Phosphoinositol 3-kinase, a novel target molecule for the inhibitory effects of juglone on TPA-induced cell transformation

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Abstract. Juglone (5-hydroxy-1,4-naphthalenedione) from black walnut trees induces apoptosis and inhibits proliferation of various malignant cells. Here, we investigated whether juglone affects 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell transformation through the phosphoinositol 3-kinase (PI3K) pathway. The results showed that TPA- and endothelial growth factor (EGF)-induced anchorage-independent colony formation were suppressed in a dose-dependent manner by treatment of JB6 CI41 mouse skin epidermal cells with juglone (2.5 and 5 μM). We demonstrated that juglone suppressed PI3K activity via direct binding to PI3K by sepharose 4B pull-down assay and western blot analysis. Juglone significantly suppressed TPA-induced protein kinase B (AKT) and c-Jun phosphorylation and c-fos activation, but not mitogen-activated protein-kinase kinase (MEK), extracellular signaling-regulated kinase (ERK) or 90 kDa ribosomal protein S6 kinase (RSK) phosphorylation. Juglone significantly blocked activator protein-1 (AP-1) and cyclooxygenase-2 (COX-2) activation more than the PI3K inhibitors LY294002 and wortmannin. Overall, these results showed the anticancer efficacy of juglone targeting PI3K to prevent TPA-induced tumorigenesis.

Introduction

Juglone (5-hydroxy-1,4-naphthalenedione) is a quinone pigment that occurs naturally in the roots, leaves, nut-hulls, bark, and wood of walnut trees (1,2). Juglone is a well-known allelopathic agent that has differential effects on cell cycling and metabolism depending on the species, organ, and drug concentration (3). Juglone has therapeutic properties and is being used in commercial hair dyes (4-6). Although hair dyes are widely used, permanent hair dye colors are formed by an oxidative process involving arylamines, giving rise to concerns about the potential adverse health effects of long-term exposure, particularly cancer. Some aromatic amines in hair dyes are mutagenic *in vitro* (7) and carcinogenic in animals and humans (8,9). Therefore, use of hair dyes has been suggested as a risk factor for several types of cancer (10).

Many types of cancer are closely connected with peptidyl prolyl isomerase (Pin1). Pin1 is significantly overexpressed in human cancer and plays a positive role in cell proliferation or transformation during oncogenesis (11,12). More than 10 years ago, juglone, which is a Pin1 inhibitor (13), was used in several cancer cell studies (14-16). But, no reports are available about the molecular mechanism of juglone and its association with the antitumorigenesis effect in skin cancer cell development.

Development of cancer cells is associated with the phosphoinositol 3-kinase (PI3K) pathway (17,18) which mediates signal transduction and gene expression effects that contribute to tumorigenesis (19). Actually, PI3K plays a major role not only in tumor growth but also in the potential response of a tumor to cancer treatment (20). Moreover, previous studies have demonstrated that PI3K is required for 12-O-tetradecanoylphorbol-13-acetate (TPA) or endothelial growth factor (EGF)-induced cell transformation (1,21). Therefore, studies about the molecular influence of juglone on PI3K signaling in skin cancer cell development are needed.

Here, we studied the molecular mechanism of juglone as a potential target for tumor suppression by studying the PI3K pathway during TPA- or EGF-induced JB6 CI41 mouse skin epidermal cell transformation. The goal of this research was to develop chemotherapy drugs against malignant cancer cells.

Materials and methods

Materials. Anti-protein kinase B (AKT), anti-phospho-AKT (Ser473), anti-phospho-c-Jun (Ser73), anti-c-Jun, anti-c-fos, anti-mitogen-activated protein kinase kinase (MEK), and

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anti-phospho-MEK (Ser217/221), anti-extracellular signaling-regulated kinase (ERK), anti-phospho-ERKs (Thr202/Tyr204), anti-RSK, and anti-phospho-p90RSK (Ser380) were purchased from Cell Signaling Technology, Inc. (Charlottesville, VA, USA). The PI3K active protein was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-β-actin, juglone, LY294002, wortmannin, and TPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture medium and other supplements were purchased from Life Technologies (Rockville, MD, USA). CNBr-Sepharose 4B and [γ-^{32}P] ATP were obtained from GE Healthcare (Piscataway, NJ, USA). The CellTiter 96 AQueous One Solution Cell Proliferation Assay kit and the luciferase assay substrate were obtained from Promega (Madison, WI, USA).

Cell culture. JB6 CI41 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine and 25 μg/ml gentamicin at 37˚C in a 5% CO_2 incubator. The JB6 CI41 cell line was stably transfected with activator protein-1 (AP-1) and cyclooxygenase-2 (COX-2) luciferase reporter plasmids obtained from Dr Zigang Dong (The Hormel Institute, University of Minnesota, Austin, MN) (22).

MTS assay. The effect of juglone on cell viability was estimated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit following the manufacturer's instructions. Cells were seeded in a 96-well plate for 24 h and then incubated with different concentrations of juglone (1, 2.5 and 5 μM) for 24 and 48 h. The assay solution was added to each well for 3 h at 37˚C in 5% CO_2, and absorbance at 490 nm was recorded using the GloMax-Multi Microplate Multimode Reader (Promega).

Anchorage-independent cell growth assay. Cells ($8x10^3$) were exposed to TPA or EGF with or without various concentrations of juglone in 1 ml of 0.33% basal medium Eagle's (BME) agar over 3 ml of 0.5% BME agar containing 10% FBS. The cultures were maintained at 37˚C in a 5% CO_2 incubator for 12 days and then the cell colonies were automatically counted using a microscope and the Image-Pro plus software program v.4 (Media Cybernetics, Silver Spring, MD, USA).

In vitro juglone-Sepharose 4B pull-down assay. This method has been described previously (23,24). Briefly, PI3K active protein (2 μg) was reacted with Sepharose 4B beads or juglone-Sepharose 4B beads in reaction buffer [50 mM Tris, pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 mM phenylmethylsulfonyl fluoride (PMSF), and 1X proteinase inhibitor cocktail]. After overnight incubation with gently rocking at 4˚C, the beads were washed five times with washing buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40 and 0.02 mM PMSF) and proteins bound to the beads were analyzed by western blot analysis.

Phosphoinositide 3-kinase activity assay. Kinase assays were conducted as reported previously (23). Briefly, active PI3K protein (100 ng) was incubated with juglone for 10 min at 30˚C. The mixtures were incubated with phosphatidylinositol (PI) (10 μg) (Avanti Polar Lipids, Alabaster, AL, USA) for 5 min. Subsequently, the mixture was incubated at room temperature in reaction buffer [100 mM HEPES (pH 7.6), 50 mM $MgCl_2$, 250 μM-ATP] containing 10 μCi of [γ-^{32}P]-ATP for 10 min at 30˚C. The reaction was terminated by adding 15 μl of 4 M HCl and 130 μl chloroform-methanol (1:1, v/v). A 30 μl aliquot of the lower chloroform phase was spotted onto a 1% potassium oxalate-coated silica gel plate that was previously activated for 1 h at 110˚C. The resulting ^{32}P-labeled phosphatidylinositol-3-phosphate (PI3P) was separated by thin-layer chromatography and radiolabeled spots were analyzed by autoradiography.

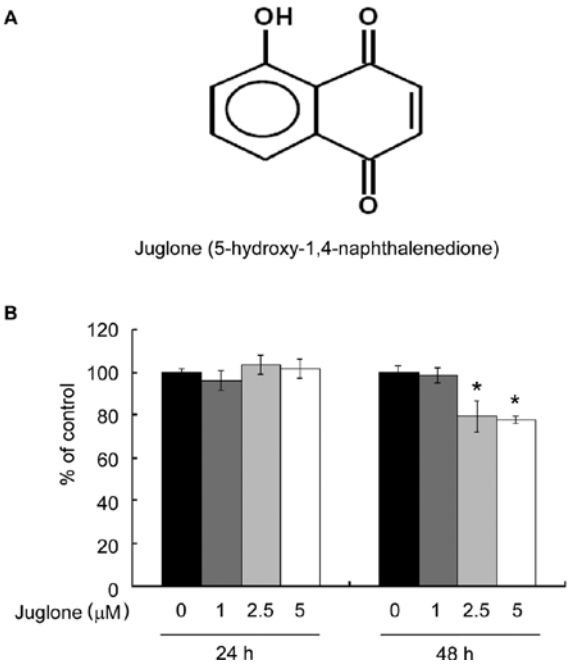

Figure 1. Effects of juglone on cell viability in JB6 CI41 mouse skin epidermal cells. (A) The chemical structure of juglone. (B) Cell viability effect of juglone on JB6 CI41 cells. JB6 CI41 cells ($1x10^3$ cells/200 μl) were treated with juglone (1, 2.5 and 5 μM) in 5% FBS-MEM for various times. Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit. Data are mean ± SD of three independent experiments performed in triplicate. The asterisk indicates a significant difference compared with the negative control ($P<0.05$).

Western blot analysis. After the cells were seeded in 10-cm dishes for 48 h, they were starved in 0.1% FBS/MEM medium for 24 h. Cells were pre-treated with juglone (1, 2.5 and 5 μM) for 1 h before they were exposed to TPA (20 ng/ml) or EGF (10 ng/ml) for an additional 24 h. The harvested cells were disrupted, and protein supernatant fractions were subjected to SDS-PAGE and then transferred to membranes and blocked with 5% skim milk followed by hybridization with specific antibodies. Proteins were exposed to horseradish peroxidase (HRP)-conjugated secondary antibody and visualized by Amersham ECL Plus Western Blotting Detection reagents (GE Healthcare).

Luciferase assay to determine AP-1 or COX-2 transactivation. AP-1 or COX-2 luciferase reporter JB6 CI41 cells were cultured in a 24-well plate for 24 h. Then, they were starved

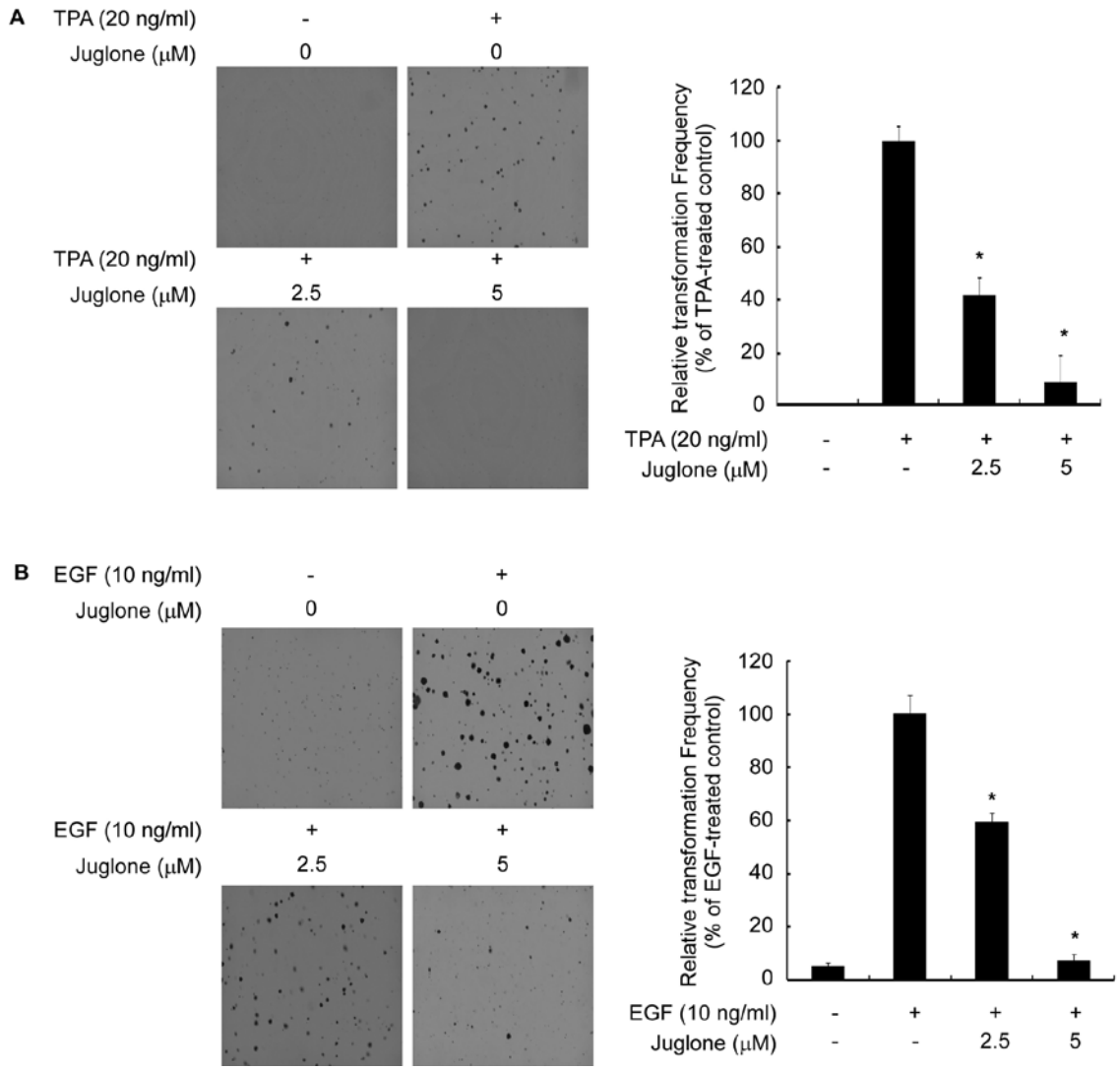

Figure 2. The effect of juglone on TPA- or EGF-induced anchorage-independent colony formation in JB6 CI41 cells. JB6 CI41 cells were treated as described in Materials and methods and cell colonies were counted after 12 days. Then, the cell colonies were automatically counted by a computerized microscope system. *P<0.001, significant difference between groups treated with TPA or EGF and the group treated with TPA or EGF and juglone. Data are mean ± SD of three independent experiments in triplicates.

in 0.1% FBS/MEM medium for an additional 24 h in a 5% CO_2 incubator. The cells were treated for 1 h with juglone or a PI3K inhibitor (LY294002 or wortmannin) and then exposed to TPA (20 ng/ml) or EGF (10 ng/ml) and harvested after 24 h. Luciferase activity was assessed using the Luciferase Assay kit as recommended by the manufacturer (Promega). Cells were disrupted with 200 μl of lysis buffer, and luciferase activity was measured using Luminoskan Ascent (Thermo Electron, Helsinki, Finland).

Statistical analysis. Data are reported as mean ± SD of at least three independent experiments performed in triplicate. Data were analyzed for statistical significance using a one-way analysis of variance. A value of P<0.05 was considered to denote significant differences.

Results

Effect of juglone on JB6 CI41 cell viability. Recent studies have demonstrated that juglone induces cell death in skin cancer (B16F1, mouse melanoma cells) (25). The viability effects of juglone on JB6 CI41 mouse skin epidermal cells during 24 and 48 h were determined by the MTS assay at various concentrations (1, 2.5 and 5 μM). Juglone had no affect on JB6 CI41 cell toxicity at any dose or time point assessed (Fig. 1B).

Juglone significantly suppresses TPA- or EGF-induced cell transformation. JB6 CI41 cells are useful for studying the molecular mechanisms of tumor promotion and antitumor agents (26). We examined the inhibitory activities of juglone on TPA- or EGF-induced neoplastic JB6 CI41 cell transformation. Treatment with juglone (2.5 and 5 μM) significantly inhibited 50-90% of neoplastic transformation as compared to TPA- or EGF-induced transformation without juglone. These results indicate that juglone is a potent inhibitor of TPA- or EGF-induced transformation in JB6 CI41 cells (Fig. 2).

Phosphoinositide 3-kinase is a potential target of juglone. The PI3K pathway is one of the most important pathways in cancer

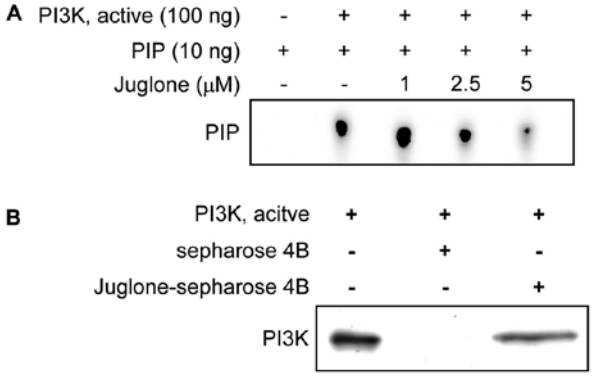

Figure 3. The effect of juglone on PI3K activity *in vitro*. (A) Juglone strongly inhibited PI3K activity. Active PI3K protein (100 ng) was pre-incubated with juglone for 10 min at 30˚C. The resulting ^{32}P-labelled PI3P was visualized as described in Materials and methods. (B) Juglone directly bound with PI3K *in vitro*. The *in vitro* binding of juglone with active PI3K protein was confirmed by a pull-down assay using juglone-Sepharose 4B beads and subsequent western blot analysis.

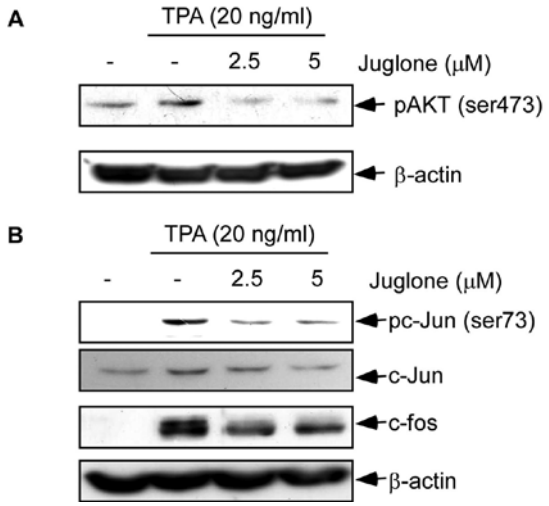

Figure 4. The effect of juglone on TPA-induced AKT, c-Jun, and c-fos activation in JB6 CI41 cells. Juglone inhibited TPA-induced (A) AKT, c-Jun, and (B) c-fos activation. JB6 CI41 cells were treated with juglone at 2.5 and 5 μM for 1 h before being treated with TPA (20 ng/ml) and harvested after 30 min. The protein expression levels of phosphorylated and total AKT, c-Jun and c-fos proteins were analyzed by western blot analysis. Equal loading of proteins was determined by incubating the same membrane with anti-β-actin antibody.

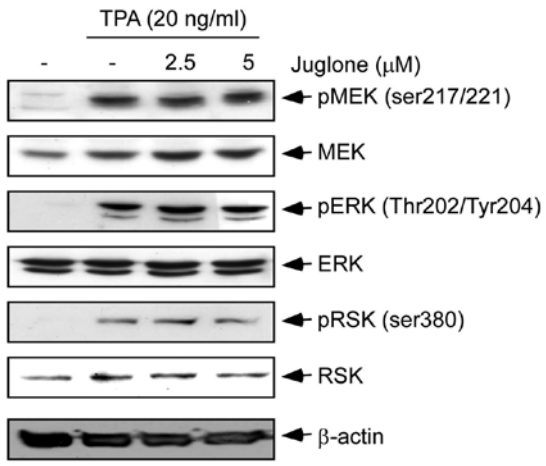

Figure 5. The effect of juglone on TPA-induced phosphorylation of MEK, ERK and RSK. JB6 CI41 cells were treated with juglone at various concentrations (1, 2.5 and 5 μM) for 1 h and then exposed to 20 ng/ml TPA for 30 min. The levels of phosphorylated and total MEK, ERK, and RSK protein detected by western blot analysis. Cellular extract (30 μg/lane) was separated on a 10% SDS-PAGE gel using the indicated antibodies. Equal loading and protein transfer were confirmed with anti-β-actin antibody. Data are representative of three independent experiments.

metabolism and growth (27). Moreover, previous studies have demonstrated that PI3K is required for TPA- or EGF-induced cell transformation (1,21). Therefore, we studied the effect of juglone on PI3K activity to investigate the molecular target of juglone. The PI3K assay data indicated that juglone (5 μM) significantly inhibited PI3K activities *in vitro* (Fig. 3A). A direct interaction of juglone-Sepharose 4B beads with active PI3K was demonstrated by the *in vitro* pull-down assay (Fig. 3B). Our results suggest that juglone strongly suppressed PI3K activity by directly binding to PI3K.

Juglone blocks TPA-induced activation of AKT but not MEK. Our results indicate that juglone specifically binds with PI3K and subsequently inhibits PI3K activation. Therefore, we investigated the influence of juglone on TPA-induced activation of AKT. TPA-induced phosphorylation of AKT was almost inhibited by treatment with juglone (2.5 or 5 μM) (Fig. 4A). We examined the effect of juglone on the activation of several downstream kinases in JB6 CI41 cells. TPA-induced c-Jun and c-fos activation was significantly suppressed by juglone in a dose-dependent manner (Fig. 4B). Previous studies have indicated that the MEK signaling pathway is involved in TPA-induced JB6 CI41 cell transformation (26,28). Therefore, we confirmed the influence of juglone on the MEK signaling pathway. TPA-induced phosphorylation of MEK was not inhibited by juglone. Moreover, juglone had no effect on TPA-induced phosphorylation of ERK and RSK, a downstream kinase of MEK (Fig. 5).

Juglone attenuates TPA- or EGF- induced transactivation of AP-1 and COX-2. Multiple signal transduction pathways including AKT are activated by TPA (29,30) or EGF (31) and increased AKT phosphorylation leads to AP-1 and COX-2 activation (32) which modulate carcinogenesis (33). Therefore, to determine whether the suppression of transformation by juglone involves inhibition of AP-1 and COX-2 activities, we measured AP-1 and COX-2 transactivation using JB6 CI41 cell lines stably transfected with an AP-1 or COX-2 luciferase plasmid. We determined that TPA-induced AP-1 or COX-2 activity was achieved using juglone compared with LY294002 or wortmannin. LY294002 inhibits PI3K by competing with ATP for its substrate binding site (34) and wortmannin also inhibits PI3K activity (35). The results showed that wortmannin (5 μM) inhibited TPA- or EGF-induced AP-1 activity, whereas no significant inhibition was shown by COX-2 (Fig. 6). LY294002 (20 μM) significantly suppressed the activity of TPA- or EGF-induced COX-2, but not for AP-1. Juglone inhibited TPA- or EGF-induced transactivation of either AP-1 or COX-2 in a dose-dependent manner. AP-1 activity is regulated at the level of c-Jun and c-fos gene transcription by protein-protein interactions and also through Jun and fos post-translational protein modifications (36-38).

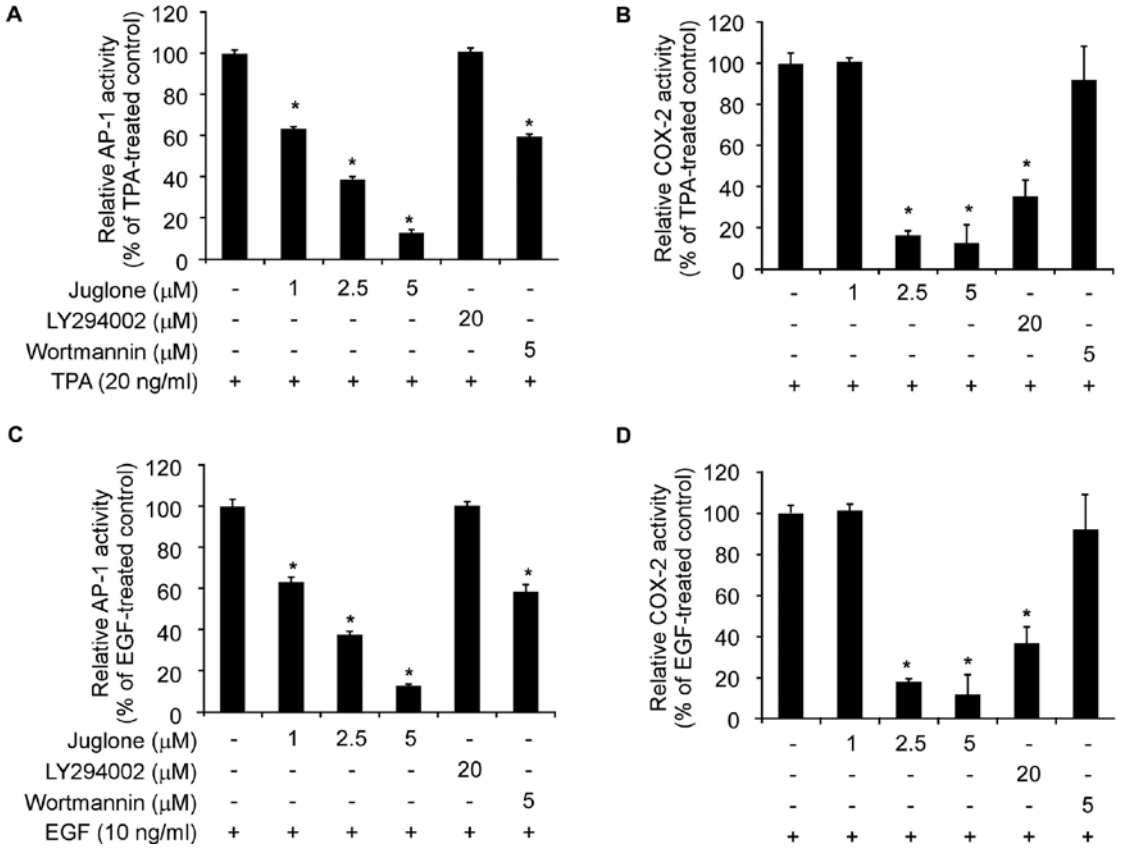

Figure 6. The effect of juglone on TPA- or EGF-induced AP-1 and COX-2 activation. The effect of juglone, LY294002 and wortmannin on (A) TPA- or (C) EGF-induced AP-1 and (B and D) COX-2 transactivation. Cells were seeded in 24-well plates for 24 h and then starved by culturing in 0.1% FBS-MEM for 24 h. Cells were pre-treated with juglone, wortmannin, or LY294002 for 1 h and than incubated with TPA (20 ng/ml) or EGF (10 ng/ml) for 24 h. Luciferase activity was measured, and AP-1 and COX-2 promoter activity was expressed as the percent inhibition relative to cells treated with TPA or EGF alone. Data are representative of triplicate samples from three independent experiments. *P<0.001, significant change relative to cells activated with TPA or EGF.

TPA-induced c-Jun and c-fos was inhibited by juglone (Fig. 4C). Juglone strongly suppressed TPA- or EGF-induced AP-1 and COX-2 luciferase activity compared with LY294002 and wortmannin by inhibiting c-Jun and c-fos.

Discussion

Juglone is one of the oldest known allelopathic compounds in the history of cultivation and is used as an active ingredient in herbal remedies and commercial dyes (4,16) (Fig. 1A). Juglone is used extensively to overcome resistance to chemotherapeutic agents in cancer due to their cytotoxicity (39). For example, the bark, branches and exocarp of the immature green fruit of this medicinal plant have been used to treat gastric cancer, liver cancer, lung cancer and other types of cancer (40). Juglone is also a potent cytotoxic agent *in vitro* in human tumor cell lines, including human colon carcinoma (HCT-15) cells, human leukemia (HL-60) cells, and doxorubicin-resistant human leukemia (HL-60R) cells (5,41). Therefore, juglone has been previously used for its therapeutic properties. Accumulated evidence suggests that juglone has chemopreventative or chemotherapeutic effects on various types of cancer.

In this study, we investigated the molecular mechanism of juglone as a potential target for tumor suppression. JB6 CI41 cells are useful for studying the molecular mechanisms of tumor promotion and antitumor agents (26). Therefore, we primarily determined the cell viability effects of juglone on the proliferation of JB6 CI41 cells using the MTS assay. We confirmed that juglone did not significantly inhibit JB6 CI41 cell growth in either a dose- or time-dependent manner (Fig. 1B). Next, we examined the inhibitory activities of juglone on TPA- or EGF-induced neoplastic JB6 CI41 cell transformation, as both TPA and EGF are well-known tumor promotion agents used to study malignant cell transformation in cell and animal models of cancer (42). Approximately 50-90% of TPA- or EGF-induced cell transformation was inhibited by juglone as compared to neoplastic transformation without juglone (Fig. 2). These results suggest that juglone plays an important role as an antitumor agent in TPA- or EGF-induced tumorigenesis. Thus, we examined a juglone pull-down assay to identify juglone molecular targets in tumorigenesis and confirmed that juglone specifically binds with PI3K (Fig. 3B). Moreover, the PI3K activity assay results showed that juglone (5 μM) significantly inhibited PI3K activity *in vitro* (Fig. 3A), indicating that PI3K activity can be directly regulated by juglone treatment.

PI3K is a major signaling component downstream of many growth factor receptor tyrosine kinases and PI3K family members act as cellular sensors to relay mitogenic signals to internal cellular effectors (43). In particular, PI3K/AKT affects the expression of genes that contribute to tumorigenesis and is activated by many types of cellular stimuli and toxic insults (44). Previous studies have demonstrated that PI3K/AKT signaling is

associated with the development of diseases, such as cancer (18). Actually, PI3K/AKT signaling plays a major role not only in tumor growth but also in the potential response of a tumor to cancer treatment (20). Therefore, we investigated the influence of juglone on TPA-induced activation of AKT, c-Jun, and c-fos, which are downstream PI3K/AKT signaling molecules. The results showed that juglone significantly inhibited TPA-induced phosphorylation of AKT (Fig. 4A) and phosphorylation of c-Jun and c-fos (Fig. 4B). However, TPA-induced phosphorylation of MEK, ERK and RSK, downstream ERK kinases were not inhibited (Fig. 5). These results suggest that juglone leads to a decrease in TPA-induced cell transformation by blocking the PI3K/AKT signaling by inhibiting c-Jun and c-fos. c-Jun and c-fos, which form dimeric complexes such as Jun-Jun or Jun-fos are components of the AP-1 family (45). AP-1 is a major eukaryotic transcription factor involved in regulating COX-2 expression (46-48) which plays an important role in tumorigenesis (49). According to a previous study, TPA-induced AP-1 transactivation and transformation in JB6 CI41 cells and JB6 CI41 cell promotion-sensitive cell transformation is induced by TPA (21). We determined whether TPA or EGF could induce AP-1 or COX-2 activity and promote TPA- or EGF-induced AP-1 or COX-2 activity in JB6 CI41 cells by exposing JB6 CI41 cells to TPA (20 ng/ml) or EGF (10 ng/ml) (Fig. 6). Additionally, juglone activity was compared to that of LY294002 and wortmannin, which are widely used specific PI3K inhibitors (50). Juglone significantly inhibited TPA- and EGF-induced AP-1 (Fig. 6A and C) and COX-2 (Fig. 6B and D) activity compared with that of LY294002 and wortmannin. These results indicate that enhancement of TPA-induced PI3K activity could be markedly blocked more by juglone than that of LY294002 or wortmannin.

In summary, juglone was effective in inhibiting the neoplastic transformation induced by TPA or EGF in JB6 CI41 cells. This inhibition was mediated primarily by blocking the PI3K/AKT signaling pathway and subsequent suppression of AP-1 and COX-2 activity. Juglone inhibited PI3K activity by directly binding with PI3K. Furthermore, juglone directly blocked TPA- or EGF-induced PI3K activation. Given the critical role of PI3K in carcinogenesis, these results suggest that PI3K is a potent molecular target of juglone and may provide the molecular basis to develop chemotherapeutic drugs that target cancerous cells. Additionally, juglone could be a useful chemical for industrial and medical products such as hair dyes, skin care balms, and anticancer foods.

Acknowledgements

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