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Arecoline-induced pro-fibrotic proteins in LLC-PK1 cells are dependent on c-Jun N-terminal kinase

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ABSTRACT

Areca nut (AN) chewing is associated with chronic kidney disease (CKD). However, the molecular mechanisms of AN-induced CKD are not known. Thus, we studied the effects of arecoline, a major alkaloid of AN, on proximal tubule (LLC-PK1) cells in terms of cytotoxicity, fibrosis, transforming growth factor-β (TGF-β) and c-Jun N-terminal kinase (JNK).

We found that arecoline dose (0.1–0.5 mM) and time (24–72 h)-dependently induced cytotoxicity without causing cell death. Arecoline (0.25 mM) also time-dependently (24–72 h) increased fibronectin and plasminogen activator inhibitor-1 (PAI1) protein expressions. Arecoline (0.25 mM) time-dependently (24–72 h) increased TGF-β gene transcriptional activity and supernatant levels of active TGF-β1. Moreover, arecoline (0.25 mM) activated JNK while SP600125 (a JNK inhibitor) attenuated arecoline-induced TGF-β gene transcriptional activity. SP600125, but not SB431542 (a TGF-β receptor type I kinase inhibitor), attenuated arecoline-induced fibronectin and PAI1 protein expressions. Finally, tubulointerstitial fibrosis occurred and renal cortical expressions of fibronectin and PAI1 proteins increased in arecoline-fed mice at 24 weeks.

We concluded that arecoline induced tubulointerstitial fibrosis in mice while arecoline-induced TGF-β and pro-fibrotic proteins (fibronectin, PAI1) are dependent on JNK in LLC-PK1 cells.

1. Introduction

Areca nut (AN, also known as betel quid) chewing is the 4th most common addictive habit in the world, including Taiwan (Guh et al., 2007, 2006). AN chewing is associated with oral and oropharyngeal cancers, diabetes mellitus, cardiovascular disease, hypertension, hyperlipidemia, metabolic syndrome and hepatocellular carcinoma (Garg et al., 2014; Guh et al., 2007, 2006). Moreover, recent studies found that AN chewing is also associated with chronic kidney disease (CKD) (Chou et al., 2009; Hsu et al., 2011; Kang et al., 2007).

CKD is characterized by progressive renal fibrosis including glomerulosclerosis and tubulointerstitial fibrosis, which involves injuries to renal tubular epithelial cells with an accumulation of plasminogen activator inhibitor-1 (PAI1) and extracellular matrices such as collagen and fibronectin, etc. (Zeisberg and Neilson 2010). Two of the pivotal factors in the pathogenesis of tubulointerstitial fibrosis are transforming growth factor-β (TGF-β) (Loeffler and Wolf, 2014; Zeisberg and Neilson, 2010) and inflammation (Campanholle et al., 2013; Zeisberg and Neilson, 2010). TGF-β activates the Smad and the non-Smad pathways such as c-Jun N-terminal kinase (JNK) (Loeffler and Wolf, 2014). Interestingly, JNK is involved in inflammation and tubulointerstitial fibrosis (Ma et al., 2009). Moreover, cyclooxygenase 2 (COX2) is one of the mediators of inflammatory renal injuries (Harris, 2013).

Abbreviations: CKD, chronic kidney disease; TGF-β, transforming growth factor-β; JNK, c-Jun N-terminal kinase; PAI1, plasminogen activator inhibitor-1; COX2, cyclooxygenase 2; AN, areca nut; ERK, extracellular signal-regulated kinase; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide; SEM, standard errors of the mean.

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Arecoline is a major alkaloid of AN, which induces cytotoxicity in various cells (Chou et al., 2008; Lee et al., 2006). For example, we have shown that arecoline induced cytotoxicity in hepatocytes by inducing TGF-β (Chou et al., 2008). Moreover, arecoline increases pro-fibrotic PAI1, JNK and COX2 in some cells (Chen and Chang, 2012; Harvey et al., 1986; Hung et al., 2011; Tsai et al., 2003, 2014).

Thus, we studied the mechanisms of AN-induced tubulointerstitial fibrosis by studying the effects of arecoline on mice and the effects of arecoline on porcine proximal tubule (LLC-PK1) cells in terms of TGF-β and JNK.

2. Materials and methods

2.1. Cell culture and reagents

LLC-PK1 cells, a porcine cell line analogous to proximal tubule of kidney, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, containing 5.5 mM D-glucose) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Gibco, Grand Island, NY) in a humidified 5% CO_2 incubator at 37 °C.

Arecoline hydrobromide (referred to as arecoline thereafter), SB203580 (a p38 kinase inhibitor), PD98059 (an extracellular signal-regulated kinase ERK1/2 inhibitor), SB431542 (a TGF-β receptor type I kinase inhibitor), SP600125 (a JNK inhibitor) and all other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless stated otherwise. These inhibitors were dissolved in dimethyl sulfoxide (DMSO, final concentration 0.02%).

2.2. Plasmids

The human TGF-β1 promoter-luciferase construct phTG5-Luc was a gift of Dr. Virelizier (Michelson et al., 1994). The phTG5-Luc contains the human TGF-β1 promoter, which measures the transcriptional activity of TGF-β1.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assay

MTT assays were used to measure cytotoxicity according to the manufacturer's instruction. Briefly, LLC-PK1 cells (4×10^3/well) were seeded for 24 h in 24-well plates. Then, cells were treated with arecoline for the indicated time, followed by adding sterile MTT solution for another 4 h at 37 °C. In the presence of DMSO, spectrometric absorbance (optical density, OD) at 540 nm was measured for formazan dye.

LDH-cytotoxicity assay kit II (BioVision Inc., Milpitas, CA) was used to measure cell death according to the manufacturer's instructions. Cell death was expressed as the relative spectrometric absorbance at 450 nm compared to the medium blank where cell lysis solution was used as the maximum cell death.

2.4. Flow cytometry

Apoptosis or necrosis was measured by flow cytometry using the Dead cell apoptosis kit (Annexin V Alexa Fluor®488 & PI, Invitrogen Corp., Carlsbad, CA) according to the manufacurer's instructions. Briefly, cells were washed, centrifuged, resuspended and adding Alexa Fluor®488 annexin V and propidium iodide. The mixture staining cells were incubated for 15 min in the dark at room temperature. Then the mixture was added with the binding buffer and measured by the COULTER® EPICS XL Flow cytometer (Beckman Coulter Inc., Brea, CA) and the results were analyzed using the WinMDI software.

2.5. Immunoblotting

Collagen I (col1α1) and fibronectin antibodies were obtained from Merck Millipore Corp. (Darmstadt, Germany). COX2 antibody was obtained from Abcam Inc. (Cambridge, Mass). PAI1 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, p-p38, p38, p-JNK and JNK antibodies were obtained from Cell Signaling Technology (Danvers, CA), α-tubulin antibody was obtained from NeoMarkers Inc. (Fremont, CA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX). Control rabbit IgG was purchased from ICN Biochemicals Inc. (Costa Mesa, CA).

Immunoblotting was performed as described in our previous study (Huang et al., 1999). Briefly, protein concentration in cell lysates was quantified with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (50 μg) were resolved by 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After blocking step, the membranes were incubated with primary antibodies for 2 h and the membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies. The protein bands were developed by using the enhanced chemiluminescence system (PerkinElmer, Inc., Boston, MA). Mouse kidney cortices for immunoblotting were sliced, lysed and homogenized by using the Tissuelyser (Qiagen Inc., Valencia, CA).

2.6. Assay for active TGF-β1 and promoter activity of TGF-β

These assays were performed in triplicate in three independent experiments. TGF-β1 in cell culture supernatants was measured by an enzyme-linked immunosorbent assay (LEGEND MAX Total TGF-β1 ELISA kit, BioLengend, Inc., San Diego, CA) according to the manufacturer's instructions. Active TGF-β1 was measured before acidification. TGF-β promoter activity was measured by the phTG5-Luc plasmid. Briefly, cells were seeded on 6-well plates (8×10^4 cells/well). After 24 h, cells were transfected with 2 μg of phTG5-Luc and co-transfected with 0.2 μg of the Renilla luciferase control vector (pRL-SV40, obtained from Promega, Corp., Madison, WI) by using Lipofectamine 2000 (Invitrogen Corp. Carsbad, CA). Transfected cells were stimulated with arecoline for 24–72 h. Dual-Luciferase Reporter 1000 assay systems (Promega Corp., Madison, WI) and the Stop & Glo Reagent were used according to the manufacturer's instructions. Luciferase activity was measured by using Dynatech ML1000 luminometer (Dynatech Laboratories, Inc., Chantilly, VA).

2.7. Mouse experiments

Among the male ICR mice (8-week old, body weight 20 g, BioLASCO, Taipei, Taiwan), five were controls and five were fed with arecoline (6.11 mg/kg/day in drinking water) (Choudhury and Sharan, 2010) for 24 weeks (Liu et al., 2011). Mice were anesthetized by chloral hydrate (400 mg/kg intraperitoneally) and perfused through the heart with normal saline and then phosphate buffered solution (pH 7.4) containing 4% paraformaldehyde for 15 min. Kidneys were removed and immersed in 4% paraformaldehyde (Shin et al., 2000).

Kidney microscopic morphology was assessed by hematoxylin & eosin stain (BioTNA Biotech Inc., Kaohsiung, Taiwan). Briefly, tissue sections measuring 3 μm were created from the paraffinized blocks, de-paraffinized in xylene and rehydrated in a graded alcohol series, and stained with hematoxylin for 3 min. After washing and put into 80% ethanol for 2 min, sections were stained with eosin for 30 s. Renal fibrosis was assessed by Masson's trichrome stain (HT15 kit, Sigma-Aldrich Co., St. Louis, MO) (Farris

and Alpers, 2014) according to the manufacturer's instructions and scored by an ImageJ plugin, which scored the blue stains as the percentage of red stains (Kennedy et al., 2006).

Immunohistochemistry was performed as described before (Ko et al., 2013; Shin et al., 2000). Briefly, formalin-fixed and paraffin-embedded tissues were cut into 4 μm thickness, deparaffinised, and immersed with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and then microwaved in 10 mM citrate buffer, pH 6.0, to unmask the epitopes. The sections were incubated with antibodies and stained by the Dako REAL EnVision (Detection System Peroxidase/DAB+, Dako Corp., Carpinteria, CA) and counterstained with hematoxylin. Immunohistochemistry was scored by the "IHC Profiler" (Varghese et al., 2014) ImageJ plugin. The IHC Profiler scores degree of staining as 0 (negative), 1 (low positive), 2 (positive) and 3 (high positive). All animal procedures were approved and done in accordance with the national guidelines and the guidelines by the Kaohsiung Medical University Animal Experiment Committee which were equivalent to the NIH Guide for the Care and Use of Laboratory Animals.

2.8. Statistical analyses

The data were expressed as the mean ± standard errors of the mean (SEM). Unpaired Student's *t*-tests were used for the comparison between two groups (Norman, 2010). One-way analysis of variance (ANOVA) followed by unpaired Student's *t*-tests was applied for the comparison between more than two groups (Lew, 2007). A P value of less than 0.05 was considered to be statistically significant, otherwise it was non-significant (NS).

3. Results

3.1. Arecoline induced cytotoxicity without causing cell death in LLC-PK1 cells

Dose- and time-dependent effects of arecoline on cytotoxicity and cell death were measured by MTT assay and LDH assay, respectively. As shown in Fig. 1A, arecoline (0.1 mM) induced cytotoxicity at 72 h while arecoline (0.25–0.5 mM) induced cytotoxicity at 48–72 h, although arecoline (0.1–0.5 mM) did not cause cell death at 24–72 h (Fig. 1B). Arecoline (0.25 mM) also did not cause apoptosis or necrosis at 24–72 h (the combined percentage of apoptotic or necrotic cells were 10 ± 0.7, 8.6 ± 3.6, 6.2 ± 1.6, and 5.33 ± 0.74% at 0, 24 h, 48 h and 72 h, respectively. P = NS by one-way ANOVA) (Supplementary Fig. 1A). However, arecoline (1 mM) caused apoptosis or necrosis at 24–48 h (Supplementary Fig. 1B).

3.2. Arecoline increased TGF-β gene transcriptional activity and levels of active TGF-β1

Because we have found that arecoline-induced cytotoxicity in hepatocytes is dependent on TGF-β (Chou et al., 2008), we studied the effects of arecoline on TGF-β gene transcriptional activity (by transient transfection of the TGF-β promoter construct phTG5-Luc) and levels of active *TGF-β1* by ELISA. We found that arecoline (0.25 mM) time-dependently (24–72 h) increased both gene transcriptional activity of TGF-β (Fig. 2A) and supernatant levels of active TGF-β1 (Fig. 2B).

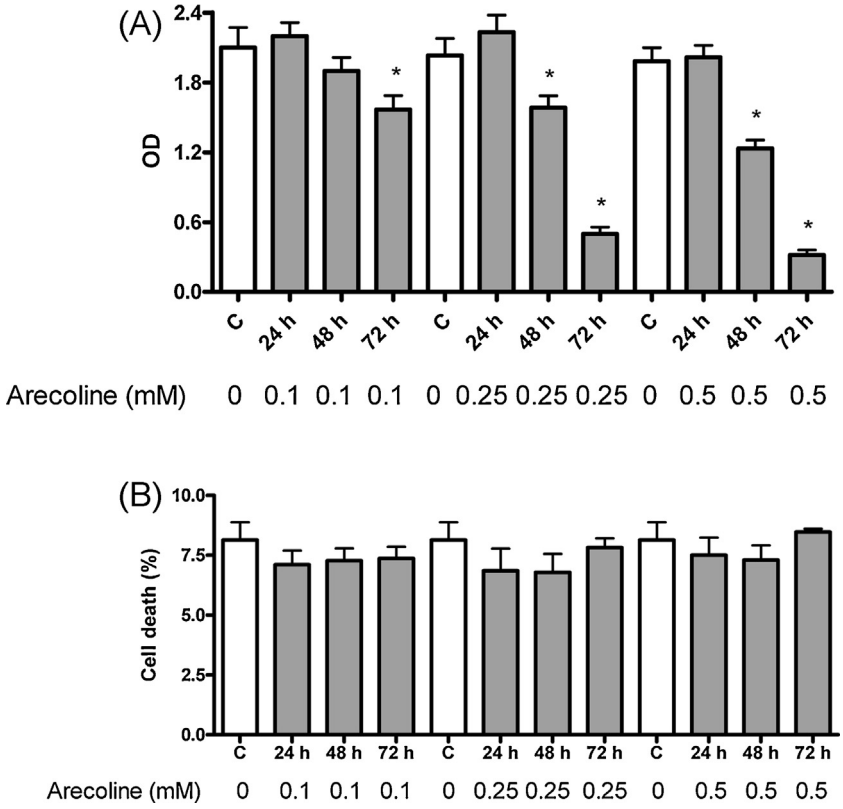

Fig. 1. Dose- and time-dependent effects of arecoline on cytotoxicity and cell death in LLC-PK1 cells.
Cytotoxicity was measured by the MTT assay and cell death was measured by the LDH-cytotoxicity assay kit II (BioVision Inc., Milpitas, CA). Cells (4×10^3/well) were seeded for 24 h in 24-well plates and were treated with arecoline (0.1–0.5 mM, gray bars) for the indicated times. (A) Effects of arecoline on cytotoxicity. (B) Effects of arecoline on cell death. The results were expressed as the means ± SEM of three independent experiments in triplicate. *: $P < 0.05$ versus control (C, blank bars).

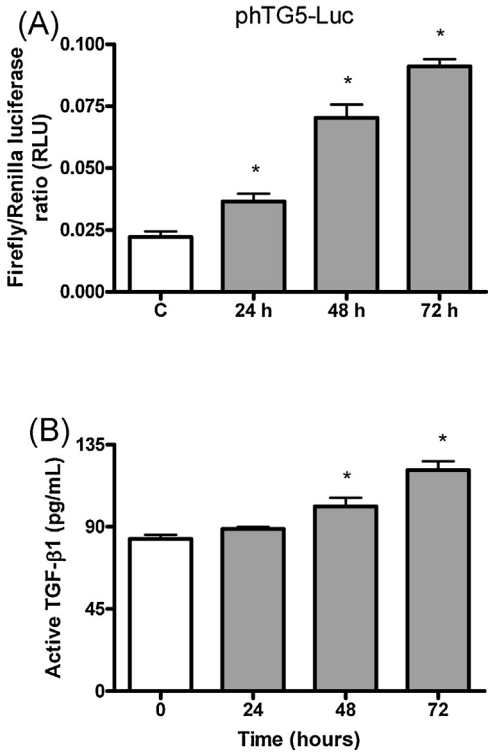

Fig. 2. Time-dependent effects of arecoline on TGF-β transcriptional activity and supernatant levels of active TGF-β1 in LLC-PK1 cells.
TGF-β transcriptional activity was measured by the TGF-β1 promoter-luciferase construct phTG5-Luc plasmid transfections and transfection efficiency was normalized by co-transfection with the Renilla luciferase plasmid (pRL-SV40) in the dual luciferase assay. Transient transfection of LLC-PK1 cells with 2 μg of phTG5-Luc plasmid was achieved by using Lipofectamine 2000. After 6 h of transfection, cells were treated with 0.25 mM arecoline (gray bars) for 24–72 h. Luciferase activity was measured by the Dual-Luciferase Reporter 1000 assay systems (Promega Corp., Madison, WI). Firefly luciferase activity was normalized by Renilla luciferase activity in cell lysates. (A) Effects of arecoline on TGF-β transcriptional activity. (B) Active TGF-β1 in cell culture supernatants was measured by an enzyme-linked immunosorbent assay (LEGEND MAX Total TGF-β1 ELISA kit, BioLengend, Inc., San Diego, CA) before acidification. Effects of arecoline on supernatant levels of active TGF-β1 were shown. The results were expressed as the means ± SEM of three independent experiments in triplicate. *: $P < 0.05$ versus C (control, blank bars).

3.3. Effects of arecoline on pro-fibrotic proteins and pro-inflammatory protein COX2

Renal fibrosis and tubulointerstitial fibrosis are characterized by the accumulation of extracellular matrices (Zeisberg and Neilson, 2010). Moreover, COX2 is one of the mediators of inflammatory renal injuries (Harris, 2013). Thus, fibronectin, collagen I, PAI1 and COX2 were measured by immunoblotting. We found that arecoline (0.25 mM) increased fibronectin protein expression at 24–48 h while increasing PAI1 protein expression at 48–72 h (Fig. 3). However, arecoline (0.25 mM) did not affect collagen I or COX2 protein expression at 24–72 h (Fig. 3).

3.4. Arecoline induced TGF-β and pro-fibrotic proteins via JNK

Because induction of TGF-β in proximal tubular cells is dependent on ERK1/2, p38 kinase and JNK (Rui et al., 2012; Zhang et al., 2006), we assessed the roles of these effectors by PD98059 (an ERK1/2 inhibitor), SB203580 (a p38 kinase inhibitor) and SP600125 (a JNK inhibitor) on TGF-β gene transcriptional activity. We found that SB203580 had cytotoxicity if used alone (Fig. 4A). However, SB203580, PD98059 and SP600124 did not have cytotoxic effects when used in combination with arecoline (Fig. 4A). Moreover, SP600125, but not SB203580 or PD98059, attenuated arecoline (0.25 mM)-induced TGF-β gene transcription (Fig. 4B). Finally, SP600125, but not SB431542 (a TGF-β receptor I kinase inhibitor), attenuated arecoline (0.25 mM)-induced fibronectin and PAI1 protein expression at 48 h (Fig. 4C). Note that the effects of SP600125 were not due to decreased cell viability because SP600125 did not affect cytotoxicity.

The effects of PD98059, SB203580 and SP600125 on p-ERK1/2, p-p38 and p-JNK were also assessed. We found that arecoline (0.25 mM) increased p-ERK1/2 (but not p-p38 or p-JNK) expression at 48 h (supplementary Fig. 4′A). Moreover, PD98059 attenuated arecoline (0.25 mM)-induced p-ERK1/2 expression while SB2035800 decreased p-p38 and SP600125 decreased p-JNK expression at 48 h (supplementary Fig. 4′A). Finally, arecoline (0.25 mM) time-dependently induced p-JNK expression at 15 min (supplementary Fig. 4′B).

3.5. Arecoline increased tubulointerstitial fibrosis and increased renal cortical fibronectin and PAI1 expressions in mice

To determine the chronic effects of arecoline on tubulointerstitial fibrosis *in vivo*, we assessed the effects of arecoline feeding (6.11 mg/kg/day in drinking water) on five mice at 24 weeks and compared with those of the five control mice. Fig. 5 showed the results of renal pathology in one control and one arecoline-fed mice and Fig. 6 showed the immunoblotting results of two randomly chosen control mice and four randomly chosen arecoline-fed mice. We found that arecoline increased tubulointerstitial fibrosis (Masson's trichrome stain, 7.7 ± 1% versus 2.2 ± 0.3% in 10 randomly chosen fields in each mouse, $P < 0.01$) at 24 weeks, although glomerular and tubulointerstital morphologies (hematoxylin & eosin stain) were not affected (Fig. 5).

Additionally, arecoline increased renal cortical fibronectin and PAI1 protein expressions at 24 weeks by immunoblotting (Fig. 6). In immunohistochemistry (supplementary Fig. 5), we found that arecoline did not affect tubulointerstitial expression of fibronectin at 24 weeks (1.1 ± 0.1 versus 1.02 ± 0.02 in 10 randomly chosen

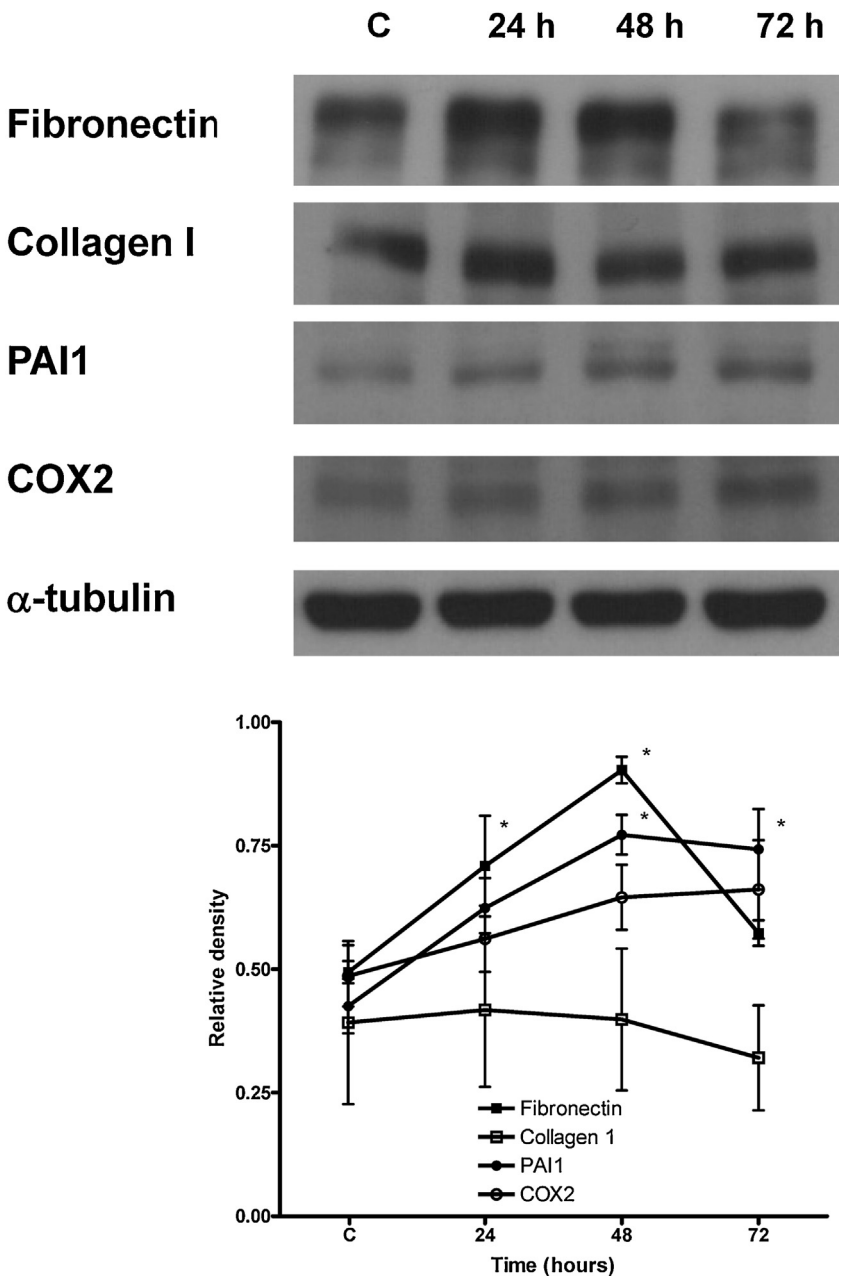

Fig. 3. Time-dependent effects of arecoline on pro-fibrotic proteins in LLC-PK1 cells.
Fibronectin, collagen I, PAI1 and COX2 proteins were measured by immunoblotting with α-tubulin as an internal control. Cells were treated with 0.25 mM arecoline for 24–72 h. The results were expressed as the means ± SEM of four independent experiments. Lanes 2–4 were compared to lane 1 (C). *: $P < 0.05$ versus lane 1.

fields in each mouse, P = NS). However, arecoline increased tubulointerstitial PAI1 protein expression at 24 weeks (1.7 ± 0.11 versus 0.97 ± 0.03 in 10 randomly chosen fields in each mouse, $P < 0.01$).

4. Discussion

This is the first demonstration that arecoline induced cytotoxicity while increasing TGF-β and pro-fibrotic proteins in proximal tubular cells. Moreover, arecoline-induced TGF-β and pro-fibrotic proteins were dependent on JNK whereas arecoline-induced pro-fibrotic proteins were not dependent on TGF-β type I receptor kinase. Finally, arecoline induced chronic tubulointerstitial fibrosis in mice. The findings provide new insights into the mechanisms of AN-induced renal fibrosis.

We used arecoline at a dose of 0.1–0.5 mM because the mean salivary arecoline is 0.2 mM (0.12–0.3 mM) during chewing in BN chewers by the liquid chromatography tandem mass spectrometry analysis (Franke et al., 2015). Our findings that arecoline induced cytotoxicity while increasing TGF-β in LLC-PK1 cells are similar to previous studies showing that arecoline induced cytotoxicity while increasing TGF-β in keratinocytes and hepatocytes (Chou et al., 2008; Khan et al., 2012). In contrast, our finding that arecoline-induced TGF-β is dependent on JNK (but not ERK1/2 or p38 kinase) is a novel one. This finding is similar to a previous study showing that arecoline activates JNK in endothelial cells (Hung et al., 2011) while aristolochic acid (a toxin to renal tubules)-induced TGF-β is dependent on JNK in proximal tubular cells (Rui et al., 2012).

Both TGF-β and JNK are involved in the pathogenesis of tubulointerstitial fibrosis (Loeffler and Wolf, 2014; Ma et al., 2007;

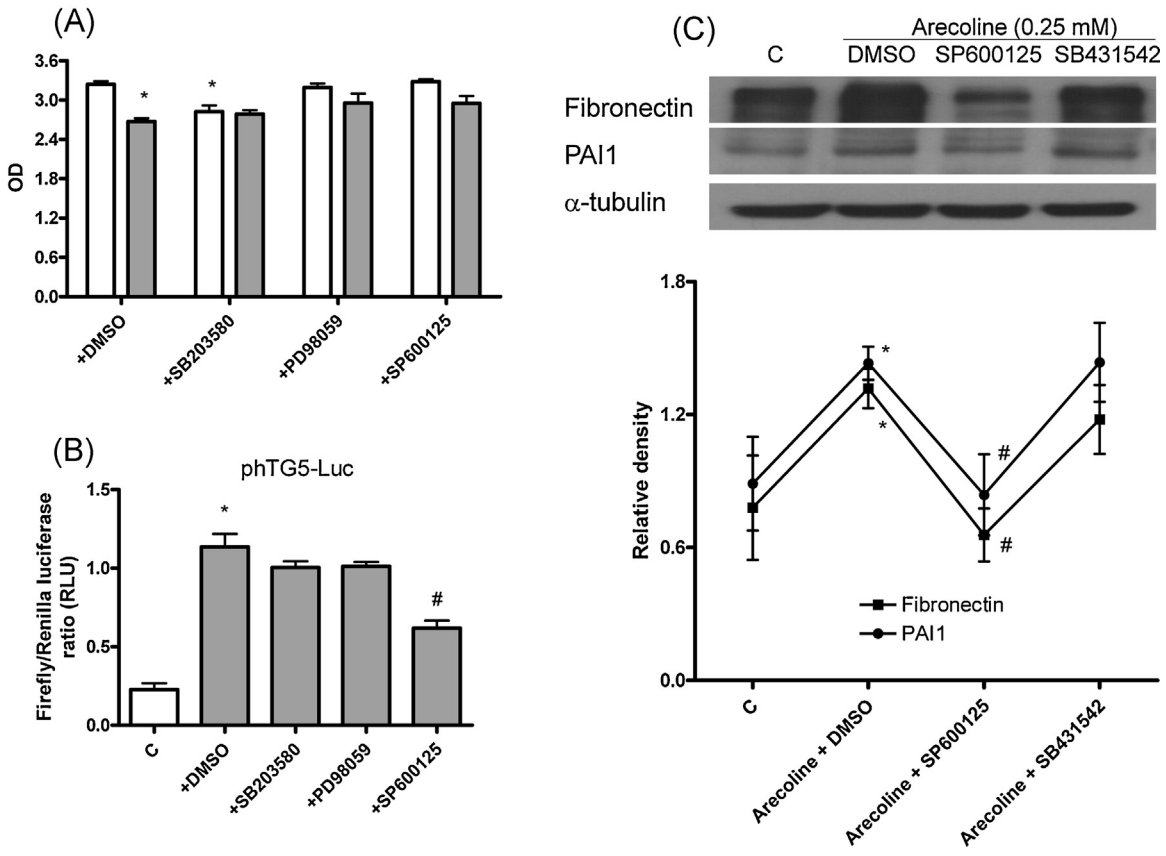

Fig. 4. Effects of various inhibitors on arecoline-induced cytotoxicity, TGF-β transcriptional activity and pro-fibrotic proteins in LLC-PK1 cells.
(A) Effects of SB203580 (5 μM), PD98059 (5 μM) or SP600125 (20 μM) signaling inhibitors (dissolved in DMSO) on cytotoxicity (measured by the MTT assay) alone (blank bars) or in combination with arecoline (0.25 mM, gray bars). (B) Effects of signaling inhibitors on arecoline (0.25 mM)-induced TGF-β transcriptional activity, which was measured by the TGF-β1 promoter-luciferase construct phTG5-Luc. Transient transfection of LLC-PK1 cells with 2 μg of phTG5-Luc was achieved by using Lipofectamine 2000. After 6 h of transfection, cells were treated with 0.02% DMSO (Control or C, blank bar) or 0.25 mM arecoline (gray bars) for 48 h. Firefly luciferase activity was normalized by the Renilla luciferase activity in cell lysates. Lanes 3–5 were compared to lane 2. (C) Effects of SB431542 (10 μM) or SP600125 (20 μM) on arecoline (0.25 mM)-induced fibronectin and PAI1 proteins were measured by immunoblotting with α-tubulin as an internal control. Cells were treated with 0.02% DMSO (control or C) or 0.25 mM arecoline for 48 h. Lanes 3–4 were compared to lane 2. The results were expressed as the means ± SEM of four independent experiments. *: $P < 0.05$ versus lane 1. #: $P < 0.05$ versus lane 2.

Zeisberg and Neilson, 2010). However, we found that arecoline did not affect collagen I at 24–72 h. This finding is similar to a previous study showing that arecoline did not affect collagen I expression in fibroblasts (Xia et al., 2009). In contrast, we found that arecoline increased two pro-fibrotic proteins (fibronectin and PAI1), but not the inflammatory mediator COX2, of renal fibrosis (Honma et al., 2014). We found that fibronectin returned to the control level at 72 h after arecoline treatment. Note that this phenomenon is common in time-dependent biologic effects and that fibronectin has a half-life of 20–30 h (Pussell et al., 1985). Next, we studied the roles of TGF-β and JNK in arecoline-induced pro-fibrotic proteins (fibronectin, PAI1).

Previous studies have already found that arecoline increases fibronectin and PAI1 in various cells (Harvey et al., 1986; Hung et al., 2011; Tsai et al., 2003, 2014). We further found that arecoline induced p-JNK and that arecoline-induced fibronectin and PAI1 were dependent on JNK. These findings were similar to previous studies showing that JNK inhibitors attenuate renal fibronectin and PAI1 expressions in diabetic nephropathy (Wang et al., 2015). Note that JNK is an inflammatory mediator (Kyriakis and Avruch, 2012) and is one of the non-Smad TGF-β signaling pathways (Loeffler and Wolf, 2014).

In contrast, our finding that arecoline-induced fibronectin and PAI1 were not dependent on TGF-β receptor type I kinase was compatible with the notion that TGF-β-activated JNK does not depend on TGF-β receptor type I kinase (Sorrentino et al., 2008). For example, SB431542 (TGF-β receptor type I kinase inhibitor) does not inhibit JNK (Inman et al., 2002). Interestingly, TGF-β-activated kinase 1 (which is activated by TGF-β, interleukin-1, tumor necrosis factor-α and toll-like receptor 4) has been shown to be a major upstream activator of JNK in renal inflammation and fibrosis (Ma et al., 2011). Finally, we found that arecoline increased tubulointerstitial fibrosis and increased renal cortical expression of pro-fibrotic proteins (fibronectin and PAI1) in mice at 24 weeks. This finding was similar to our finding that arecoline increased fibronectin and PAI1 protein expressions in LLC-PK1 cells. Note that arecoline increased tubulointerstitial expression of PAI1, but not fibronectin at 24 weeks. This finding differed from the finding that arecoline increased renal cortical expressions of fibronectin and PAI1 proteins in immunoblotting. This discrepancy can be explained by the sampling bias inherent in renal biopsy or immunohistochemistry thereof because it only consists of a small fraction of the kidney (Kretzler et al., 2002). In contrast, renal tubules contribute to the majority of renal cortical proteins. For example, glomerular volume is only 0.14 mm^3 whereas kidney volume is 141 mm^3 in mice (Guo et al., 2005). In other words, renal tubules constitute the bulk of renal cortex.

In conclusion, arecoline contributes to AN-induced CKD in that arecoline induces chronic tubulointerstitial fibrosis in mice. Moreover, arecoline injures proximal tubular cells by inducing

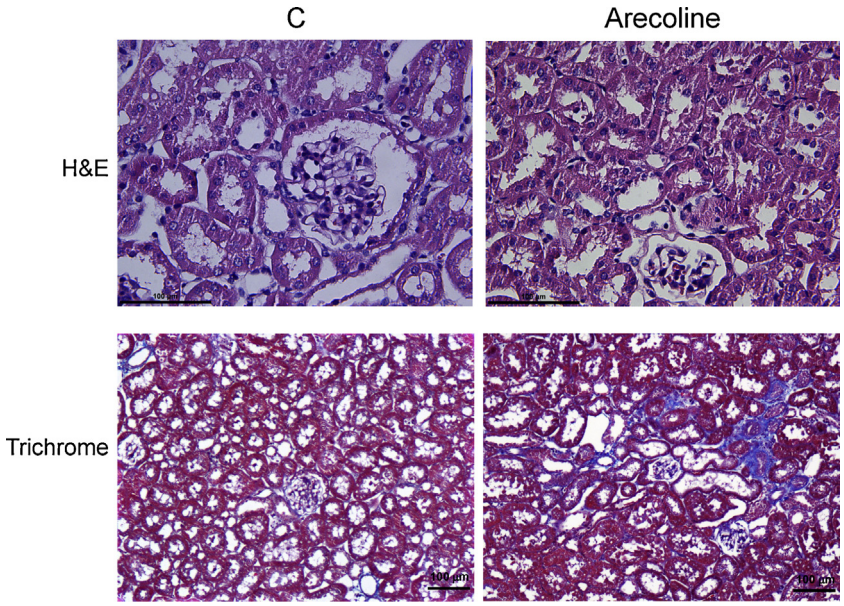

Fig. 5. Effects of arecoline on tubulointerstitial fibrosis in mice.
Among the male ICR mice (8-week old, body weight 20 g), five were controls and five were fed with arecoline (6.11 mg/kg/day in drinking water) for 24 weeks. Hematoxylin and eosin (H&E, upper panels) and Masson's trichrome (lower panels) stains were performed in formalin-fixed and paraffin-embedded tissues which were cut into 3 μm thickness, deparaffinised and rehydrated. A representative figure of the control (C, left panels) mice or the arecoline-fed (Arecoline, right panels) mice was shown.

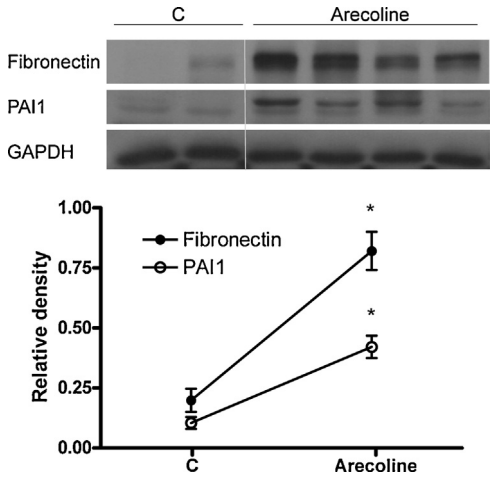

Fig. 6. Effects of arecoline on renal cortical expressions of fibronectin and PAI1 proteins in mice.
Among the male ICR mice (8-week old, body weight 20 g), five were controls and five were fed with arecoline (6.11 mg/kg/day in drinking water) for 24 weeks. Renal cortical fibronectin and PAI1 proteins were measured by immunoblotting with GAPDH as an internal control for two randomly chosen control mice and four randomly chosen arecoline-fed mice. The results were expressed as the means ± SEM of three independent experiments. *: $P < 0.05$ versus C (control).

cytotoxicity while increasing TGF-β and pro-fibrotic proteins (fibronectin and PAI1). Finally, arecoline-induced TGF-β, fibronectin and PAI1 are dependent on JNK. Thus, JNK may be a novel target for arecoline-induced renal fibrosis.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.02.004.

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