Anti-Fibrotic Effects of DL-Glyceraldehyde in Hepatic Stellate Cells via Activation of ERK-JNK-Caspase-3 Signaling Axis

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Abstract

During liver injury, hepatic stellate cells can differentiate into myofibroblast-like structures, which are more susceptible to proliferation, migration, and extracellular matrix generation, leading to liver fibrosis. Anaerobic glycolysis is associated with activated stellate cells and glyceraldehyde (GA) is an inhibitor of glucose metabolism. Therefore, this study aimed to investigate the anti-fibrotic effects of GA in human stellate LX-2 cells. In this study, we used cell viability, morphological analysis, fluorescence-activated cell sorting (FACS), western blotting, and qRT-PCR techniques to elucidate the molecular mechanism underlying the anti-fibrotic effects of GA in LX-2 cells. The results showed that GA significantly reduced cell density and inhibited cell proliferation and lactate levels in LX-2 cells but not in Hep-G2 cells. We found that GA prominently increased the activation of caspase-3/9 for apoptosis induction, and a pan-caspase inhibitor, Z-VAD-fmk, attenuated the cell death and apoptosis effects of GA, suggesting caspase-dependent cell death. Moreover, GA strongly elevated reactive oxygen species (ROS) production and notably increased the phosphorylation of ERK and JNK. Interestingly, it dramatically reduced α-SMA and collagen type I protein and mRNA expression levels in LX-2 cells. Thus, inhibition of ERK and JNK activation significantly rescued GA-induced cell growth suppression and apoptosis in LX-2 cells. Collectively, the current study provides important information demonstrating the anti-fibrotic effects of GA, a glycolytic metabolite, and demonstrates the therapeutic potency of metabolic factors in liver fibrosis.

Key Words: Hepatic stellate cells, DL-glyceraldehyde, Apoptosis, MAPKs, α-SMA, Collagen type-I
treatment of liver fibrosis, but none meet the criteria for final validation. Therefore, there is still a need for a highly effective and target-specific antifibrotic agent. Mounting evidence suggests that inhibiting the continuous activation and growth of HSCs, thus inducing apoptosis, may be an effective therapeutic strategy to inhibit liver fibrosis (Movassaghi et al., 2013; Kuo et al., 2018). Apoptosis is an event of programmed cell death, characterized by morphological changes, cell shrinkage, and DNA breakdown. The activation of some enzymes, such as the caspase family, is closely linked to apoptosis (Samsuzzaman and Jang, 2022). In addition, mitogen-activated protein kinases (MAPKs) play an important role in activating apoptosis in HSCs (Lee et al., 2021).

DL-glyceraldehyde (GA) is one of the simplest aldotrioses and is an important metabolite produced by the normal metabolic cycle. It is a vigorous glycolytic inhibitor known for its impressive association with glycolysis (Woodward and Hudson, 1954; Loiseau et al., 1985; Dwarkanath and Jain, 1989). Enhanced glycolysis and oxidative status have been reported in activated HSCs compared with inactivated HSCs. Moreover, the prevention of HSC activation by targeting metabolic pathways has been demonstrated (Chen et al., 2012; Du et al., 2018). In addition, some metabolic factors have been reported to have protective effects against HSC activation and liver fibrosis. The anti-cancer effects of GA in the brain and liver have been confirmed. The glycolysis inhibitory effects of GA have been reported previously (Holmes, 1934; Ashford, 1937; Needham and Nowiński, 1937). However, the effect of free GA on activated HSCs has not yet been elucidated. Based on previous studies, we assumed that GA might inhibit cell proliferation and induce apoptosis in activated HSCs. In this study, we investigated the possible underlying mechanism of action of GA in activated HSCs.

MATERIALS AND METHODS

Reagents

GA, 2',7'-dichlorofluorescein diacetate (DCF-DA), 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), and monoclonal anti-α-tubulin antibody were purchased from Sigma (St. Louis, MO, USA). Phospho-ERK1/2 (p-ERK1/2), ERK, p38, phospho-p38 (p-p38), JNK, phospho-JNK (p-JNK), and PARP were purchased from Cell Signaling Technology (Danvers, MA, USA). An apoptosis detection kit (Annexin V-fluorescein isothiocyanate (FITC)) was obtained from BD Biosciences (San Diego, CA, USA). ERK inhibitor (U012), JNK inhibitor (SP600125), and p38 inhibitor (SB203580) were purchased from Cell Signaling Technology. All enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (MN, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Cytiva HyClone Laboratories (UT, USA).

Cell culture

LX-2 (human HSC) and Hep-G2 cells stored in a liquid nitrogen tank were inoculated in DMEM and Eagle’s Minimum Essential Medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin (100 U/mL), and streptomycin (100 μg/mL). Then, the cells were allowed to grow in a 5% CO₂ atmosphere at 37°C.

Cell viability assay

LX-2 and Hep-G2 cells were seeded in 96-well plates and allowed to grow overnight till 80% confluency. Additionally, the cells were pretreated with ERK, JNK, P38, and pan-caspase inhibitors for 2 h; then, GA (0.25, 0.5, and 0.75 mM) was added to the cells and incubated for a further 22 h. After every treatment, the conditioned medium was removed, and then, 100 μL MTT solution was added to each well and incubated for 1 h. Then, the supernatant was removed from each well, the formed formazan was dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured using a spectrophotometer at 570 nm (Molecular Devices, San Jose, CA, USA).

Cell morphology analysis

Cells were seeded in 48 well cell culture plates overnight. When the cell confluency reached 70-80%, the cells were treated with or without GA at various concentrations (0.25, 0.5, and 0.75 mM) for an additional 24 h. The cells were washed with phosphate-buffered saline (PBS) and images of intact cells were captured using phase contrast electron microscopy equipped with Nikon digital camera (Nikon, Tokyo, Japan).

Cell proliferation assay

To investigate the effects of GA on LX-2 cell proliferation, a BrdU cell proliferation assay kit (Cell Signaling Technology) was used, following the manufacturer’s protocol. LX-2 cells were seeded in 96-well cell culture plates and incubated for 24 h. The cells were then treated with different concentrations (0.25, 0.5, and 0.75 mM) of GA for 24 h. Fixed cells were detected by incubating with the anti-BrdU detection antibody for 1 h. Next, horseradish peroxidase (HRP)-conjugated secondary antibody and substrate solution were added to the wells, and the reaction was stopped by adding the stop solution. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices).

Measurement of lactate production

L-lactate production in GA-treated or non-treated LX-2 cells was analyzed using an L-lactate assay kit (EnzyChrom™, Hayward, CA, USA), according to the manufacturer’s protocol. First, the cells were seeded in a 6-well cell culture plate for 24 h and treated with or without different concentrations of GA for 24 h. The culture medium (CM) was collected and transferred to a 96-well clear-bottom plate. The L-lactate standard was prepared by dilution in serum-free cell culture media. Then, 20 μL of the standard solution was added to 20 μL of CM in a 96-well plate. The reaction mixture was prepared by mixing 60 μL of assay buffer, 14 μL of MTT, 10 μL of NAD, 1 μL of enzyme A, and 1 μL of enzyme B. Finally, 80 μL of the reaction mixture was added to each well containing the standard and sample. Absorbance was measured at 565 nm using a microplate reader (Molecular Devices).

Detection of Apoptosis

After LX-2 treatment with GA for 24 h, apoptosis was assessed using an Annexin V-FITC kit (BD Biosciences) following the manufacturer’s instructions. Briefly, cells were seeded in 60-mm plates in 3 mL cell culture medium and left to proliferate overnight. Then, GA at 0.25, 0.5, and 0.75 mM prepared to a 96-well plate. After every treatment, the conditioned medium was removed, and then, 100 μL MTT solution was added to each well and incubated for 1 h. Then, the supernatant was removed from each well, the formed formazan was dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured using a spectrophotometer at 570 nm (Molecular Devices, San Jose, CA, USA).
twice with cold PBS, and resuspended in 1X binding buffer. When binding was complete, 5 μL of Annexin V-FITC and 5 μL propidium iodide solution were added to the cells and incubated for 15 min in the dark at RT. Finally, cell apoptosis was analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA) within 1 h.

**Detection of reactive oxygen species (ROS) production**

ROS generation was detected using the DCF-DA staining assay kit, according to the manufacturer’s protocol, with slight modifications. For ROS measurement, the cells were seeded and allowed to grow for 24 h. The cells were then treated with different concentrations (0.25, 0.5, and 0.75 mM) of GA for 2 h. The conditioned medium was then removed from each well, and DCF-DA staining solution was added to the cells and incubated for 30 min at 37°C. The stained cells were washed with 1X phosphate buffer and analyzed with flow cytometry (Becton Dickinson). In flow cytometry, DCF was excited at 488 nm laser and detected at 535 nm.

**Western blotting**

Western blotting was performed to evaluate the protein expression status or changes in the presence or absence of GA in LX-2 cells. Cells were treated with or without different concentrations (0.25, 0.5, and 0.75 mM) of GA for 24 h. The treated cells were harvested and lysed in a protein lysis buffer named PRO-PREP™ protein extraction solution, which also contained a phosphatase inhibitor mixture. Protein quantification was performed using a bichinoninic acid (BCA) protein assay kit, with bovine serum albumin as a standard. Equal amounts (40 μg) of protein were loaded in each lane, and proteins were separated using 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane using the WSE-4204 HorizeBLOT 4M-R semi-dry transfer system (Merk Millipore, Ireland). After transfer, the proteins were blocked with 5% skimmed milk prepared in Tris-buffered saline-Tween 20 (TBST) solution for 1.5 h and washed three times with TBST before incubating the proteins with their respective primary antibodies overnight at 4°C on a shaker. After overnight incubation, the primary antibodies were removed and washed again with TBST, and HRP-conjugated secondary antibodies were added for another 1.5 h at room temperature on an orbital shaker. Protein expression and band intensity were detected using the ChemiDoc XRS+ imaging system (Biorad, Hercules, CA, USA).

**Quantitative real-time PCR (qRT-PCR)**

LX-2 cells were seeded and treated with GA for 24 h. Then, total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s protocol. After extraction, the RNA concentration was measured using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA), and 1 μg of total RNA was used to synthesize cDNA using an RT master mix (TAKARA, Shiga, Japan). The cDNA was then amplified using TB SYBER Premix Ex Taq™ in a real-time qPCR system. The real time PCR program was run for 30 s at 95°C following 40 PCR cycles of 5 s at 94°C, 15 s at 95°C and 1 min at 60°C with the final extension at 95°C for 15 min. The expression level of each gene was normalized to that of the housekeeping gene GAPDH.

The following gene-specific primers were used for amplification: Col1a1 (sense) 5'-CATGAGCGGAAGCTAACC-3', (antisense) 5'-TGTCGAGATACAGATCAAGC-3', SMA (sense) 5'-CAGGGGAGTAATGGTTGGAAT-3', (antisense) 5'-TCTCAAACTATCTGGTCTA-3'. The fold change of genes mRNA expression was calculated by the equation 2^{-ΔΔCt}.

**Statistical analysis**

All experimental data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Biological data were analyzed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance was used to identify the statistical differences between the control and treated groups. Statistical significance was set at p<0.05.

**RESULTS**

**GA treatment decreased the viability, proliferation, and lactate production of LX-cells**

To determine the specificity of GA toxicity, we used two types of hepatic cell lines: Hep-G2 and Lx-2. Both cell lines were treated with or without different concentrations (0.25, 0.5, and 0.75 mM) of GA for 24 h. The control culture medium contained 0.1% DMSO. Morphological analysis showed that GA treatment changed the morphology of LX-2 cells to a quiescent-like morphology, whereas Hep-G2 cell morphology remained unchanged in the control and treated panels (Fig. 1A, 1B). The morphology of the cells was observed using an electron microscope. GA treatment significantly reduced the viability of LX-2 cells at 0.75 mM as compared to the control, but did not show any effects at the same concentration on Hep-G2 cells in terms of viability (Fig. 1C, 1D). Moreover, we assessed the effects of GA on the proliferation and L-lactate production of LX-2 cells. The results indicated that GA significantly inhibited proliferation and L-lactate production at 0.75 mM in LX-2 cells as compared to that in the control or other GA-treated groups (Fig. 1E, 1F). Based on these results, we found that GA reduced cell viability, altered cell morphology, and inhibited cell proliferation and L-lactate production in LX-2 cells.

**GA treatment induced apoptosis and modulated the activity of caspase 3/9 and PARP in LX-2 cells**

To investigate whether the GA-induced cell toxicity is related to apoptosis, the dissemination of apoptosis in LX-2 cells was assessed using the Annexin V-FITC assay kit using fluorescence-activated cell sorting (FACS) analysis. Flow cytometry analysis revealed that the number of apoptotic cells was significantly higher in the GA-treated cells than that in the control and the number of viable LX-2 cells exposed to 0.75 mM GA was reduced compared to that of the control, suggesting that GA induced cell apoptosis (Fig. 2A, 2B). Next, we examined the potential effects of GA on the expression of different apoptotic markers, such as caspases and PARP, in LX-2 cells using western blotting analysis. Caspase activity was detected by measuring the expression levels of the pro and active forms of caspases after treatment with various concentrations of GA. In the case of PARP, the pro- and cleaved- form of PARP was examined in the presence or absence of GA in LX-2 cells. GA at 0.25 and 0.5 mM did not increase the levels of the active forms
of caspase 3 and caspase 9 nor those of the cleaved form of PARP in LX-2 cells. However, the active forms of caspase 3 and caspase 9 and the cleaved form of PARP were remarkably visible upon GA treatment at 0.75 mM in LX-2 cells (Fig. 2C, 2D). The expression levels of the housekeeping protein α-tubulin remained the same in all GA treatment groups under the same conditions.

GA induced cell death in LX-2 cells by regulating caspase activation

Given that caspase activation was observed in the presence of GA in LX-2 cells, we explored the role of caspase activation in GA-triggered apoptosis and growth suppression. We used a pan-caspase inhibitor (z-VAD-FMK) to identify the caspase-inhibitory effects of GA in LX-2 cells. As shown in Fig. 2, z-VAD-FMK at 20 μM strongly protected against GA-induced cell death (Fig. 2E). Western blotting analysis confirmed that z-VAD-FMK effectively inhibited the cleaved form of PARP in LX-2 cells (Fig. 2F, 2G). These results suggest that GA-induced toxicity may occur via the caspase activation pathway in LX-2 cells.

GA regulated ROS generation and MAPK signaling in LX-2 cells

Next, we assessed ROS production in the presence of GA in LX-2 cells, as ROS production is notably linked to apoptosis in cells. Treatment with GA induced ROS production at 0.75 mM GA concentration in LX-2 cells (Fig. 3A, 3B). It has been reported that ROS can act as an upstream regulator of MAPK, which helps activate MAPK in terms of apoptosis induction. Further, we examined the MAPK activation status in the presence or absence of GA at several concentrations in LX-2 cells. GA treatment at 0.25 and 0.5 mM did not elevate the level of the phosphorylated form of MAPK as compared to that in the control. However, GA exposure at 0.75 mM significantly increased the expression levels of phosphorylated forms of ERK and JNK as compared to that in the control, while their total form and p-38 expression remained constant in LX-2 cells (Fig. 3C, 3D).

GA altered the levels of fibrosis marker in LX-2 cells

To determine the effect of GA on fibrosis markers in LX-2 cells, the cells were exposed to different concentrations of GA for 24 h. Our data revealed that GA at 0.25 mM did not significantly induce a reduction in α-SMA and COL1A-1 protein expression as compared to those in the control. However, the protein expression levels of α-SMA notably decreased in cells treated with 0.5 mM and 0.75 mM GA as compared to the control. Similarly, GA treatment at 0.75 mM dramatically facilitated the decline of COL1A-1 protein expression levels in LX-2 cells compared to that in the control (Fig. 4A-4C). To confirm the mRNA expression levels of α-SMA and COL1A-1 in the presence or absence of GA in LX-2 cells, we performed qRT-PCR analysis. As shown in Fig. 4D and 4E, GA greatly
Fig. 2. GA induced apoptosis in LX-2 cells. Cells were seeded and treated with different concentrations of GA (0.25, 0.5, and 0.75 mM) for 24 h. (A) Apoptosis was detected using Annexin V-staining kit (PI) by flow cytometry. (a) control group (b) 0.25 mM of GA (c) 0.5 mM of GA. (D) Cells were seeded and treated with or without GA at 0.75 mM in duplicate for 1 h. Cell lysates were prepared, and the phosphorylated and total protein expression of caspase-3, caspase-9, and PARP by western blotting. Effects of Z-VAD-fmk on the GA induce growth inhibition and apoptosis in LX-2 cells. Cells were pretreated with Z-VAD-fmk for 1 h and then exposed to GA at 0.75 mM for a further 23 h. (E) Cell viability was measured by MTT assay. (F) Cells harvested from control, 0.75 mM of GA, 20 mM of Z-VAD, and Z-VAD+GA groups were lysed and analyzed the pro and cleaved protein expression PARP by western blotting. (G) Densitometry graph of (F). **p<0.01 vs. control, *p<0.05 vs. only GA treatment.

Fig. 3. GA induced ROS production and MAPK activation in LX-2 cells. Cells were seeded and treated with different concentrations of GA (0.25, 0.5, and 0.75 mM) for 24 h. (A) ROS production was analyzed by flow cytometry. (a) M1=control, M2=GA 0.25 mM (b) M1=control, M2=GA 0.5 mM (c) M1=control, M2=GA 0.75 mM. (B) Quantitative analysis of representative cytograms of (A) (DCF-DA staining. (C) Cells were seeded and treated with or without different concentrations of GA (0.25, 0.5, and 0.75 mM) for 1 h. Cell lysates were prepared, and the phosphorylated and total protein expression levels of MAPK (ERK, JNK, and P-38) were analyzed via western blotting. (D) Cells were seeded and treated with or without GA at 0.75 mM in duplicate for 1 h. Cell lysates were prepared, and the phosphorylated and total protein expression levels of MAPK (ERK, JNK, and P-38) were analyzed via western blotting. **p<0.001 vs. control.
reduced the mRNA expression levels of α-SMA and COL1A-1 compared to those in the untreated group. Additionally, we examined whether GA modulates the phosphorylated levels of Smad2/3 in the presence or absence of Tgf-β1 in LX-2 cells by treating LX-2 cells with 10 ng of Tgf-β1 and 0.75 mM of GA for 1 h. Western blotting data revealed that Tgf-β1 significantly induced the phosphorylation of Smad3 as compared to the control, while Smad2 levels did not change in LX-2 cells. However, GA treatment significantly reduced phosphorylated Smad3 levels in GA and Tgf-β1 combined treated cells as compared to GA or Tgf-β1 alone treated LX-2 cells (Fig. 4F, 4G). These results indicate that GA greatly altered the expression of fibrosis markers in HSCs in the presence or absence of Tgf-β1.

**GA induced apoptosis in LX-2 cells through the activation of MAPK signaling**

To clarify the role of MAPK activation in GA-induced apoptotic cell death, LX-2 cells were exposed to GA in the presence or absence of MAPK inhibitors UO126, SB203580, and SP600125 against ERK, p38, and JNK, respectively. The cells were first pre-treated with MAPK inhibitors for 2 h and then exposed to GA (0.75 mM) for 22 h. The levels of apoptosis were analyzed by cell viability assay, morphological analysis, and western blotting techniques using caspase 3 and PARP antibodies. The cell viability assay results showed that UO126 and SP600125 pre-treatment at 20 μM reduced GA-induced toxicity in LX-2 cells, whereas SB203580 treatment did not have any protective effects on LX-2 cells (Fig. 5A, 5B). Additionally, treatment with UO126 and SP600125 reduced GA-induced changes in cell morphology and growth suppression. Notably, the protein levels of cleaved caspase 3 and PARP induced by GA decreased in the presence of UO126 and SP600125 in LX-2 cells (Fig. 5C, 5D). These data reveal the involvement of MAPK activation in GA-induced apoptosis induction in activated LX-2 cells.

**DISCUSSION**

Liver fibrosis is a pathological progression of liver injury and continues to be the leading cause of liver cancer. It affects patients’ health worldwide and is strongly linked to tremendous morbidity and mortality (Wang et al., 2018; Cortes et al., 2018).
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2019). Many studies have reported that liver fibrosis is a reversible wound healing process that can lead to irreversible liver cirrhosis (Saeed et al., 2018). In the normal state, HSCs remain inactivated and serve as a proper storage site for lipid droplets and vitamin A. Upon exposure to several stimuli such as viral infections, alcoholism, drug toxicity, genetic metabolic problems, and autoimmune disease exposure, they undergo notable changes and are activated, followed by the expression of fibrotic factors such as α-SMA and accumulation of collagen (Bataller and Brenner, 2005; Chen et al., 2017). Many underlying approaches have been used to suppress HSC activation and cell death, including the induction of apoptosis, inhibition of the phosphatidylinositol 3-kinase (PI3K)–Akt pathway, and activation of MAPK proteins. An effective and early treatment can prevent the conversion of fibrotic liver to liver cirrhosis. However, the effects of free intracellular GA on activated stellate cells have not yet been proven. Notably, metabolites such as methylglyoxal (MG), produced during glycolysis, even at lower concentrations, have hermetic roles in cell growth, proliferation, and apoptosis in cancer (Nokin et al., 2019). GA is a very important metabolic by-product that is mostly generated in hepatocytes due to the high fructose metabolism rate in the liver. Studies have suggested that GA-derived glycated products are associated with many events of NASH, infertility, and cancer (Hyogo et al., 2007; Jinno et al., 2011; Kan et al., 2015). Interestingly, GA-derived glycated products were not found in steatosis or healthy patients, indicating that free GA may have beneficial effects during the fibrotic process at the beginning of NASH pathogenesis. Nonetheless, we assume that free GA before glycation could play a potential role in preventing fibrotic aggression in hepatocytes as part of the liver homeostasis process.

The appropriate intracellular and in vivo concentrations of GA remain undetermined. To clarify the effects of GA on HSCs, we used millimolar concentrations of GA. A previous study demonstrated that GA inhibits the growth of neuroblastoma cells at 1 mM after 24 h of treatment (Sakamoto et al., 1972). Moreover, it reduces the growth of several types of rodent neoplasms. A recent study has demonstrated that GA causes hepatocarcinoma cell death via necrosis and caspase-3 modification. Millimolar concentrations of extracellular GA was used in all experiments using Hep-G2 cells (Sakasai-Sakai et al., 2017). In addition, GA does not affect the normal liver, kidney, or brain, even at higher concentrations (Kapoor et al., 2014). Therefore, we used extracellular GA at millimolar concentrations in all of our experiments.

In the present study, we investigated the effects of extracellular GA on HSC viability. We used two types of hepatic cells: Hep-G2 and LX-2. The results suggested that GA did not affect hepatic Hep-G2 cells at the concentrations used in our study, but greatly reduced cell density and changed the morphology of HSCs. This event strongly revealed the inhibitory effects of GA. Cell proliferation and lactate production are also inhibited by GA in activated HSCs (aHSCs), suggesting that glycolytic status is modulated in aHSCs. Inhibition of glycolysis regulates the proliferation of activated human primary

![Fig. 5. Involvement of MAPK in GA induced apoptosis in LX-2 cells. Cells were pretreated with ERK inhibitor (U0126), p38 inhibitor (SB203580), and JNK inhibitor (SP600125) for 2 h and then exposed to GA at 0.75 mM for a further 22 h. (A) Cell viability was measured by MTT assay. (B) Morphological analysis of control, 0.75 mM of GA, U016+GA, and SP600125+GA groups were done under phase electron microscopy. (C) Whole-cell lysates harvested from control, 0.75 mM of GA, U016+GA, SB203580+GA, and SP600125+GA groups were prepared and analyzed for the pro and cleaved protein expression of caspase-3, and PARP by western blotting. (D) Densitometry graph of (C). ***p<0.001 vs. control, **p<0.0001 vs. control, *p<0.01, #p<0.001 vs. only GA treatment.](#)
HSCs. In line with our findings, 2-deoxy glucose (2D), a glycolysis inhibitor, strongly inhibited proliferation in human aHSCs and reduced fibrosis marker levels in rat aHSCs (Smith-Cortinez et al., 2020). Glycolysis inhibition may be a crucial factor involved in the antiproliferative effects of GA in aHSCs. Apoptosis is a new and generous form of programmed cell death that plays a key role in many diseases. Thus, inhibition of HSC activation and induction of apoptosis in activated HSCs has become a hot research topic in terms of inhibiting liver fibrosis. Apoptosis induction is mainly mediated through intrinsic and extrinsic pathways. In both pathways, caspases are activated and participate in apoptosis by cleaving their downstream factors, such as PARP and other important proteins (Zaman). At the basal level, caspases are synthesized in an inactive zymogen form; however, upon activation, they play a significant role in apoptosis (Samsuzzaman and Jang, 2022). Next, we investigated whether GA-induced reduction in cell density or morphological changes were associated with the activation of the apoptosis pathway. GA treatment strongly cleaved caspase 3, caspase 9, and PARP. Next, z-VAD-FMK was used to confirm the GA-induced cell effects via the caspase activation pathway. ROS can act as upstream regulators of MAPK, which can further induce apoptosis in cells (Huang et al., 2008). Of note, it was reported that MAPK signaling proteins are crucially involved in apoptosis, cell growth, and proliferation. ERK, JNK, and p-38 proteins of MAPK have the ability to induce apoptosis by interacting with p-53 and mitochondrial membrane proteins (Huang et al., 2008). In addition, JNK and p-38 activation is known to upregulate the pro-apoptotic process, but they do not always work in tune (Kuo et al., 2018). To comply with these notions, we also examined the impact of GA on ROS production and MAPK proteins. In our study, GA significantly increased ROS production and activated MAPK proteins such as ERK, JNK, and p-38. Under normal conditions, HSCs retain lipid droplets and exhibit a quiescent-like structure. Upon exposure to various endogenous or exogenous stimuli, HSCs are activated into myofibroblast-like structures and express several fibrotic markers. Liver fibrosis is characterized by the upregulation of α-SMA and Col1-a1 in the liver (Higashi et al., 2017). Thus, GA treatment strongly inhibited the protein and mRNA levels of α-SMA and Col1-a1 and reversed them to a quiescent structure. Transforming growth factor-j1 (TGF-j1) is known for its profibrotic function, mainly through the activation of the Smad2/3 signaling pathway (Derynck and Budi, 2019). GA also inhibited Smad signaling, even in the presence of TGF-j1 in HSCs. Finally, we elucidated the mechanism of GA-induced apoptosis in LX-2 cells. Based on cell viability and western blotting assays, we found that ERK and JNK activation by GA was involved in apoptosis induction.

In conclusion, the anti-fibrotic effects and plausible molecular mechanisms of GA have been demonstrated in the human hepatic stellate cell LX-2 cell line. GA significantly decreased the cell density, proliferation, and lactate production of activated LX-2 cells by inducing apoptosis through caspase enzyme activation and upregulation of phosphorylated forms of ERK and JNK proteins. GA also dramatically suppressed primary fibrotic markers α-SMA and Col1-a1 at the protein and mRNA levels. This study provides information on the beneficial effects of secondary metabolites produced during metabolism in our body and potential development of a new anti-fibrotic drug by targeting or modifying the chemical structure of these kinds of secondary metabolites. Further research is required on enzymes involved in the production of GA. It is necessary to consider whether enzymes, along with GA, may be more important in liver fibrosis.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGMENTS**

This research was supported by a grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2018R1D1A1B07049500).

**AUTHOR CONTRIBUTIONS**

Conceptualization, design, experiment, Formal analysis, writing draft preparation: M.S, Funding acquisition, Supervision, review, and editing: KSY.

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https://doi.org/10.4062/biomolther.2022.131