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ORIGINAL PAPER

Gamma-linolenic acid inhibits hepatic PAI-1 expression by inhibiting p38 MAPK-dependent activator protein and mitochondria-mediated apoptosis pathway

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Abstract Fibrosis is induced by the excessive and abnormal deposition of extracellular matrix (ECM) with various growth factors in tissues. Transforming growth factor beta 1 (TGF-β1), plays a role in inducing apoptosis, modulates fibrosis, and ECM accumulation. Plasminogen activator inhibitor 1 (PAI-1) plays an important role in the development hepatic fibrosis. The overexpression of PAI-1 induces ECM accumulation, the main hallmark of chronic liver diseases. Death of hepatocytes is a characteristic feature of chronic liver disease due to various causes. The TGF-β1-mediated apoptotic pathway is regarded as a promising therapeutic target in hepatic fibrosis. Gamma-linolenic acid (GLA) is of special interest as it possesses anti-fibrosis, anti-inflammatory, and anti-cancer properties. However, the precise mechanism for GLA in chronic liver disease is not still clear. The aim of the present study was to determine whether GLA prevents hepatic PAI-1 expression and apoptosis through the inhibition of TGF-β1-mediated molecular mediators. GLA attenuated TGF-β1-stimulated PAI-1 expression, and inhibited PAI-1 promoter activity in AML12 cells. This effect was mediated by Smad3/4, the p38 pathways. We also found that GLA suppressed TGF-β1-induced apoptotic activation of the Bcl-2 family and caspase family of proteins, which resulted in the inhibition of poly-ADP-ribose polymerase 1 cleavage. GLA ameliorates the pro-fibrotic and pro-apoptotic effects of TGF-β1 in hepatocytes, suggesting GLA exerts a protective effect on hepatocytes and has a therapeutic potential for the treatment of chronic liver disease.

Keywords Transforming growth factor-β1 · Hepatic fibrosis · Plasminogen activator inhibitor-1 · Hepatocyte · Gamma-linolenic acid · Apoptosis

Abbreviations

TGF	Transforming growth factor
ECM	Extracellular matrix
PAI	Plasminogen activator inhibitor
PARP	Poly-ADP-ribose polymerase
Apaf	Apoptosis induced factor
MAPK	Mitogen-activated protein kinase
TβR	Transmembrane serine/threonine kinase receptors

Introduction

Transforming growth factor (TGF)-β family members are pleiotropic factors implicated in diverse biological processes, such as embryonic development, tumor genesis, inflammation, fibrosis, cell apoptosis, proliferation, and epithelial–mesenchymal transition [1]. Hepatic fibrosis is characterized by progressive inflammation, and involves several liver cell types and the activation of multiple signaling mechanisms that result in the deposition of extracellular matrix (ECM) [2]. The expression of TGF-β1 is increased in various types of experimental and human liver diseases, ranging from hepatocyte apoptosis, cholestatic liver disease, hepatitis, and liver cirrhosis [3, 4]. In addition, TGF-β1 stabilizes ECM proteins by stimulating the expression of protease inhibitors, including plasminogen

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activator inhibitor 1 (PAI-1) [5]. Suppression of TGF-β1 signaling has therefore been utilized in several therapeutic approaches to prevent hepatic fibrosis [3].

The TGF-β1-mediated apoptotic pathway is regarded as a promising therapeutic target in hepatic fibrosis [6]. In vitro and in vivo studies have also shown that TGF-β1 controls hepatocyte growth directly by inducing apoptosis [7]. Hepatocyte apoptosis was also shown to contribute to liver inflammation and fibrosis [8]. TGF-β1 induces apoptosis through reactive oxygen species (ROS) generation, Bcl-2 family activation, mitochondrial permeability transition, and caspase activation [9]. Mitochondrial-mediated apoptotic pathway is a major pathway involved in apoptosis, which requires the release of mitochondrial cytochrome c. Released cytochrome c binds to apoptosis induced factor (Apaf-1) in the presence of ATP and activates the caspase-3/-9 signaling cascade, leading to the apoptotic destruction of the cell [10]. Triggering apoptosis results in a cascade of caspases activation, in which the last activated caspases digest cellular substrates resulting in morphological changes and cell death [11].

PAI-1 is the main physiological inhibitor of the tissue and urokinase plasminogen activator and is considered to be the most important inhibitor of fibrinolysis [12, 13]. Previous studies have identified a new potential role of PAI-1 in hepatic fibrosis [5, 14]. PAI-1 is an acute phase protein that can be induced during inflammation [15, 16]. TGF-β1-induced PAI-1 expression directly influences the overall tissue site's proteolytic balance and is a critical determinant in directed cell movement, provisional matrix remodeling, and ECM invasion/accumulation. PAI-1 expression by The TGF-β1 is often increased in patients with diseases mellitus [17]. In addition, PAI-1 appears to influence apoptosis by decreasing cell adhesion as well as its effect on intracellular signaling [18]. As such, the overexpression of PAI-1 to induce ECM accumulation is the main hallmark of chronic liver diseases, including carious fibrosis diseases, making PAI-1 a good candidate for the regulation of liver fibrosis [5]. Thus, exploring the mechanisms of PAI-1 regulation and apoptosis is important in the development of new and efficacious therapies for the treatment of cirrhosis, portal hypertension, liver cancer, and other liver diseases [19].

Gamma-linolenic acid (GLA), an n-6 GLA, are found mostly in the plant seed oils of evening primrose, borage, black currant and hemp [20]. In a previous study, GLA was shown to abrogate renal fibrosis in a 5/6 nephrectomy model [21], and other investigations demonstrated that GLA treatment improved autoimmune diseases and diabetic neuropathy via an anti-inflammatory mechanism [22]. A recent study has indicated that GLA exerts anti-inflammatory and anti-fibrotic effects in experimental diabetic nephropathy and in high-glucose-stimulated renal cells [23]. GLA has also been shown to have a preventive effect against conjugated linoleic acid-induced fatty liver in mice [24]. In addition, exogenous GLA suppressed cell proliferation in hepatocellular carcinoma cell lines [25]. However, the molecular mechanisms of anti-apoptotic and anti-fibrotic effects of GLA have not been fully elucidated in hepatocytes.

The present study tested the hypothesis that GLA protects against TGF-β1-induced hepatocyte cell death and fibrosis via the inhibition of apoptosis signaling. Here, we report that GLA prevents hepatic fibrosis and apoptosis through the inhibition of multiple TGF-β1-mediated molecular mediators involved in hepatic injury.

Materials and methods

AML12 hepatocyte culture

A nontumorigenic mouse hepatocyte cell line, AML12 (America Tissue Culture Collection, CRT-2254; ATCC, VA, USA), was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 medium (Gibco, NY, USA) containing 5 μg/ml ITS premix (Sigma-Aldrich, MO, USA), 40 ng/ml dexamethasone (Sigma-Aldrich), and 10 % fetal bovine serum (FBS, Gibco). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2. For serum stimulation, cells at ~80–90 % confluence were made quiescent in serum-free media.

Lactate dehydrogenase (LDH) release assay

The cytotoxicity induced by TGF-β1 was assessed by LDH release into the cell medium. Following treatment as indicated, the cell medium was collected and centrifuged at 1,000 rpm for 5 min to obtain a cell-free supernatant. The activity of LDH in the medium was determined using the LDH Cytotoxicity Assay Kit (Takara Biomedicals, Otsu, Japan) according to the manufacturer's instructions. Absorbance was recorded at 490 nm using a microplate spectrophotometer system (iMark, Bio-Rad Laboratories, CA, USA) and the results are expressed as a percentage of cytotoxicity ((treated cells O.D. − control cells O.D.)/(maximum LDH release O.D. − control cells O.D.) × 100). Reagents did not interfere with the determination of LDH.

Morphological examination

Morphological changes in the cells were observed under an inverted phase-contrast microscope (Leica, Germany). The photographs were taken at 200× or 400× magnificantion using a digital camera.

Protein isolation and immunoblot analysis

Mitochondrial and cytosolic protein fractions were obtained as described [26]. The protein concentration was determined with a Bio-Rad Bradford kit (Bio-Rad Laboratories). The samples were boiled for 5 min and equal volumes (30 µl per lane) were loaded on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the separation the proteins were transferred to a nitrocellulose membrane during 1 h at 4 °C and blocked overnight with PBS-T (0.1 % (v/v) Tween-20, 5 % (w/v) powdered milk in 137 nm NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4) at 4 °C. Immune complexes were detected with a horseradish peroxidase (HRP)-conjugated secondary antibody and were visualized by an enhanced chemiluminescence (ECL) detection system (Bio-Rad Laboratories). The primary antibodies used in this study were probed with anti-PAI-1, anti-caspase-9, -3, anti-Bcl-2, anti-Bax, anti-BcL-xL, anti-VDAC, anti-cytochrome c, anti-PARP-1, anti-p38 and anti-phospho-p38 (Thr^{180}/Tyr^{182}) (Cell signaling, MA, USA), Type-I collagen and fibronectin (Abcam, Cambridge, MA, USA), anti-GAPDH (Santa Cruz, CA, USA). The luminescent signals were analyzed using an ImageQuant LAS 4000 Scanner of GE Healthcare (Piscataway, NJ, USA).

RNA isolation and RT-PCR

The total RNA was extracted from AML12 hepatocytes with TRIzol reagent (Invtrogen Co, Grand Island, NT, US) according to the manufacturer's instructions. Briefly mentioned, cells were dissolved in TRIzol and mixed with chloroform and subsequently centrifuged at 12,000 rpm for 20 min. Iso-propylalcohol was added into the supernatant to the isolated total RNA. The purity and quantity of the RNA preparation were measured at optical densities of 260 and 280 nm. First strand cDNA was synthesized with oligo-d(T) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Aliquots of cDNA were used for PCR using primer sets specific to PAI-1, type-I collagen, fibronectin and GAPDH as a control. Used primers are as follows: PAI-1 sense: 5′-CAC AAG TCT GAT GGC AGC AC-3′, antisense: 5′-CAG GCA TGC CCA ACT TCT C-3′; type-I collagen sense: 5′-ACG GCT GCA CGA GTC ACA C-3′, antisense: 5′-GGC AGG CGG GAG GTC TT-3′; fibronectin sense: 5′-GTC AGT GTC TCC AGT GTC TAC-3′, antisense: 5′- TGG CTT GCT GGC CAA TCA GT-3′; GAPDH sense: 5′ TTG CAG TGG CAA AGT GGA GA-3′, antisense: 5′-CGT GGT TCA CAC CCA TCA CAA-3′. The PCR products were analyzed by agarose gel and visualized by ethidium bromide staining. The fluorescent signals were analyzed by using the ImageQuant LAS 4000 Scanner of GE Healthcare.

DNA transfection and luciferase assay

Reporter gene activity was evaluated by cell-based analysis methods for assaying Smad3/4 and PAI-1 activity. To measure TGF-β1 signaling the TGF-β1-sensitive reporter construct, 100 ng of $(CAGA)_{12}$-Luc reporter, which encodes 12 copies of the CAGA canonical Smad DNA binding sequence [27]. The PAI-1 promoter construct (p800neo-Luc, 200 ng/well) was used in transfection assays [28]. The cells were cotransfected with plasmid constructs and 1 µg of the pCMA-β-galactosidase plasmid for 12 h with Lipofectamine 2,000 transfection reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After 12 h incubation in fresh medium, the enzyme activities of luciferase and β-galactosidase were determined using commercial kits (Promega, Madison, WI, USA), according to the manufacturer's protocol. Luciferase activity was calculated as luciferase activity normalized with β-galactosidase activity in each cell lysate.

MAPK inhibitors treatment and siRNA transfection

AML12 cells were pre-treated with mitogen-activated protein kinase (MAPK) inhibitors (Calbiochem, CA, USA) such as ERK1/2-specific inhibitor: PD98059 (50 µM), JNK-specific inhibitor: SP600125 (20 µM), and p38-specific inhibitor: SB203580 (20 µM). After 30 min, the cells were treated and co-cultured with TGF-β1 for 12 h. Cells were transfected with control siRNA (Santa Cruz, CA, USA) and p38 MAPK siRNA (Cell Signaling Technology, MA, USA) using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instruction.

Electrophoretic mobility shift analysis (EMSA)

A DIG Gel shift kit (Roche, Mannheim, Germany) was used for EMSA. The Smad3/4, AP-1 and SP-1 oligonucleotide probe (Smad3/4: 5′-TCG AGA GCC AGA CAA TTA GCC-3′; AP-1: 5′-CGC TTG ATG AGT CAG CCG GAA-3′; SP-1: 5′-CTT GAA CCC CGC CCC TGT CTT-3′—only sense sense strands are shown; consensus sequences for Smad3/4, AP-1 and SP-1 was underlined) containing the Smad3/4, AP-1 and SP-1 DNA binding motif was end-labeled with DIG-ddUTP. For the binding reaction, 10 µg of nuclear protein were incubated at room temperature for 30 min with a Dig-labeled probe. The DNA–protein complexes were separated by electrophoresis in 6 % non-denaturing polyacrylamide gels using 0.25 × Tris–borate-EDTA as a running buffer. After electrophoresis, the gels were transferred to nylon membranes and detected chemiluminescent. The luminescent signals were analyzed using an ImageQuant LAS 4000 Scanner of GE Healthcare.

Annexin V/PI staining

Apoptotic cells were differentiated from viable or necrotic cells using a combined staining of Annexin V-FITC, and PI (Invitrogen). AML12 cells were preated with GLA for 30 min and then exposed to TGF-β1 for 12 h. After the cells were trypsinized, they were washed in PBS and resuspended in annexin-binding buffer. The cell suspension was incubated with FITC-conjugated anti-annexin V and propidium iodide (PI) for 15 min in the dark. AML12 cells were counted using a BD FACSVerse™ (BD Biosciences, NJ, USA) and analyzed by Flowing 2.5 version software.

Cell cycle analysis

AML12 cells were plated at a density of 1×10^5 cells/ml in a total volume of 10 ml and exposed for 12 h at 37 °C to TGF-β1 or GLA. After treatment, cells were collected by centrifugation for 5 min at 1,000 rpm, resuspended in 300 µl of ice-cold phosphate-buffered saline (PBS), fixed in ice-cold 95 % ethanol and stored at −20 °C for at least 30 min. Fixed cells were collected by centrifugation and resuspended in PBS containing RNase A (500 UI/ml). Samples were kept at room temperature for 30 min. To determine cellular DNA content, cells were stained with PI (5 µg/ml), incubated for 20 min on ice, and then analyzed in duplicate by flow cytometry using a BD FACSVerse and analyzed by Flowing 2.5 version software.

Hoechst 33342 fluorescence staining

For the analysis of apoptotic cell death, the nuclei of the cells were stained with a Hoechst 33342 (ImmunoChemistry, MN, USA) solution. Blue fluorescence was visualized by using fluorescence microscopy (excitation/emission = 330–380 nm/460 nm). Slides were mounted using ProLong® Gold antifade reagent (Molecular Probes® by Life Technologies™, CA, USA). Immunolabeling was examined by an Axio Observer A1 microscope (Carl Zeiss, Sartrouville, France).

JC-1 mitochondrial transmembrane potential assay

To measure the mitochondrial transmembrane potential, JC-1 dye (Stratagene, CA, USA), a sensitive fluorescent probe, was used. Fluorescence microscopy with a 488 nm filter was used for the excitation of JC-1. Emission filters of 535 and 595 nm were used to quantify the population of mitochondria with green (JC-1 monomers) and red (JC-1 aggregates) fluorescence, respectively. Immunolabeling was examined by an Axio Observer A1 microscope (Carl Zeiss, Sartrouville, France) and BD FACSVerse flow cytometer.

Statistical analysis

The data are presented as means values ± standard error (S.E) of the mean of at least three separate experiments. Comparisons were made using Student's t test. For all analysis, a two-sided p value <0.05 was considered to indicate statistical significance.

Results

Effects of GLA on cell toxicity in AML12 cells

We investigated the effects of TGF-β1 on cell cytotoxicity by measurement of LDH release. The LDH assay signifies membrane integrity and direct measurement of cell death. At 12 h after treatment, LDH release treated with 5 ng/ml TGF-β1 increased by about 67 % as compared to normal control cells (Fig. 1a). As shown in Fig. 1b, treatment of cell with GLA ranging from 5 to 50 µM was not cytotoxic. Adding 30 µM of GLA significantly decreased the LDH released of AML12 cells treated with TGF-β1 compared to only treating them with TGF-β1. Based on these results, the optimal GLA treatment for use in subsequent experiments on injured AML12 cells was 30 µM. We next examined the morphology of TGF-β1-treated AML12 cells and GLA treated AML12 cells by phase-contrast microscopy. As shown in Fig. 1c, control AML12 cells maintained in media alone exhibited a rounded cobblestone appearance. When exposed to TGF-β1 for 12 h, the cells appeared to have detached from the dish, with cell rounding, cytoplasmic blebbing, and an irregular shape. However, cells pretreated with GLA before TGF-β1 were in much better condition than cells treated with TGF-β1 alone. Flow cytometric analyses showed that 70.13 % of the TGF-β1-treated AML12 cells stained positive for annexin V. However, only 52.65 % cell treated with 30 µM GLA prior to TGF-β1 stained positive for annexin V. There results suggested that GLA increases cell viability and inhibits apoptosis of TGF-β1 treated AML12 cells.

GLA inhibits TGF-β1-stimulated profibrotic gene and the TGF-β1/Smad signaling pathway

To determine whether GLA inhibits TGF-β1-stimulated fibrotic gene expression, AML12 cells were pretreated with GLA for 30 min and then exposed to TGF-β1 for 12 h. GLA attenuated TGF-β1-stimulated PAI-1 mRNA expression in a dose-dependent manner (Fig. 2a). A similar suppressive effect on PAI-1 expression by GLA was also confirmed by immunoblot. To investigate the mechanism for suppression of PAI-1 transcription by GLA, we examined the effect of GLA on PAI-1 promoter activity by

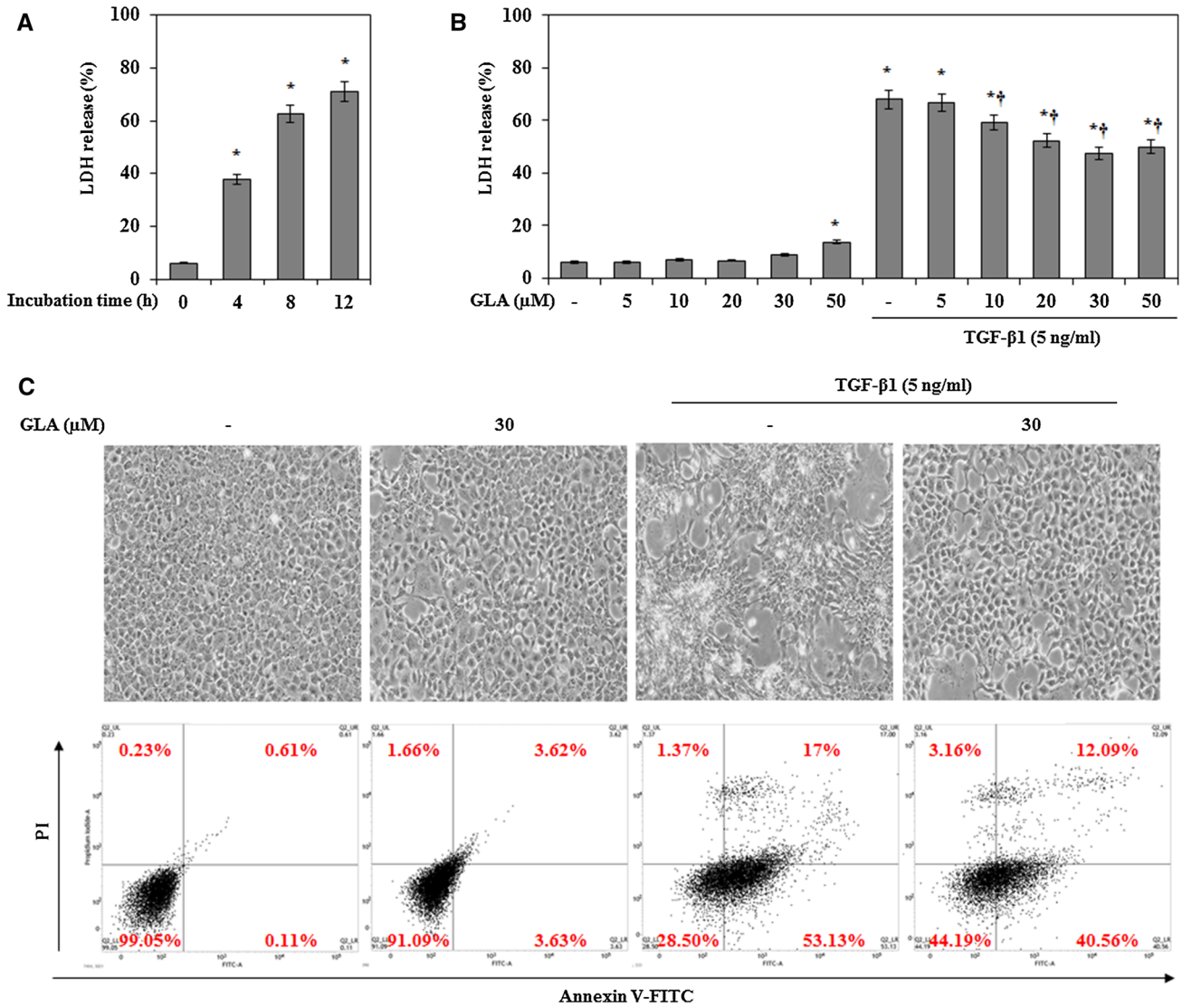

Fig. 1 Effects of GLA on TGF-β1-induced AML12 cells. **a** Time-dependent effect of TGF-β1 on the LDH release of AML12 cells. LDH release was then calculated to indicate cell toxicology for 12 h. **b** Dose-dependent effect of GLA on the LDH release of AML12 cells. AML12 cells were pretreated with GLA for 30 min and then exposed to TGF-β1 for 12 h. **c** *Upper panel* shows the phase-contrast photograph of changes in the cellular morphology of the AML12 cells. Magnifications ×200. *Lower panel* shows the flow cytometric analysis. Quadrants: *lower left* live cells, *lower right* early apoptotic cells, *upper left* necrotic cells, *upper right* late apoptotic cells. The data are representative of three similar experiment and quantified as mean values ± S.E. $*p < 0.05$ compared to normal control, $\dagger p < 0.05$ compared to TGF-β1 treatment

transiently transfecting AML12 cells with the luciferase reporter plasmid containing the PAI-1 promoter sequence. As shown in Fig. 2b, GLA significantly inhibited TGF-β1-stimulated PAI-1 promoter activity in a dose-dependent manner. In addition, GLA inhibited TGF-β1-stimulated type-I collagen and fibronectin mRNA and protein expression (Fig. 2c). This result was confirmed in the profibrotic factor expression by immunoblot. Smads are important intracellular mediators for TGF-β1 induced responses due to the regulation of target genes transcription [29]. Smad3 is main downstream mediator of TGF-β1 signaling, which involves PAI-1 gene expression [30]. The TGF-β1-induced Smad3/4 was shown to directly bind to the CAGA box, and the heterodimeric complex of Smad3/4 was shown to bind to GACACC or GTCTAGAC sequence existing in the PAI-1 promoter region, and to upregulate human PAI-1 promoter activity [31]. To further investigate GLA's effect on Smad3/4 DNA binding activity in TGF-β1-induced AML12 cells, nuclear extracts prepared from isolated AML12 cells were analyzed for their Smad3/4 DNA binding activity (Fig. 3a). The Smad3/4 DNA binding activity was significantly increased by TGF-β1, which

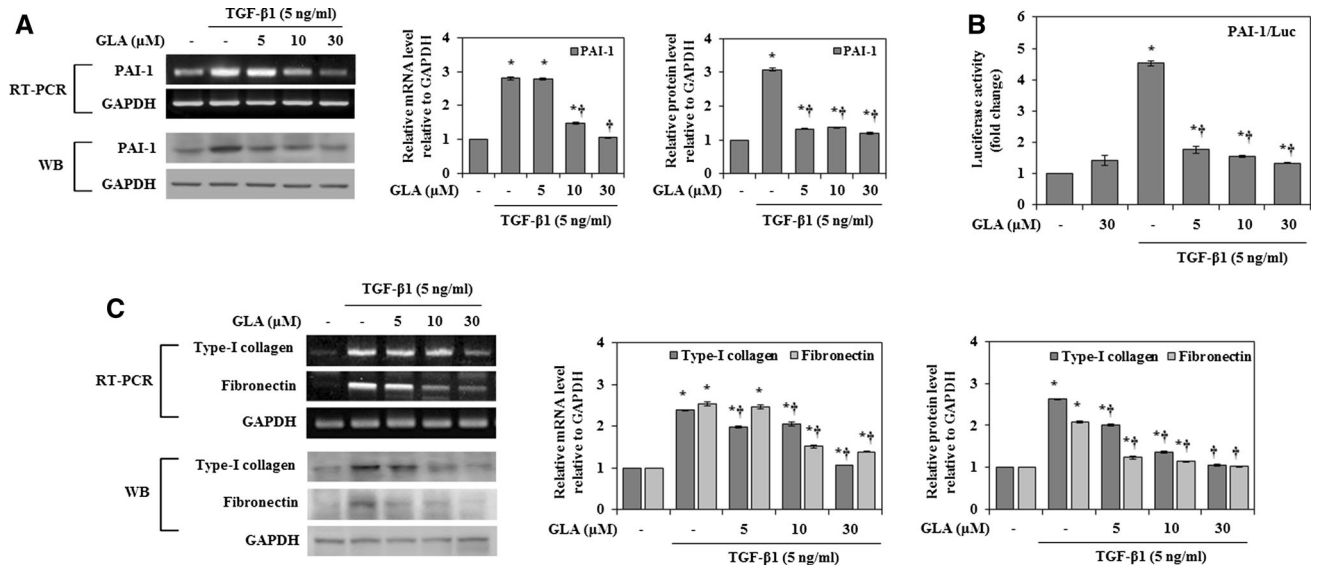

Fig. 2 Inhibition of TGF-β1-induced PAI-1 expression and profibrotic factors by GLA. AML12 cells were pretreated with GLA for 30 min and then exposed to TGF-β1 for 12 h. **a** TGF-β1-induced PAI-1 expression was analyzed by RT-PCR and immunoblot. **b** Effect of GLA on TGF-β1-stimulated PAI-1 promoter activity in AML12 cells. AML12 cells were transfected with PAI-1 promoter-containing reporter vector (p800-luc) and incubated with GLA in the absence or presence TGF-β1. **c** GLA inhibits the TGF-β1-mediated profibrotic factors mRNA and protein expression. GAPDH was used to confirm equal sample loading. The data are representative of three similar experiment and quantified as mean values ± S.E. $*p < 0.05$ compared to normal control, $\dagger p < 0.05$ compared to TGF-β1 treatment

was inhibited by GLA in a dose-dependent manner. The inhibitory effect of GLA on TGF-β1-dependent gene transcription of the $(CAGA)_{12}$-Lux reporter was dose-dependent (Fig. 3b). To further investigate the intracellular signal transduction mechanism, we treated TGF-β1-stimulated cells with GLA. As shown in Fig. 3c, phosphorylation of Smad2 and Smad3 were inhibited by GLA in a dose-dependent manner. These results suggest that GLA inhibits fibrotic gene expression through downregulation of the TGF-β1/Smad signaling pathway.

GLA inhibits TGF-β1-stimulated MAPK activity

Accumulating evidence indicates that MAPK mediates PAI-1 expression via both TGF-β1-dependent and TGF-β1-independent pathways [32–34]. TGF-β1 activates the MAPK cascade including ERK1/2, JNK1/2, and p38 [28]. We therefore aimed to identify the precise MAPK signaling pathway involved in GLA inhibition of TGF-β1-induced PAI-1 expression, utilizing specific kinase inhibitors for ERK (PD98059), JNK (SP600125), and p38 (SB203580). As shown in Fig. 4a, SB203580 or 30 μM GLA most strongly inhibited PAI-1 expression in TGF-β1-induced AML12 cells. Next, we examined the involvement of MAP kinases in TGF-β1-stimulated-PAI-1 expression in AML12 cells. GLA most strongly inhibited TGF-β1-induced phosphorylation of p38 in dose-dependent manner (Fig. 4b). There was little change in the levels of ERK phosphorylation in the presence of GLA. To confirm the p38 MAPK-dependent PAI-1 up-regulation, AML12 cells were pre-treated with p38 siRNA. After transfection with control and p38 siRNA for 24 h, phospho-p38 and p38 expression was decreased in TGF-β1-induced AML12 cells (Fig. 4c). However, transfection with control siRNA did not have any effect on p38 accumulation. Next, we examined the effects of GLA on TGF-β1 induced PAI-1 induction. As shown in Fig. 4d, pre-treatment with p38 siRNA inhibited the expression of PAI-1. Furthermore, 30 μM GLA suppressed PAI-1 expression in TGF-β1-induced AML12 cells. Mouse PAI-1 promoter contains multiple transcription factor binding sites such as Smad binding elements, AP-1, and SP-1 binding sites [33]. We then examined the effects of GLA on the DNA binding activity of AP-1 and SP-1, the primary transcription factors involved in stimulating the PAI-1 promoter downstream of the active MAPK. As shown in Fig. 4e, the enhancement of AP-1 and SP-1 DNA binding activity was markedly decreased after treatment with 30 μM GLA. Together, these data further suggest that inhibition of p38 pathway signaling, which inhibit the binding of transcription factors AP-1 and SP-1, may underlie the suppression of TGF-β-induced PAI-1 gene transcription by GLA.

Effects of GLA on TGF-β1-induced apoptosis

TGF-β1 induces apoptosis in AML12 cells, which is associated with the activation of p38 MAPK signaling pathway [35]. To determine if cells were killed via

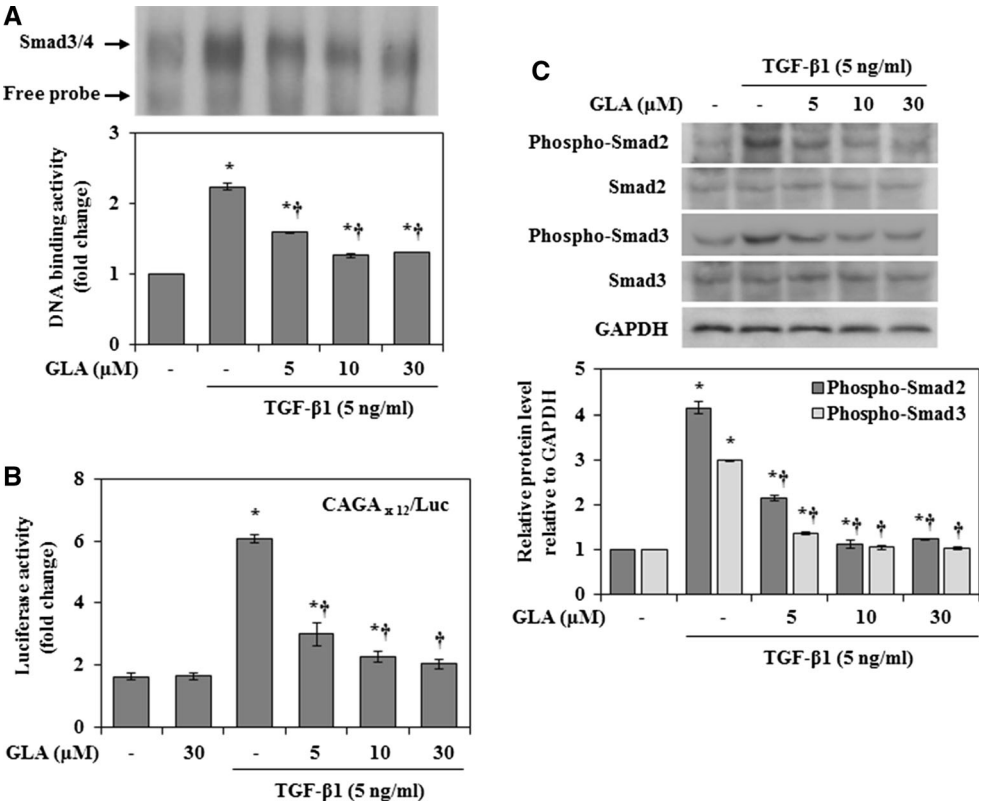

Fig. 3 GLA inhibits the TGF-β1/Smad signaling pathway. **a** Electrophoretic mobility shift assay of the effect of GLA on the TGF-β1-stimulated DNA binding activity of Smad3/4. **b** GLA inhibits TGF-β1-dependent transcriptional activity of the $CAGA_{x12}$-Luc reporter in a dose-dependent manner. **c** Immunoblot analysis of the effect of GLA on the TGF-β1-stimulated phosphorylation of Smad2 and Smad3 in AML12 cells. GAPDH was used to confirm equal sample loading. The data are representative of three similar experiment and quantified as mean values ± S.E. $*p < 0.05$ compared to normal control, $\dagger p < 0.05$ compared to TGF-β1 treatment

apoptotic processes, we examined the nuclear morphology and cycle distribution of GLA-treated and TGF-β1-treated AML12 cells. Because of a change in membrane permeability, early apoptotic cells displayed an increased uptake of Hoechst 33342 compared with live cells (Fig. 5a). AML12 cells treated with TGF-β1 were detached from the dish, with cell rounding, irregular shape, decreased cell number, and increased fragmented nuclei compared with normal control cells. However, AML12 cells treated with 30 μM GLA were in much better condition and were increased in number, compared with cells treated with TGF-β1 alone, as demonstrated by photographs revealing the presence of condensed nuclei. The cell cycle distribution was analyzed by flow cytometric analysis to better understand GLA's inhibitory effect on the growth of AML12 cells. When AML12 cells were treated with TGF-β1, the G0 phase increased from 2.1 % of the normal control to 61.9 % as demonstrated in Fig. 5b. The percentage of cells in the G1 phase was decreased by 44.7 % with TGF-β1 treatment compared to 68.3 % of the normal control cells. In contrast, the G1 phase was increased by 48.7 % with 30 μM GLA treatment compared with TGF-β1. These results suggest that GLA causes G1 phase arrest in AML12 cells, confirming that GLA protects them against TGF-β1 treatment.

During apoptosis, several key events occur in mitochondria, including the loss of mitochondrial transmembrane potential, decreased ratio of Bcl-2/Bax and cytochrome c release [36]. Also, we used JC-1 staining to measure mitochondrial membrane potential to investigate whether mitochondrial membrane integrity is affected by GLA (Fig. 5c). Treatment with 30 μM GLA clearly decreased the amount of mitochondria, which was observed by the decreased membrane potential in TGF-β1-treated AML12 cells. A similar suppressive effect on mitochondrial membrane integrity by GLA was also confirmed by flow cytometric analyses. When AML12 cells were treated with TGF-β1, green fluorescence increased from 4.67 % of the normal control to 56.58 % as demonstrated in Fig. 5c. The percentage of cells that fluoresced red decreased to 51.59 % after TGF-β1 treatment compared with normal control cells (95.33 %). In contrast, the red fluorescence increased to 70.98 % after 30 μM GLA treatment compared with TGF-β1. Taken together, these results indicate that GLA protects hepatocytes against mitochondrial injury caused by TGF-β1.

To evaluate the effects of TGF-β1 on the production of apoptotic proteins related to mitochondria, we measured Bcl-2 family activation in TGF-β1-treated AML12 cells by immunoblot analysis of mitochondrial and cytosolic proteins isolated from the cells. As shown in Fig. 6a, the pro-apoptotic protein Bax was significantly decreased, while the anti-apoptotic proteins Bcl-2 and Bcl-xL were increased in a dose-dependent manner by GLA. The

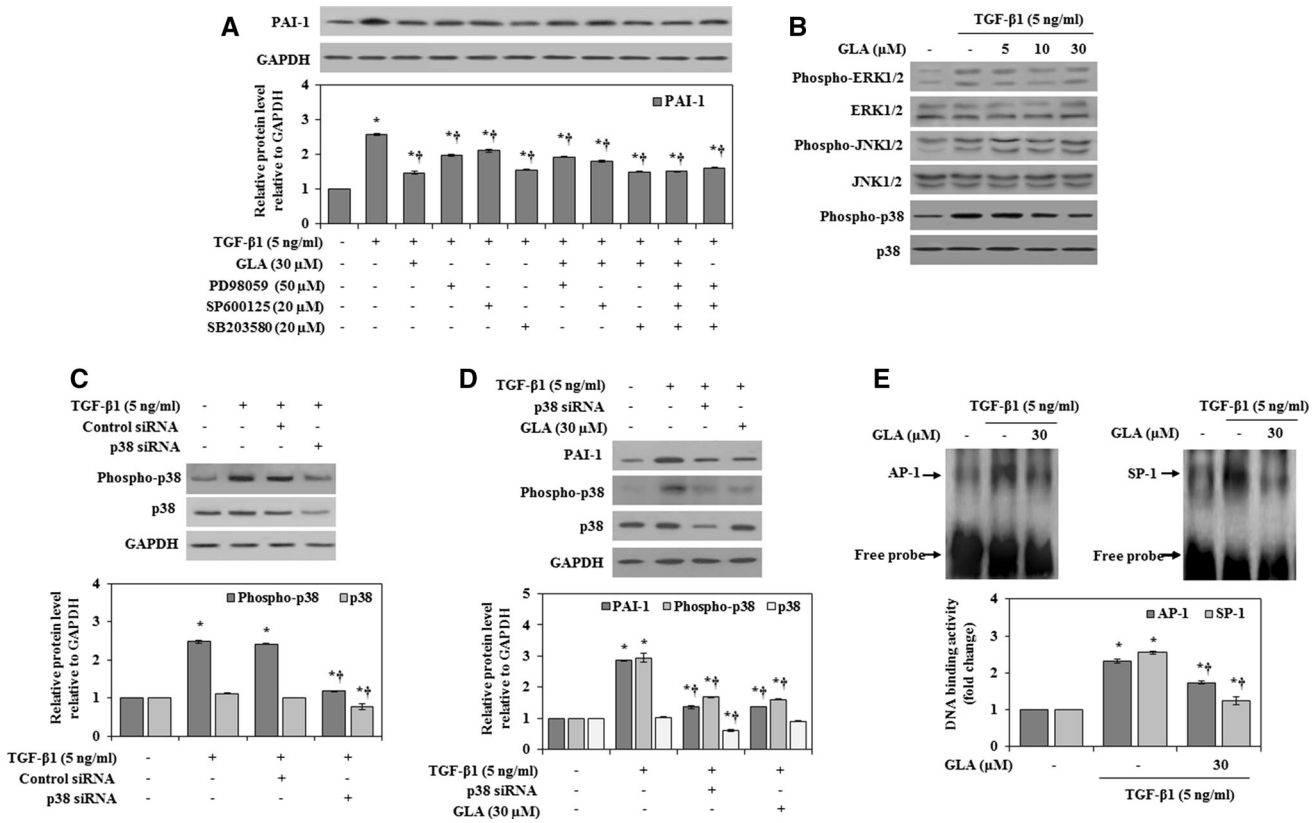

Fig. 4 GLA effectively inhibits the MAPK signaling pathway in TGF-β1-treated AML12 cells. **a** AML 12 cells were pre-treated with inhibitors of p38 (SB203580), ERK (PD98059), and JNK (SP600125) or with GLA for 30 min and then exposed to TGF-β1 for 12 h. **b** Immunoblot analysis of the effect of GLA on the TGF-β1-stimulated MAPK in AML12 cells. **c** AML12 cells were transfected with control or specific p38 siRNA and then treated with TGF-β1 for 12 h. The expression of p38 was suppressed by p38 siRNA treatment in TGF-β1-treated AML12 cells. **d** TGF-β1-induced PAI-1 expression suppressed by p38 siRNA and GLA. **e** Electrophoretic mobility shift assay of the effect of GLA on the TGF-β1-stimulated DNA binding activity of AP-1 and SP-1. GAPDH was used to confirm equal sample loading. The data are representative of three similar experiment and quantified as mean values ± S.E. $*p < 0.05$ compared to normal control, $\dagger p < 0.05$ compared to TGF-β1 treatment

cytosol fraction of untreated control cells contained negligible amounts of Apaf-1 and cytochrome c. In cells undergoing apoptosis, cytochrome c and Apaf-1 are released from the intermembrane space and into the cytoplasm. The release of cytochrome c and Apaf-1 were significantly inhibited in a dose-dependent manner by GLA (Fig. 6b). Most forms of apoptosis are mediated by the proteolytic action of caspase. Since caspase activation plays an important role in apoptosis [37], we investigated the activation of caspase-9 and caspase-3 after the AML12 cells were treated with GLA for 12 h (Fig. 6c). TGF-β1-treated AML12 cells showed an increased cleavage of caspase-9 (37 kDa) and caspase-3 (17 kDa). The addition of GLA inhibited TGF-β1-induced proteolytic fragmentation of caspase-9 and caspase-3 in a dose-dependent manner. PARP-1, a zinc dependent DNA binding protein that recognizes DNA strand breaks and can be a marker of apoptosis [38], was inhibited by GLA, which was in agreement with the observed nuclear morphological changes and caspase activation. Taken together, these findings strongly indicate that GLA protected the mitochondrial apoptosis pathway associated with activation of caspase cascades in AML12 cells.

Discussion

In the present study, we investigated whether GLA inhibited PAI-1 expression and apoptosis in liver cells. GLA decreased TGF-β1-induced PAI-1 expression in liver cells through inhibiting the activity of TGF-β1-mediated molecular mediators Smad3, AP-1 and SP-1. Moreover, GLA protected cells from apoptosis, as evaluated in assays characterizing the DNA fragmentation, chromatin condensation, nuclear disintegration and mitochondrial membrane potential.

TGF-β1 is the most established mediator of fibrosis [39] and apoptosis [10], which it stimulates via Smad-dependent

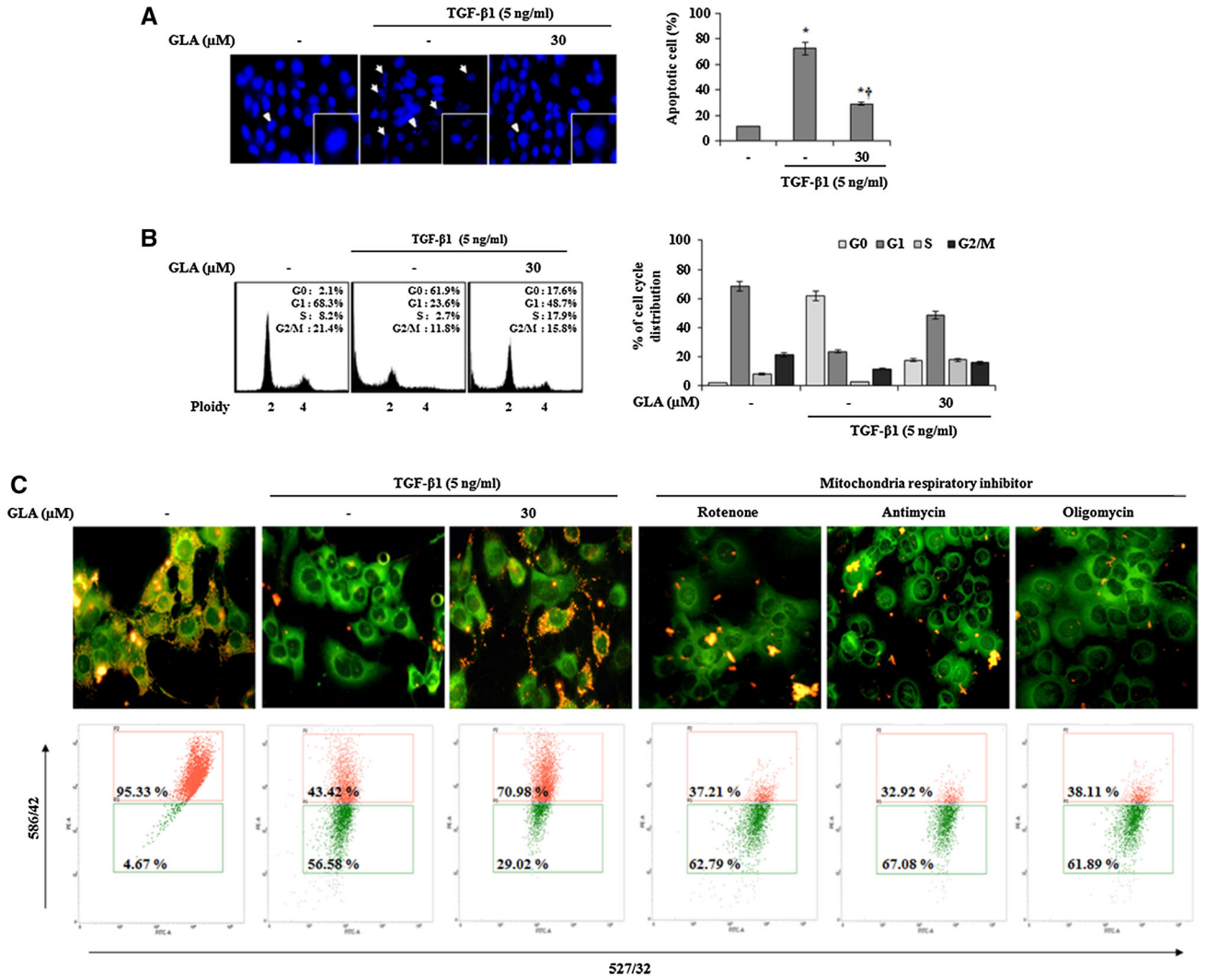

Fig. 5 GLA protects TGF-β1-induced apoptosis in AML12 cells. **a** Fluorescent microscopy showing nuclei by Hoechst 33342 stain *blue color*. *Arrows* indicate cells with condensed chromatin. Magnifications ×400, triangle region ×600. *Right panel* The data are expressed as the percentage of apoptotic cells. **b** Cell cycle distribution was analyzed by flow cytometry. *Right panel* Cell cycle distribution was quantified using a densitometer. **c** AML12 cells were evaluated by morphological criteria after JC-1 mitochondria staining (*red* and *green*) by using fluorescent microscope and flow cytometry. Mitochondria respiratory inhibitor; rotenone, antimycin and oligomycin. Magnification ×400. The data are representative of three similar experiment and quantified as mean values ± S.E. $*p < 0.05$ compared to normal control, $\dagger p < 0.05$ compared to TGF-β1 treatment

activation and Smad-independent induction of MAPK pathways [7, 40]. PAI-1 is thought to promote tissue fibrosis by inhibiting plasmin- and metalloproteinase-mediated ECM degradation [41], and has been implicated in experimental cholestatic liver injury [42, 43].

Several studies have shown that GLA supplementation plays a therapeutic role in the clinical management of patients with inflammatory disorders [44]. A recent study suggested GLA exerts anti-inflammatory and anti-fibrotic effects in experimental diabetic nephropathy and in high glucose stimulated renal cells [23]. Also, it was reported that GLA plays a role in the tumor progression [25], regulation of macrophages [45] and prevention of fatty liver in mice [24]. Another omega-6 fatty acid, arachidonic acid (AA) found in meat and fish, plays an important role in inflammation and vascular dysfunction [46]. A recent study indicated that AA induces human brain endothelial cells mitochondrial apoptosis via signaling pathway involving p38-MAPK and intracellular calcium signaling [47]. Other investigations demonstrated that increased AA levels are associated with reduced pro-inflammatory IL-6 and IL-1 levels and increased anti-inflammatory tumor necrosis factor-beta [48]. We found that GLA reduced the expression ECM proteins, including fibronectin and type-I collagen and inhibited PAI-1 expression in hepatic fibrosis in vitro.

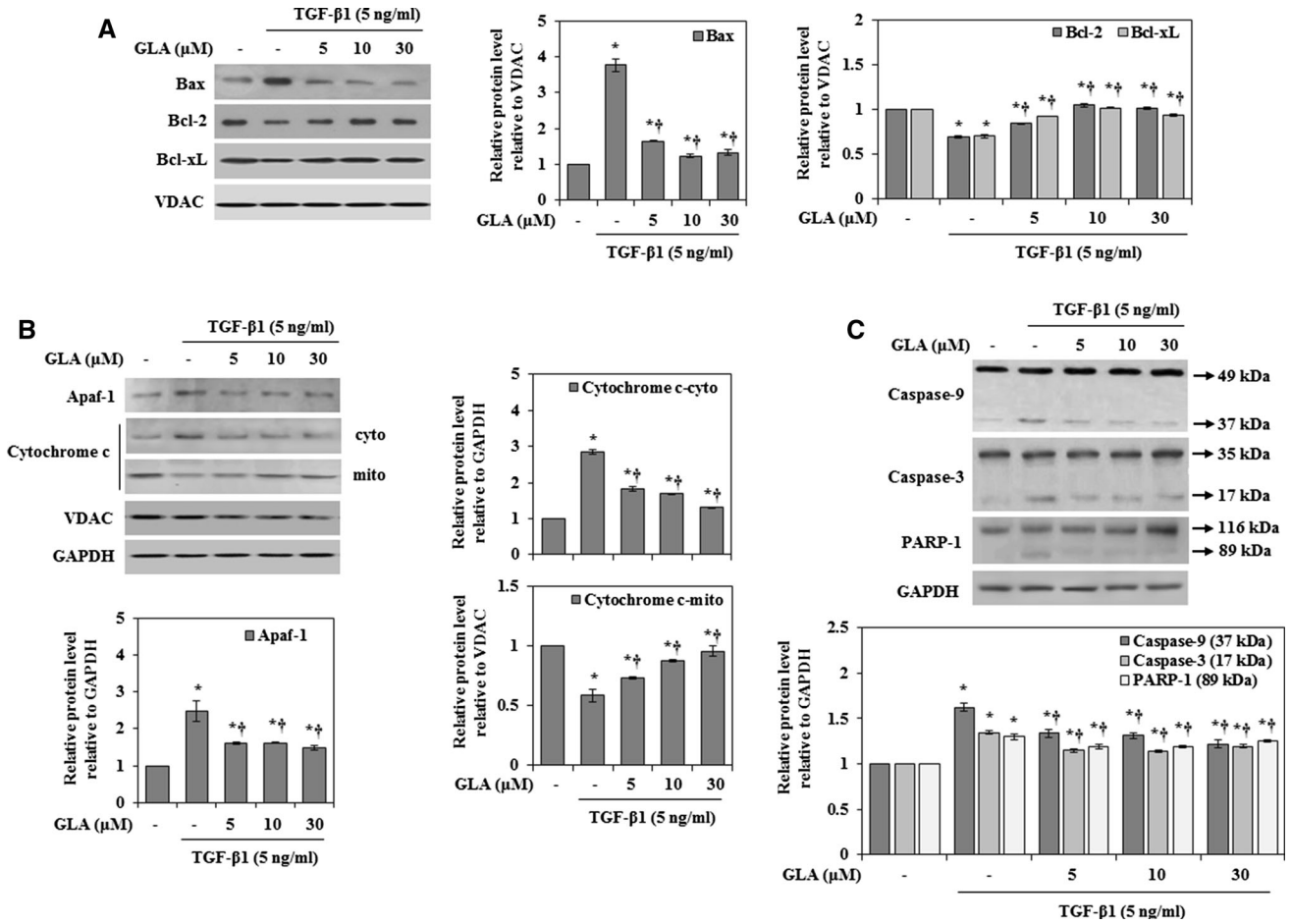

Fig. 6 GLA inhibits TGF-β1-induced cell death through mitochondria-related apoptotic pathway. GLA protects TGF-β1-induced cell death through Bcl-2 family (**a**), release of mitochondrial apoptogenic factors (**b**), and Caspase family (**c**). Immunoblot of mitochondrial fractions of Bcl-2 family, cytochrome c, and cytosol fractions of cytochrome c and Apaf-1 examined. VDAC was used mitochondrial loading control and GAPDH was used to confirm equal sample loading. The data are representative of three similar experiment and quantified as mean values ± S.E. $*p < 0.05$ compared to normal control, $\dagger p < 0.05$ compared to TGF-β1 treatment

TGF-β1 regulates PAI-1 at the level of transcription. The TβR type I and type II receptors are transmembrane Ser/Thr kinase receptors that phosphorylate the receptor-associated Smad proteins Smad2/3. Once phosphorylated, the heterotrimeric complex of Smad3/4 enter the nucleus where they activate the expression of target genes, including PAI-1 [49]. In the present study, the GLA potently attenuated TGF-β1 induced-Smad3/4 complex nuclear translocation and TGF-β1-dependent gene transcription of the $(CAGA)_{12}$-Lux reporter, which raises the possibility GLA may directly or indirectly modify key proteins in the TGF-β1 signaling pathway.

The MAPK signaling pathways also contribute to PAI-1 expression [43]. Several recent studies have demonstrated that MAPKs are involved in TGF-β1-induced PAI-1 expression in various tissues [33], which prompted us to examine whether the effects of GLA were mediated by components of the MAPK pathways. TGF-β1 activates the MAPK cascade, including p38. The specific inhibitor of p38 MAPK strongly inhibited the TGF-β1-induced apoptosis and PAI-1 promoter activity [35]. SB203580 (p38 inhibitor) or GLA specifically inhibited the expression of PAI-1. Also we found GLA significantly inhibited phosphorylation of p38 in TGF-β1 treated AML12 cells in a dose-dependent manner. Furthermore, GLA inhibited the TGF-β1-stimulated DNA binding activity of AP-1 and SP-1, which are the primary transcription factors involved in the stimulation of the PAI-1 promoter downstream of the active MAPK [33].

TGF-β1-induced apoptosis in AML12 cells can be blocked by inhibiting protein synthesis [29]. The p38 MAPK has been shown to mediate apoptosis induced by many stimuli [35]. We evaluated the effect of GLA on TGF-β1 induced apoptosis in AML12 cells to gain a better understanding of the molecular mechanisms involved in GLA's anti-apoptotic effects. GLA protected cells from

apoptosis, as evaluated due to assays characterizing the chromatin condensation, nuclear disintegration and mitochondrial membrane potential. The Bcl-2 family is induces the into anti-apoptotic proteins Bcl-2 and BcL-xL, which reduce the level of cytochrome c release [50] and pro-apoptotic proteins such as Bax and Bak, which induce the release of cytochrome c and loss of mitochondrial membrane potential [51]. Therefore, the ratio of pro-apoptotic and anti-apoptotic activities of the Bcl-2 family may be pivotal in the release of cytochrome c from the mitochondria into the cytosol. In the present study, Bcl-2 and Bcl-xL was shown to be up-regulated, whereas Bax was down-regulated by GLA in TGF-β1 induced apoptosis. The protective effects became more pronounced when the cells were treated with GLA. The activation of initial caspases is known to trigger the activation of effecter caspases, like caspase-3 and subsequently inducing apoptosis [7]. In the presence of extensive DNA damage, PARP can play a role in caspase-dependent apoptotic cell death [38]. It was also reported PARP is cleaved before or concomitant the degradation of nuclear DNA into nucleosomal fragments, and the presence of PARP inhibitors delays or prevents apoptosis [11]. Our results demonstrated that the cleavage of PARP was decreased through the inhibition of caspase-3 cleaved by GLA.

In conclusion, our findings suggest that GLA ameliorates the pro-fibrotic and pro-apoptotic effects of TGF-β1 in hepatocytes, thereby exerting a protective effect on hepatocytes and possibly having therapeutic potential for the treatment of chronic liver disease.

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