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NF-κB-activated tissue transglutaminase is involved in ethanol-induced hepatic injury and the possible role of propolis in preventing fibrogenesis

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Abstract

The increased expression and cross-linking activity of tissue transglutaminase (tTG) have been demonstrated in acute liver injury and fibrosis. We focused on the molecular mechanisms that contribute to ethanol-induced tTG expression and investigated the efficacy of propolis components in preventing both the tTG expression *in vitro* and fibrogenesis *in vivo*. We demonstrate herein that both ERK1/2 and PI3K/Akt pathways can regulate the effects of ethanol on NF-κB-dependent transcription and these signaling pathways may be involved in activation of ethanol-mediated tTG expression. We also found that administration of pinocembrin (PIN), one of the major components of propolis, inhibited tTG activation and significantly prevented the development of thioacetamide (TAA)-induced liver cirrhosis. The present study suggests that tTG may be an important member of the cascade of factors necessary for ethanol-induced liver fibrogenesis and PIN could serve as an anti-fibrogenic agent.

Keywords: Tissue transglutaminase; NF-κB; Ethanol; Fibrogenesis; Propolis

1. Introduction

Chronic injury leading to fibrosis in liver occurs in response to a variety of insults, including viral hepatitis (especially hepatitis B and C), alcohol abuse, drugs, and other factors (Friedman, 1998). Liver fibrosis, the formation of scar-like tissue in response to liver damage, is reversible, whereas cirrhosis, the end stage consequence of fibrosis, is generally irreversible. Thus, efforts to understand fibrosis focus primarily on events that lead to the early accumulation of scarring, in hopes of identifying therapeutic targets to slow its progression (Bataller and Brenner, 2005; Friedman, 2000). The final common pathway in all types of liver fibrosis, regardless of etiology, is the increased deposition of extracellular matrix (ECM) proteins, such as collagens, proteoglycans, fibronectin, and a variety of glycoproteins (Friedman, 1993). One factor that may prove to be significant in the process of liver fibrogenesis is tissue transglutaminase (tTG) (Mirza et al., 1997). Transglutaminase exists in both extracellular and intracellular forms and has been detected in many different kinds of cells, body fluids and tissues (Greenberg et al., 1991). The tTG is a member of the transglutaminase family that is involved in the cross-linking of ECM protein (Martinez et al., 1994; Upchurch et al., 1987). Previous reports demonstrated that the cross-linking activity of tTG is increased in humans with acute liver injury and in model systems of liver injury and fibrosis (Mirza et al., 1997; Wu et al., 2000).

Abbreviations: tTG, tissue transglutaminase; TAA, thioacetamide; ECM, extracellular matrix; NF-κB, nuclear factor-κB; ERK1/2, extracellular signal regulated kinase 1/2; MAPK, mitogen-activated protein kinase; PI3K/Akt, phosphatidylinositol 3-kinase/amino kinase terminal; LCM, Laser capture microdissection; RT-PCR, reverse transcriptase-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation analysis; PIN, pinocembrin.

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Alcoholic liver injury is a complex process involving several injury mechanisms and multiple cellular targets (Nanji and Zakim, 1996). Ethanol oxidation and ethanol-induced injury occur in hepatocytes; thus, determining the mechanism by which ethanol primes and sensitizes hepatocytes is of primary importance (Tsukamoto and Lu, 2001). It is well established that ethanol exposure inhibits hepatocyte regeneration in *in vitro* and *in vivo* systems (Carter and Wands, 1985; Diehl et al., 1988). Although some associations have been found, the mechanism by which this inhibition occurs has not been delineated. One proposed hypothesis is that ethanol decreases putrescine levels in the liver and that putrescine administration abrogates this inhibition of liver regeneration by ethanol administration (Diehl et al., 1990). The mechanism by which putrescine inhibits hepatocyte regeneration may be through its known action as a competitive substrate inhibitor of tTG activity (Wu et al., 2000). However, the detailed molecular regulation responsible for ethanol-induced tTG gene expression has never been reported.

Nuclear factor-κB (NF-κB) is a multisubunit transcription factor that can rapidly activate the expression of genes involved in inflammatory, immune, and acute phase responses (Baeuerle, 1991). In most cells, including "unstimulated" hepatocytes, NF-κB is inactive (under normal conditions it is bound to inhibitory I-κB proteins that mask a nuclear localization signal) and retained in the cytosol (Freedman et al., 1992; Perkins, 2000). Once activated, the complex enters the nucleus and binds to its consensus sequence, thus activating its downstream genes. A number of proteins are involved in cell signaling, including members of the Src kinase family and G-proteins. Members of this kinase family are found to be activated by a wide variety of agents via a cascade of kinase/effector molecules, which include extracellular signal regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) (Robinson and Cobb, 1997; Wu and Cederbaum, 2003). Members of the phosphatidylinositol 3-kinase/amino kinase terminal (PI3K/Akt) are also involved in the transduction of extracellular signals (Cantley, 2002; Toker and Cantley, 1997). Kinases in the PI3K/Akt and p38 MAPK pathways have been reported to act in the regulation of nuclear translocation of NF-κB (Madrid et al., 2001; Sizemore et al., 1999).

In this study, we focused on the molecular mechanisms that contribute to ethanol-induced tTG expression in human hepatoma cell lines and present a novel therapeutic strategy to attenuate hepatic fibrosis. The efficacy of several natural compounds was tested in preventing the tTG expression *in vitro* and the thioacetamide (TAA)-induced liver fibrogenesis (Muller et al., 1988) *in vivo*. We demonstrate herein that both ERK1/2 and PI3K/Akt pathways can regulate the effects of ethanol on NF-κB-dependent transcription, and these signaling pathways may be involved in activation of ethanol-mediated gene expression of tTG. We also demonstrated that PIN, one of the major components of propolis, inhibited tTG activation, and that administration of propolis prevented the development of TAA-induced liver cirrhosis.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade. Pinocembrin (PIN) was from Sigma Chemical Company (St. Louis, MO, USA). Protease inhibitors (phenylmethyl sulfonyl fluoride (PMSF), pepstatin A, leupeptin, and aprotinin) were acquired from Sigma–Aldrich, Inc. (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin solution, and fungizone were purchased from Gibco-Life Technologies (Paisley, UK). The chemical inhibitors were obtained from various sources as indicated: The NF-κB (TLCK), ERK1/2 (PD98059), and the PI3K (LY294002) inhibitors were from Calbiochem (San Diego, CA, USA). The tea polyphenollic compounds were gifts from Dr. Lin, J.K. The following monoclonal antibodies were obtained from various sources as indicated: anti-p38, anti-p-p38, anti-NFκB, anti- AKT and anti-p-AKT, anti-GAPDH monoclonal antibodies (Santa Cruz Biotechnology, CA), anti-ERK and anti-p-ERK monoclonal antibodies (Transduction Laboratories, Lexington, KY).

2.2. Cell lines and cell culture

The Hep 3B and Hep G2 cell lines were derived from human hepatocellular carcinoma (ATCC HB-8064 and HB-8065) (Knowles et al., 1980). In Hep 3B cells, the p53 gene was partially deleted (Bressac et al., 1990). The cell lines were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 50 μg/mL gentamycin and 0.3 mg/mL glutamine in a humidified incubator (37 °C, 5% CO_2).

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The RT-PCR assays for tTG gene expression were performed as described below. The Hep 3B and Hep G2 cells were treated with 10–100 mM ethanol for the indicated time points. The cells were harvested separately after ethanol treatment. Total RNAs were isolated from cells using Trizol reagent as recommended by manufacturer (Life Technologies, Inc.). The cDNA was amplified from 1 μg of total RNA using a SuperScript one-step RT-PCR with platinum Taq system (Life Technologies, Inc.). PCR was conducted for 30 cycles in thermal controller. Primers used for amplification were as follows: tTG-specific primer, tTG-f 5′-TGAATAGTGACAAGGTGTACTGGCA-3′ and tTG-r 5′-GTGGCCTGAGACATTGAGCAGCAT-3′. The GAPDH specific PCR products from the same RNA samples were amplified and served as internal controls. Primers GAPDH-f 5′-ACCACAGTCCATGCCATCAC-3′ and GAPDH-r 5′-TCCACCACCCTGTTGCTGTA-3′ were used for amplification of GAPDH. Each amplification cycle consisted of 0.5 min at 94 °C for denaturation, 0.5 min at 55 °C for primer annealing, and 1 min at 72 °C for extension. In all of the amplification procedures, we included reverse transcriptase-free control assays consisting of the amplification mixture, the RNA sample, and distilled water in place of reverse transcriptase to check for possible contamination of the RNA samples with DNA. After PCR amplification, the fragments were stained with ethidium bromide and analyzed by agarose gel electrophoresis.

2.4. Western blotting analysis

Western blotting analysis was performed as described previously (Ho et al., 2001). Briefly, cell lysates were prepared, electrotransferred, immunoblotted with antibodies, and then visualized by incubating with the colorigenic substrates (nitroblue tetrazolium, NBT and 5-bromo-4-chloro-3-indolyl phosphate, BCIP) (Sigma Chemical Co., St. Louis, MO). The expression of either GAPDH was used as control for equal protein loading.

2.5. Immunofluorescence assay

Hep 3B and Hep G2 cells were incubated in 0.05% DMSO (control), or treated with ethanol (10 mM) for the indicated time points. Cells were immunostained with 1:100 diluted monoclonal anti-NF-κB antibody (Santa Cruz, CA,

USA) for 2 h. After washing with PBS/Tween 20 for three times, monoclonal antibody was visualized with goat-anti-mouse IgG antibody labeled with FITC (green) for 1 h. Propidium iodide at concentration of 100 ng/ml for 5 min was used for nuclear staining. Stained cells were imaged by confocal scanning microscopy (Olympus, Tokyo, Japan) using excitation/emission wavelengths of 458 nm/488 nm and 543 nm/633 nm for FITC and MitoTracker/propidium iodide, respectively.

2.6. Electrophoretic mobility shift assay (EMSA) (Uen et al., 2007)

The double-stranded DNA probe used in the experiment contained the tTG promoter 5′-AGTTGAG-GGGACTTTCCCAGG-3′ with NF-κB consensus binding site. The radiolabeled DNA (4 ng, 100,000 cpm) was incubated with nuclear extract in 15 μl of binding buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 200 mM NaCl, 1 μg probe DNA) on ice for 5 min. The samples were electrophoresed in a 5% polyacrylamide gel at room temperature for 3 h at 130 V. The gel was then dried on Whatman 3 M paper and exposed to Fuji X-ray films at −70 °C.

2.7. Preparation of nuclear and cytoplasmic fractions

Nuclear and cytoplasmic fractions from control (DMSO-treated) and ethanol-treated Hep 3B cells were prepared as described previously (Singh et al., 2004). Briefly, cells were harvested by scraping and rinsed twice in ice-cold PBS. The cells were then swollen in ice-cold hypotonic lysis buffer (20 mM HEPES, pH 7.1, 5 mM KCl, 1 mM MgCl2, 10 mM *N*-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 2 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml antipain) for 10 min. The cells were lysed by 20 strokes in a Dounce homogenizer, and the nuclei were cleared by centrifugation (400 × *g*, 10 min). After this step, the supernatant (cytosolic fraction) was concentrated and stored at −80 °C. The nuclear extract was prepared using the same lysis buffer and stored at −80 °C prior to Western blot analysis. The blot was stripped and reprobed with β-actin or PCNA antibody to ensure equal protein loading as well as to rule out cross contamination of cytoplasmic and nuclear fractions (data not shown).

2.8. Chromatin immunoprecipitation analysis (ChIP)

ChIP assays of cultured cells were performed as described previously (Wilkinson et al., 2005). Briefly, the cells were fixed with finial concentration of 1% formaldehyde directly to cell culture media at 25 °C for 15 min. Stop the crosslinking reaction with 0.125 M glycine for 5 min and collected the cells into a new tube. The cell lysates were sonicated three times with 10-S bursts to yield DNA fragments around 1000 bp in size. The NF-κB specific antibody (Santa Cruz Biotechnology Inc) was used for the immunoprecipitation reactions. Primers specific for detection of the NF-κB binding motifs of the tTG gene were adapted with sense primer 5′-GCATTGAGACCCTCCTCA-3′ and anti-sense primer 5′-CAAAGCGGGCTATAAGTTAG-3′ for 32 cycles. The PCR products were then proceed with agarose gel analysis.

2.9. Preparation of propolis soluble extracts (Quiroga et al., 2006)

Propolis was gathered from colonies of *Apis mellifera*, hand gathered in March 2005 from an apiary located at 2000 m above sea level in Amaicha del Valle, Tucumaĭn, Argentina and stored at 4 °C in the dark. The sample was cut into small pieces after cooling at −20 °C and extracted with 96% ethanol (1 g of propolis per 10 ml of 96% ethanol). This suspension was kept at room temperature for a period of 5 days in the dark with occasional shaking and centrifuged at 15,000 × *g* for 15 min to eliminate ethanol insoluble substances. Then, the mixture was frozen at −20 °C for 2 h and centrifuged at 15,000 × *g* for 15 min to discard waxes and gums. The solvent of the supernatant was evaporated under reduced pressure at 40 °C in a rotary evaporator until constant weight. Residue was dissolved in 96% ethanol up to a concentration of 63.6 mg/ml (w/v). The obtained brownish but transparent preparation was named partially purified propolis extract (PPPE). It was stored at 4 °C in the dark until it was determined for HPLC analysis.

2.10. Reverse-phase HPLC analysis of propolis polyphenols and PIN

The PPPE was filtered for analysis by HPLC. In all operations, an IB-SIL RP 18 column (5 μm, 250 mm × 4.6 mm, Phenomenex) equipped with a Gilson 118 UV detector was used. An aliquot was injected via a Rheodyne valve fitted with a 20-μl loop. The mobile phase was acetonitrile and 0.1% trifluoracetic acid (45%:55%, v/v), and the flow rate was 1 mL/min. Detection was achieved at 290 nm. Reference compounds commercially obtained were co-chromatographed with the experimental sample to confirm the HPLC retention time. UV–vis absorption spectra were compared with literature data and analytical grade standards (Quiroga et al., 2006).

2.11. In vivo TAA-induced liver cirrhosis model

For the induction of liver cirrhosis, rats were given intraperitoneal (i.p.) injections of TAA (200 mg/kg) twice a week for 12 weeks (Hori et al., 1993). Two groups of ten rats each were exposed to (a) TAA (200 mg/kg) alone for 12 weeks; or (b) TAA (200 mg/kg) plus 0.3% propolis diet supplement for 12 weeks. A third group received only the normal diet and served as control. Percentage of fibrosis in the liver tissue was determined using a computer assisted automated image analyzer by analyzing 20 random fields per slide and calculating the ratio of connective tissue to the whole liver area.

2.12. Transglutaminase activity assay

The activity of tTG in cell homogenates was measured by the incorporation of ^{14}C-labeled putrescine (Amersham Biosciences, Buckinghamshire, UK) into *N*,*N*′-dimethylcasein as previously described by Lorand et al. (1972). One unit of tTG activity equals to 1 nmol of putrescine incorporated per h.

2.13. Statistics

Statistical analysis was carried out using analysis of variance (ANOVA)—one-way analysis of variance with Student–Newman correction, and the Student's *t*-test. Significance was assumed for values of $P < 0.05$.

3. Results

3.1. Ethanol-induced tTG mRNA expression in Hep G2/Hep 3B cell lines

RT-PCR analysis was performed for detection of the mRNA expression levels of tTG treated with ethanol in two human liver cancer cell lines, Hep G2 and Hep 3B. As shown in Fig. 1A, ethanol (10 mM) induced a significant increase of tTG mRNA level in Hep 3B cells at 5 h and then decreased at 10 h. However, no change of tTG gene expression in Hep G2 cells could be found with the same treatment. When the exposure time was extended to 24 h, a dose-dependent effect of ethanol (1–100 mM) on the tTG gene expression in Hep 3B cells was found (Fig. 1B). To mimic the long-term effect of ethanol consumption on tTG gene expression in human liver cancer cells, the Hep 3B cells were treated with 10 mM ethanol in the cultured medium repeatedly. Different passages (p10–p50) were verified for tTG mRNA levels (Fig. 1C). The results indicated that with longer passage times, higher background levels of the tTG gene were detected (Fig. 1C). In addition, the tTG gene expression could be further induced by 10 mM ethanol in the long-term treated Hep 3B cells.

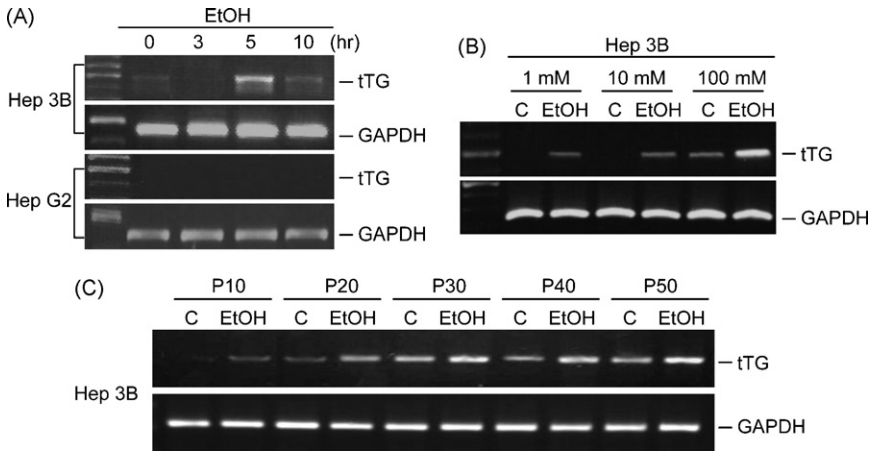

Fig. 1. Dose- and time-dependent effects of ethanol on the expression of tTG in human hepatocellular carcinoma cells. (A) Human Hep G2 and Hep 3B cells were treated with ethanol (10 mM) time-dependently. RT-PCR analysis was performed for detection of the expression levels of tTG and GAPDH as described in Section 2. (B) Human Hep 3B cells were treated for 24 h with ethanol in a dose-dependent manner. RT-PCR analysis was performed for detection of the expression levels of tTG and GAPDH. (C) Different passages (from p10–p50) of human Hep 3B cells were treated with 10 mM ethanol. RT-PCR analysis was performed for detection of the expression levels of tTG and GAPDH. The ethanol (10 mM)-treated Hep 3B cells were harvested from the dishes with trypsin-EDTA and defined as one passage.

3.2. Comparison of the effects of ethanol-induced NF-κB activation and tTG gene expression in Hep G2/Hep 3B cell lines

NF-κB is present in the cytoplasm in an inactive form, which is activated by many environmental perturbations. Once activated, the complex enters the nucleus and binds to its consensus sequence, thus activating a number of genes. Because NF-κB plays an important role in regulating a variety range of cell functions, its biochemical roles in Hep G2/Hep 3B cells treated with ethanol were determined. The Western blot in Fig. 2A shows that in Hep 3B cells under treatment with 10 mM ethanol for 15–90 min, the NF-κB proteins were transferring from cytoplasm to nucleus at 30 and 60 min. To determine the effect of ethanol on NF-κB/DNA binding activity, nuclear extracts were prepared from DMSO- and ethanol-treated Hep 3B cells for 60 min. Chromatin immunoprecipitation was performed by using the NF-κB specific antibody to precipitate the NF-κB-associated DNA complex sequence. Increased binding of NF-κB to the tTG promoter was detected by PCR analysis in the ethanol-treated Hep 3B cells (Fig. 2B, lanes 3–5). Confocal microscopic analysis further confirmed that NF-κB proteins are expressed in the nucleus of Hep 3B cells treated with ethanol (10 mM) for 60 min (Fig. 2C, left) whereas the same treatment failed to induce the translocation of NF-κB in Hep G2 cells (Fig. 2C, right).

To further prove NF-κB is responsible for tTG expression in Hep 3B cells, the nuclear extracts were prepared from ethanol-treated cells, and the NF-κB transcriptional activity was detected by EMSA. The results indicated an increase of NF-κB transcriptional activity in Hep 3B, but not in Hep G2 cells treated with ethanol (Fig. 2D). Administration of TLCK, an NF-κB inhibitor, showed a significant decrease of the tTG expression in Hep 3B cells treated with ethanol (Fig. 2E).

3.3. Mitogenic signaling was related to ethanol-induced NF-κB activation in Hep 3B cells

To dissect the signaling pathway involved in NF-κB activation, the mitogenic signalings including PI3K/AKT, ERK1/2 and p38MAPK pathways in ethanol-treated Hep 3B cells were examined by immunoblotting assay using antibodies specific for the phosphorylated (active form) AKT, ERK1/2, or p38. Fig. 3A and B demonstrate that both ERK1/2 and AKT were activated 5–30 min after ethanol treatment. Ethanol (10 mM) rapidly increased the phosphorylation of ERK1/2 time-dependently whereas total ERK1/2 proteins were not altered in the Hep 3B cells. Phosphorylation of AKT could also be seen at 5 and 10 min after ethanol treatment. However, ethanol did not affect the phosphorylation of p38 in Hep 3B cells. To further scrutinize the kinases involved in NF-κB activation, specific inhibitors of ERK1/2 (PD98059) and AKT (LY294002) were used. Pretreatment of Hep 3B cells with LY294002 and PD98059 resulted in an inhibition of the phosphorylated ERK1/2 levels and the nucleus translocation of NF-κB induced by ethanol (Fig. 3C). We also demonstrated that administration of PIN, one of the major components of propolis, significantly inhibited the ERK1/2 phosphorylation treated with ethanol (Fig. 3D).

3.4. Polyphenolic compounds inhibit tTG mRNA expression and enzyme activity induced by ethanol

Polyphenolic compounds included EGCG, PIN and Myricetin were tested for their efficacy in preventing the tTG mRNA expression induced by ethanol. Our results demonstrated that PIN possesses the strongest inhibition effect than EGCG and myricetin in the Hep 3B cells (Fig. 4B). The tTG enzyme activity was assayed based on the putrescine incorporation as described in Section 2. When Hep 3B cells were treated with 10 mM ethanol for 3–24 h, the tTG enzyme activity reached a maximum at 9 h and returned to the control level at 15 h (Fig. 4C).

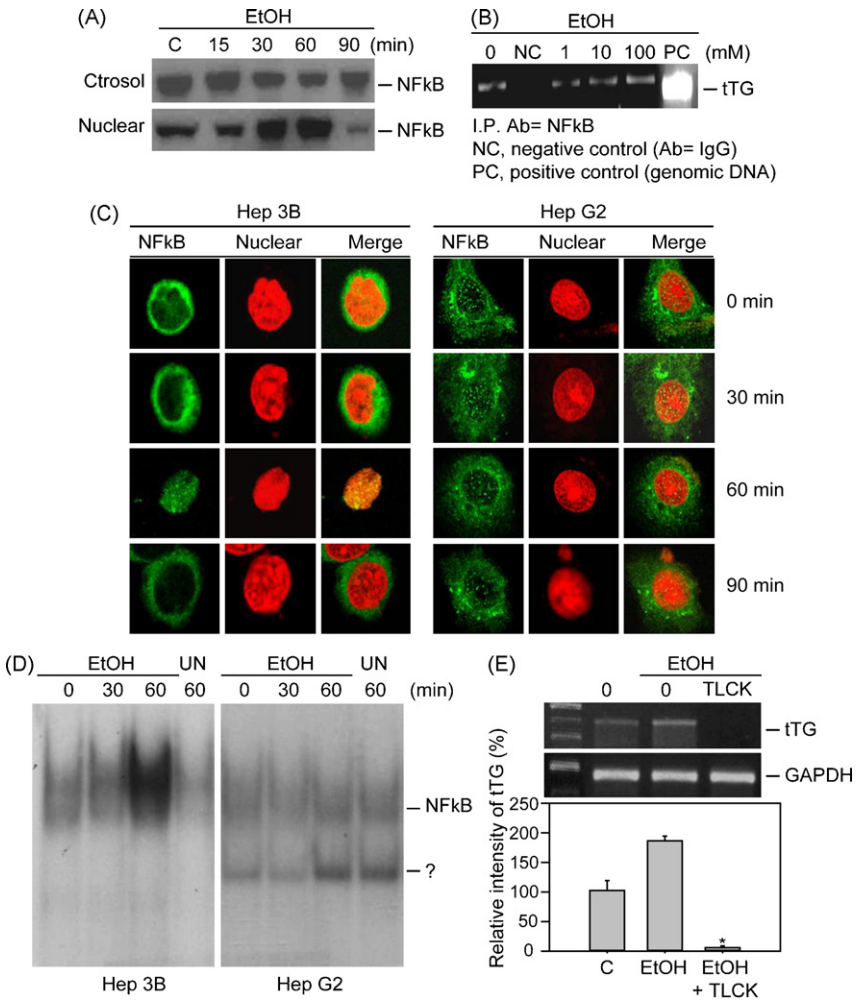

Fig. 2. NF-κB was preferentially activated in Hep 3B cells in response to ethanol treatment. (A) Subcellular fractionation was performed in human Hep 3B cells exposed to ethanol (10 mM) for 15–90 min. The NF-κB expression levels in the nuclear and cytoplasmic fractions were then determined by immunoblotting analysis. Blots were stripped and reprobed with anti-β-actin and anti-PCNA antibodies to normalize for equal protein loading as well as to rule out cross contamination of the nuclear and cytoplasmic fractions (data not shown). (B) For the ChIP assay, Hep 3B cells were treated with ethanol (1–100 mM) for 24 h. Nuclear extracts were sorted, sonicated, and then immunoprecipitated with antibody specific against NF-κB. The precipitated protein-DNA complex was then isolated and PCR was performed to identify tTG promoter sequence as described in Section 2. The PCR products were observed by agarose gel analysis. Shown is a representative ChIP of two experiments with similar results. NC: non-immunized immunoglobulin was used as a negative control, PC: genomic DNA was used as a template for tTG promoter and PCR reaction was performed as a positive control. (C) Confocal microscopic analysis for nuclear/cytoplasmic distribution of NF-κB in ethanol-treated Hep 3B (left panels) and Hep G2 cells (right panels). Cells were treated with ethanol (10 mM) for the indicated time points and then stained with anti-NF-κB antibody (green) or P.I. (red). (D) Human Hep G2 and Hep 3B cells were treated with ethanol (10 mM) for the indicated time points. Preparation of the nuclear extracts and electrophoretic mobility gel shift assay were described in Section 2. Increased DNA binding activity between NF-κB protein and its consensus binding site with tTG promoter DNA probe were observed in the Hep 3B (left) cells but not in the Hep G2 (right) cells. (E) The Hep 3B cells were treated with ethanol (10 mM) for 5 h in the presence or absence of the NF-κB specific inhibitor (20 μM TLCK pretreated for 1 h). Cells were then harvested and RT-PCR analysis was performed for observation of the tTG and GAPDH expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Thus, 9 h was chosen as the optimal time point for evaluation of the tTG enzyme activity after ethanol treatment. The inhibition effects of PIN on ethanol-induced tTG enzyme activity were further tested, and it was found that 10 μM PIN inhibited the tTG enzyme activity induced by 10 mM ethanol (Fig. 4D). In addition, pretreatment of the specific inhibitors of ERK1/2 (PD98059), AKT (LY294002) and NF-κB (TLCK) to the Hep 3B cells also showed an inhibition effect on ethanol-induced tTG enzyme activity, indicating that these signaling pathways are involved in the ethanol-induced tTG enzyme activity (Fig. 4D).

3.5. Prevention of chemical-induced hepatic fibrosis in rat by propolis

To determine whether the PIN was present in the propolis samples which were found to have adapted in the animal experiment, HPLC analysis was performed in which the PIN was administered as a standard compound. The HPLC profile demonstrated that the elution time at 18.80 min was represented as a PIN peak in each analysis (Fig. 5A, upper). The supposed PIN peak was detected in the propolis extracts (Fig. 5A, middle). The results shown in Fig. 5A (lower) revealed that the highest peak

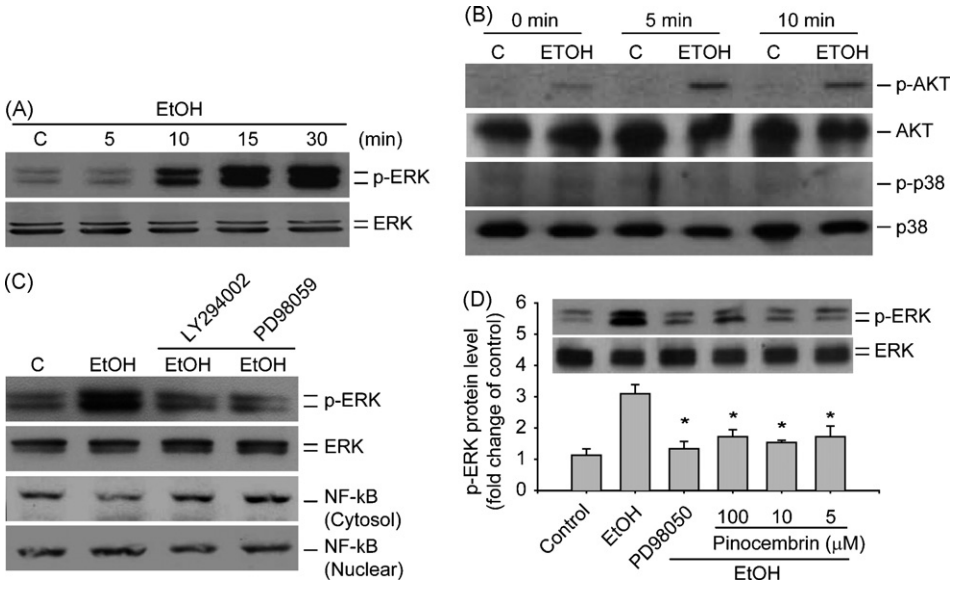

Fig. 3. Ethanol-induced NF-κB activation was attenuated by PIN through inhibition of the AKT and ERK pathways. (A and B) Human Hep 3B cells were treated with ethanol (10 mM) for the indicated time points. After treatment, the cells were harvested and immunoblotting analysis was performed. The protein expression levels of (A), ERK/p-ERK and (B), AKT/p-AKT and p38/p-p38 were determined, respectively. (C), Human Hep 3B cells were pretreated with LY294002 (50 μM) or PD98059 (10 μM) for 1 h. After treatment, the cells were then treated with 10 mM ethanol for an additional 30 min. The cells were harvested and the nuclear and cytosolic fractions were isolated for protein extraction and the levels of the NF-κB were determined by immunoblotting analysis. The total protein lysates were also harvested for ERK/p-ERK detection. (D), Human Hep 3B cells were pretreated with LY294002 (50 μM), PD98059 (10 μM) or PIN (5–100 μM) for 1 h. After treatment, the cells were then treated with 10 mM ethanol for additional 30 min. The cells were harvested and the protein levels of the ERK/p-ERK were detected by immunoblotting analysis.

was PIN co-injected with reagent standard and propolis extracts. The concentration of PIN was calculated and demonstrated that the percentage of PIN in total propolis compounds was 0.16% (w/w).

The potency of propolis was tested in preventing hepatic fibrosis induced in rats chronically treated with TAA. Fibrosis and/or cirrhosis were observed in all animals treated with TAA. Fig. 5B shows the gross appearance of the representative liver in animals treated with vehicle or TAA. There was a significant decrease of cirrhosis incidence in the propolis administration group. Fig. 5C shows the H.E. staining for gross morphology observation and the specific Masson staining for determination of specific fibrotic region in liver tissues. The administration of TAA for 12 weeks induced extensive micro- and macronodular cirrhosis with signs of cell necrosis in hepatocytes. Propolis administration drastically reduced the histological signs of cell damage (Fig. 5C, green arrowheads). When histologic features were analyzed, a 25% fibrosis index was found in the livers of TAA-treated animals, whereas only a 14% fibrosis index was found in the propolis combined TAA group (Fig. 5D).

4. Discussions

Alcohol abuse accounts for more than half of the prevalence of liver fibrosis and cirrhosis in the western world. Fibrosis of the liver is a wound-healing process that occurs in response to persistent hepatocellular injury (Siegmund and Brenner, 2005). One of the important functions of tTG is to catalyze the covalent cross-linking of ECM proteins. There is evidence suggesting that tTG may be involved in the specialized processing of matrix that occurs during wound healing and other remodeling processes (Griffin et al., 1979; Upchurch et al., 1991). In this study, we describe the initial investigation of detail molecular mechanisms of ethanol-related tTG expression and its association in fibrotic liver disease. We also found that tTG gene expression is markedly enhanced in microdissected human hepatocellular carcinoma cells by laser capture microdissection from tumor tissue compared with normal liver cells (data not shown). Additionally, our results also demonstrate that the expression levels of tTG could be significantly induced by acute or long-term treatment with ethanol in human liver cancer cell line Hep 3B (Fig. 1). Similar results were reported in a previous study showing that ethanol treatment led to enhanced tTG cross-linking activity (Wu et al., 2000). These findings implied an important role of tTG in the pathogenesis of alcoholic liver injury. Although the precise function of tTG in the process of ethanol-related fibrogenesis has not been delineated by this study, several of its already established actions, such as TGF-β activation and inhibition of hepatocyte proliferation, may be relevant to the pathogenesis of alcoholic liver fibrosis (Kojima et al., 1993; Wu et al., 2000).

It has been shown that tTG can be induced directly by NF-κB activation in liver cells because the tTG promoter has an NF-κB binding motif (Mirza et al., 1997). However, NF-κB binding does not appear to be the only factor that causes enhanced tTG gene expression. Not surprisingly, a number of cytokines associated with hepatic injury also appear to activate the expression of the tTG gene (Nanji et al., 1999). The exact causal relationship between activation of NF-κB and alcoholic liver injury remains speculative, but a possible mechanism for such a relationship is

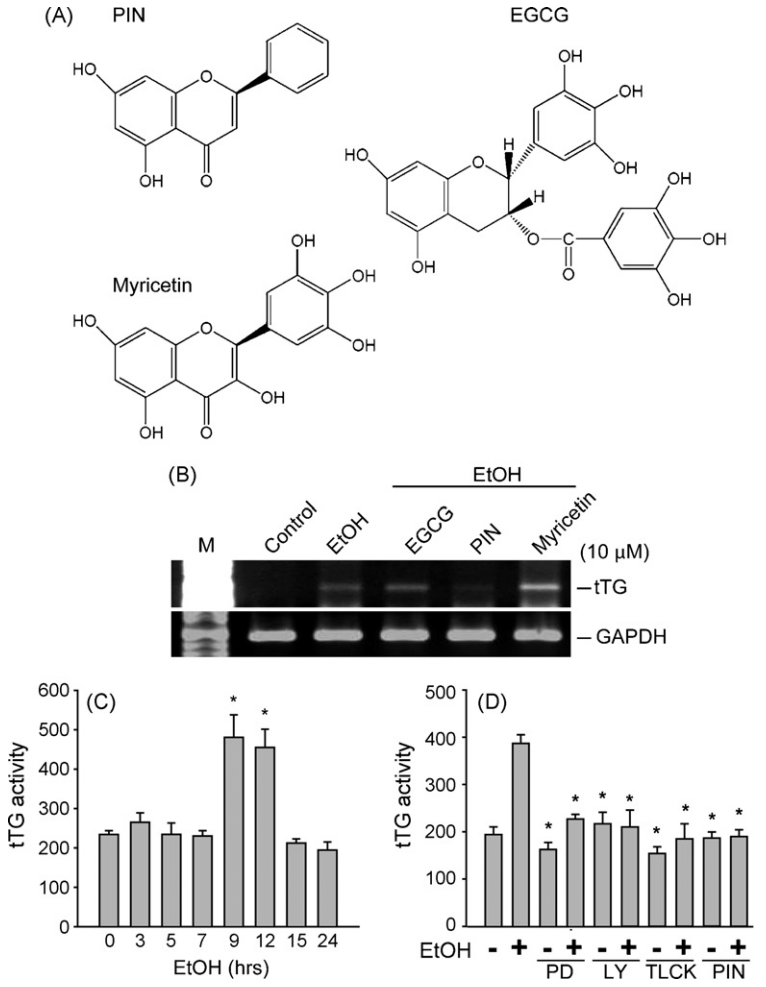

Fig. 4. Polyphenolic compounds inhibit the ethanol-induced tTG mRNA expression and its enzyme activity. (A) Chemical structures of PIN, EGCG and myricetin. (B) Human Hep 3B cells were pretreated with EGCG, PIN and myricetin (10 μM each) for 1 h. After treatment, the cells were then treated with 10 mM ethanol for additional 5 h. The cells were harvested and the mRNA levels of the tTG and GAPDH were determined by RT-PCR analysis. (C) Human Hep 3B cells were treated with ethanol (10 mM) in a time-dependent manner. The tTG enzyme activity was determined as described in Section 2 and represented as μmole of [^{3}H] putrescine incorporated/μg protein/min. (D) Human Hep 3B cells were pretreated with PIN (10 μM), TLCK (20 μM), LY294002 (50 μM), or PD98059 (10 μM) for 1 h. After treatment, the cells were then treated with 10 mM ethanol for an additional 9 h. The cells were then harvested and the tTG activity was determined as described in Section 2. PD, PD98059; LY, LY294002; PIN.

that NF-κB, acting as an intermediate, increases the expression of cytokines and fibrosis-related genes. Our data suggest that nuclear translocation of the transcription factor NF-κB is associated with the tTG expression in Hep 3B liver cells exposed to ethanol. Activation of NF-κB occurs secondarily to the proteolytic degradation of IkBα, allowing free NF-κB to translocate to the nucleus and initiate gene transcription (Baeuerle and Baltimore, 1996; May and Ghosh, 1997). The present results are similar to those in another report (Roman et al., 2000), which showed that activation of NF-κB could be found in hepatoma cells treated with acetaldehyde, the major metabolite of ethanol. Additionally, activation of NF-κB and increased expression of proinflammatory cytokines could also be found in experimental alcoholic liver disease in the rat (Nanji et al., 1999).

Different signaling pathways appear to mediate both overlapping and distinct effects in the activation of both PI3K/Akt and the MAPK cascades (Cantley, 2002), contributing to the stimulation of NF-κB-dependent transcription in hepatic cells. It was demonstrated that PI3K/Akt can stimulate the transcriptional activity of NF-κB by targeting the basal levels of nuclear NF-κB (Sizemore et al., 1999). In addition, the p38 MAPK and ERK1/2 pathways were suggested to be required for NF-κB activation (Madrid et al., 2001). In the present study, we demonstrate that exposure of cultured Hep 3B cells to ethanol can markedly, and in a time-dependent manner, activate the p-ERK and PI3K/Akt pathways, whereas the p38 MAPK was not involved in ethanol-mediated cell activation. Furthermore, we used pharmacological inhibitors of ERK1/2 and PI3K/Akt to demonstrate that ERK1/2 and PI3K/Akt signaling pathways play a critical role in the regulation of ethanol-induced activation of NF-κB. Indeed, ethanol-induced NF-κB transcriptional activity in Hep 3B cells was inhibited by LY294002, an inhibitor of PI3K/Akt. We also provide evidence that the ERK1/2 inhibitor PD98059 significantly attenuated the ethanol-induced NF-κB activity (Fig. 3). The relationship between signaling pathways, including PI3K/Akt, ERK1/2 and NF-κB activation, remains

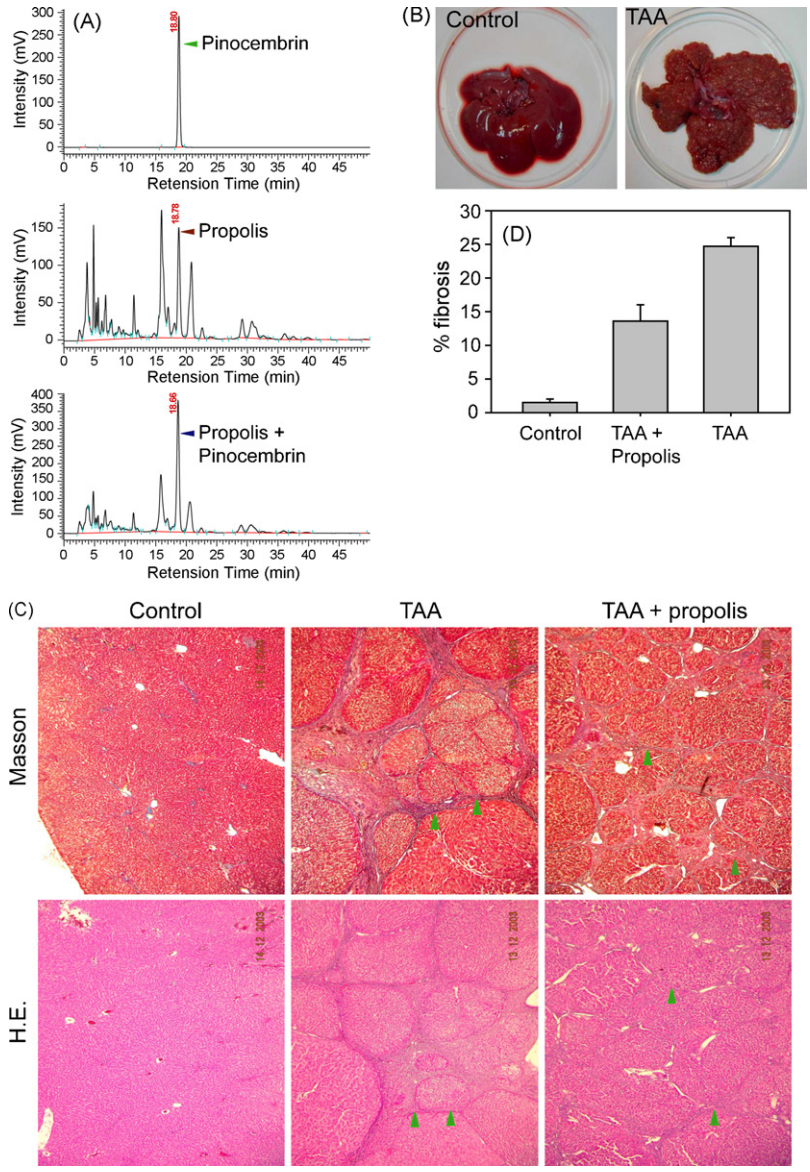

Fig. 5. Propolis components inhibit chemical-induced liver fibrosis *in vivo*. (A) Isocratic HPLC separation of PIN in propolis ethanol extracts. The following sample solutions were analyzed: top: the authentic standard compound (PIN, 10 ng) was injected into HPLC system and detected with a UV detector (290 nm). The elution time of the PIN was detected at 18.80 min in each analysis. Middle: the propolis extracts (10 μl) were injected into HPLC system and detected with UV 290 nm. The suspected elution time of PIN in propolis extracts was detected at 18.78 min. Bottom: the propolis extract was coinjected with PIN standard compound and the highest peak shown here was detected using a UV detector set at 290 nm. The quantitative analysis demonstrated that the PIN comprises 0.16% of the total propolis extracts (data not shown). (B) The gross appearance of the representative liver in animals treated with vehicle (left) or TAA (right). (C) The liver tissues were cut into 5–7 μm thick slices and serial sections were stained with H.E. (bottom) for gross morphology observation, or with the specific Masson staining method for determination of specific fibrotic region in liver tissues. (D) Percentage of fibrosis ratio, as obtained by computerized image analysis of multiple liver sections from all animals with different treatments.

poorly understood and may differ between cell types and in response to different stimuli (Heck et al., 1999; Karin and Ben-Neriah, 2000; Madrid et al., 2000). Moreover, these pathways may act sequentially and may also demonstrate cross-talk (Carter and Hunninghake, 2000; Surapisitchat et al., 2001). Our results provide evidence that ethanol mediated activation of both PI3K/Akt and ERK1/2 signaling can trigger activation of NF-κB and expression of inflammatory mediators, or fibrosis-related genes such as tTG.

Antioxidants represent a potential group of therapeutic agents for alcoholic liver disease. They likely provide beneficial effects for hepatocytes via desensitization against oxidative stress, while inhibiting priming mechanisms for expression of proinflammatory and cytotoxic mediators via suppression of NF-κB (Hill et al., 1999; McClain et al., 1998). Although progress has been made in the management of liver cirrhosis and its complications, preventive strategies should be beneficial in reducing the burden of this disease (Iredale, 2003; Karsan et al., 2004). In the present study, we examined whether treatment with antioxidants would affect the ethanol-induced tTG mRNA expression and its enzyme activity in Hep 3B cells. We found that among PIN, EGCG and myricetin, PIN possesses higher inhibition effect

compared with other tested compounds (Fig. 4). PIN is one of the major components of propolis. Collected from various natural plant sources, propolis is a resinous substance extensively eaten to improve health and to prevent diseases such as heart disease, diabetes, and cancer both as a dietary supplement and in applications within the pharmaceutical industry (Kumazawa et al., 2004). Recently, much attention has been paid to PIN because of its benefits for human health due to anti-inflammatory, antioxidant, anti-thrombotic, antimicrobial, anti-allergic, hepatoprotective, anti-viral, cancer chemopreventive, and anti-asthmatic activities (Hwang et al., 2003; Sala et al., 2003). However, few, if any, studies have been conducted on its effects on liver fibrosis/cirrhosis, especially in animal models, until now.

TAA, although not toxic itself, is metabolized into potent hepatotoxins by hepatic cytochromes (Hunter et al., 1977). These can produce liver injury through the formation of highly reactive compounds (Balkan et al., 2001; Bruck et al., 2001) and possibly also by activating NF-κB (Bruck et al., 2004). It is assumed that free radical-mediated lipid peroxidation contributes to the development of TAA-induced liver fibrosis (Muller et al., 1991). The form of hepatic damage produced by TAA depends on the dosage and duration of its administration. With respect to biochemical and morphological characteristics, TAA-induced rat liver cirrhosis has been shown to resemble the human disease, thus, it may serve as a suitable animal model (Muller et al., 1988). Quantification analysis showed that the PIN concentrations compose 0.16% of the total propolis extracts used in the current animal study (Fig. 5A). One of the objectives of the present study was to assess whether dietary propolis could attenuate the development of liver cirrhosis in rats due to chronic TAA administration. Rats that received bi-weekly, i.p. injections of TAA for 12 weeks developed hepatic cirrhosis, manifested by gross macroscopic appearance and liver histopathology. These indices of fibrosis and cirrhosis were significantly lower in the rats that were administered propolis concomitantly with TAA. In fact, the pathological changes were virtually prevented by propolis (Fig. 5). The present results suggest the possibility of propolis/PIN application to chronic liver damages due to free radical-mediated lipid peroxidation or other unknown factors. There are many unanswered questions concerning hepatic fibrosis, including which cellular elements regulate the process, what the temporal sequence of their interactions is, and how the various elements interact to facilitate the process. Although we have demonstrated that propolis/PIN inhibited the ethanol-induced tTG mRNA expression and its enzyme activity in Hep 3B cells, the precise mechanism by which propolis/PIN prevents liver fibrosis induced by chronic TAA administration is still unclear and needs further investigation.

In summary, the present study suggests that tTG is an important member of the cascade of factors necessary for ethanol-induced liver fibrogenesis. Both ERK1/2 and PI3K/Akt pathways can regulate the effects of ethanol on NF-κB-dependent transcription and also the activation of ethanol-mediated gene expression of tTG. Administration of propolis/PIN inhibited tTG activation and prevented the development of TAA-induced liver cirrhosis. As propolis is safe for consumption by humans, it may have a beneficial role in chronic liver diseases caused by ongoing hepatic damages.

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