Biochemical and Biophysical Research Communications 427 (2012) 24–29

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Withaferin A inhibits JAK/STAT3 signaling and induces apoptosis of human renal carcinoma Caki cells

Hee Jung Um [a,1], Kyoung-jin Min [a,1], Dong Eun Kim [b], Taeg Kyu Kwon [a,*]

[a] Department of Immunology, School of Medicine, Keimyung University, 2800 Dalgubeoldaero, Dalseo-Gu, Daegu 704-701, South Korea
[b] Department of Otolaryngology, School of Medicine, Keimyung University, 2800 Dalgubeoldaero, Dalseo-Gu, Daegu 704-701, South Korea

ARTICLE INFO

Article history:
Received 27 August 2012
Available online 12 September 2012

Keywords:
Withaferin A
STAT3
JAK2
Apoptosis

ABSTRACT

Withaferin A, the active component of *Withania somnifera*, causes cytotoxicity in a variety of tumor cell lines. In this study, we show that withaferin A inhibits constitutive and IL-6-induced phosphorylation of STAT3 (on Tyr705), but not IFN-γ-induced STAT1 phosphorylation. Withaferin A-induced down-regulation of STAT3 activation is associated with a reduction in Janus-activated kinase 2 (JAK2) activity. Withaferin A also down-regulates the expression of STAT3 regulated genes such as Bcl-xL, Bcl-2, cyclin D1 and survivin. The apoptotic effect of withaferin A in Caki human renal cancer cells was investigated. Withaferin A induced dose-dependent apoptotic cell death in Caki cells, as measured by FACS analysis and PARP cleavage. Furthermore, overexpression of STAT3 attenuated withaferin A-induced apoptosis. Taken together, the present study provides strong evidence that down-regulation of the STAT3 signaling pathway mediates withaferin A-induced apoptosis.

1. Introduction

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family, is a key signal transduction protein that mediates the signaling of many cytokines, hormones, growth factors, and oncoproteins [1]. STAT3 is constitutively activated in most human solid tumors. Persistent activation of STAT3 signaling has been demonstrated to influence important processes in tumors such as survival, proliferation, apoptosis, angiogenesis, and invasion [2–4]. Activated STAT3 binds to consensus sequences ($TTCN_{2-4}GAA$), known as gamma activated sites (GAS), and modulates the expression of target genes that are involved in various physiological functions including apoptosis (survivin, Bcl-xL, and HSP27), cell cycle regulation (cyclin D1, c-fos, and c-myc), and angiogenesis (VEGF) [5].

Withaferin A, a steroidal lactone purified from the Indian medicinal plant *Withania somnifera*, exhibits inhibitory effects against several different cancer types [6]. Withaferin A induces apoptosis through reactive oxygen species (ROS) generation [7], Par-4 induction [8] and p38 MAP kinase activation [9]. Withaferin A was also shown to cause FOXO3a- and Bim-dependent apoptosis [10]. In our previous study, we found that withaferin A induced Akt inactivation and endoplasmic reticulum (ER) stress [11,12]. We also found that the withaferin A sensitized TRAIL-mediated apoptosis through up-regulation of death receptor 5 (DR5) and down-regulation of c-FLIP [13]. However, the cellular and molecular mechanisms underlying withaferin A-induced apoptosis are not fully understood.

In this study, we investigated whether withaferin A could modulate the constitutive and IL-6-inducible STAT3 pathways in Caki cells, leading to the induction of apoptosis. In addition, STAT3 overexpression attenuated withaferin A-induced apoptosis. The present study clearly demonstrates that withaferin A-induced apoptosis is associated, at least in part, with down-regulation of the STAT3 signaling pathway.

2. Materials and methods

2.1. Cells and materials

Caki, and MDA-MB 231 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 20 mM Hepes buffer, and 100 μg/ml gentamicin. Anti-phospho-STAT3 (Tyr705), anti-STAT3, anti-cyclin D1, anti-Bcl-2, anti-Bcl-xL, anti-survivin, anti-PARP, anti-Ref-1 and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-STAT1 (Tyr701), anti-STAT1, anti-phospho-Src (Tyr416), anti-Src, anti-phospho-JAK2 (Tyr1007/1008), and anti-JAK2 antibodies were obtained from Transduction Laboratories, (Lexington, KY). Withaferin A was purchased from

* Corresponding author. Fax: +82 53 5803882.
E-mail address: kwontk@dsmc.or.kr (T.K. Kwon).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.133

Biomol (Biomol Research Laboratories, Inc., PA, USA). All of the other chemicals were obtained from Sigma Chemical Co.

2.2. Western blot analysis

The cells were washed with cold PBS and lysed on ice in a modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2 mM EDTA). The lysates were centrifuged at 10,000*g* for 10 min at 4 °C, and the supernatant fractions were collected. The proteins were separated by SDS–PAGE and transferred to an Immobilon-P membrane. The specific proteins were detected using an enhanced chemiluminescence (ECL) Western Blotting kit according to the manufacturer's instructions.

2.3. Flow cytometry analysis

The cells were suspended in 100 μl of phosphate-buffered saline (PBS), and 200 μl of 95% ethanol was added during a vortex step. The cells were incubated at 4 °C for 1 h, washed with PBS and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. The incubation was continued at 37 °C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting on a FACScan flow cytometer for relative DNA content based on red fluorescence.

2.4. DNA transfection

The transient transfections were performed in 6-well plates. One day before transfection, Caki cells were plated at approximately 60–80% confluence. The STAT3 plasmids were transfected into cells using the Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA). Twenty-four hours after the transfection, the cells were treated with withaferin A for 18 h, and the sub-G1 phase was determined by flow cytometry.

2.5. Immunocytochemistry

The cells were fixed with 100% methanol at −20 °C for 20 min, washed in PBS, and incubated with 10% FBS for 30 min. Next, the cells were incubated for 2 h at room temperature with an anti-STAT3 antibody, washed with PBS, and reacted with a fluorescence-conjugated secondary antibody for 1 h at room temperature. After the cells were washed with PBS, 300 nM DAPI (4′-6′-diamidino-2-phenylindole, Roche, Germany) was added to the cells for 5 min, and the cells were mounted with the ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). Finally, the cells were examined under a fluorescence microscope (Zeiss, Goettingen, Germany).

2.6. Preparation of nuclear extracts

Following the required treatments, Caki cells were trypsinized and suspended in buffer A (10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After incubation on ice for 30 min, cells were centrifuged at 2500 rpm for 3 min to obtain a nuclear pellet. Supernatant fractions were collected as the cytosol extract. Buffer C (20 mM HEPES at pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) was added, followed by rotation for 30 min at 4 °C. The resulting lysates were centrifuged at 12,000 rpm at 4 °C for 5 min. Supernatant fractions were collected as the nuclear extract.

2.7. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

The preparation of nuclear extracts from the control or drug-treated cells was carried out as described previously [14]. The sequence of the double-stranded oligonucleotide used to detect the DNA-binding activities of STAT3 is as follows: STAT3, 5′-GAT CCT TCT GGG AAT TCC TAG ATC-3′. The reaction mixture for EMSA contained 20 mM Tris–HCl (pH 7.6), 1 mM dithiothreitol, 2 mM MgCl2, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 μg poly (dI-dC) and 5 μg nuclear proteins. Unlabeled wild type oligo-nucleotide was added into the reaction mixture and incubated for 10 min at room temperature. A [^{32}P]-labeled probe DNA (300,000 cpm) was added, and the binding reaction was allowed to proceed for another 20 min. The mixtures were resolved on an 8% polyacrylamide gels at 150 V for 4 h. Then, the gels were dried and subjected to autoradiography.

2.8. Cell viability assay

Cell proliferation was detected by 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) (Wel Gene, Seoul, Korea) assay. Cells were seeded on a 96-well plate (2×10^4 cells/100 μl/well) for 24 h. Cells were divided into a control group and a withaferin A-treated group. Absorbance (A) was detected with an enzyme calibrator at 450 nm. Cell viability was calculated as follows: (A of study group/A of control group) × 100%. The experiment was repeated twice with three wells for each concentration.

2.9. Cell death assessment by DNA fragmentation assays

The cell death detection ELISA plus kit (Boerhringer Mannheim, Indianapolis, IN) was used for assessing apoptotic activity by detecting fragmented DNA within the nucleus in withaferin A-treated cells. Briefly, each culture plate was centrifuged for 10 min at 200*g*, the supernatant was removed, and the pellet was lysed for 30 min. After centrifuging the plate again at 200*g* for 10 min, the collected supernatant containing cytoplasmic histone-associated DNA fragments was incubated with an immobilized anti-histone antibody, and the reaction products were determined by spectrophotometry. Finally, absorbance at 405 and 490 nm (reference wavelength), upon incubating with a peroxidase substrate for 5 min, was determined with a microplate reader. Signals in the wells containing the subsstrate only were subtracted as background.

2.10. Statistical analysis

The data were analyzed with a one-way ANOVA, followed by post hoc comparisons (Student–Newman–Keuls) using the Statistical Package for Social Sciences 8.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Withaferin A inhibits constitutive STAT3 phosphorylation in Caki cells

We previously reported that withaferin A inhibited expression of Bcl-xL, a target gene of STAT3 [11]. To determine whether withaferin A inhibits STAT3 activation, we incubated Caki cells with various concentrations of withaferin A for 3 h. We examined the activation of STAT3 by Western Blot analysis using an antibody that recognized phosphorylation of STAT at tyrosine 705. As shown in Fig. 1A, withaferin A inhibited constitutive STAT3 phosphoryla-

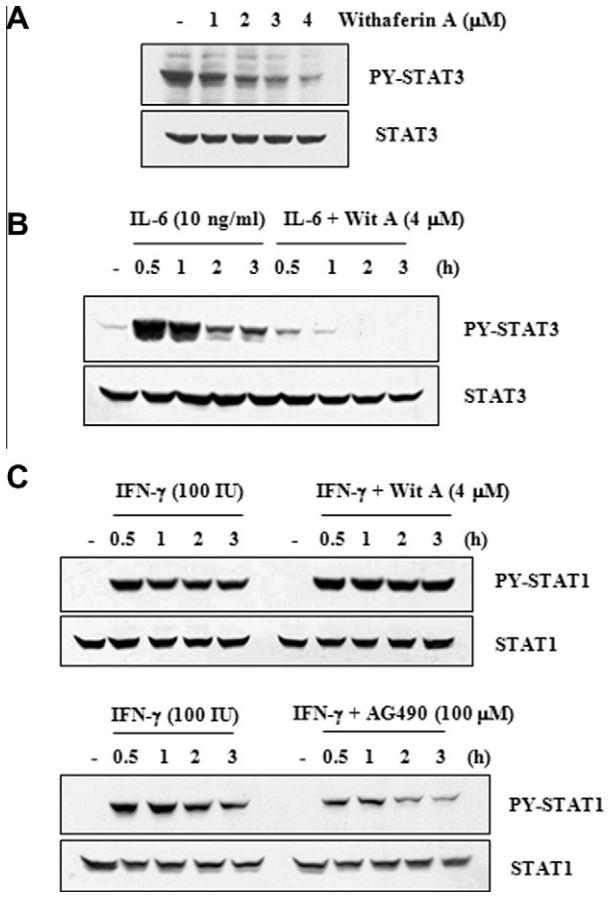

Fig. 1. Withaferin A inhibits phosphorylation of STAT3 but not STAT1. (A) Caki cells were treated with the indicated concentrations of withaferin A for 3 h. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western Blot for phospho-STAT3 (Tyr705) and STAT3 as a control for protein loading. (B) MDA-MD 231 cells were treated with IL-6 (10 ng/ml) in the presence or absence of withaferin A for the indicated times. Whole cell extracts were prepared and phospho-STAT3 was detected by Western blot analysis. (C) Caki cells were treated with IFN-γ (100 IU/ml) in the presence or absence of withaferin A (top) or AG490 (bottom) for the indicated times. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western Blot for phospho-STAT1 (Tyr701) and STAT1 as a control for protein loading.

tion of Caki cells in a dose-dependent manner. Withaferin A had no effect on STAT3 protein expression (Fig. 1A, lower panel). In addition, to determine whether withaferin A inhibits inducible STAT3 activation, MDA-MD 231 breast cancer cells were pretreated with or without withaferin A (4 μM), followed by treatment with IL-6 (10 ng/ml) for the indicated time periods. IL-6 treatment induced a strong transient increase in phosphorylated STAT3 levels, which peaked at 30 min and declined thereafter. Withaferin A decreased the levels of IL-6-induced phosphorylation of STAT3 to the resting state within 1 h (Fig. 1B). These results demonstrate that withaferin A inhibits both constitutive and IL-6-induced STAT3 activation. We sought to further clarify the underlying mechanisms of the inhibitory specificity of STAT3 phosphorylation in withaferin A-treated cells. To study this, Caki cells were pretreated with or without withaferin A (4 μM), followed by a treatment with interferon γ (IFN γ) for the indicated time periods. As shown in Fig. 1C, withaferin A did not inhibit IFN-γ-induced STAT1 phosphorylation. However, AG-490 (a tyrosine kinase inhibitor) inhibited IFN-γ-induced STAT1 phosphorylation in a time-dependent manner. The results indicate that withaferin A specifically inhibits STAT3 tyrosine phosphorylation.

3.2. Withaferin A inhibits nucleus localization of STAT3 and STAT3 DNA binding activity

Phosphorylation of STAT3 on Tyr705 results in homodimerization or heterodimerization of STAT3, enabling nuclear localization and DNA binding [1]. To investigate the effect of withaferin A on STAT3 nuclear localization, we used immunocytochemistry. As shown in Fig. 2A, withaferin A inhibited the translocation of STAT3 to the nucleus in Caki cells. Furthermore, we confirmed that withaferin A inhibited STAT3 nuclear localization using nuclear extraction in Caki cells (Fig. 2B). Next, we investigated whether withaferin A inhibits STAT3 DNA binding activity. An EMSA analysis of nuclear extracts prepared from Caki cells showed that withaferin A caused a decrease in STAT3 DNA binding activity in a dose-dependent manner (Fig. 2C). These data clearly indicate that withaferin A-induced STAT3 dephosphorylation is associated with STAT3 DNA binding activity.

3.3. Withaferin A inhibits constitutive JAK2 activation

STAT3 has been reported to be activated by soluble tyrosine kinases in the Src kinase and Janus kinase families [15]. Because soluble tyrosine kinases (JAK family and Src family kinases) have been implicated in STAT3 activation [16], we investigated whether withaferin A-induced inhibition of STAT3 tyrosine phosphorylation could be due to inhibition of these tyrosine kinases. We found that withaferin A suppressed the constitutive phosphorylation of JAK2 in a time-dependent manner. However, the level of phosphorylation of c-Src kinase did not significantly change under the same conditions. These results collectively indicate that withaferin A-induced STAT3 dephosphorylation is associated with JAK2 inhibition. In addition, we investigated whether withaferin A-induced STAT3 dephosphorylation could be due to protein tyrosine phosphatase (PTPase) activation. Pretreatment with sodium pervanadate (a broad-acting tyrosine phosphatase inhibitor) blocked dephosphorylation of STAT3 after treatment with withaferin A (Fig. 3B).

3.4. Withaferin A induces apoptosis in Caki cells

To investigate the relationship between suppression of STAT3 phosphorylation and apoptosis, Caki cells were treated with the indicated concentrations of withaferin A for 18 h, and apoptotic cells (sub-G1 population) were determined by flow cytometry. As shown in Fig. 4A, treatment of Caki cells with withaferin A resulted in a markedly increased accumulation of sub-G1 phase cells and proteolytic cleavage of PARP in a dose-dependent manner. Next, we examined STAT3 downstream anti-apoptotic proteins including Bcl-2, Bcl-xL, and survivin. We also examined cell cycle regulator proteins, such as cyclin D1. Expression levels of Bcl-2, Bcl-xL, survivin and cyclin D1 were decreased in a time-dependent manner in the Caki cells treated with 4 μM withaferin A. To further address the significance of STAT3 activation in withaferin A-induced apoptosis, we assessed whether overexpression of STAT3 could rescue withaferin A-induced apoptosis. Caki cells were transfected with the STAT3 plasmid for 24 h. The cells were then treated with withaferin A for 18 h, and we examined the sub-G1 population. As shown in Fig. 4C, overexpression of STAT3 markedly reduced the withaferin A-induced sub-G1 phase population. We next measured the expression levels STAT3 and PARP cleavage. PARP cleavage was inhibited by ectopic expression of STAT3 (Fig. 4C). Furthermore, we confirmed that ectopic expression of STAT3 reversed withaferin A-mediated cell death using XTT assay and DNA fragmentation assay (Fig. 4D and E). Taken together, these results indicate that the JAK/STAT pathway plays a role in regulating withaferin A-induced apoptosis of Caki cells.

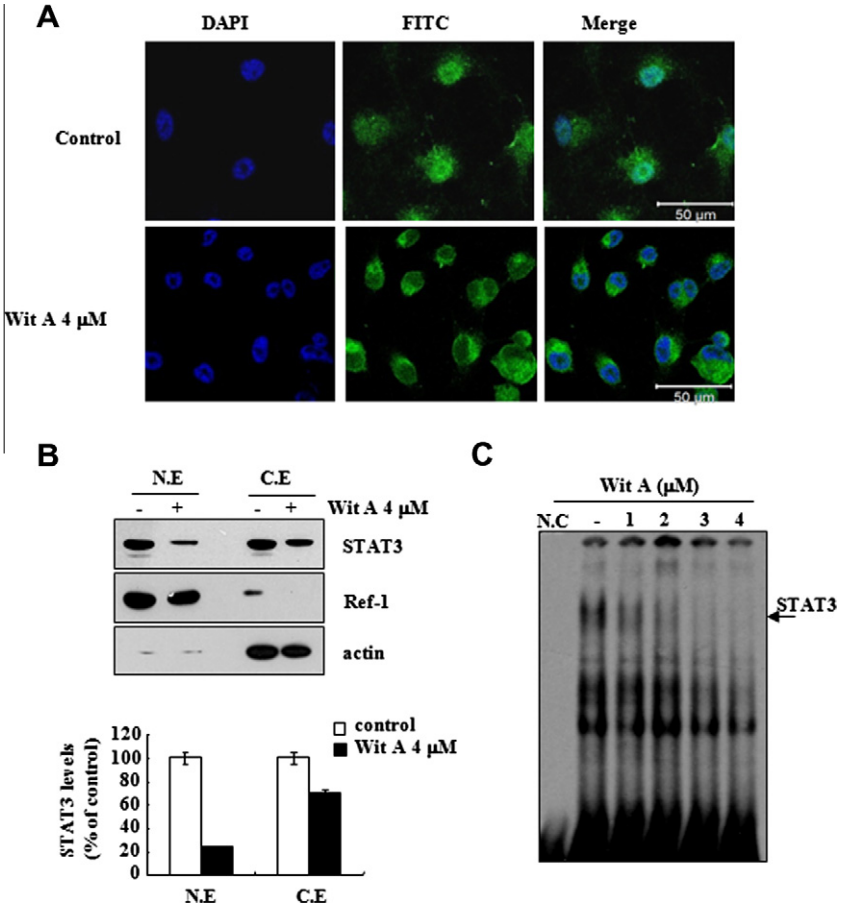

Fig. 2. Withaferin A inhibits STAT3 nuclear translocation and DNA binding activity. (A) Caki cells were treated with or without 4 μM withaferin A for 6 h. The cells were fixed and labeled with an anti-STAT3 antibody, followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The nuclei were visualized by DAPI staining as described in Section 2. The fluorescent images were obtained using a Zeiss fluorescence microscope. (Size bar = 50 μm). (B) Caki cells were treated with or without 4 μM withaferin A for 6 h. Nuclear extracts (N.E) and cytosol extracts (C.E) were analyzed for the detection of STAT3 nuclear translocation. The bar graph shows quantitation of STAT3 protein levels, normalized to Ref-1 (N.E) and actin (C.E) levels, respectively. Ref-1 was used as a nuclear extract marker. (C) Withaferin A inhibits STAT3 DNA binding. Caki cells were treated with the indicated concentrations of withaferin A for 6 h and analyzed for STAT3 DNA binding by EMSA.

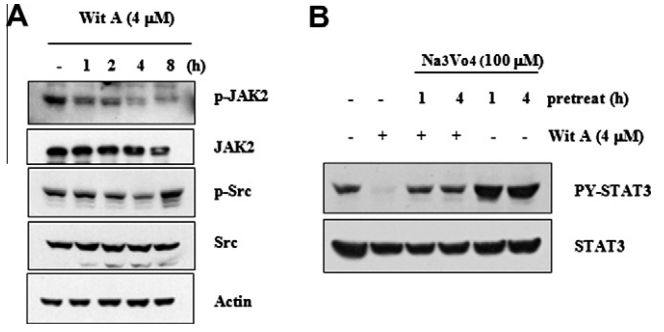

Fig. 3. The effect of withaferin A on c-Src and JAK2 activation. (A) Caki cells were treated with withaferin A (4 μM) for the indicated time points. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to a membrane, and probed with specific antibodies (anti-phospho-Src (Tyr416), anti-Src, anti-phospho-JAK2 (Tyr1007/1008), and anti-JAK2 antibodies). A representative study is shown. Two additional experiments yielded similar results. (B) Caki cells were pretreated with sodium pervanadate (100 μM) for 1 or 4 h followed by treatment with 4 μM withaferin A for 3 h. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western Blot for phospho-STAT3 (Tyr705) and STAT3 as a control for protein loading.

4. Discussion

Withaferin A is reported to have anti-inflammation and anti-tumor properties [17–19]. However, the molecular mechanism involved in withaferin A-induced apoptosis is not well established. In this study, we investigated the effect of withaferin A on the apoptotic pathway in Caki cells. We provide important evidence, which suggests that withaferin A inhibits STAT3 activation and induces apoptosis in Caki cells. This view is supported by the following findings: (a) withaferin A inhibits both constitutive and inducible STAT3 activation; (b) withaferin A effectively inhibits JAK2 phosphorylation; (c) withaferin A suppresses the expression of STAT3-regulated genes and anti-apoptotic gene products including survivin, Bcl-2 and Bcl-xL.

There is increasing evidence that STAT3 is constitutively activated in various types of carcinoma, sarcoma, lymphoma and leukemia. Furthermore, STAT3 also possesses oncogenic potential and anti-apoptotic activity [20]. STAT3 activation requires phosphorylation, which results in dimerization, nuclear translocation, DNA binding, and transcriptional activation of target genes [21]. To investigate the mechanism of withaferin A-induced STAT3 inhibition in Caki cells, we analyzed the activation of upstream protein kinases such as JAK and Src. As shown in Fig. 3A, only phosphorylation of JAK2 was inhibited by treatment with withaferin A. Therefore, it is likely that inhibition of STAT3 phosphorylation at Tyr705 was due to inhibition of JAK2 activity. Interestingly, withaferin A-induced inhibition of tyrosine phosphorylation was only observed for STAT3, but not STAT1 (Fig. 1C). These results suggest that withaferin A specifically inhibits phosphorylation of STAT3 at Tyr705. STAT3 phosphorylation plays an important role in the invasion,

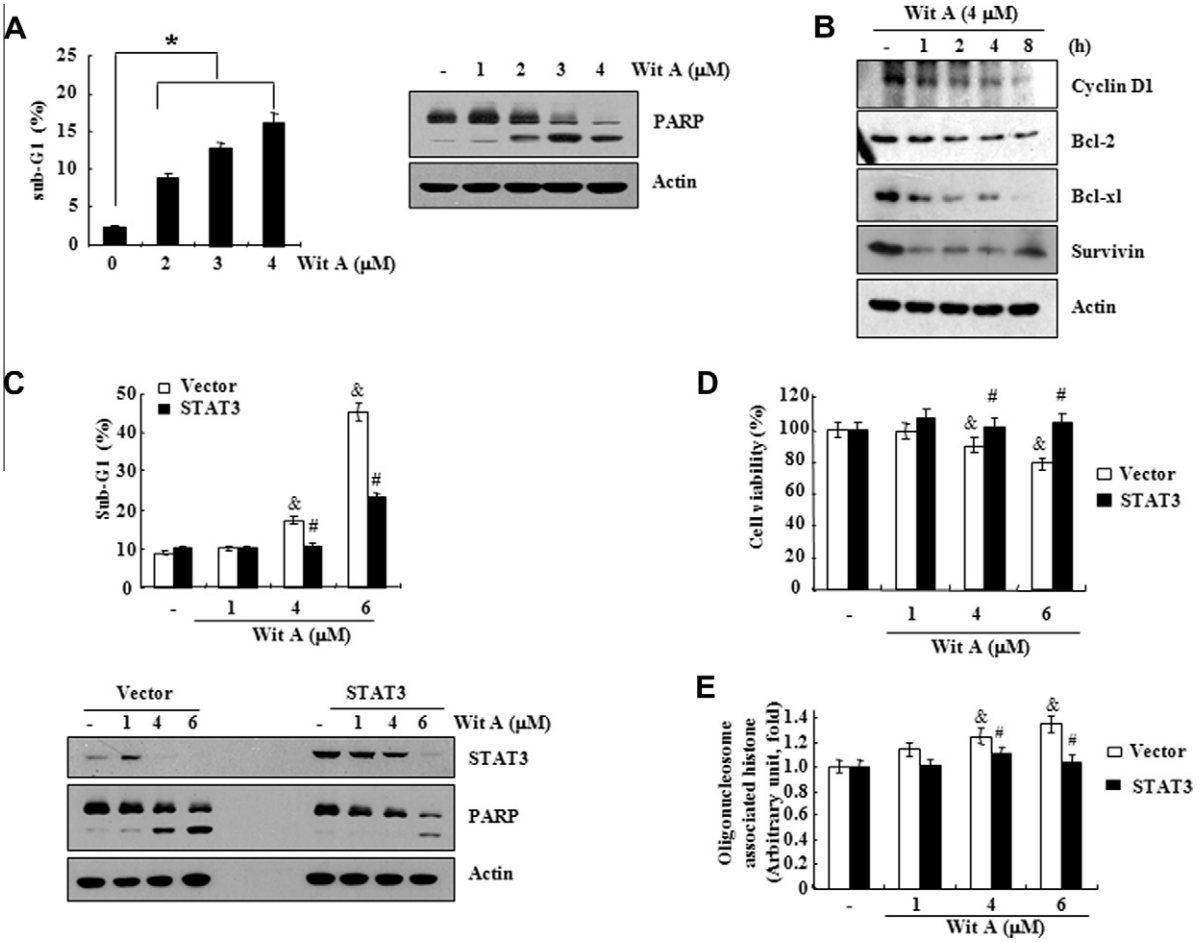

Fig. 4. The effect of STAT3 overexpression on withaferin A-induced apoptosis. (A) Caki cells were treated for 18 h with the indicated concentrations of withaferin A. Apoptosis was analyzed using the sub-G1 fraction measured by FACS. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western Blot for PARP. The data are the mean values obtained from three independent experiments and the bars represent the standard deviation. A Student's *t*-test for unpaired values was used. * indicates $p < 0.05$ vs control cells. (B) Caki cells were treated with 4 μM withaferin A for the indicated time points. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to a membrane, and probed with specific antibodies against cyclin D1, Bcl-2, Bcl-xL, and survivin. Actin was used as a control to normalize the samples. (C) Caki cells were transfected with the STAT3 expression plasmid. Twenty-four hours after transfection, the cells were treated with the indicated concentration of withaferin A for 18 h. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE gel electrophoresis, transferred to a membrane and probed with specific antibodies against anti-PARP, anti-STAT3 or anti-actin. Actin was used as a loading control. Apoptosis was analyzed using the sub-G1 fraction measured by FACS. (D and E) Caki cells were transfected with the STAT3 expression plasmid. Twenty-four hours after transfection, the cells were treated with the indicated concentration of withaferin A for 18 h. Cell viability was determined by XTT assay (D). Fragmented DNA was determined by the DNA fragmentation detection kit (E). & indicates $p < 0.05$ vs control cells. # indicates $p < 0.05$ vs vector cells. The data shown are the means ± S.D. ($n = 3$).

angiogenesis and proliferation of tumor cells [3,4]. Lee et al. recently reported that withaferin A has inhibitory effects against both constitutive and IL-6-induced activation of STAT3 in breast cancer cells [22]. However, IL-6 stimulation showed minimal impact on withaferin A-induced apoptosis [22].

Downstream target genes of activated STAT3 include survivin, Bcl-xL, and Bcl-2. Deregulated expression of these target genes influences apoptosis [23,24]. Our results indicate that withaferin A suppresses Bcl-2, survivin, and Bcl-xL expression in a time-dependent manner (Fig. 4B). Down-regulation of Bcl-2, survivin, and Bcl-xL likely contributes to the ability of withaferin A to induce cell death in Caki cells. Simonian et al. reported that Bcl-xL is expressed in various tumors and modulates the sensitivity of tumor cells to chemotherapeutic agents and gamma-irradiation [25]. Furthermore, withaferin A-induced apoptosis and PARP cleavage were markedly inhibited when STAT3 was overexpressed (Fig. 4C). We also examined that effect of ectopic overexpression of STAT3 on withaferin A-induced cell death in other tumor cell types, such as Caski cell (human cervical cancer cell line) and A549 (human lung adenocarcinoma epithelial cells). Overexpression of STAT3 reduced the withaferin A-induced sub-G1 phase population and PARP cleavage in Caski and A549 cells (Supplementary figure 1). These data suggested that withaferin A might target STAT3 in Caki cells.

In conclusion, this study demonstrated that withaferin A induced apoptosis via down-regulation of the STAT3 signaling pathway. Because withaferin A causes apoptosis, it may be a candidate for a cancer chemopreventative or chemotherapeutic agent.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.133.

References

[1] J.E. Darnell Jr., STATs and gene regulation, Science 277 (1997) 1630–1635.
[2] T. Bowman, R. Garcia, J. Turkson, R. Jove, STATs in oncogenesis, Oncogene 19 (2000) 2474–2488.
[3] M. Kujawski, M. Kortylewski, H. Lee, A. Herrmann, H. Kay, H. Yu, STAT3 mediates myeloid cell-dependent tumor angiogenesis in mice, J. Clin. Invest. 118 (2008) 3367–3377.

[4] E. Devarajan, S. Huang, STAT3 as a central regulator of tumor metastases, Curr. Mol. Med. 9 (2009) 626–633.
[5] T. Kisseleva, S. Bhattacharya, J. Braunstein, C.W. Schindler, Signaling through the JAK/STAT pathway, recent advances and future challenges, Gene 285 (2002) 1–24.
[6] L.C. Mishra, B.B. Singh, S. Dagenais, Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): a review, Altern. Med. Rev. 5 (2000) 334–346.
[7] F. Malik, A. Kumar, S. Bhushan, S. Khan, A. Bhatia, K.A. Suri, G.N. Qazi, J. Singh, Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concomitant protection by N-acetyl cysteine, Apoptosis 12 (2007) 2115–2133.
[8] S. Srinivasan, R.S. Ranga, R. Burikhanov, S.S. Han, D. Chendil, Par-4-dependent apoptosis by the dietary compound withaferin A in prostate cancer cells, Cancer Res. 67 (2007) 246–253.
[9] C. Mandal, A. Dutta, A. Mallick, S. Chandra, L. Misra, R.S. Sangwan, C. Mandal, Withaferin A induces apoptosis by activating p38 mitogen-activated protein kinase signaling cascade in leukemic cells of lymphoid and myeloid origin through mitochondrial death cascade, Apoptosis 13 (2008) 1450–1464.
[10] S.D. Stan, E.R. Hahm, R. Warin, S.V. Singh, Withaferin A causes FOXO3a- and Bim-dependent apoptosis and inhibits growth of human breast cancer cells in vivo, Cancer Res. 68 (2008) 7661–7669.
[11] J.H. Oh, T.J. Lee, S.H. Kim, Y.H. Choi, S.H. Lee, J.M. Lee, Y.H. Kim, J.W. Park, T.K. Kwon, Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation, Apoptosis 13 (2008) 1494–1504.
[12] M.J. Choi, E.J. Park, K.J. Min, J.W. Park, T.K. Kwon, Endoplasmic reticulum stress mediates withaferin A-induced apoptosis in human renal carcinoma cells, Toxicol. In Vitro 25 (2011) 692–698.
[13] T.J. Lee, H.J. Um, D.S. Min, J.W. Park, K.S. Choi, T.K. Kwon, Withaferin A sensitizes TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of c-FLIP, Free Radic. Biol. Med. 46 (2009) 1639–1649.
[14] W.K. Baek, J.W. Park, J.H. Lim, S.I. Suh, M.H. Suh, E. Gabrielson, T.K. Kwon, Molecular cloning and characterization of the human budding uninhibited by benomyl (BUB3) promoter, Gene 295 (2002) 117–123.
[15] J.N. Ihle, STATs: signal transducers and activators of transcription, Cell 84 (1996) 331–334.
[16] J.J. O'Shea, M. Gadina, R.D. Schreiber, Cytokine signaling in 2002: new surprises in the Jak/Stat pathway, Cell 109 (2002) 121–131.
[17] R. Agarwal, S. Diwanay, P. Patki, B. Patwardhan, Studies on immunomodulatory activity of *Withania somnifera* (ashwagandha) extracts in experimental immune inflammation, J. Ethnopharmacol. 67 (1999) 27–35.
[18] H. Yang, G. Shi, Q.P. Dou, The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from "Indian winter cherry", Mol. Pharmacol. 71 (2007) 426–437.
[19] N. Sen, B. Banerjee, B.B. Das, A. Ganguly, T. Sen, S. Pramanik, S. Mukhopadhyay, H.K. Majumder, Apoptosis is induced in leishmanial cells by a novel protein kinase inhibitor withaferin A and is facilitated by apoptotic topoisomerase I-DNA complex, Cell Death Differ. 14 (2007) 358–367.
[20] D.J. Kim, K.S. Chan, S. Sano, J. Digiovanni, Signal transducer and activator of transcription 3 (STAT3) in epithelial carcinogenesis, Mol. Carcinog. 46 (2007) 725–731.
[21] B. Groner, P. Lucks, C. Borghouts, The function of STAT3 in tumor cells and their microenvironment, Semin. Cell Dev. Biol. 19 (2008) 341–350.
[22] J. Lee, E.R. Hahm, S.V. Singh, Withaferin A inhibits activation of signal transducer and activator of transcription 3 in human breast cancer cells, Carcinogenesis 31 (2010) 1991–1998.
[23] M. Bhutani, A.K. Pathak, A.S. Nair, A.B. Kunnumakkara, S. Guha, G. Sethi, B.B. Aggarwal, Capsaicin is a novel blocker of constitutive and interleukin-6-inducible STAT3 activation, Clin. Cancer Res. 13 (2007) 3024–3032.
[24] H. Xiong, Z.G. Zhang, X.Q. Tian, D.F. Sun, Q.C. Liang, Y.J. Zhang, R. Lu, Y.X. Chen, J.Y. Fang, Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells, Neoplasia 10 (2008) 287–297.
[25] P.L. Simonian, D.A. Grillot, G. Nuñez, Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death, Blood 90 (1997) 1208–1216.