLC-PK1 cells, associated with an increased intracellular calcium concentration. We show in this study that AA has an impact to the Ca^{2+} homeostasis of MDCK cells. It evokes a rapid rise in the $[Ca^{2+}]_i$ through both release of ER Ca^{2+} stores and influx of extracellular Ca^{2+} (Fig. 3), suggesting a role for AA in activating Ca^{2+}-release channels and/or inhibiting Ca^{2+}-ATPases. More importantly, we demonstrate that AA-induced increase in $[Ca^{2+}]_i$ may subsequently cause ER stress and mitochondria stress, resulting in activation of caspases and finally apoptosis (Fig. 6). We show that the Ca^{2+} antagonists, including calbindin-D_{28k} and BAPTA-AM, are capable of ameliorating ER stress and mitochondria stress, and thereby enhance the resistance of the cells to AA (Fig. 4). These results support a casual role of increased $[Ca^{2+}]_i$ in AA-induced cytotoxicity and its links with ER stress and mitochondria stress. Furthermore, we show that overexpression of the anti-apoptotic protein Bcl-2 in combination with the intracellular Ca^{2+} chelator BAPTA-AM can provide renal tubular cells with almost full protection against AA-induced cytotoxicity (Fig. 5B), underscoring the significance of ER stress and mitochondria stress in AA-induced apoptosis. Bcl-2 is known to reside not only in mitochondrial membranes but also in ER membranes, where it decreases the steady-state free Ca^{2+} concentration in the ER [8]. Therefore, the

cytoprotective effect of Bcl-2 against AA-induced cytotoxicity, as shown in Fig. 5, could be resulted from its effects on preventing mitochondrial permeability transition and/or decreasing the ER Ca^{2+} load.

Ca^{2+}, the cellular messengers that control every aspects of cell and tissue physiology, can be turned into death signals when delivered at the wrong time and place. It has become clear that cellular Ca^{2+} overload, or perturbation of intracellular Ca^{2+} compartmentalization, can cause cytotoxicity and trigger either apoptotic or necrotic cell death [2]. A consensus that the Ca^{2+} load of the ER determines the cell's sensitivity to apoptotic stress has gradually emerged. Procedures that increase the ER Ca^{2+} load sensitize cells to apoptotic stress [8]. In addition to ER Ca^{2+} load, the ability of cells to transfer from the ER to the mitochondria is another critical factor for cell's sensitivity to apoptosis. For example, Bax/Bak overexpression favors the transfer of Ca^{2+} from ER to the mitochondria and induces cell death [48]. Conversely, cells deficient in both Bax and Bak are resistant to a wide range of apoptotic stimuli and show a decrease in ER Ca^{2+} load [3]. Therefore, the high sensitivity of the proximal tubular epithelial cells to AA-induced cytotoxicity may be because of their high ER Ca^{2+} load and/or high Ca^{2+} transferring from ER to the mitochondria. In fact, AA was shown to increase expression of Bax, resulting in a decrease of the Bcl-xL/Bax ratio and induction of apoptosis in murine renal proximal tubular cells [28].

The immunosuppressive agent cyclosporine A (CsA) is widely used for the management of organ transplantation and in the treatment of various autoimmune disorders. We and others have shown that CsA has a direct toxicity to renal tubular cells, leading to their apoptosis [49, 50]. However, CsA can also attenuate apoptosis of some non-renal cells by preventing mitochondrial permeability transition [30, 32, 46]. This differential effect of CsA on cell survival is somewhat like the effect of AA on renal *versus* non-renal cells. In our previous report [50], we showed that CsA-induced apoptosis of renal tubular cells could be enhanced by diltiazem, a Ca^{2+} channel antagonist, and in contrast, ameliorated by A23187, a Ca^{2+} ionophore, suggesting a possible regulation of CsA cytotoxicity by intracellular Ca^{2+} level. This finding at the first glance seems contradict to the concept proposed in this study that increased $[Ca^{2+}]_i$ is harmful to renal tubular cells. It should be noted that although Ca^{2+} release from the ER can be a signal for execution of apoptosis, it could also activate cell survival pathways involving proteins such as the transcriptional factor NFAT [51] and NF-κB [52]. In fact, in contrast to AA, CsA does not induce a rapid increase in $[Ca^{2+}]_i$ (data not shown). Therefore, it is possible that increased $[Ca^{2+}]_i$ by Ca^{2+} ionophore might counteract the inhibitory effect of CsA on calcineurin [53], a Ca^{2+}-dependent protein phosphatase important for dephosphorylation (i.e. activation) of NFAT.

The renal tubular epithelium is often found to be the main target of many cytotoxic agents, suggesting high sensitivity of renal tubular cells to apoptotic stress. Since the Ca^{2+} content of the ER could determine the cell's sensitivity to apoptotic stress (as discussed above), the ability of renal tubular cells to maintain appropriate ER Ca^{2+} load may not be as efficient as other types of cells. Therefore, the cytotoxicity of a given medicine to renal tubular cells might be significantly modulated by Ca^{2+} metabolism modulators, including Ca^{2+} channel antagonists, Ca^{2+} ionophores, and Ca^{2+} chelators.

Conclusion

Aristolochic acid (AA) has been demonstrated to play a causal role in Chinese herbs nephropathy. Here we demonstrate that AA evokes a rapid rise in the intracellular Ca^{2+} concentration of cultured renal tubular cells, which in turn causes ER stress and mitochondria stress, resulting in activation of caspases and finally apoptosis. The AA-induced apoptosis can be significantly suppressed by Ca^{2+} antagonists, supporting a critical role for Ca^{2+} signals in AA-induced cytotoxicity.

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