JNK-dependent AP-1 activation is required for aristolochic acid-induced TGF-β1 synthesis in human renal proximal epithelial cells

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Submitted 7 October 2011; accepted in final form 20 March 2012

Rui HL, Wang YY, Cheng H, Chen YP. JNK-dependent AP-1 activation is required for aristolochic acid-induced TGF-β1 synthesis in human renal proximal epithelial cells. Am J Physiol Renal Physiol 302: F1569–F1575, 2012. First published March 21, 2012; doi:10.1152/ajprenal.00560.2011.—Chronic aristolochic acid nephropathy (CAAN) is a chronic and progressive tubulointerstitial nephropathy characterized by extensive interstitial fibrosis. Aristolochic acid (AA) could induce overexpression of transforming growth factor-β1 (TGF-β1) in a human renal proximal tubule epithelial cells line (HKC), which has been implicated in the pathogenesis of CAAN. The present studies in HKC cells showed 1) AA could activate JNK in time- and dose-dependent manners and JNK inhibitor SP600125 could inhibit AA-induced TGF-β1 promoter activity and TGF-β1 synthesis; 2) AA-induced JNK activation and TGF-β1 synthesis were significantly inhibited by kinase-inactive mutants of MEKK4, MKK4, or MKK7; 3) AA could upregulate luciferase activity derived by a wild-type TGF-β1 promoter, but not by an AP-1 binding-deficient TGF-β1 promoter; and 4) AA could upregulate expression of c-Fos, phospho-c-Jun, and phospho-ATF2. The above data suggest AA-induced TGF-β1 overexpression in HKC cells may be mainly mediated by the JNK signaling pathway. Both the upstream kinases of JNK including MEKK4, MKK4, and MKK7, and the downstream transcription factor of JNK, AP-1, may also participate in this process.

transforming growth factor-β1; MEKK4; MKK4; MKK7; c-Jun

ARISTOLOCHIC ACID (AA), a component in some kinds of Chinese herbs including Aristolochia fangchi, Aristolochia manshurienisis, and Aristolochia debilis (5), has been demonstrated to play an unequivocal pathogenic role in aristolochic acid nephropathy (AAN), so-called Chinese herb nephropathy. Recently, there were strong evidences that AA is the cause of Balkan endemic nephropathy and the associated urothelial malignancies (1, 6, 10). Chronic aristolochic acid nephropathy (CAAN) is characterized by extensive interstitial fibrosis with tubular atrophy and ischemic, wrinkled glomeruli (3, 17). Both clinical and laboratory findings suggest that the proximal tubule is one of the direct targets of AA injury (4). The attack of AA on proximal tubular epithelial cells might induce the cytokine secretion promoting extracellular matrix (ECM) synthesis and epithelial-mesenchymal transformation (20, 29).

Among fibrogenic cytokines, transforming growth factor-β1 (TGF-β1) is the most prominent. It modulates the expression of ECM in several kinds of renal cells, including proximal tubular epithelial cells, and is considered a determinant of ECM accumulation in renal interstitial fibrosis (7, 8, 18). Our previous studies in vitro and in vivo have also shown that TGF-β1 is critical for the AA-induced cell injury in cultured proximal tubular epithelial cells (25, 29) and progressive renal interstitial fibrosis in a CAAN animal model (28, 32). However, little is known about the molecular pathogenic mechanism of TGF-β1 overexpression in AAN (16, 31).

The promoter region of the human TGF-β1 gene contains heptamerous consensus sequences, known as the TPA-responsive element (TRE), for the binding of transcription factor activating protein-1 (AP-1) (13, 22, 26). AP-1 is a heterodimeric complex that principally comprises members of the Jun and Fos nuclear oncprotein families in mammalian cells (26). AP-1 can be activated and phosphorylated at the N-terminal region of the c-Jun by JNK, a member of the MAPK family (22). Upon activation, AP-1 binds to TRE and induces transcription of a variety of genes involved in multiple cellular processes such as proliferation, survival, differentiation, transformation, and apoptosis (22, 26). Regulation of AP-1 activity is complex and occurs at various levels including dimmer-composition, transcriptional and posttranscriptional events, and interaction with accessory proteins (26).

The present study is designed to better understand the molecular pathogenic mechanism of the AA-induced TGF-β1 overexpression in proximal tubular epithelial cells. Our research results showed that the TGF-β1 overexpression might be mainly mediated by the JNK signaling pathway, the upstream kinases of JNK including MEKK4, MKK4, and MKK7, and the downstream transcription factor of JNK, such as AP-1, may also participate in this process.

MATERIALS AND METHODS

Cell culture. The human proximal tubular epithelial cell line (HKC), which was originally developed by Prof. L. C. Racusen (21), was kindly provided by Prof. F. L. Zheng (24). HKC cells were incubated and passaged in 75-cm² cell culture flasks containing DMEM-F12 (GIBCO BRL) culture medium with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin) at 37°C in humidified air with 5% CO₂. When cells were confluent, the cell growth was arrested in serum-free medium for 24 h. All experiments were subsequently performed under serum-free conditions.

ELISA. Cell culture supernatants were assayed by the TGF-β1 Emax ImmunoAssay System according to the manufacturer’s instructions (Promega). In brief, the supernatants were activated with 1 N HCl followed by neutralization with 1.2 N NaOH/0.5 M HEPES to measure total TGF-β1. Samples were plated on 96-well plates coated with monoclonal anti-human TGF-β1 antibody and incubated for 1 h at room temperature. After vigorous washing, plates were incubated in turn with polyclonal TGF-β1 antibody and a TGF-β1 horseradish peroxidase conjugate for 2 h, respectively, and TMB One solution was added. The reaction was stopped by adding stop solution. Absorbance at 450-nm wavelength in each well was measured, and the concentration of TGF-β1 was calculated by comparison with the standard curve.
expression was calculated according to the formula 2(Rt - Ct) = 2-ΔΔCt, where Rt is the threshold cycle number for the reference gene observed in the test sample.

The human TGF-β 1 promoter cloning

TGF-β1 promoter

F1: GTTGGAAAGCCTCTTCTTCC
R1: CCAGGGGGGCGCTCAAGAGCC
-1292

TGF-β1 promoter

F2: TCCCAAGCTTGGTTCTCCTCCG
R2: CGGAAGGAAGACGAGGCTGGA
-1262

TRE mutant 1

F3: GCTTCCGCTTGGTTCTGATCC
R3: GGGTATGACACCAAGGGGACC
-1217

TRE mutant 2

F4: CGCATGTGAGAGAGAGAGAG
R4: GGAGTATGACACCAAGGGGACC
-1196

RT-PCR analysis. Total RNA of each sample was isolated from HKC cells with classic total RNA isolation kits (Sangong Biotechnology), and equal amounts of mRNA (2 μg) from different samples were reverse transcribed to CDNA with Moloney Murine Leukemia Virus reverse transcriptase (Promega), respectively. Real-time PCR was performed in a 0.5-ml Eppendorf tube containing 2 μl of cDNA, 1 μM of each primer, and 47 μl Power SYBR Green real-time PCR master mix (Toyobo). Gene-specific primers were designed using GenBank and detailed as follows: TGF-β1, forward: 5’-GGATCCACGAGCGGGCCCGGGAGGCTCAGAGCCG-3’, reverse: 5’-TGCGGCCCACGTAGTACACG-3’; and β-actin, forward: 5’-GGAGCAATGATCCTGATCTC-3’, reverse: 5’-CCTTCTTGGCATGGACTCTGGT-3’. The specificity of the primers was verified by melting curves, and amplified products were sequenced to ensure the validity. Quantitative real-time RT-PCR was performed using ABI7500 (Applied Biosystems, Foster City, CA). A thermal cycling profile consisted of a preincubation step at 95°C for 60 s, followed by 40 cycles of denaturation (95°C, 15 s), annealing (60°C, 15 s), and extension (72°C, 45 s). Reactions were performed in triplicate, and threshold cycle numbers were averaged. Nontemplate control was used as a negative control. Samples were collected with normalization to β-actin. Gene expression was calculated according to the formula 2(Rt - Eo) × 10ⁿ, where Rt is the threshold cycle number for the reference gene observed in the test sample, and Eo is the threshold cycle number for the experimental gene observed in the test sample.

Construction of plasmids. Expression vectors of heme agglutinin (HA)-tagged kinase-inactive mutants of MEKK1 (HA-MEKK1-C1255M), ASK1 (HA-ASK1-K709R), TAK1 (HA-TAK1-K63W), MEKK4 (HA-MEKK4-K4-KM), MKK4 (HA-MKK4-DN), and MKK7 (HA-MKK7-DN) were gifts kindly provided by Dr. Sheng-cai Lin (30). The human TGF-β1-luciferase reporter gene pGL3-hTGF1-WT was generated by insertion of nucleotides -1292 to -740 of the human TGF-β1 promoter (+1 as the translational starting site) into the KpnI and BglII sites of the vector pGL3-Basic (Promega), which contains the firefly (Photinus pyralis) luciferase gene. To generate the AP-1 binding-deficient TGF-β1 promoter, two TREs of human TGF-β1 promoter (−1254 to −1248 and −1207 to −1201) were mutated by a two-time SOE (splicing by overlapping extension) PCR (Table 1). Primers F1, R1, F2, and human TGF-β1 promoter (−1292 to −740) were used for the first-time SOE PCR; primers F1, R1, F3, R3, and the product of first-time SOE PCR were used for the second-time SOE PCR. The product of second-time SOE PCR was sequenced and fused into the pGL3-basic vector to generate mutated human TGF-β1-luciferase reporter gene pGL3-hTGF1-MT.

Table 1. Primers used for wild-type and mutant TGF-β1 promoter cloning

<table>
<thead>
<tr>
<th>Primers (5’-3’)</th>
<th>Start Position</th>
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<tr>
<td>TGF-β1 promoter</td>
<td>F1: GTTGGAAAGCCTCTTCTTCC</td>
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<tr>
<td></td>
<td>R1: CCAGGGGGGCGCTCAAGAGCC</td>
</tr>
<tr>
<td>TRE mutant 1</td>
<td>F3: GCTTCCGCTTGGTTCTGATCC</td>
</tr>
<tr>
<td>TGF-β1 promoter</td>
<td>R3: GGGTATGACACCAAGGGGACC</td>
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Western blot assay. HKC cell lysates in cell lysis buffer (Cell Signaling Technology) in six-well plates were harvested, sonicated five times for 1 s each, and centrifuged at 13,000 rpm for 20 min at 4°C. The boiled protein in samples was separated by SDS-10% PAGE and transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk in phosphate-buffered saline with 0.1% Tween 20 for 1 h, the membranes were probed with either rabbit anti-total JNK, anti-phospho-JNK, anti-c-Fos, anti-phospho-c-Jun, anti-phospho-ATF2 antibody (Cell Signaling Technology), or mouse anti-β-actin antibody (Sigma-Aldrich), anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, and then incubated with horseradish peroxidase-labeled anti-rabbit or anti-mouse antibody. The bound antibodies were visualized by an enhanced chemiluminescence kit (Roche Diagnostics). The relative phospho-JNK level was expressed as the phospho-JNK/total JNK ratio. The relative level of c-Fos, phospho-c-Jun, or phospho-AATF2/β-actin ratio.

Statistical analysis. The values are represented as means ± SD. One-way ANOVA was used to test the differences among groups. Statistical significance was defined as *P < 0.05.
RESULTS

Activation of JNK in HKC by AA in time- and dose-dependent manners. JNK activity was analyzed by Western blotting using anti-JNK and anti-phospho JNK antibodies. As shown in Fig. 1A, HKC cells were incubated with 10 mg/l AA in different durations, and the activity of JNK was significantly increased at 15, 30, 60, and 120 min ($P < 0.05$ or $P < 0.01$). The relative phospho-JNK level reached the peak at 15 min and returned to normal at 240 min. HKC cells were also incubated with 2.5, 5. or 10 mg/l AA for 30 min, respectively, and the activity of JNK was significantly increased ($P < 0.05$ or $P < 0.01$). The phosphorylated JNK levels gradually went up from exposure to 2.5–10.0 mg/l AA (Fig. 1B). The results suggest that JNK is activated by AA in dose- and time-dependent manners.

Effects of SP600125 on AA-upregulated TGF-β1 protein and gene expression. To determine the role of members of the MAPK family in AA-induced TGF-β1 synthesis, we examined whether their inhibitors (U0126 for ERK, SP600125 for JNK, and SB203580 for p38) could inhibit AA-induced TGF-β1 production. HKC cells were incubated in normal media or media containing 10 mg/l AA alone and 10 mg/l AA with 15 μmol/l U0126, SP600125, or SB203580 for 36 h, respectively. The TGF-β1 protein in the supernatant of cell culture was measured by ELISA, and it was significantly increased after 10 mg/l AA stimulation compared with that without AA stimulation ($P < 0.01$). AA-induced TGF-β1 production was significantly inhibited by exposure to 15 μmol/l SP600125 ($P < 0.01$), but not by U0126 and SB203580 ($P > 0.05$) (Fig. 2A).

To confirm the results of protein assays, the gene expression of TGF-β1 in HKC cells was also analyzed by relatively quantitative real-time RT-PCR. TGF-β1 mRNA expression was significantly upregulated after 10 mg/l AA stimulation for 12 h ($P < 0.01$), and the upregulated TGF-β1 mRNA expression was significantly inhibited by 15 μmol/l SP600125 ($P < 0.01$), but not by U0126 or SB203580 (Fig. 2B).

Effects of MAP3Ks and MAP2Ks on AA-induced JNK activation. To explore which members of MAP3Ks and MAP2Ks are involved in AA-induced JNK activation, HKC cells were transiently transfected with 2 μg kinase-inactive mutant of ASK1 (HA-ASK1-K709R), MEKK1 (HA-MEKK1-C-KM), MEKK4 (HA-MEKK4-C-KM), or TAK1 (HA-TAK1-K63W), respectively, and with 2 μg of pCDNA3.1 vector as a control. 2 μg kinase-inactive mutant of MKK4 (HA-MKK4-DN), MKK7 (HA-MKK7-DN), or vector control was transfected into HKC cells, respectively. After 24 h, HKC cells were stimulated with 10 mg/l AA for 30 min. Then, the cell lysates were analyzed by Western blotting and the relative phos-JNK levels were measured. Experiments were repeated 5 times independently. Values are means ± SD. *$P < 0.05$, **$P < 0.01$ compared with control group. #$P < 0.05$, ##$P < 0.01$ vs. AA alone group.

Fig. 2. Effects of SP600125 on AA-upregulated TGF-β1 protein and gene expression. HKC cells were incubated in normal media or media containing 10 mg/l AA alone or 10 mg/l AA with 15 μmol/l U0126, SP600125, or SB203580 for 36 h, respectively. The TGF-β1 protein in the supernatant of cell culture was measured by ELISA. B: after 12 h, cells were lysed and the mRNA expression of TGF-β1 and β-actin was analyzed by relative quantitative real-time RT-PCR. Experiments were repeated 6 times independently. Values are means ± SD. *$P < 0.05$, **$P < 0.01$ vs. control group. #$P < 0.05$, ##$P < 0.01$ vs. AA alone group.

Fig. 3. Effects of MAP3Ks and MAP2Ks on AA-induced JNK activation. A: HKC cells were transiently transfected with 2 μg kinase-inactive mutant of ASK1 (HA-ASK1-K709R), MEKK1 (HA-MEKK1-C-KM), MEKK4 (HA-MEKK4-C-KM), or TAK1 (HA-TAK1-K63W), respectively, and with 2 μg of pCDNA3.1 vector as a control. B: 2 μg kinase-inactive mutant of MKK4 (HA-MKK4-DN), MKK7 (HA-MKK7-DN), or vector control was transfected into HKC cells, respectively. After 24 h, HKC cells were stimulated with 10 mg/l AA for 30 min. Then, the cell lysates were analyzed by Western blotting and the relative phos-JNK levels were measured. Experiments were repeated 5 times independently. Values are means ± SD. *$P < 0.05$, **$P < 0.01$ compared with control group. #$P < 0.05$, ##$P < 0.01$ vs. AA alone group.
cells were separately transfected with HA-tagged kinase-inactive mutants of MEKK1, ASK1, or TAK1, and then JNK activity was measured by Western blot analysis.

Results showed the overexpression of kinase-inactive mutants of MEKK1, ASK1, or TAK1 did not inhibit AA-induced JNK activation (Fig. 3A), but kinase-inactive mutants of MEKK4, MKK4, or MKK7 reduced AA-induced JNK activation (P < 0.05 or P < 0.01) (Fig. 3A and B). These results suggest that MEKK4, MKK4, and MKK7 are necessary for AA-induced JNK activation.

Effects of MAP3Ks and MAP2Ks on AA-induced TGF-β1 protein production. To explore the effects of members of MAP3Ks and MAP2Ks on AA-induced TGF-β1 production, the TGF-β1 protein synthesis by HKC cells was examined with ELISA. Consistent with the results of JNK activation, AA-induced TGF-β1 protein synthesis was also significantly inhibited by kinase-inactive mutants of MEKK4, MKK4, or MKK7 (P < 0.05 or P < 0.01) (Fig. 4), but not by kinase-inactive mutants of MEKK1, ASK1, or TAK1.

Effects of AA on TGF-β1 promoter activity. It was examined whether AA could influence TGF-β1 promoter activity. The wild-type human TGF-β1 promoter (fragment −1292/−740) (Fig. 5A) was fused into the pGL3-basic vector to generate luciferase reporter gene pGL3-hTGF-1WT. As shown in Fig. 5B, upon AA stimulation, luciferase activity was significantly increased to 1.9–2.0-fold compared with control (P < 0.01). TGF-β1 EXPRESSION IN HKC

Fig. 4. Effects of MAP3Ks and MAP2Ks on AA-induced TGF-β1 protein production. HKC cells were transiently transfected with 2 μg kinase-inactive mutant of ASK1, MEKK1, MEKK4, TAK1, MKK4, MKK7, or vector control, respectively. After 24 h, HKC cells were treated with 10 mg/ml AA for 36 h. The culture media were collected and measured for TGF-β1 concentration by ELISA. Experiments were repeated 6 times independently. Values are means ± SD. **P < 0.01 compared with control group. #P < 0.05, ##P < 0.01 vs. control group.

Effects of AA on TGF-β1 promoter activity. To explore the possible role of the MAPK signaling pathway in AA-enhanced TGF-β1 promoter activity, it was also tested whether the inhibitors of members of the MAPK family (U0126 for ERK, SP600125 for JNK, and SB203580 for p38) could inhibit AA-induced TGF-β1 promoter activity. As shown in Fig. 6, the luciferase activity driven by the wild-type TGF-β1 promoter was significantly decreased in the 15 μmol/l SP600125 group (P < 0.01), but not in the U0126 and SB203580 groups, which suggests the JNK signaling pathway is involved in AA-induced TGF-β1 expression at the transcriptional level.

Effects of AA on AP-1 proteins. To study the changes in AP-1 proteins in HKC cells stimulated by 10 mg/l AA, Western blot analysis was performed. As shown in Fig. 7, the amount of c-Fos was significantly increased and reached the peak in cell extracts after incubation with AA for 6 h (P < 0.01). In addition, phospho-c-Jun and phospho-ATF-2 levels were also significantly increased in cell extracts after incubation with AA for 2 h (P < 0.05 or P < 0.01) and reached the peak after incubation with AA for 8 and 6 h, respectively.

DISCUSSION

The main results of this study in HKC were as follows. AA activated JNK in time and dose-dependent manners. SP600125 could inhibit AA-induced TGF-β1 mRNA expression and protein production. Overexpression of kinase-inactive mutants of MEKK4, MKK4, and MKK7, respectively, also inhibited JNK activation and TGF-β1 protein production. AA activated the TGF-β1 promoter through JNK- and AP-1-dependent pathways.
mechanisms. AA could upregulate expression of AP-1 components c-fos, phos-c-Jun, and phos-ATF-2.

Recently, several studies found that AA enters renal proximal tubular epithelial cells and plays its role through anion transporter OAT1/3 on the cell membrane (2, 27), but the intracellular signaling pathway which mediates AA-induced responses has not been fully understood. Our previous study showed that AA could directly upregulate expression of TGF-β1 and interstitial collagen in cultured proximal tubular epithelial cells (28), which might be critical in AA-induced renal interstitial fibrosis. Thus the principal purpose of this study was to explore the intercellular signaling pathways that are involved in AA-induced TGF-β1 expression.

The involvement of JNK in AA-induced TGF-β1 expression was confirmed by the following two independent results in this study: AA enhanced the phosphorylation of JNK; JNK inhibitor SP600125 downregulated TGF-β1 mRNA expression and protein production as well as TGF-β1 promoter activity induced by AA. After AA stimulation, phosphorylated JNK levels reached the maxima at 15 min and were reduced to the baseline level at 240 min. These data are consistent with those obtained with AA stimulation of the NRK52E cell line reported by Zhou et al. (31). In addition, Han et al. (11) reported that aldosterone-induced TGF-β1 expression in glomerular mesangial cells was regulated by ERK and JNK, and either ERK inhibitor PD98059 or JNK inhibitor SP600125 could inhibit TGF-β1 production. We also found activation of ERK in HKC after AA stimulation (data not shown), but blockade of ERK by...
U0126 was not able to inhibit AA-induced TGF-β1 production, suggesting that AA-activated ERK is not involved in the process of TGF-β1 production.

Our data showed that AA-induced JNK activation and TGF-β1 production could be inhibited by overexpression of kinase-inactive mutants of MEKK4, MKK4, or MKK7, suggesting MEKK4, MKK4, and MKK7 might be upstream of JNK in AA-induced TGF-β1 production. Recently, the roles of MEKK4, MKK7, and MKK4 in some cell responses were also reported. Sun et al. (23) reported that MEKK4 could stimulate JNK and p38 activation in the human prostate adenocarcinoma cell line after tumor necrosis factor-related apoptosis-inducing ligand treatment, which might contribute to cell apoptosis. Nakagawa et al. (19) reported MKK4 and MKK7 sequentially phosphorylated tyrosine and threonine residues of JNK, respectively, and phosphorylation of both residues would lead to synergistic JNK activation in stress-stimulated embryonic stem cells, which might play an important role in stress signaling. However, to our knowledge, no data about the role of MEKK4, MKK4, or MKK7 in TGF-β1 expression were reported previously.

Which transcription factors mediate the AA-induced transcription of the TGF-β1 gene? Actually, there are at least three different binding elements in the TGF-β1 promoter, which could bind to SP-1, AP-1, and NF-κB, respectively (14). The observation that AP-1 and NF-κB mediates TGF-β1 gene expression has been reported (15). Since c-Jun, a component of AP-1, is the substrate of JNK, it was assumed by us that AP-1 might mediate transcription of TGF-β1 as downstream of JNK. Using a luciferase reporter assay, we found AA increased activity of the wild-type TGF-β1 promoter, but not the AP-1 binding-deficient TGF-β1 promoter. In addition, we found that JNK inhibitor SP600125 significantly reduced the AA-enhanced TGF-β1 promoter activity of the wild-type TGF-β1 promoter. Thus it could be considered that AP-1 is the transcription factor which mediates the TGF-β1 transcription as downstream of JNK.

AP-1 is a dimeric complex that comprises Jun, Fos, and ATF proteins in mammalian cells (26). Our study showed that AA could increase the amount of the AP-1 factor c-fos and enhance the activity of the AP-1 factors c-Jun and ATF-2 by increasing their phosphorylation levels. After AA stimulation, phos-JNK, c-Fos, phos-ATF2, and phos-c-Jun were activated sequentially and reached maximal expression at 15 min, 6 h, 6 h, and 8 h, respectively. Protein expression of TGF-β1 was measured at 24 h. It seems that upregulation of phos-JNK is more rapid than that of c-Fos, phos-ATF2, or phos-c-Jun. The rapid activation of JNK has also been observed in other studies (9, 12). These results provided further evidence that AP-1 is involved in AA-induced, JNK-dependent TGF-β1 gene expression.

In conclusion, our results suggest that AA-induced TGF-β1 expression might be mainly mediated by the JNK signaling pathway; the upstream kinases of JNK including MEKK4, MKK4, and MKK7, and the downstream transcription factor of JNK, AP-1, all participate in this process (Fig. 8). Our above data reveal a link between AA stimulation and the enhanced expression of the fibrogenic cytokine TGF-β1 on a molecular basis, providing an improved insight into the pathogenetic mechanism of the development of CAAN.

REFERENCES


