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Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH_2-terminal kinase-dependent activation of mitochondrial pathway

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Abstract

Genipin, the aglycone of geniposide, exhibits anti-inflammatory and anti-angiogenic activities. Here we demonstrate that genipin induces apoptotic cell death in FaO rat hepatoma cells and human hepatocarcinoma Hep3B cells, detected by morphological cellular changes, caspase activation and release of cytochrome *c*. During genipin-induced apoptosis, reactive oxygen species (ROS) level was elevated, and *N*-acetyl-L-cysteine (NAC) and glutathione (GSH) suppressed activation of caspase-3, -7 and -9. Stress-activated protein kinase/c-Jun NH_2-terminal kinase 1/2(SAPK/JNK1/2) but neither MEK1/2 nor p38 MAPK was activated in genipin-treated hepatoma cells. SP600125, an SAPK/JNK1/2 inhibitor, markedly suppressed apoptotic cell death in the genipin-treated cells. The FaO cells stably transfected with a dominant-negative c-Jun, TAM67, was less susceptible to apoptotic cell death triggered by genipin. Diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, inhibited ROS generation, apoptotic cell death, caspase-3 activation and JNK activation. Consistently, the stable expression of Nox1-C, a C-terminal region of Nox1 unable to generate ROS, blocked the formation of TUNEL-positive apoptotic cells, and activation of caspase-3 and JNK in FaO cells treated with genipin. Our observations imply that genipin signaling to apoptosis of hepatoma cells is mediated via NADPH oxidase-dependent generation of ROS, which leads to downstream of JNK.

Keywords: Genipin; Apoptosis; Caspase; NADPH oxidase; Reactive oxygen species; Mitochondria

1. Introduction

Geniposide is one of the major iridoid glycosides in gardenia fruit used as an oriental folk medicine, and is hydrolyzed to the aglycone genipin by β-D-glycosidases in the intestines and the liver [1]. Geniposide has been shown to possess detoxifying, antioxidant, anticarcinogenic and anti-angiogenic activities [2,3]. Penta-acetyl geniposide, an acetylated derivative of geniposide, was reported to inhibit the growth of rat C6 glioma cells in culture and in the bearing rats [4], and to induce apoptosis of the same tumor cells through the activation of protein kinase Cδ [5]. Although genipin was not studied in detail, its several pharmacological actions have been documented in recent years. Genipin shows neuritogenic effect by activating mitogen-activated protein kinase (MAPK) through the soluble guanylate cyclase-cGMP-dependent protein kinase signaling pathway in PC12h cells, a cell line derived from rat pheochromocytoma [6,7]. Recently, it has also been shown to possess anti-inflammatory, anti-angiogenic and NO production-inhibitory properties [8].

Abbreviations: GSH, reduced glutathione; DPI, diphenyleneiodonium; HA, hemagglutinin; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorohydrofluorescein diacetate; FACS, fluorescence-activated cell sorting; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; SAPK, stress-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositol 3-kinase; JNK, c-Jun NH_2-terminal kinase; TGF-β1, transforming growth factor β1; PBS, phosphate-buffered saline

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Reactive oxygen species (ROS), such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), have been shown to induce various biological processes, including apoptosis [9]. This implies that the redox state of a cell is a crucial factor in deciding its susceptibility to apoptotic stimuli [10]. ROS at low concentration play the role of an intracellular messenger in many molecular events, including cell proliferation and apoptosis, while the production of large amounts of ROS contributes to apoptosis [11]. Recent studies indicate that hepatoma cell growth is ROS-dependent, and fluctuation of the intracellular redox state regulates hepatoma cell growth through Akt phosphorylation and PI3K/Akt pathway, resulting in a broad array of responses from cellular proliferation to apoptosis [12]. GD3, a disialoganglioside implicated in cell growth and proliferation, mediates regulation of cell proliferation and apoptosis via the recruitment of ROS in human aortic smooth muscle cells [13]. Although some of the compounds to evoke oxidative stress, including peroxisome proliferatiors, are not free radicals themselves, they cause the generation of ROS, rendering cells more susceptible to apoptosis [14,15]. Cellular antioxidants such as glutathione (GSH) and thioredoxin are suggested to mediate apoptosis via the regulation of ROS levels [16].

NADPH oxidase catalyzes the reduction of molecular oxygen to superoxide, which is then converted into other oxidants, and is crucial especially for the killing of bacteria and fungi in phagocytes [17]. ROS generated by NADPH oxidase are supposed to participate in the induction of apoptosis in neutrophils [18] and leukemia cells [19]. The phagocytic NADPH oxidase consists of five protein components, namely two transmembrane flavocytochrome *b* components ($gp91^{phox}$ and $p22^{phox}$) and three cytosolic components ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) [20]. The Nox family protein Nox1/3/4/5 and Duox1/2 in nonphagocytic cells were identified to be homologous to $gp91^{phox}$ (Nox2), the catalytic subunit of phagocytic NADPH oxidase [21]. Nox1 is regulated by NOXO1 (Nox organizer 1) and NOXA1 (Nox activator 1), homologs of $p47^{phox}$ and $p67^{phox}$, respectively [22]. For Nox1, NOXO1 cooperates with NOXA1 to regulate the catalytic subunit [2].

In the present study, we demonstrate that genipin induces apoptosis in human hepatocarcinoma Hep3B and rat hepatoma FaO cell lines, which is mediated via NADPH oxidase-dependent generation of ROS leading to the activation of stress-activated protein kinase/c-Jun NH_2-terminal kinase 1/2(SAPK/JNK1/2).

2. Materials and methods

2.1. Cell culture and generation of stable cell lines

FaO rat hepatoma and human hepatocarcinoma Hep3B cell lines, obtained from American Type Culture Collection (Manassas, VA), were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For stable expression of dominant negative c-Jun (pcDNA3 c-Jun (TAM67), kindly provided by Dr. M.J. Birrer, National Cancer Institute, Rockville, MD, USA) and dominant-negative Nox1-C (pcDNA3.0-HA-Nox1-C, kindly provided by Dr Y.S. Bae, Ewha Womans University, Seoul, Korea) mutants, FaO cells were transfected with each of the plasmids using Lipofectamin Plus reagent (Gibco BRL, Grand Island, NY). One day after transfection, cells were selected for neomycin resistance. After 2 weeks of selection, neomycin-resistant and fluorescence-positive colonies were isolated, expanded, and analyzed.

2.2. Caspase-3 assay

Caspase-3 activity in cytosolic extracts was determined with a spectrophotometric assay, as described previously [23]. Briefly, the peptide substrate *N*-acetyl-Asp-Glu-Val-Asp-ρ-nitroanilide (Ac-DEVD-ρNA) was added to the cell lysates in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol) and incubated at 37°C. The cleavage of the substrate was monitored at 405 nm.

2.3. Flow cytometric analysis

For flow cytometric assay [24], hepatoma cells were grown in six-well plates and incubated for 24 h at 37°C, and then treated with genipin, geniposide or TGF-β1. After 24 h, cells were harvested and washed twice with PBS (pH 7.4). After fixing in 80% ethanol for 30 min, cells were washed twice and resuspended in PBS (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml propodium iodide (PI), and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed by a FACScan cytometer (Program CellQuest, BD Biosciences).

2.4. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay

Control and HA-Nox1-C-expressing FaO cells were plated at 5×10^4 cells/eight-well chamber slide (Nalge Nunc International, Rochester, NY) and incubated for 24 h. The cells were treated with genipin for 24 h and fixed with 4% paraformaldehyde (pH 7.4) for 10 min. Apoptotic cells were assessed by measuring DNA fragmentation in a standard TUNEL assay according to the instructions with the kit (In Situ Cell Death Detection Kit, POD; Roche Molecular Biochemicals).

2.5. Immunoblot analysis

Whole-cell extracts were obtained in 1% Triton X-100 lysis buffer (50 mM Tris–Cl, pH 8.0, 150 mM sodium

chloride, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Western blotting was performed using anti-phospho-JNK (G9; Cell Signaling Technology, Beverly, MA), anti-JNK (56G8; Cell Signaling Technology, Beverly, MA), anti-phospho-MEK1/2 (166F8; Cell Signaling Technology, Beverly, MA), anti-MEK1/2 (47E6; Cell Signaling Technology, Beverly, MA), anti-phospho-p38 (3D7; Cell Signaling Technology, Beverly, MA), anti-p38 (5F11; Cell Signaling Technology, Beverly, MA), anti-phospho-c-Jun (54B3; Cell Signaling Technology, Beverly, MA), anti-cleaved caspase-3 (5A1; Cell Signaling Technology, Beverly, MA), anti-cleaved caspase-7 (Cell Signaling Technology, Beverly, MA), anti-cleaved caspase-9 (Cell Signaling Technology, Beverly, MA), anti-cytochrome *c* (7H8.2C12; PharMingen, San Diego, CA), and anti-β-actin (AC-15; Sigma, St. Louis, MO) antibodies. Protein samples were heated at 95°C for 5 min and analyzed by SDS-PAGE. Immunoblot signals were developed by Super Signal Ultra chemiluminescent reagent (Pierce Biotechnology, Rockford, IL).

2.6. Analysis of cytochrome c release

For mitochondrial cytochrome *c* release assay [25], hepatoma cells were scraped off in isotonic isolation buffer (10 mM HEPES, pH 7.6, 1 mM EDTA, 250 mM sucrose), collected by centrifugation at 2500 × *g* for 5 min at 4°C, and resuspended in hypotonic isolation buffer (10 mM HEPES, pH 7.6, 1 mM EDTA, 50 mM sucrose). Cells were disrupted by passing through a 27-gauge needle 5–10 times and checked for cracked cells by trypan blue staining. Hypertonic isolation buffer (10 mM HEPES, pH 7.6, 1 mM EDTA, 450 mM sucrose) was added to balance the buffer's tonicity. Samples were centrifuged at 100,000 × *g* at 4°C for 1 h, and supernatants containing the cytosolic proteins were recovered, and analyzed by Western blotting.

2.7. Measurement of intracellular ROS

For analysis of intracellular ROS, the redox-sensitive fluorescent probe DCFH-DA was used, as previously described [26]. Cells were incubated with 5 μM DCFH-DA for 30 min at 37°C. The harvested cells were immediately analyzed by a flow cytometry.

2.8. Northern blot analysis

Total RNA was isolated with TRIZOL reagent (GIBCO-BRL, Grand Island, NY) according to manufacturer's protocol. Ten micrograms of RNA was eletrophoresed on a 1.0% agarose gel containing 0.66 M formaldehyde, transferred to a Duralon-UV membrane, and cross-linked with a UV Stratalinker (Stratagene, La Jolla, CA). Blots were prehybridized and hybridized in 1% bovine serum albumin, 7% (w/v) SDS, 0.5 M sodium phosphate, 1 mM EDTA at 65°C. To detect c-Jun (TAM67) transcripts, cDNA of c-Jun was labeled by PRIME-IT random primer labeling kit purchased from Stratagene.

2.9. Statistical analysis

All data presented under Section 3 are expressed as means ± S.D., and representative of three or more independent experiments. Statistical analysis was assessed by Student's *t*-test for paired data. Results were considered significant at $P < 0.05$, and asterisks in figures denote statistically significant differences.

3. Results

3.1. Apoptosis of hepatoma cells by genipin

To assess that genipin induces apoptosis of hepatoma cells, FaO rat hepatoma cells and human hepatocarcinoma Hep3B cells were exposed to 200 μM genipin or 200 μM geniposide for 24 h. A fluorescence-activated cell sorter (FACS) analysis was conducted for treated FaO cells. The cell death percentage of genipin-treated FaO cells counted in sub-G_1 phase was similar to the TGF-β1-treated cells (Fig. 1A). TGF-β1 was used as an apoptotic-positive control in hepatocytes [25]. However, FACS analysis showed that the cell death percentage of geniposide-treated FaO cells was similar to that of control cells (Fig. 1A). Exposure of both hepatoma cells to 200 μM genipin for 24 h resulted in the appearance of apoptotic bodies, whereas their exposure to the same concentration of geniposide was unable to give apoptotic bodies (Fig. 1B). To clarify whether the mitochondria-dependent pathway is involved in the genipin-induced apoptosis, the efflux of cytochrome *c* from mitochondria to cytosol was examined in genipin-treated FaO cells by Western blotting. As shown in (Fig. 1C), cytochrome *c* content increased significantly in the cytosol of genipin-treated FaO cells. Taken together, genipin, but not geniposide, induces apoptosis of hepatoma cells.

3.2. Caspase activation in genipin-induced apoptosis

Caspases, known as crucial mediators of apoptosis, depends on proteolytic activation of the procaspase forms to enzymatically active forms. The sequential activation of caspases in the transduction of apoptotic signal is also characteristic of the mitochondrial apoptotic pathway [27]. Caspase-3 activity time-dependently increased in Hep3B cell treated with 200 μM genipin but not with 200 μM geniposide (Fig. 2A). When FaO cells were treated with 200 μM genipin for 6, 12 or 24 h, significant proteolytic cleavage of procaspase-3 was detected using Western

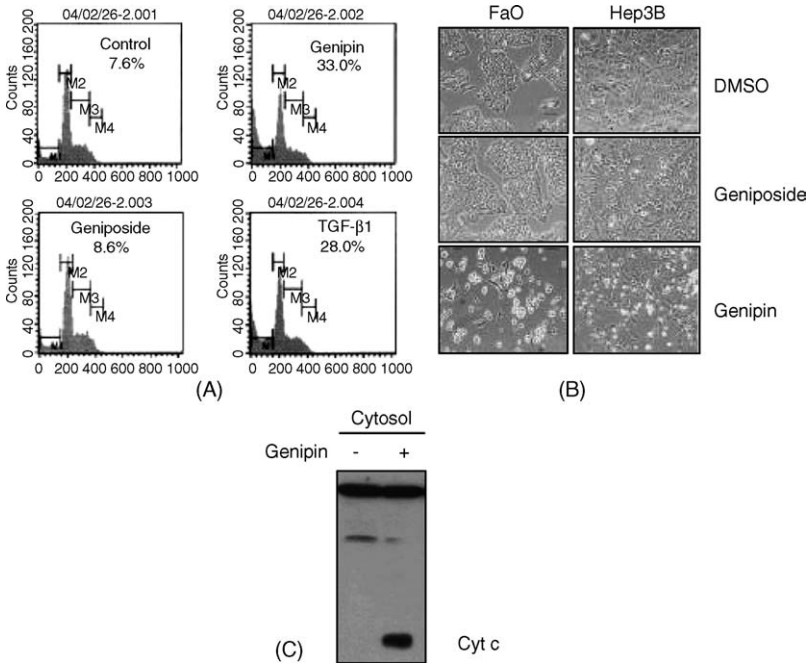

Fig. 1. Genipin-induced apoptosis in hepatoma cells. (A) FaO cells were treated with 200 μM genipin, 200 μM geniposide, 5 ng/ml TGF-β1 or vehicle for 24 h prior to flow cytometric analysis. Cell death is expressed as the percentage of cells counted in sub-G_1 phase. (B) Changes in cellular morphologies were observed by phase-contrast microscopy (magnification, 200×). FaO and Hep3B cells were treated with 200 μM genipin or 200 μM geniposide for 24 h. (C) Release of cytochrome *c* from mitochondria in FaO cells treated with 200 μM genipin, which was detected by Western blot analysis.

blotting (Fig. 2B). As expected, TGF-β1, a potent inducer of apoptosis in hepatocytes, also induced proteolytic activation of caspase-3 in FaO cells. Caspase-7, one of downstream caspases, was observed to be similarly activated in the same cells after the treatment with genipin but not with geniposide (Fig. 2B). These results suggest that activation of caspase-3 and caspase-7 is ultimately responsible for genipin-induced apoptotic process in hepatoma cells.

3.3. Participation of ROS in genipin-induced apoptosis

Although the source of reactive oxygen species (ROS) has not been completely clarified yet, ROS has been recognized as an important mediator of the stress response in many cell types and also involved in MAPK activation [16]. To demonstrate whether ROS participates in genipin-induced apoptosis, two antioxidants, *N*-acetyl-L-cysteine (NAC) and glutathione (GSH), were added into Hep3B cells growing in the presence of genipin. Concurrent treatment with NAC or GSH markedly diminished caspase-3 activity in genipin-treated Hep3B cells (Fig. 3A). The two antioxidants significantly reduced activation of caspase-3 and caspase-7, which elevated in genipin-treated hepatoma cells (Figs. 2 and 3B). Proteolytic activation of caspase-9, usually following the release of cytochrome *c* from mitochondria, also decreased in genipin-treated Hep3B cells in the presence of NAC or GSH (Fig. 3B). The redox-sensitive fluorescent dye DCFH-DA reacts with

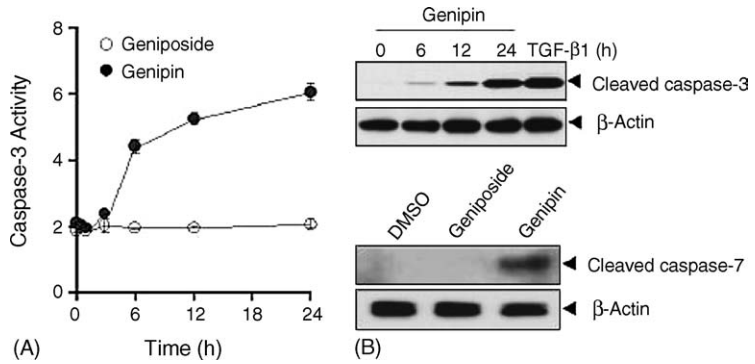

Fig. 2. Activation of caspase-3 and -7 by genipin treatment. (A) Caspase-3 activity in Hep3B cells treated with 200 μM genipin or 200 μM geniposide. The enzymatic activity is represented as ΔA_{405}/min/mg protein. (B) Immunoblot for cleaved caspase-3 and -7. For the detection of cleaved caspase-3, FaO cells were treated with 200 μM genipin for the varying times. For the detection of cleaved caspase-7, FaO cells were treated with 200 μM genipin or 200 μM geniposide for 24 h.

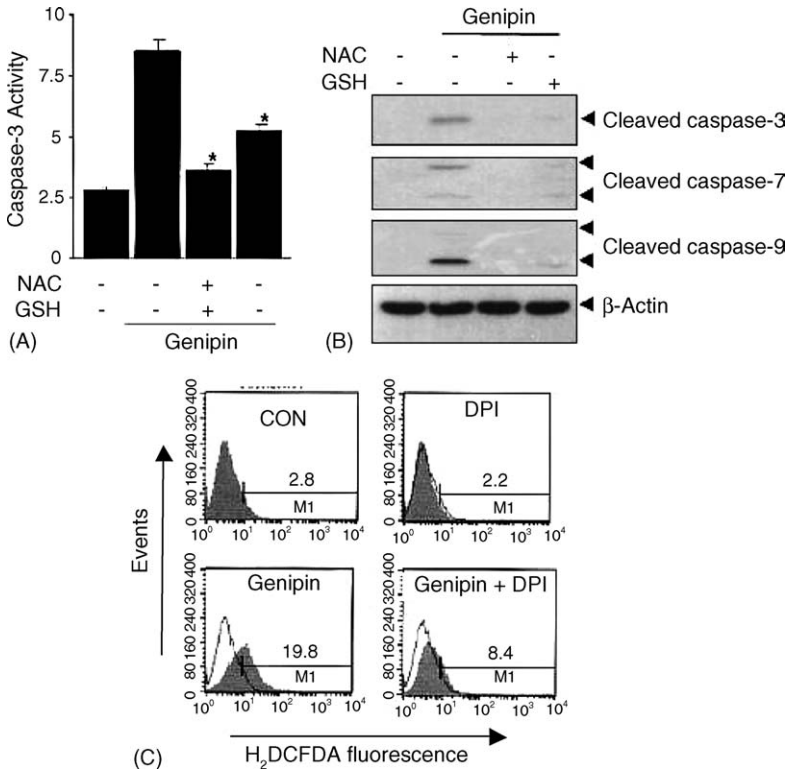

Fig. 3. Effects of *N*-acetyl-L-cysteine (NAC) and glutathione (GSH) on genipin-induced caspase activation. Hep3B cells were cultured for 24 h with or without 200 μM genipin in the presence (+) or absence (−) of 5 mM NAC or 3 mM GSH, and subjected to caspase-3 assay (A) and immunoblot analysis (B) using antibodies specific to the cleaved forms of caspase-3, -7 and -9. (C) Enhanced production of ROS in Hep3B cells treated with genipin. Hep3B cells were pretreated with the redox-sensitive fluorescent dye DCFH-DA for 0.5 h, and then, treated with 200 μM genipin in the presence or absence of diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase.

a wide range of ROS, including species derived from H_2O_2 such as $OH^{\bullet}$. DCFH-DA is hydrolyzed by intracellular esterases to a non-fluorescent product, which is readily oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF) by ROS [28]. Therefore, DCFH-DA was used to measure ROS level in genipin-treated Hep3B cells using FACS analysis. As shown in (Fig. 3C), ROS level notably increased in Hep3B cells treated with 200 μM genipin. Collectively, these results imply that ROS generation takes part in genipin-induced apoptotic process in hepatoma cells.

3.4. Activation of SAPK/JNK1/2 in genipin-induced apoptosis

To identify the involvement of extracellular signal-regulated kinase 1/2(ERK1/2), stress-activated protein kinase/c-Jun NH_2-terminal kinase 1/2(SAPK/JNK1/2) or p38 mitogen-activated protein kinase (p38 MAPK), the levels of phosphorylated and total MEK1/2, SAPK/JNK1/2 and p38 MAPK were determined in FaO cells treated with 200 μM genipin for the various times. Phosphorylation of SAPK/JNK1/2 was clearly detected in genipin-treated FaO cells, whereas MEK1/2 and p38 MAPK were not activated after genipin treatment (Fig. 4). Activation of SAPK/

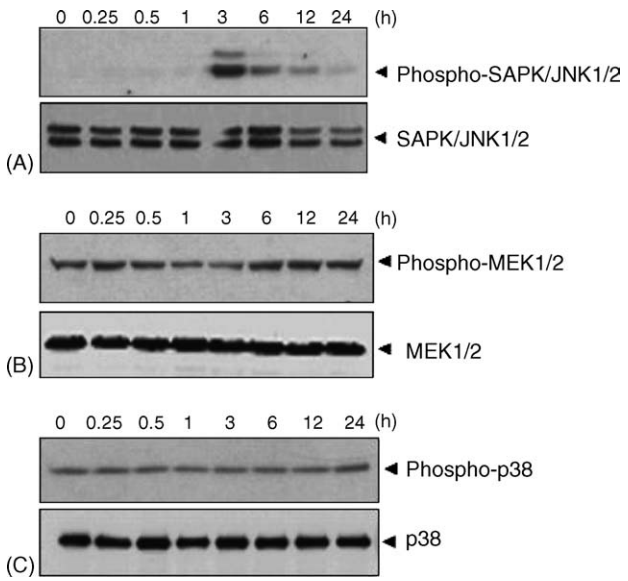

Fig. 4. Effects of genipin treatment on the activation of SAPK/JNK1/2 (A), MEK1/2 (B) and p38 MAPK (C). FaO cells were treated with 200 μM genipin for the times indicated in the figure. Cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using antibodies specific to regular and phosphorylated forms of SAPK/JNK1/2, MEK1/2 and p38 MAPK.

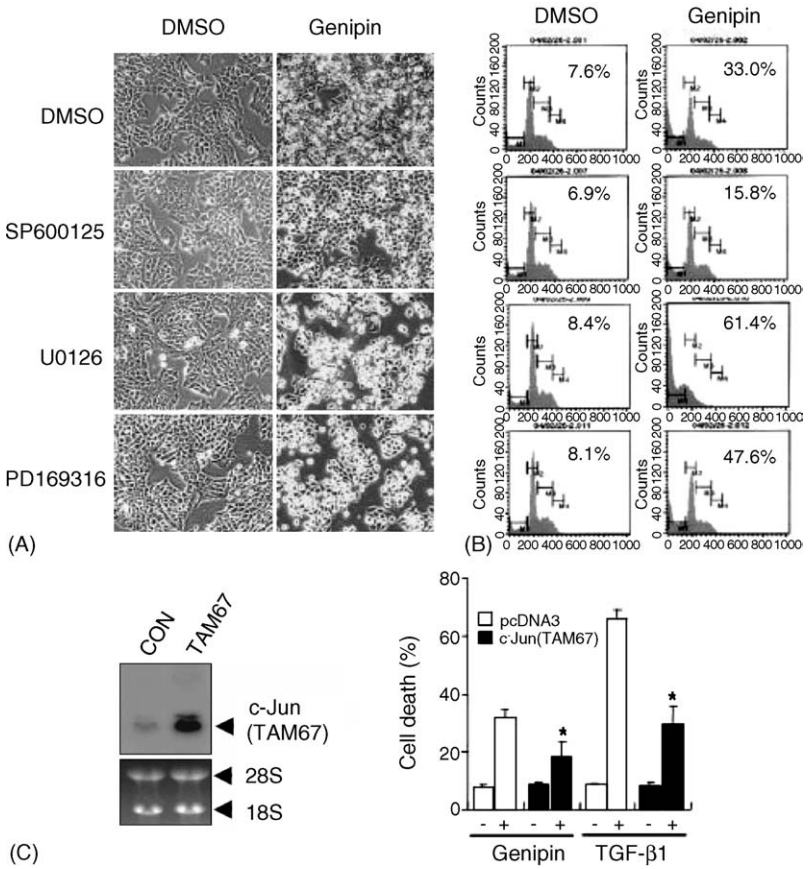

Fig. 5. Effects of JNK1/2, MEK1/2 and p38 MAPK inhibitors on genipin-induced apoptosis. FaO cells were treated with 200 μM genipin or vehicle for 24 h in the absence (control) or presence of JNK1/2 inhibitor SP600125 (20 μM), MEK1/2 inhibitor U0126 (10 μM) or p38 MAPK inhibitor PD169316 (5 μM). (A) Changes in cellular morphologies. (B) Flow cytometric analysis. (C) FaO cells, which were stably transfected with the mammalian vector encoding a dominant-negative c-Jun, TAM67, were treated with 200 μM genipin or 5 ng/ml TGF-β1 for 24 h. Northern blot analysis was done to identify the TAM67 mRNA. TUNEL-positive apoptotic cells were counted, and the percentage of apoptotic cells were graphed.

JNK1/2 during genipin-induced apoptosis was further confirmed by the use of SP600125 (an SAPK/JNK1/2 inhibitor), U0126 (an MEK inhibitor) and PD169316 (a p38 MAPK inhibitor). SP600125 markedly suppressed the formation of apoptotic bodies in genipin-treated FaO cells (Fig. 5A). In contrast, the other inhibitors, U0126 and PD169316, were unable to suppress the formation of apoptotic bodies in the same conditions (Fig. 5A). Upon FACS analysis, SP600125 only inhibited cell death of genipin-treated FaO cells (Fig. 5B). Additional evidence on the participation of SAPK/JNK1/2 in genipin-induced apoptosis was obtained using a dominant-negative c-Jun, TAM67 [29]. The number of TUNEL-positive apoptotic cells was reduced by 48.6% of stage-matched controls in FaO cells stably transfected with the mammalian vector encoding TAM67 (Fig. 5C). Taken together, the SAPK/JNK1/2 pathway mediates genipin-induced apoptosis in hepatoma cells.

3.5. Requirement for NADPH oxidase in genipin-induced apoptosis

Since the redox state of a cell is a crucial factor in determining its susceptibility to apoptosis, both ROS and antioxidants play a critical role in regulating cell death following the application of some apoptotic stimuli. Although mitochondria are the major source of ROS production in most cells, NADPH oxidase, located primarily at the plasma membrane of a variety of cells including phagocytes and hepatocytes, generates ROS playing a regulatory role in apoptosis. Generation of ROS in genipin-induced apoptosis has already been shown in (Fig. 3C). However, DPI, an inhibitor of NADPH oxidase, considerably suppressed the generation of ROS in genipin-treated Hep3B cells (Fig. 3C). This result indirectly suggests that NADPH oxidase plays a role in the generation of ROS in genipin-induced apoptosis. DPI significantly suppressed genipin-induced apoptosis in FaO cells (Fig. 6A), and decreased caspase-3 activity enhanced during genipin-induced apoptosis (Fig. 6B). DPI also inhibited phosphorylation of c-Jun by genipin, which resulted from SAPK/JNK1/2 activation in genipin-induced apoptosis (Fig. 6C). Nox1-C gene containing C-terminal residues 217 to 550 is unable to support the induction of ROS generation [21]. FaO cells stably transfected with pcDNA3.0-HA-Nox1-C [21] appeared to be less susceptible to genipin-induced apoptosis in FaO cells, which had

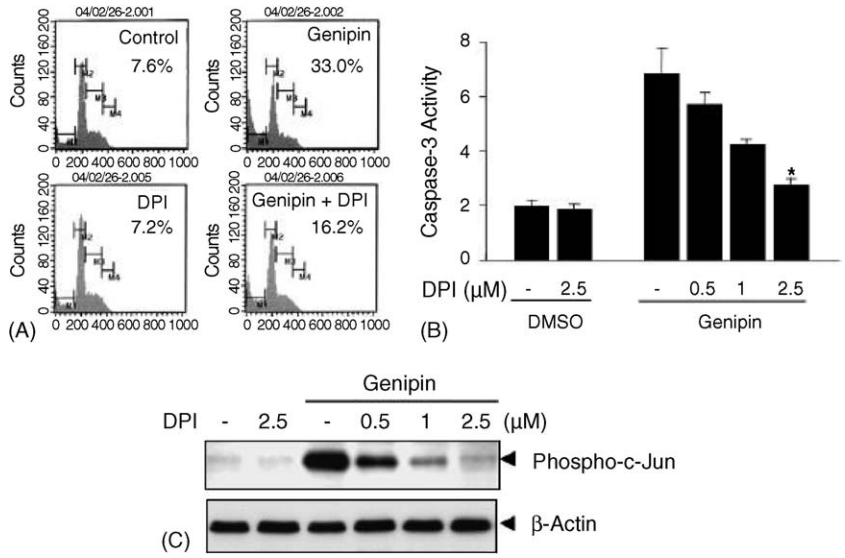

Fig. 6. Effects of DPI, an inhibitor of NADPH oxidase, on genipin-induced apoptosis. FaO cells were treated with 200 μM genipin for 3 h in the absence or presence of varying concentrations of DPI. (A) Flow cytometric analysis. (B) Changes in caspase-3 activity. (C) Phosphorylation of c-Jun, a substrate of SAPK/JNK1/2.

been detected by TUNEL assay (Fig. 7A), and gave rise to decreased proteolytic activation of caspase-3 in the presence of genipin compared to control cells (Fig. 7B). Less phosphorylation of c-Jun in the presence of genipin was observed in Nox1-C-expressing FaO cells (Fig. 7C). These results suggest that activation of SAPK/JNK1/2 in genipin-induced apoptosis requires ROS generation by NADPH oxidase. Taken together, NADPH oxidase is required for genipin-induced apoptosis in hepatoma cells.

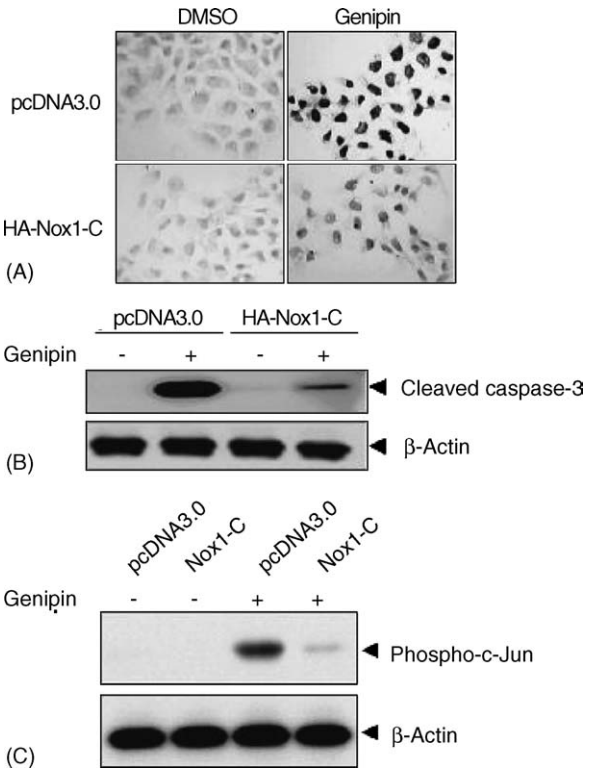

Fig. 7. Requirement of NADPH oxidase on genipin-induced apoptosis of hepatoma cells. Control and Nox1-C-expressing FaO cells were treated with 200 μM genipin for 24 h. pcDNA3.0-HA-Nox1-C [21] encodes Nox1-C containing C-terminal residues 217–550, which is unable to support the induction of ROS generation. (A) Detection of TUNEL-positive apoptotic cells. (B) Activation of caspase-3. (C) Phosphorylation of c-Jun, a substrate of SAPK/JNK1/2.

4. Discussion

Apoptosis, programmed cell death, is known to be essential to develop and maintain homeostasis during cell growth and elimination of damaged cells in multicellular organisms. Disregulation of apoptosis, a genetically and evolutionarily highly conserved process, is one of principal mechanisms leading to many hepatic diseases, and failure of apoptosis is regarded as a major determinant in development of hepatocellular carcinoma [30]. The present study demonstrates that genipin, a plant-derived iridoid, induces cell death in the two different hepatoma cell lines. Based on FACS analysis, changes in cellular morphologies, and caspase activation, the cell death of hepatoma cells by genipin is confirmed to be apoptosis. However, geniposide, an iridoid glycoside, is unable to induce apoptosis in the hepatoma cells. Penta-acetyl geniposide, produced by acetylation of geniposide, has been recently shown to induce apoptosis in rat C6 glioma cells through the activation but not the synthesis of protein kinase Cδ [5,31]. Inability of geniposide to induce apoptosis may arise from the presence of its free hydroxyl groups in D-glucose moiety. Similarly, the flavonoid myricitin but not its respective glycoside, myricitrin, reduces the viability of human leukemia HL-60 cells via apoptosis, which occurred through a mitochondrial-dependent, ROS-independent pathway [32]. Quercetin, but not its glycosides quercitrin or rutin, possesses effective preventive ability on

H_2O_2-induced apoptosis [33]. During apoptotic process, cytochrome *c* is released from mitochondria to cytosol, due to increased membrane permeability as a result of interaction between translocated Bax and pore proteins on the mitochondrial membrane, and the released cytochrome *c* complexes with pro-caspase 9 and Apaf-1 to induce caspase activation [34]. As shown in (Fig. 1C), cytochrome *c* is significantly released from mitochondria to cytosol in genipin-treated FaO cells, suggesting that genipin-induced apoptosis occurs in the mitochondrial apoptotic pathway.

ROS including H_2O_2 activates a variety of intracellular signaling cascades closely associated with both cell death and cell survival pathways [35,36]. It has been proposed that ROS plays a role as a mediator of apoptosis [37]. ROS was recently shown to induce apoptosis by regulating the phosphorylation and ubiquitination of Bcl-2 family proteins, resulting in increased pro-apoptotic protein levels and decreased anti-apototic protein expression [38]. It was suggested that the increased ROS production in choline deficiency-induced apoptosis of rat hepatocytes might be related to dysfunction of the mitochondrial respiratory chain [39]. A few natural products such as curcumin [37] and phycocyanin [40] have already been shown to generate ROS mediating their pro-apoptotic effects preceded by down-regulation of the anti-apoptotic Bcl-2. NAC reduced dissipation of the mitochondrial membrane potential, caspase 9 activation, and apotosis, indicating a role for hepatocarcinogen-induced ROS in human HepG2 cells [41]. Hence, antioxidants such as GSH have been shown to contain a regulatory role in the initiation of apoptosis. As shown in Fig. 3C, ROS greatly increased in Hep3B cells treated with 200 μM genipin. NAC and GSH, two different antioxidants, markedly suppressed genipin-induced enhancement of caspase-3 activity and proteolytic activation of caspase-3, -7 and -9 (Fig. 3A and B). These results suggest that the generation of ROS mediates genipin-induced apoptosis in hepatoma cells.

The mitogen-activated protein kinase (MAPK) family of proteins comprises three signal transduction pathways each with distinct terminal kinases, including extracellular-regulated kinase 1/2(ERK1/2), stress-activated protein kinase/c-Jun NH_2-terminal kinase 1/2(SAPK/JNK1/2) and p38 MAPK [42,43]. The ERK pathway is preferentially activated by mitogens and growth factors, and ERK1/2 is considered to be a survival mediator involved in the protective action of growth factors against cell death [44]. SAPK/JNK1/2 and p38 MAPK pathways are generally activated by stress agents, and are implicated as key regulators of stress-induced apoptosis in different cell types [45,46]. However, the induction of cell death can be mediated via ERK1/2 [44], whereas SAPK/JNK1/2 and p38 MAPK activation may protect cells against the induction of cell death [47,48]. Genipin resulted in an up-regulation of SAPK/JNK1/2 activation, but no effects on p38 MAPK and MEK1/2 activation in FaO cells (Fig. 4). The involvement of SAPK/JNK1/2 in genipin-induced apoptosis in hepatoma cells was further supported by the use of SP600125 (an SAPK/JNK1/2 inhibitor) and a dominant-negative c-Jun, TAM67 (Fig. 5). These results imply that genipin induces apoptosis in hepatoma cells via the SAPK/JNK1/2 pathway. Previous studies have shown that H_2O_2 stimulates robust increases in ERK1/2, SAPK/JNK1/2 and protein kinase B (PKB) in human SH-SY5Y neuroblastoma cells, and inhibition of the ERK1/2 pathway protects SH-SY5Y cells from H_2O_2-induced cell death [36]. SAPK/JNK1/2 and p38 MAPK activation was observed following enhanced ROS production in oncogenically transformed NIH3T3 cells [49]. ROS generated during hyperoxia mediates cell death of lung epithelium via ERK1/2 activation [50]. These findings and our results propose that activation of SAPK/JNK1/2, ERK1/2and/or p38 MAPK following ROS generation may depend on ROS types and levels in specific cells.

NADPH oxidase, originally identified in phagocytic cells, catalyzes the reduction of oxygen to $O_2^{-\bullet}$, which in turn leads to the production of secondary derivatives such as $OH^{\bullet}$ and H_2O_2. Therefore, NADPH oxidase may contribute to the induction of apoptosis in a variety of cells. DPI, a flavoprotein inhibitor whose targets include NADPH oxidase, decreased ROS generation caused by genipin (Fig. 3C), and accordingly, suppressed genipin-induced apoptosis in FaO cells (Fig. 6A). It also suppressed enhancement of caspase-3 activity (Fig. 6B) and phosphorylation of c-Jun, a major target of SAPK/JNK (Fig. 6C) in genipin-treated FaO cells. Participation of NADPH oxidase in genipin-induced apoptosis was additionally confirmed by the use of Nox1-C, which is unable to support the induction of ROS generation [21]. Nox4, which is mainly expressed in cultured vascular smooth muscle cells (SMCs), is overproduced by oxidative stress such as 7-ketocholesterol, leading to the activation of the unfold protein response and pro-apoptotic signaling pathway and thus to SMC death [51]. NADPH oxidase-derived ROS induces activation of ERK1/2 required for the *Entamoeba*-induced neutrophil apoptosis [52]. On the contrary, growth factor-induced ROS produced by Nox4 protects pancreatic cancer cells from apotosis, implying that this mechanism may play an important role in pancreatic cancer resistance to treatment [53]. In genipin-induced apoptosis of hepatoma cells, our results indicate that NADPH oxidase is required for changes in cellular morphologies, caspase-3 activation, formation of TUNEL-positive apoptotic cells, and phosphorylation of c-Jun. However, genipin was previously shown to suppress in vitro Fas-mediated apoptosis in primary-cultured murine hepatocytes through the inhibition of activation of caspase-3 and caspase-8 and rapid reduction of membrane permeability transition [54].

In conclusion, genipin induces apoptosis in hepatoma cells via sequential events such as stimulation of NAPH oxidase, ROS generation, activation of SAPK/JNK1/2, release of cytochrome *c* and caspase activation.

Genipin-induced apoptosis may be related with its anti-inflammatory and anti-angiogenic activities.

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