Arctigenin Enhances Chemosensitivity of Cancer Cells to Cisplatin Through Inhibition of the STAT3 Signaling Pathway

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ABSTRACT

Arctigenin is a dibenzylbutyrolactone lignan isolated from *Bardanae fructus*, *Arctium lappa* L, *Saussureamedusa*, *Torreya nucifera*, and *Ipomea cairica*. It has been reported to exhibit anti-inflammatory activities, which is mainly mediated through its inhibitory effect on nuclear transcription factor-kappaB (NF-κB). But the role of arctigenin in JAK-STAT3 signaling pathways is still unclear. In present study, we investigated the effect of arctigenin on signal transducer and activator of transcription 3 (STAT3) pathway and evaluated whether suppression of STAT3 activity by arctigenin could sensitize cancer cells to a chemotherapeutic drug cisplatin. Our results show that arctigenin significantly suppressed both constitutively activated and IL-6-induced STAT3 phosphorylation and subsequent nuclear translocation in cancer cells. Inhibition of STAT3 tyrosine phosphorylation was found to be achieved through suppression of Src, JAK1, and JAK2, while suppression of STAT3 serine phosphorylation was mediated by inhibition of ERK activation. Pervanadate reversed the arctigenin-induced downregulation of STAT3 activation, suggesting the involvement of a protein tyrosine phosphatase. Indeed, arctigenin can obviously induce the expression of the PTP SHP-2. Furthermore, the constitutive activation level of STAT3 was found to be correlated to the resistance of cancer cells to cisplatin-induced apoptosis. Arctigenin dramatically promoted cisplatin-induced cell death in cancer cells, indicating that arctigenin enhanced the sensitivity of cancer cells to cisplatin mainly via STAT3 suppression. These observations suggest a novel anticancer function of arctigenin and a potential therapeutic strategy of using arctigenin in combination with chemotherapeutic agents for cancer treatment. J. Cell. Biochem. 112: 2837–2849, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: STAT3; ARCTIGENIN; CHEMOSENSITIVITY; CISPLATIN

Signal transducers and activators of transcription (STAT) proteins are a family of seven proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6) that have been shown to play an important role in tumor cell survival and proliferation [Yu and Jove, 2004]. Among the STATs, STAT3 is often constitutively active in a variety of human cancers, including multiple myeloma [Yu et al., 2007], head and neck cancers [Song and Grandis, 2000], hepatocellular carcinoma [Yoshikawa et al., 2001], lymphomas, and leukemia [Zhang et al., 2002].

The major phosphorylation sites in STAT3 activation include tyrosine and serine residues at positions 705 and 727, respectively.

Phosphorylation of STAT3 on Tyr705 is mediated through the activation of Janus-activated kinases and Src family kinases [Yu et al., 1995], while STAT3 can be phosphorylated on Ser727 by the activation of kinases of the mitogen-activated protein kinase family [Decker and Kovarik, 2000], which enhances its transcriptional regulatory activities [Wen et al., 1995]. Fully activated STAT3 undergoes dimerization, nuclear localization and DNA binding and then regulates specific target genes [Reich and Liu, 2006]. These target genes products include antiapoptotic proteins (Bcl-2 [Real et al., 2002]; Mcl-1 [Epling-Burnette et al., 2001; Liu et al., 2003], cIAP2 [Bhattacharya and Schindler, 2003; Bhattacharya et al.,

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2005]) proliferation regulatory proteins (Cyclin D1 [Leslie et al., 2006], c-Myc [Kiuchi et al., 1999]).

STAT3 signaling pathway has recently been shown to confer resistance to chemotherapy-induced apoptosis in human tumors [Catlett-Falcone et al., 1999; Barre et al., 2007]. Thus, abrogation of STAT3 activation is believed to render tumor cells more susceptible to cancer therapeutic agents.

Arctigenin is a dibenzylbutyrolactone lignan isolated from *Bardanae fructus*, *Arctium lappa* L., *Saussureamedusa*, *Torreya nucifera*, and *Ipomea cairica*. It has been reported to exhibit anti-inflammatory activities, which is mainly mediated through its inhibitory effect on nuclear transcription factor-kappaB (NF-κB) [Cho et al., 2002]. However, whether arctigenin could inactivate other survival signaling pathways to achieve its antitumor effect remains unclear. Here we reveal a novel mechanism by which arctigenin could suppress JAK-STAT3 signaling pathway and sensitize cancer cells to cisplatin.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Actigenin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and dissolved in dimethyl sulfoxide as a 60 mM stock solution and stored at −20°C. Further dilution was done as needed in cell culture medium.3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide(MTT), cisplatin, U0126, Dimethyl sulfoxide were purchased from Sigma–Aldrich (St. Louis, MO). 4,6-Diami-dino-2-phenylindole (DAPI) was from Invitrogen (Grand Island, NY). Recombinant human IL-6 was from PreproTechn, Inc. (Rochy Hill, NJ). Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against GAPDH, Bcl-2, cIAP2, cyclin D1, Mcl-1, C-myc were obtained from Santa Cruz Biotechnology. SHP-2, H3.1 antibodies were from Signalway Antibody (Pearland, TX). Antibodies tocaspase-3, PARP, phospho-STAT3(Tyr705), phospho-STAT3 (Tyr727), STAT5, phospho-STAT5, phospho-Akt, Akt, phosphor-Src (Tyr416), Src, phospho-JAK1(Tyr1022/1023), JAK1, phospho-JAK2 (Tyr 1007/1008) and JAK2, ERK, phospho-ERK, JNK, phospho-JNK, P38, phospho-P38 were purchased from Cell Signaling Technology (Beverly, MA).

CELL LINES AND TRANSFECTION

Human cancer cells, HepG2, HeLa and K562 were obtained from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, People's Republic of China), and cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. Dominant-negative STAT3-Y705F (STAT3F) and. control plasmids were a kind gift from Dr. Xunli Xia at Huazhong University of Science and Technology, China. Transient transfection was performed by the Lipofect-AMINE 2000 reagent (Invitrogen) according to the manufacture's instructions. In all cases, the total amount of DNA was normalized by the empty control plasmids. The overall transfection efficiency for cells assessed by X-Gal staining assay was 48–50%.

WESTERN BLOT

Cells were lysed in the lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 25 mM β-glycerophoshate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF and 1 mM phenylmethylsulfonyl fluoride (PMSF) supplemented with complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) at 4°C. Cells lysates were centrifuged (15,000g) at 4°C for 15 min and then subjected to SDS–PAGE followed by transferring onto nitrocellulose membranes (Whatman). The antibody-antigen complexes were visualized by the Li-COR Odyssey Infrared Imaging System according to the manufacturer's instruction using IRDye800 flurophore-conjugated antibody (Li-COR Biosciences, Lincoln, NE). Quantification was directly performed on the blot using the Li-COR Odyssey analysis software.

MTT ASSAY

The cytotoxicity of cisplatin and actigenin on different human cancer cells was detected by MTT dye uptake method as described previously [Yang et al., 1999]. Briefly, the cells (5×10^3/ml) were incubated in a 96-well plate with actigenin or cisplatin, either individually or in combination, in a final volume of 0.2 ml for indicated for concentrations and time intervals at 37°C. Thereafter, 20 μl MTT solution (5 mg/ml in PBS) was added to each well. After a 2-h incubation at 37°C, 0.1 ml lysis buffer (20% SDS, 50% dimethylformamide) was added; incubation was continued overnight at 37°C; and then the optical density (OD) at 570 nm was measured by BioTeck plate reader.

FLOW CYTOMETRIC ANALYSIS

To determine the effect of actigenin on the cell cycle, cells were first synchronized by serum starvation and then exposed to actigenin for various durations. Thereafter cells were washed, fixed with 70% ethanol, and incubated for 30 min at 37°C with 0.1% RNase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 μg/ml propidium iodide (PI) for 30 min at room temperature. Cell distribution across the cell cycle was detected on a Guava EasyCyte™ System, and the data were analyzed using Guava Cell Cycle Software (Guava Technologies, Hayward, CA).

PREPARATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared by nuclear extraction kit (KGA826, Keygentec, Nanjing, China) according to the manufacturer's protocol. Briefly, after washed with cold PBS, cell pellets were collected by centrifugation at 800g for 5 min, and removed the supernatant. Cell pellets were lysed with ice-cold Lysis Buffer and resuspended in Regent A, and then chilled on ice for 20 min. After 3 min centrifugation (1,000g, at 4°C), pellets were resuspended in Medium Buffer A, and transferred to new eppendorf tube with Medium Buffer B, then pellets were collected immediately as nuclear extracts by centrifugation for 10 min (1,000g, at 4°C).

DETECTION OF APOPTOSIS

Cells undergoing apoptosis were evaluated by DAPI staining for morphologic changes including chromatin condensation and

nuclear shrinkage as reported previously [Shi et al., 2005]. Briefly, after various designated treatments, medium was removed, and cells were fixed with 70% ethanol at room temperature for 10 min. This was followed by staining the fixed cells with 0.3 μg/ml DAPI (in PBS) at room temperature for another 10 min and visualized under an inverted fluorescence microscope. The cell death was quantified by counting the percentage of cell with condensed nuclear among 200 randomly selected cells.

STATISTICS

Analysis of variance (ANOVA) was used to compare the results between two groups. Individual points were compared using a Student's t test and differences were considered significant for $P < 0.05$. Data are presented as means ± SD. Western blot analysis experiments were repeated three times with similar trends.

RESULTS

We investigated the effect of arctigenin on both constitutive and IL-6-inducible STAT3 activation in cancer cells. We also evaluated the effect of arctigenin on various mediators of cellular proliferation, cell survival, and apoptosis. The structure of arctigenin is shown in Figure 1A.

ARCTIGENIN SPECIFICALLY SUPPRESSES CONSTITUTIVE STAT3 PHOSPHORYLATION AND NUCLEAR TRANSLOCATION

The ability of arctigenin to modulate constitutive STAT3 activation in HepG2 cells was investigated. Cells were incubated with different concentrations and various time of arctigenin, whole-cell extracts were prepared and the phosphorylation of STAT3 was examined by Western blot analysis using antibodies which recognize phospho-STAT3 (Ser 727 and Tyr 705) and total STAT3 expression. As shown in Figure 1B,C, arctigenin inhibited the constitutive activation of STAT3 in HepG2 cells in a dose-and time-dependent manner, with maximum inhibition occurring at around 80 μM and 8 h, respectively. Arctigenin had no effect on the expression of STAT3 protein. Because it is not certain whether phosphorylation is mandatory for nuclear transport of STAT3 and its oncogenic functions [Bowman et al., 2000], we analyzed the effect of arctigenin on STAT3 nuclear translocation, pretreatment of arctigenin almost completely abolished the phospho-STAT3 nuclear translocation (Fig. 1D).

Whether arctigenin affects the activation of other STAT proteins in HepG2 cells was also investigated. Under the conditions where actigenin completely inhibited STAT3 phosphorylation, it altered neither the levels of constitutively phosphorylated STAT5 nor the expression of STAT5 proteins (Fig. 1E).

ARCTIGENIN INHIBITS IL-6-INDUCIBLE STAT3 PHOSPHORYLATION

We also examined whether arctigenin could inhibit IL-6-induced STAT3 activation in human cancer cells. As shown in Figure 2A, IL-6 stimulation on HepG2 cells was associated with marked increases of phospho-STAT3 levels, which was effectively abolished by arctigenin treatment.

ARCTIGENIN-INDUCED INHIBITION OF STAT3 PHOSPHORYLATION IS REVERSIBLE

We examined whether arctigenin-induced inhibition of STAT3 phosphorylation is reversible. HepG2 cells were first treated for 8 h with arctigenin, and then the cells were washed twice with PBS to remove arctigenin. The cells were then cultured in fresh medium for various durations, and the levels of phosphorylated STAT3 were measured. The removal of arctigenin resulted in a gradual increase in phosphorylated STAT3 (Fig. 2B). The reversal was complete by 24 h and did not involve changes in STAT3 protein levels.

ARCTIGENIN SUPPRESSES CONSTITUTIVE ACTIVATION OF c-Src, JAK1, AND JAK2

STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families, the Janus family (JAKs) [Yu et al., 1995]. Therefore, we examined the effect of constitutive activation of Src, JAKs kinase in HepG2 cells. As expected, Arctigenin markedly suppressed phosphorylation of Src, JAK1, and JAK2, whereas the levels of total Src, JAK1, and JAK2 remained unchanged (Fig. 2C).

ARCTIGENIN INHIBIT CONSTITUTIVE ACTIVATION OF Akt

As activation of Akt has also been linked with STAT3 activation [Chen et al., 1999]. We therefore investigated whether arctigenin modulates constitutive activation of Akt in HepG2 cells. We found that arctigenin affected the constitutive phosphorylation of Akt (Fig. 2D). The levels of nonphosphorylated Akt remained unchanged under these conditions.

ARCTIGENIN INHIBITS EXTRACELLULAR SIGNAL-REGULATED KINASE

Apart from tyrosine phosphorylation, STAT3 also undergoes phosphorylation at serine residues [Decker and Kovarik, 2000], we therefore investigated whether arctigenin affects constitutive activation of extracellular signal-regulated kinase (ERK)1/2 kinases in HepG2 cells. We found that arctigenin suppressed the constitutive phosphorylation of ERK1/2 kinase (Fig. 3A), whereas other members of the MAPK family such as P38, JNK had no significant effect .The levels of nonphosphorylated ERK1/2 remained unchanged under the same conditions.

To further understand the role of ERK in arctigenin-induced suppression of STAT3 phosphorylation at Ser727, a MEK1/2 inhibitor U0126 was used to inhibit ERK1/2 activation, Arctigenin alone and in combination with U0126 caused a strong decrease in Stat3 phosphorylation at Tyr705 together with modest decrease in Ser727 phosphorylation (Fig. 3B). Thus, arctigenin-induced decrease in STAT3 phosphorylation at Ser727 may be involved in suppression of ERK activation.

TYROSINE PHOSPHATASES SHP-2 ARE INVOLVED IN ARCTIGENIN-INDUCED INHIBITION OF STAT3 ACTIVATION

Because protein tyrosine phosphatases have also been implicated in STAT3 activation [Han et al., 2006], we determined whether arctigenin-induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a protein tyrosine phosphatase (PTPase). Treatment of HepG2 cells with the broad-acting tyrosine
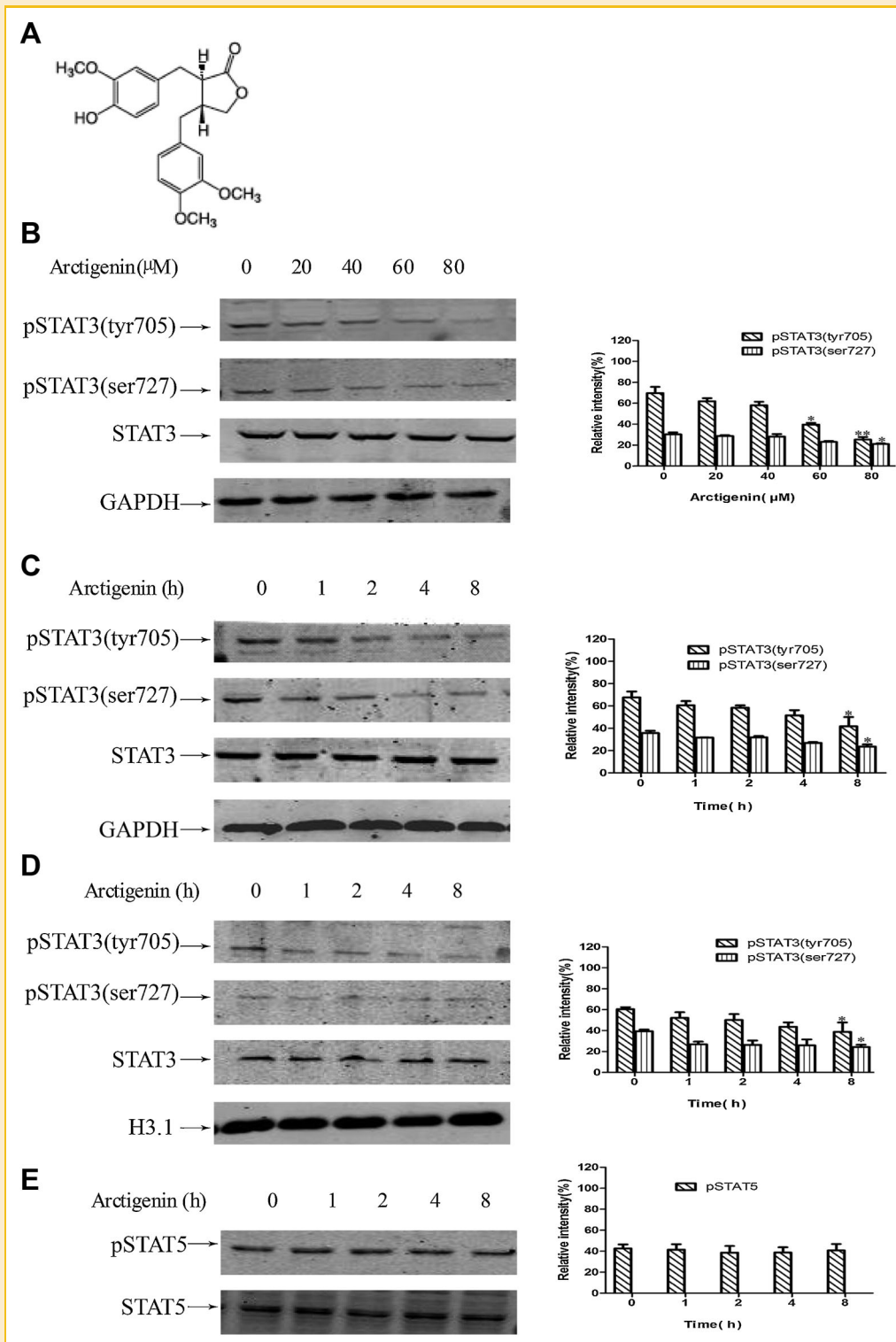
Fig. 1. Arctigenin inhibits constitutively active STAT3 in HepG2 cells. A: The chemical structure of arctigenin. B: Arctigenin suppresses phospho–STAT3 levels in a dose–dependent manner. HepG2 cells (2×10^6/ml) were treated with the indicated concentrations of arctigenin for 8 h, after which whole–cell extracts were prepared, and 30 μg of protein was resolved on 12% SDS–PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho–STAT3. C: Arctigenin suppresses phospho–STAT3 levels in a time–dependent manner. HepG2 cells (2×10^6/ml) were treated with 80 μM arctigenin for the indicated durations and analyzed for phospho–STAT3 levels. D: Arctigenin inhibits nuclear translocation. Nuclear lysates were immunoblotted with the indicated antibodies. H3.1 was used as loading controls for nucleus subfraction. E: Arctigenin had no effect on phospho–STAT5 and STAT5 protein expression. HepG2 cells (2×10^6/ml) were treated with 80 μM arctigenin for the indicated times. Whole–cell extracts were examined by Western blot using antibodies against phospho–STAT5 and STAT5. Data are representatives of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$.
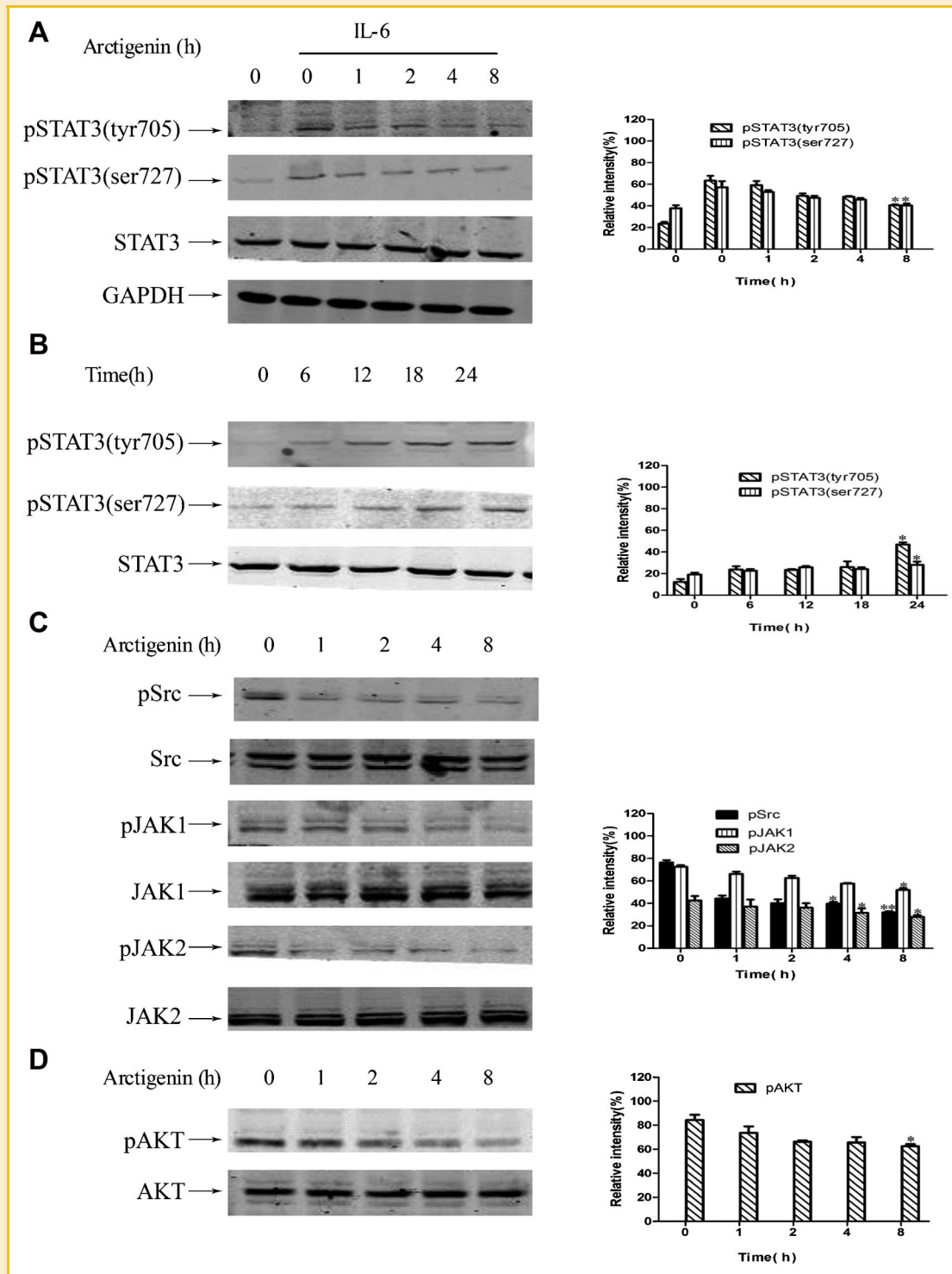
Fig. 2. A: Arctigenin downregulates IL-6-induced phospho-STAT3, HepG2 cells (2×10^6/ml) were treated with 80 μM arctigenin for the indicated times and then stimulated with IL-6 (20 ng/ml) for 15 min. Whole-cell extracts were then prepared and analyzed for phospho-STAT3 by Western blot. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. B: Arctigenin-induced inhibition of STAT3 phosphorylation is reversible. HepG2 cells were cultured in serum free medium for 24 h and then were pre-treated with 80 μM arctigenin. After 8 h of pre-treatment, the medium was discarded and fresh medium without arctigenin was added. Phosphorylated STAT3 and total STAT3 were detected by Western blot. C: Effect of arctigenin on c-Src, JAK1, and JAK2 activity. HepG2 cells (2×10^6/ml) were treated with arctigenin (80 μM) for indicated time points, whole-cell extracts were prepared, and probed for either p-Src, p-JAK1, or p-JAK2 antibody by Western blot. The same blots were stripped and reprobed with Src, JAK1, or JAK2 antibody to verify equal protein loading. D: Arctigenin inhibit constitutive activation of Akt. HepG2 cells (2×10^6/ml) were treated with 80 μM arctigenin for the indicated times. Whole-cell extracts were then prepared and analyzed for phospho-Akt by Western blotting. The same blots were stripped and reprobed with Akt antibody to verify equal protein loading. The results shown are representative of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$.
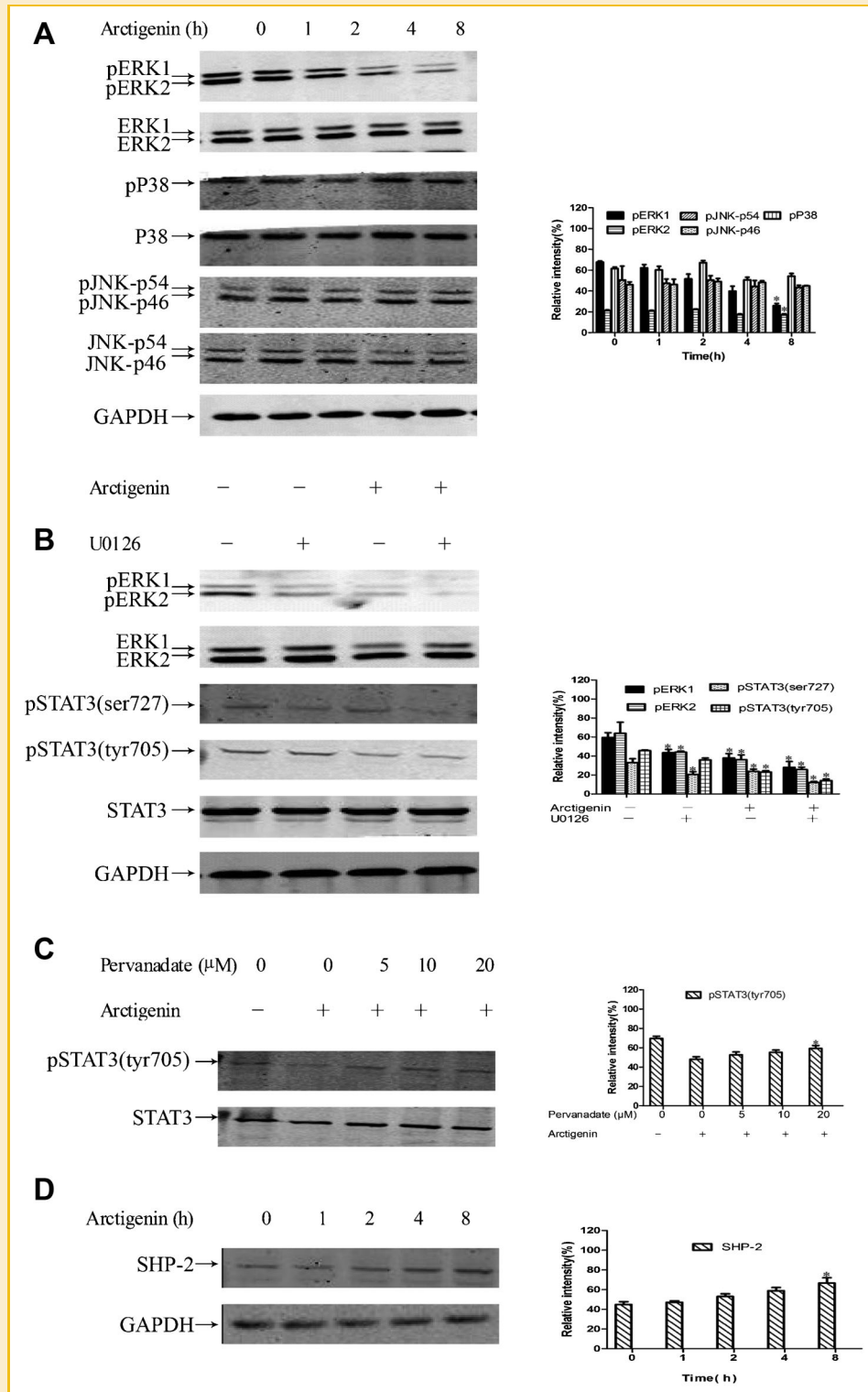
Fig. 3. A: Arctigenin inhibits ERK. HepG2 cells were treated with 80 μM arctigenin for the indicated time intervals, after which whole-cell extracts were prepared and probed with phospho-ERK1/2 antibody. The same blots were stripped and reprobed with ERK1/2 antibody. B: Arctigenin-induced dephosphorylation of STAT3 (Ser727) involved suppression of ERK activation, HepG2 cells were treated with U0126 (10 μM) for 2 h and then treated with or without arctigenin (80 μM) for 8 h. At the end of these treatments, total cell lysates were prepared and Western blot was carried out for pERK, pSTAT3 (Tyr705 and Ser727) and total ERK, STAT3. Protein loading in each case was checked by stripping and re-probing the same membranes for GAPDH. C: Pervanadate reverses the phospho-STAT3 inhibitory effect of arctigenin. HepG2 cells were first treated with the indicated concentration of pervanadate for 30 min followed by 80 μM arctigenin for 8 h, and whole-cell extracts were subjected to Western blot analysis for phospho-STAT3 and STAT3. D: Arctigenin induces the expression of SHP-2. HepG2 cells were treated with arctigenin (80 μM) for indicated time points. After treatment, whole-cell extracts were prepared and subjected to Western blot analysis for SHP-2. The same blots were stripped and reprobed with GAPDH antibody to verify equal protein loading. Data are representatives of three independent experiments. $^{*}P < 0.05$.

phosphatase inhibitor sodium pervanadate prevented the arctigenin-induced inhibition of STAT3 activation (Fig. 3C). This suggests that tyrosine phosphatases are involved in arctigenin-induced inhibition of STAT3 activation.

The PTP Src homology 2 phosphatase 2 (SHP-2) is a member of a small family of Src homology 2 (SH2) domain-containing PTPs, and has been reported to play an important role in the negative regulation of JAK/STAT signaling [You et al., 1999; Ohtani et al., 2000]. We therefore examined whether arctigenin can modulate expression of SHP-2 in HepG2 cells. We incubated cells with arctigenin for various time points. Our results suggest that the stimulation of SHP-2 expression by arctigenin was associated with the downregulation of constitutive STAT3 activation in HepG2 cells (Fig. 3D).

ARCTIGENIN DOWNREGULATES THE EXPRESSION OF cIAP2, Mcl-1, Bcl-2, C-myc, CYCLIN D1, AND CAUSES ACCUMULATION OF CELLS IN THE SUB-G1 CELL CYCLE PHASE

STAT3 activation has been shown to regulate the expression of various gene products involved in cell survival, proliferation, angiogenesis, and chemoresistance [Yu and Jove, 2004]. We found that arctigenin downregulated the expression of cIAP2, Mcl-1, Bcl-2, C-myc, cyclin D1, all of which have been reported to be regulated by STAT3 (Fig. 4A). Because D-type cyclins are required for cell progression from the G1 to the S phase of the cell cycle, we sought to determine the effect of arctigenin on cell cycle phase distribution. We found that arctigenin caused significant accumulation of cells in the sub-G1 phase, an indicator of apoptosis (Fig. 4B).

CONSTITUTIVELY ACTIVE STAT3 CONFERS RESISTANCE TO CISPLATIN-INDUCED CELL DEATH IN CANCER CELLS

Due to STAT3 resistance to chemotherapeutic drug treatment [Catlett-Falcone et al., 1999], we tested the relationship between STAT3 activation level and cellular resistance to cisplatin. HepG2, HeLa, and K562 were treated with different doses of cisplatin for 24 or 48 h, and the cell viability was detected using the MTT assay. As shown in Figure 5B, these cell lines show obvious different sensitivities to cisplatin, especially when treated for 48 h. Among them, K562 cells are the most sensitive ones, while HepG2 cells are the most resistant to cisplatin-induced cell death, which is consistent with the STAT3 phosphorylation status (Fig. 5A). Furthermore, overexpression of a dominant negative mutant of STAT3(STAT3-Y705F) completely abrogated constitutively activated STAT3 (Fig. 5C), and significantly enhanced cisplatin-induced cell growth inhibition (Fig. 5D). These data thus indicate that suppression of STAT3 would be a reasonable approach to enhance the therapeutic activity of cisplatin.

ARCTIGENIN ENHANCES CISPLATIN-INDUCED APOPTOSIS IN CANCER CELLS

We examined whether arctigenin potentiates the effects of cisplatin-induced apoptosis As shown in Figure 6A, when the cells were pretreated with arctigenin for 2 h following by cisplatin for 24 h, the cell viability of all these cell lines was reduced dramatically, comparing to the arctigenin alone and cisplatin alone treatment.

Morever, the different cancer cells with different STAT3 activation levels responded to the combined treatment of arctigenin and cisplatin had similar patterns, suggesting that the suppressed STAT3 activity by arctigenin abolishes chemoresistance of cancer cells to cisplatin.

Because cisplatin-mediated cytotoxicity is mainly executed by inducing apoptotic cell death [Kelland, 2007], we also found arctigenin could enhance the apoptotic cell death in cisplatin-treated cells (Fig. 6B), and induce cleavage of caspase-3 and its downstream substrate PARP (Fig. 6C). Thus, arctigenin would lower the apoptotic threshold and increase chemotherapeutic activity of cisplatin via suppression of STAT3.

DISCUSSION

In this study, we confirmed arctigenin suppressed both constitutive and inducible STAT3 phosphorylation in time- and dose-dependent manners and these effects were specific to STAT3, as arctigenin had no effect on STAT5 phosphorylation. Arctigenin specifically stimulated the expression of protein tyrosine phosphatase SHP-2, and it downregulated the expression of STAT3-regulated gene products, including, cIAP2, Bcl-2, cyclinD1, and Mcl-1. It induced the inhibition of proliferation, increased apoptosis, and significantly potentiated the apoptotic effects of cisplatin in some cancer cells.

As documented previously, the inhibition of the STAT3 tyrosine phosphorylation is majorly achieved via the inhibition of upstream receptor tyrosine kinases such as JAK and Src. The effects of arctigenin on STAT3 tyrosine phosphorylation are related with the suppression of Src, JAK1, and JAK2, though the involvement of other members of the JAK family such as JAK3 cannot be ruled out. Previous studies have indicated that Src and JAK1 kinase activities cooperate to mediate constitutive activation of STAT3 [Garcia et al., 2001; Jaganathan et al., 2010]. In addition to STAT3 tyrosine phosphorylation, STAT3 serine phosphorylation has been proposed to participate in the regulation of STAT3 phosphorylation. Indeed, we found that arctigenin not only inhibits STAT3 tyrosine phosphorylation but also affect STAT3 serine phosphorylation. Arctigenin-induced dephosphorylation of STAT3 (Ser727) involves suppression of ERK activation. Consistent with this result, MEK1/2-specific inhibitor U0126 was able to reduce STAT3 Ser727, not Tyr705 phosphorylation. This finding indicates that arctigenin inhibition of STAT3 signaling may be also dependent of serine phosphorylation. Nevertheless, STAT3 serine phosphorylation is much less understood, and the involved kinases are not clearly known. There are some studies suggesting that involvement of PI3K and serine phosphatase in STAT3 serine phosphorylation [Decker and Kovarik, 2000; Krasilnikov et al., 2003], More recent report suggests PKC varepsilon mediates STAT3 Ser727 phosphorylation through integration with the MAPK cascade (RAF-1, MEK1/2, and ERK1/2) [Aziz et al., 2010]. Hence, whether arctigenin-induced dephosphorylation of STAT3 serine phosphorylation is related with these kinase and phosphatase activities remains to be investigated. We also found that arctigenin inhibits Akt phosphorylation, suggested that it has an effect quite proximal to the IL-6 receptor. Thus, whether arctigenin inhibits the phosphorylation of gp130, and
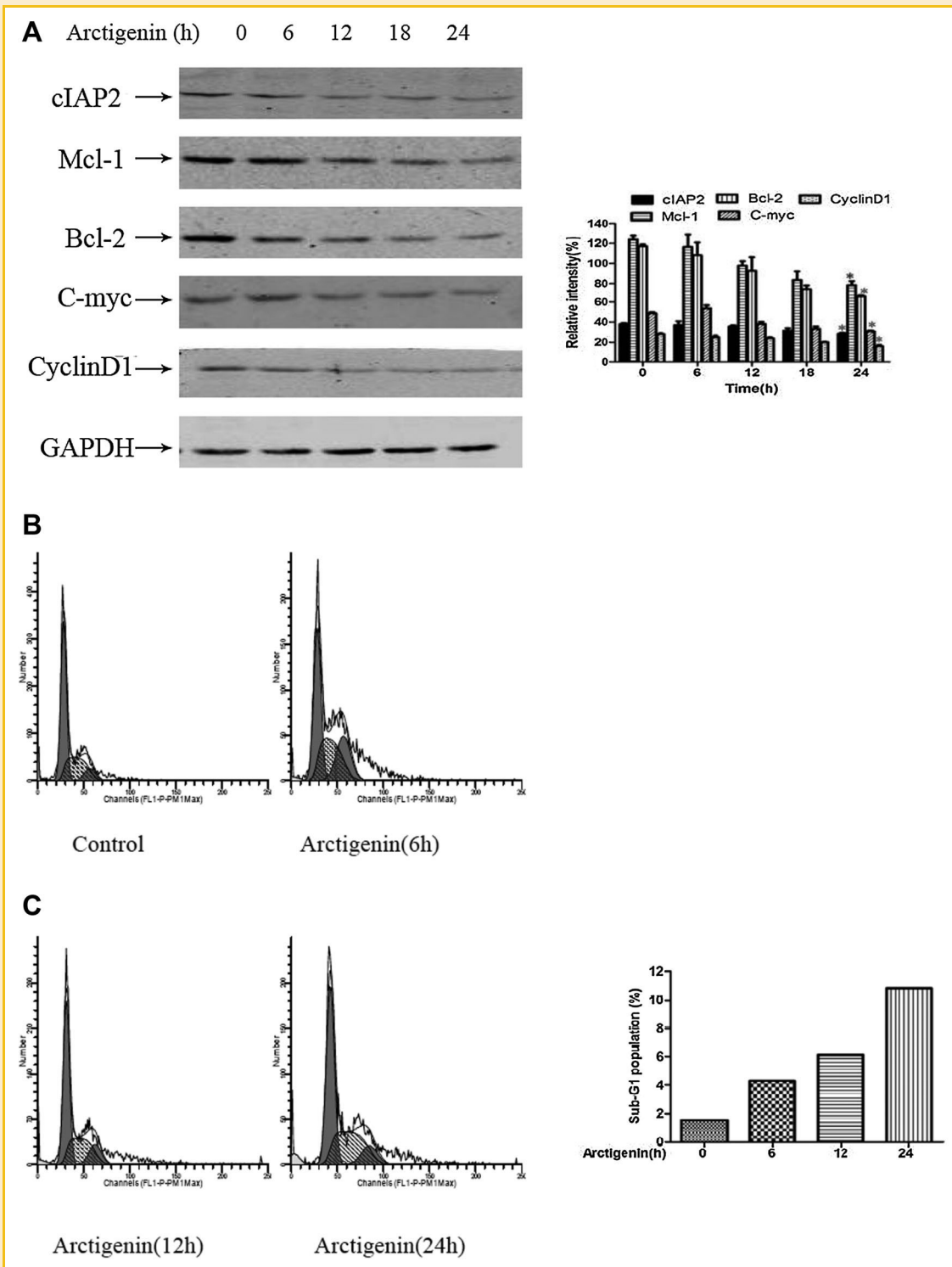
Fig. 4. Arctigenin suppresses STAT3–regulated gene products involved in proliferation, survival, and angiogenesis. A: HepG2 cells (2×10^6/ml) were treated with $80\,\mu$M arctigenin for indicated time intervals, after which whole–cell extracts were prepared and 30 μg portions of those extracts were resolved on 12% SDS–PAGE, and probed against cIAP2, Mcl–1, Bcl–2, C-myc, and cyclin D1antibodies. The same blots were stripped and reprobed with GAPDH antibody to verify equal protein loading. Data are representatives of three independent experiments. $^*P < 0.05$. B: Arctigenin causes accumulation of cells in the sub–G1 phase. HepG2 cells (2×10^6/ml) were synchronized by incubation overnight in the absence of serum and then treated with $80\,\mu$M arctigenin for the indicated times, after which the cells were washed, fixed, stained with propidiumiodine and analyzed for DNA content by flow cytometry.
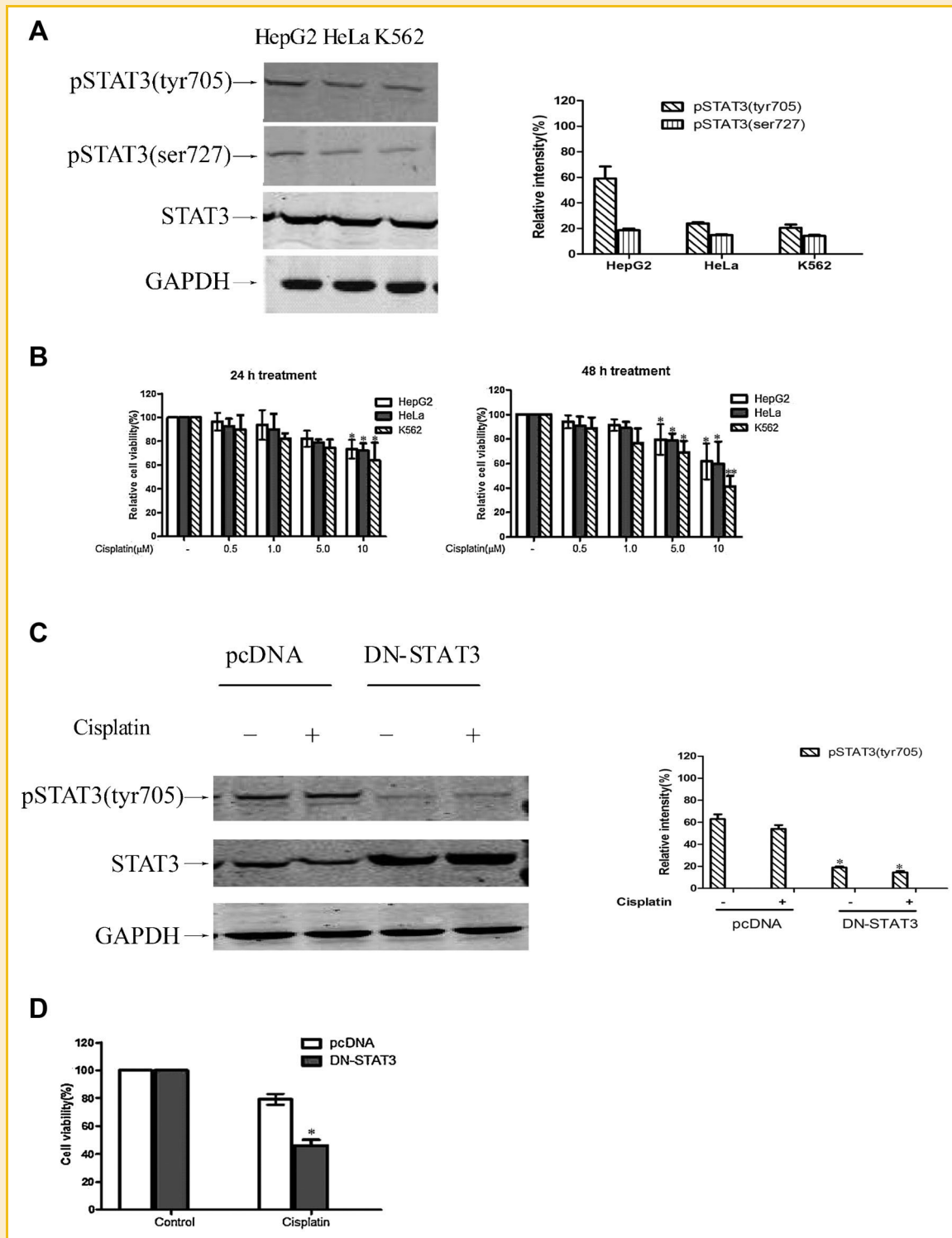
Fig. 5. Constitutively active STAT3 confers resistance to cisplatin–induced cytotoxicity in cancer cells. A: Western blot was used to detect the phospho–STAT3 and STAT3 levels in HepG2, HeLa, and K562 cells. B: HepG2, HeLa, and K562 cells were treated with indicated concentrations of cisplatin for 24 or 48 h, and cell viability was evaluated by MTT assay. Data were presented as mean ± SD from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$. C: HepG2 cells were transiently transfected with STAT3-Y705F or pcDNA which was used as transfection control, then Western blot was used to detect STAT3, phospho–STAT3, Data were presented as mean ± SD from three independent experiments. $^*P < 0.05$. D: After transfection with pcDNA or STAT3-Y705F, HepG2 cells were treated with cisplatin (1.0 μM) for 24 h, and cell viability was evaluated by MTT assay and represented by relative cell viability compared with untreated control group. Data were presented as mean ± SD from three independent experiments. $^*P < 0.05$.
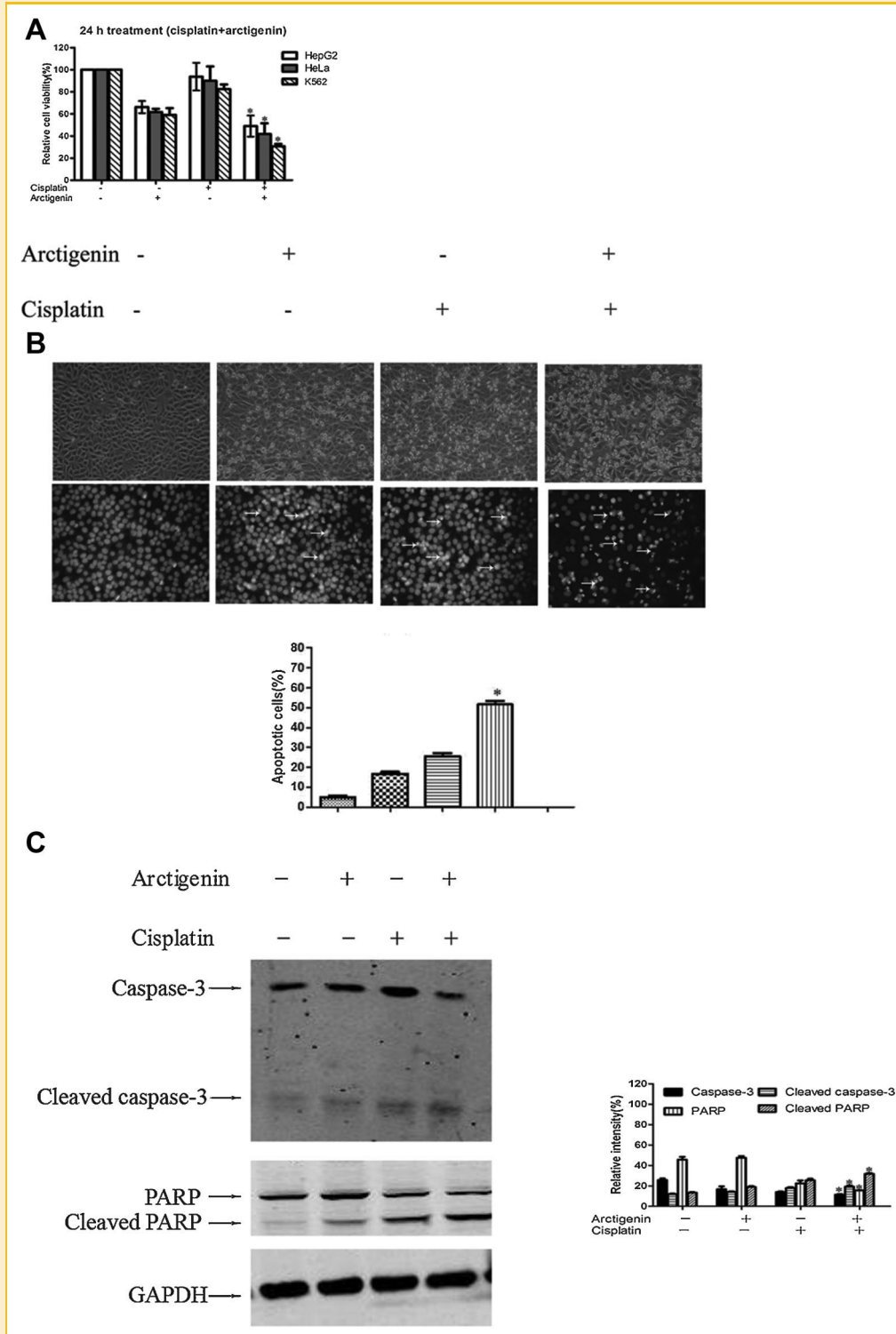
Fig. 6. Arctigenin enhances cisplatin-induced cytotoxicity in cancer cells. A: HepG2, HeLa, and K562 cells were pretreated with arctigenin (80 μM) for 2 h, followed by treatment with a subtoxic concentration of cisplatin (1.0 μM) for another 24 h. The cell viability was evaluated by MTT assay and expressed by relative cell viability compared with untreated control group, and data were presented as mean ± SD from three independent experiments. Data were presented as mean ± SD from three independent experiments. *$P < 0.05$. B: HepG2 were pretreated with the indicated concentration of arctigenin for 2 h, followed by treatment with a subtoxic concentration of cisplatin (1.0 μM) for another 24 h. Representative images of HepG2 cells with various treatments were photographed using a normal light microscope and an inverted fluorescence microscope (magnification, 200×). The percentage of apoptosis was determined using DAPI staining, apoptosis cells are indicated by white arrows. Data were presented as mean ± SD from three independent experiments, *$P < 0.05$. C: HepG2 cells were treated as indicated in panel B. Cell lysates were collected and subjected to Western blot for detecting the cleavage of caspase-3 and PARP, and GAPDH was used as a loading control. Data are representatives of three independent experiments, *$P < 0.05$.

whether arctigenin inhibits Akt phosphorylation in systems in which STAT3 is not activated cannot be ruled out.

Arctigenin was previously reported to possess powerful anti-inflammatory activity through the inhibition of NF-κB [Cho et al., 2002], But to date, there is no report of arctigenin on STAT3 signaling pathway. Whether suppression of STAT3 activation by actigenin is linked to inhibition of NF-κB activation is not clear. However, STAT3 presumably in its unphosphorylated form can bind to NF-κB, displace IκB from NF-κB and thereby facilitate NF-κB activation and nuclear entry even in the absence of conventional IKK signaling [Yang et al., 2007], Furthermore, STAT3 prolongs NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export [Lee et al., 2009]. Interestingly, erythropoietin has been shown to activate both NF-κB and STAT3 through the activation of JAK2 kinase [Digicaylioglu and Lipton, 2001]. Thus, it is possible that suppression of JAK activation is the critical target for inhibition of both NF-κB and STAT3 activation by arctigenin.

We further found that arctigenin-induced inhibition of STAT3 activation involves a protein tyrosine phosphatase (PTP). Numerous PTPs have been implicated in STAT3 signaling, including SHP-1, SHP-2, T-cell PTP, PTEN, PTP-1D, CD45, PTPe, and low molecular weight PTP [Pao et al., 2007]. SHP-2 is implicated in negative regulation of JAK/STAT signaling pathways [You et al., 1999; Ohtani et al., 2000]. Indeed, we found for the first time that arctigenin stimulates the expression of SHP-2 protein which correlated with downregulation of constitutive STAT3 phosphory-lation. In addition to PTP, STAT3 activation is also negatively regulated through other mechanisms. These involve the suppressors of cytokine signaling (SOCS), protein inhibitor of activated STAT (PIAS) [Wormald and Hilton, 2004], and ubiquitination-dependent proteosomal degradation [Daino et al., 2000], smad4 [Zhao et al., 2008]. Further investigation is required for elucidation of the molecular mechanism of these inhibitors in arctigenin-induced dephosphorylation of STAT3.

STAT3 is an attractive cancer drug target because of its overexpression in numerous cancers. Some natural agents known to be chemopreventive are quite effective in suppressing STAT3 activation [Aggarwal et al., 2009]. For instance, effect of arctigenin is indeed similar to a diosgenin that have been reported to inhibit STAT3 pathway by suppressing JAK1/2 and Src, upregulating the expression of SHP-2 [Li et al., 2010a], Interestingly, guggulsterone has been shown to upregulate the expression of SHP-1, which leads to inactivation of STAT3 [Ahn et al., 2008]. Other mechanisms have also been described. For instance, luteolin has been shown to promote the degradation of STAT3 by ubiquitination-proteasome pathway [Selvendiran et al., 2006]. Indirubin and thymoquinone were found to block STAT3 activation through inhibition of Src kinase activity and JAK [Nam et al., 2005; Li et al., 2010b], Curcumin was found to target PIAS3 [Saydmohammed et al., 2010], Cryptotanshinone inactivates STAT3 by directly binding to the SH2 domain of STAT3 [Shin et al., 2009].

Cisplatin is one of the most commonly used chemotherapeutic drugs, but its application has been limited by the presence of cellular resistance [Wu et al., 2010]. Some reports have shown that the inhibition of STAT3 could sensitize tumor cells to cisplatin-induced

cell death [Sims et al., 2009; Liu et al., 2010; Yilmaz et al., 2010]. Therefore, we postulate that arctigenin may promote chemosensi-tivity of tumor cells to cisplatin through inhibition of STAT3 activity. As expected, combination of arctigenin and cisplatin apparently induced cell death and suppressed the cell proliferation in a number of cancer cell lines. A recent study reported that cisplatin downregulates the JAK/STATS pathway through dephos-phorylation of JAK/STATS in cancer cells [Song et al., 2004], Other studies have also demonstrated that several platinum-containing compounds disrupt STAT3 signaling and suppress its biological functions [Turkson et al., 2004, 2005], Thus actigenin combined with cisplatin may synergistically affect the same survival-critical STAT3 signaling pathway and decrease the apoptotic threshold.

Arctigenin has also been tested in animals, administration of arctigenin (10 or 20 mg/kg) was injected in colon cancer xenografts in nude mice intra-tumorally and the mean tumor volumes of the controls and those treated with 20 mg/kg arctigenin were 715.78 and 472.42 mm^3, respectively, at day 14, suggested that arctigenin had a therapeutic effect on xenograft tumors at well-tolerated doses [Kim et al., 2010]. Further in vivo studies using arctigenin in combination with conventional chemotherapeutic agents for treatment of cancer remain to be fully determined.

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