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Cardamonin Suppresses TGF-β1-Induced Epithelial Mesenchymal Transition via Restoring Protein Phosphatase 2A Expression

Eun Ji Kim[1], Hyun Ji Kim[1], Mi Kyung Park[1], Gyeung Jin Kang[1], Hyun Jung Byun[1], Ho Lee[2] and Chang Hoon Lee[1,]*

[1]BK21PLUS R-FIND Team, College of Pharmacy, Dongguk University, Seoul 100-715,
[2]National Cancer Center, Goyang 410-769, Republic of Korea

Abstract

Epithelial mesenchymal transition (EMT) is the first step in metastasis and implicated in the phenotype of cancer stem cells. Therefore, understanding and controlling EMT, are essential to the prevention and cure of metastasis. In the present study, we examined, by Western blot, reverse transcription polymerase chain reaction (RT-PCR), and confocal microscopy, the effects of cardamonin (CDN) on transforming growth factor-β1 (TGF-β1)-induced EMT of A549 lung adenocarcinoma cell lines. TGF-β1 induced expression of N-cadherin and decreased expression of E-cadherin. CDN suppressed N-cadherin expression and restored E-cadherin expression. Further, TGF-β1 induced migration and invasion of A549 cancer cells, which was suppressed by CDN. TGF-β1 induced c-Jun N-terminal kinase (JNK) activation during EMT, but CDN blocked it. Protein serine/threonine phosphatase 2A (PP2A) expression in A549 cancer cells was reduced by TGF-β1 but CDN restored it. The overall data suggested that CDN suppresses TGF-β1-induced EMT via PP2A restoration, making it a potential new drug candidate that controls metastasis.

Key Words: Cardamonin, Epithelial mesenchymal transition, TGF-β1, JNK, PP2A, A549

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths, killing more than 1 million people every year worldwide (Parkin *et al*., 2005). Non-small cell lung cancer (NSCLC) is the leading cause of lung cancer death in the world (Hung *et al*., 2009; Lee *et al*., 2013). For early-stage NSCLC, surgical resection is the treatment of choice (Hung *et al*., 2009). The most common post-resection events leading to mortality are tumor recurrence and metastasis (Williams *et al*., 2006).

Epithelial mesenchymal transition (EMT) in lung cancer arises during embryonic cell layer movements and tumor cell invasion (Denlinger *et al*., 2010). An analysis of a large number of lung tumor specimens showed that the majority of primary lung cancer cells and even premalignant lesions have the mesenchymal phenotype, which is characterized by down-regulation of E-cadherin and up-regulation of N-cadherin (Prudkin *et al*., 2009). During EMT, the epithelial protein E-cadherin is down-regulated, and the mesenchymal proteins vimentin and N-cadherin are up-regulated, via transcription factors that include snail, ZEB1, and ZEB2 (Peinado *et al*., 2007).

Cytokines of the transforming growth factor-β (TGF-β) family, meanwhile, have multiple roles in the development of diseases including cancer (Kawata *et al*., 2012). Perturbations of TGF-β signaling, for example, are central to tumorigenesis and tumor progression, specifically through their effects on cellular processes including cell proliferation and cell invasion (Ikushima and Miyazono, 2010). In this way, TGF-β1 induces EMT in cancer cells, thereby enabling them to become motile and invasive (Kim *et al*., 2007; Kawata *et al*., 2012; Park *et al*., 2013b).

C-Jun N-terminal kinase (JNK) is a member of the family of mitogen-activated protein kinases (MAPK) that is well-known for its role in stress responses and apoptosis regulation (Davis, 2000). Recent studies have uncovered evidence of JNK's possible role in TGF-β1-induced EMT (Alcorn *et al*., 2008; Velden *et al*., 2011). It appears that sustained JNK activity, by regulating extracellular signal-regulated kinase activation, promotes EMT, invasion and survival of breast cancer cells (Wang *et al*., 2010). Recently, we found that transglutaminase-2 (Tgase-2) activated JNK by reduced expression of protein serine/threonine phosphatase 2A (PP2A) in TGF-β1-induced EMT (Park *et al*., 2013b).

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***Corresponding Author**
E-mail: uatheone@dongguk.edu
Tel: +82-31-961-5213, Fax: +82-31-961-5206

PP2A, is an abundant cellular enzyme with numerous substrates that modulates various cellular functions (McConnell *et al*., 2010). PP2A's catalytic subunit has the capacity to dephosphorylate serine and threonine residues (Fellner *et al*., 2003). The activities of several kinases, including JNK, are reduced by PP2A (Kins *et al*., 2003).

Cardamonin (CDN, 2′,4′-dihydroxy-6′-methoxychalcone), a well-known component of *Alpinia Katsumadai* (Wang *et al*., 2007; Park *et al*., 2013a) has anti-tumor, anti-inflammatory, anti-itching, and anti-nociceptive activities (Chow *et al*., 2012; Yadav *et al*., 2012; Park *et al*., 2014a; Park *et al*., 2014b). Studies have found that it inhibits the production of pro-inflammatory mediators in whole blood or human monocytes (Ahmad *et al*., 2006; Hatziieremia *et al*., 2006).

Several mechanism of actions of CDN were proposed to explain its diverse biological activities. For example, previous studies found that CDN inhibited mTOR, NF-κB, Wnt pathways (Park *et al*., 2013c; He *et al*., 2014; Tang *et al*., 2014). Notably, we also reported that CDN blocks cancer cell migration by inhibiting the expression and activity of transglutaminase-2 (Tgase-2), an important target for cancer and inflammation (Kim *et al*., 2006; Park *et al*., 2012; Park *et al*., 2013a). We have also showed that N-cadherin expression is dependent on Tgase-2 in TGF-β1-induced EMT (Park *et al*., 2013b). Therefore, it is necessary to determine if CDN suppresses Tgase-2 dependent N-cadherin expression during TGF-β1-induced EMT. Furthermore, it is not known whether CDN suppress the TGF-β1-induced EMT.

In the present study, we examined the effects of CDN on TGF-β1-induced EMT of A549 lung cancer cells and found that CDN inhibited Tgase-2 dependent N-cadherin expression during TGF-β1-induced EMT by restoring PP2A expression.

MATERIALS AND METHODS

Reagents

RPMI1640 and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, South Korea). TGF-β1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Mouse monoclonal anti- E-cadherin, and anti-PP2A antibodies were acquired from BD Biosciences (San Jose, CA, USA). Mouse monoclonal anti-β-Actin and rabbit polyclonal anti-N-cadherin antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-JNK, mouse monoclonal anti-phosphor-JNK and HRP-conjugated anti-mouse antibodies were purchased from Cell Signalling Technology, Inc. (Beverly, MA, USA).

Cell culture

A549 (CCL-185), a human lung adenocarcinoma cell line, was obtained from the American Type Culture Collection. The A549 cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C in a humidified, 5% CO_2 atmosphere.

Cell migration assay

Migration assays were performed in a transwell (Neuro Probe, Inc., Gaithersburg, MD, USA) coated with 10 μg/mL fibronectin. A549 cells (1×10^6 cells/mL) suspended in serum-free medium were added to the upper chamber of the transwell inserts. To the lower chamber, medium containing 3% FBS was added. After 5 h incubation, non-migrated A549 cells were scraped off the upper surface of the membrane; the migrated A549 cells on the lower surface were stained by Diff-quick and counted under four randomly chosen high-power fields (20× magnification). All of the experiments were repeated at least three times with two replicates each.

Cell-invasion assay

A cell invasion assay was performed using matrigel-coated (0.5 μg/mL) transwell inserts, as described previously. A549 cells (1×10^6 cells/mL) suspended in serum-free medium were added to the upper chamber of the transwell inserts. To the lower chamber, medium containing 10% FBS was added. After 16 h incubation, non-migrated A549 cells were scraped off the upper surface of the membrane; the cells on the lower surface were stained using the Hema 3 staining system (Fisher Scientific, Houston, TX, USA), photographed, and counted under four randomly selected fields (20× magnification). All of the experiments were repeated at least three times with two replicates each.

Western blot analysis

After incubation, the A549 cells were collected and washed twice with cold phosphate-buffered saline (PBS). They were then lysed in a buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% tritonX-100, 2 mM EDTA, 1% DOC (Deoxycholic-acid), 0.1% SDS, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT] and centrifuged to yield whole-cell lysates. The protein concentration was measured using the Bradford method. Aliquots of the lysates (20-30 μg of protein) were separated on 8-12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA) with glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 10%MeOH (v/v)]. After blocking the non-specific site with 5% non-fat dry milk, the membrane was incubated with specific primary antibody in 3% bovine serum albumin (BSA) at 4°C overnight, and then further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5,000, Santa Cruz Biotechnology Inc.) at room temperature. Immunoreactive proteins were detected using PowerOpti-ECL Western blotting detection reagent (Animal Genetics Inc., Gyeonggi, Korea).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction from A549 cells was conducted in an RNase-free environment by the Tri-Reagent method (Invitrogen), according to the manufacturer's instructions. Reverse transcription of 1 μg RNA was carried out using M-MuLV reverse transcriptase (Promega), oligo (dT) 15 primer, dNTP (0.5 μM) and 1 U RNase inhibitor. PCR was performed with an Applied Biosystems GeneAmp PCR system (Invitrogen); the amplification program included 30 cycles at 94°C (denaturing), 50-58°C (annealing) and 72°C (extension), respectively. The PCR products were electrophoresed on 1.2% agarose gel.

Confocal microscopy

A549 cells grown on coverslips and fixed in methanol (MeOH) for 10 min at room temperature were permeabilized with a 10 min wash in 0.1% Triton X-100, also at room temperature, followed by several washes in PBS with 3% BSA. Phosphor-JNK and PP2A primary antibody were incubated with the A549 coverslips overnight at 4°C, after which the antibody was removed with four washes in PBS. Species-specific

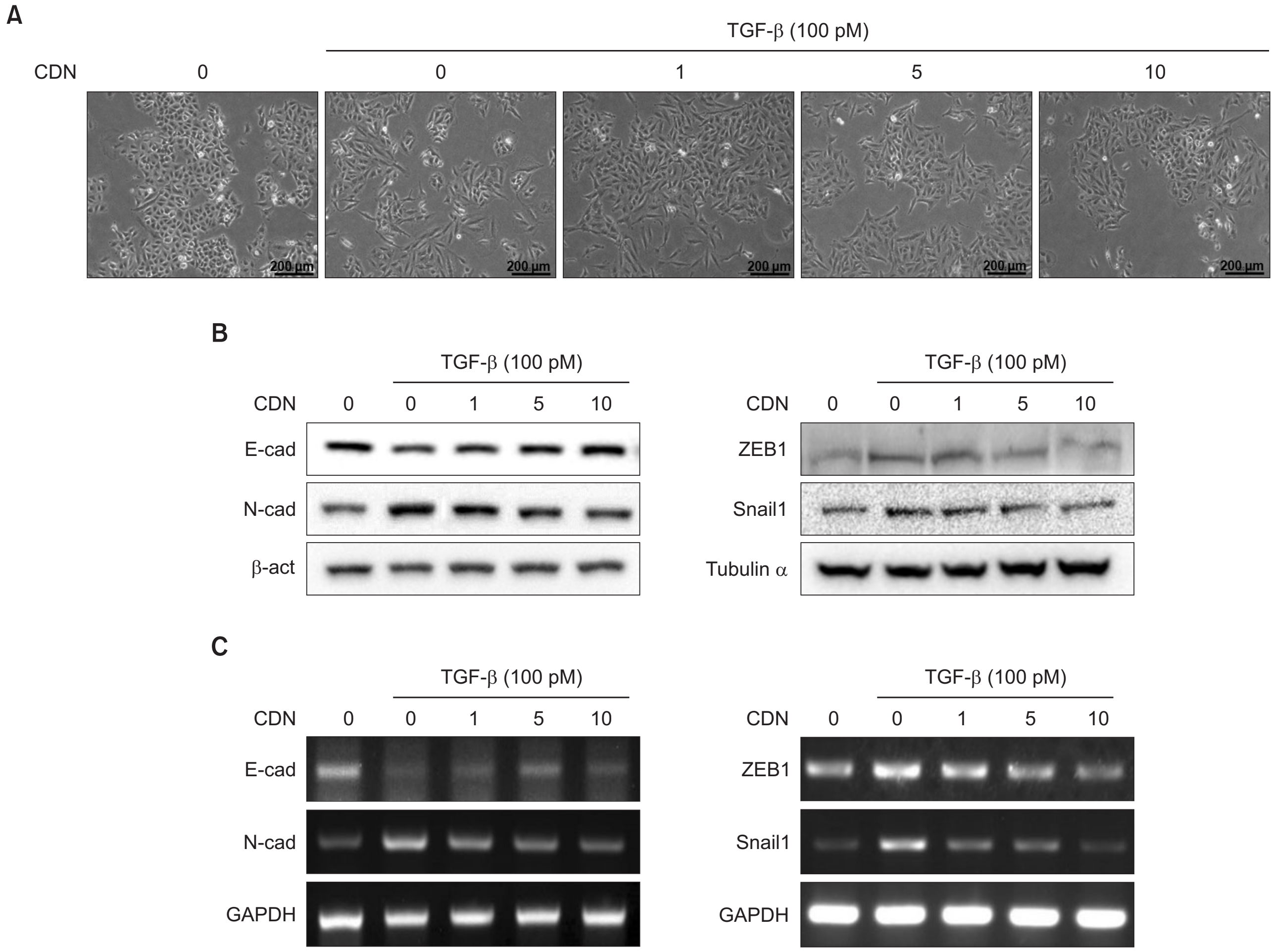

Fig. 1. CDN inhibited TGF-β-induced EMT in A549 cells. (A) Phase-contrast images of A549 cells. Cells were pre-treated with CDN (1, 5 and 10 μM) for 1h. Next, they were treated with TGF-β1 (100 pM). After 48 h, the cells were photographed using a real-time image system under Leica microscopy (20× magnification). (B) Immunoblot analysis of E-cadherin, N-cadherin and transglutaminase-2 (Tgase-2) in A549 cells treated with CDN (1, 5 and 10 μM) and TGF-β1 (100 nM) for 48h. The β-Actin was used here as an internal control. (C) Reverse transcription-PCR of E-cadherin and N-cadherin in A549 cells treated with CDN (1, 5 and 10 μM) and TGF-β1 (100 nM) for 48 h.

secondary antibodies conjugated in goat anti-mouse IgG antibody (Alexa Fluor 488, 1:500 Molecular Probes) were then reacted with the coverslips for 1 h at room temperature followed by four washes in PBS. The final samples were mounted onto slides and visualized under Nikon confocal microscopy.

Statistical analysis

Data herein are expressed as the mean ± standard deviations (S.D.) of at least three independent experiments performed in triplicate. A student's *t*-test was used to analyze the data, according to the following significance levels: $^*p<0.05$, $^{**}p<0.01$.

RESULTS

CDN suppressed TGF-β1-induced EMT in A549 lung cancer cell lines

EMT was induced in A549 lung adenocarcinoma cells by TGF-β1 (100 pM) treatment, according to the Lee's report (Lee *et al.*, 2013). TGF-β1 treatment of A549 cells induced the mesenchymal morphology, including loss of cell-to-cell contact, and CDN suppressed those mesenchymal changes (Fig.1A). TGF-β1 treatment also induced the mRNA expression of mesenchymal markers including N-cadherin and reduced the expression of epithelial markers such as E-cadherin, and induced the EMT-related transcription factors such as ZEB1 and snail1 (Fig. 1B). These results were confirmed by Western blot (Fig. 1C).

CDN suppressed TGF-β1-induced migration and invasion in A549 lung cancer cell lines

TGF-β1-induced Tgase-2 is involved in the EMT in A549 cells, leading to the migration and invasion of A549 cancer cells (Park *et al.*, 2013b). In a previous report, we showed that CDN suppresses the expression and activity of Tgase-2, leading to inhibition of HT1080 cell migration (Park *et al.*, 2013a). Therefore, we examined the effects of CDN on the migration and invasion of A549 cancer cell lines. We found that TGF-β1 induced enhanced migration and invasion in A549 lung cancer cells and that CDN dose-dependently suppressed such migration and invasion (Fig. 2).

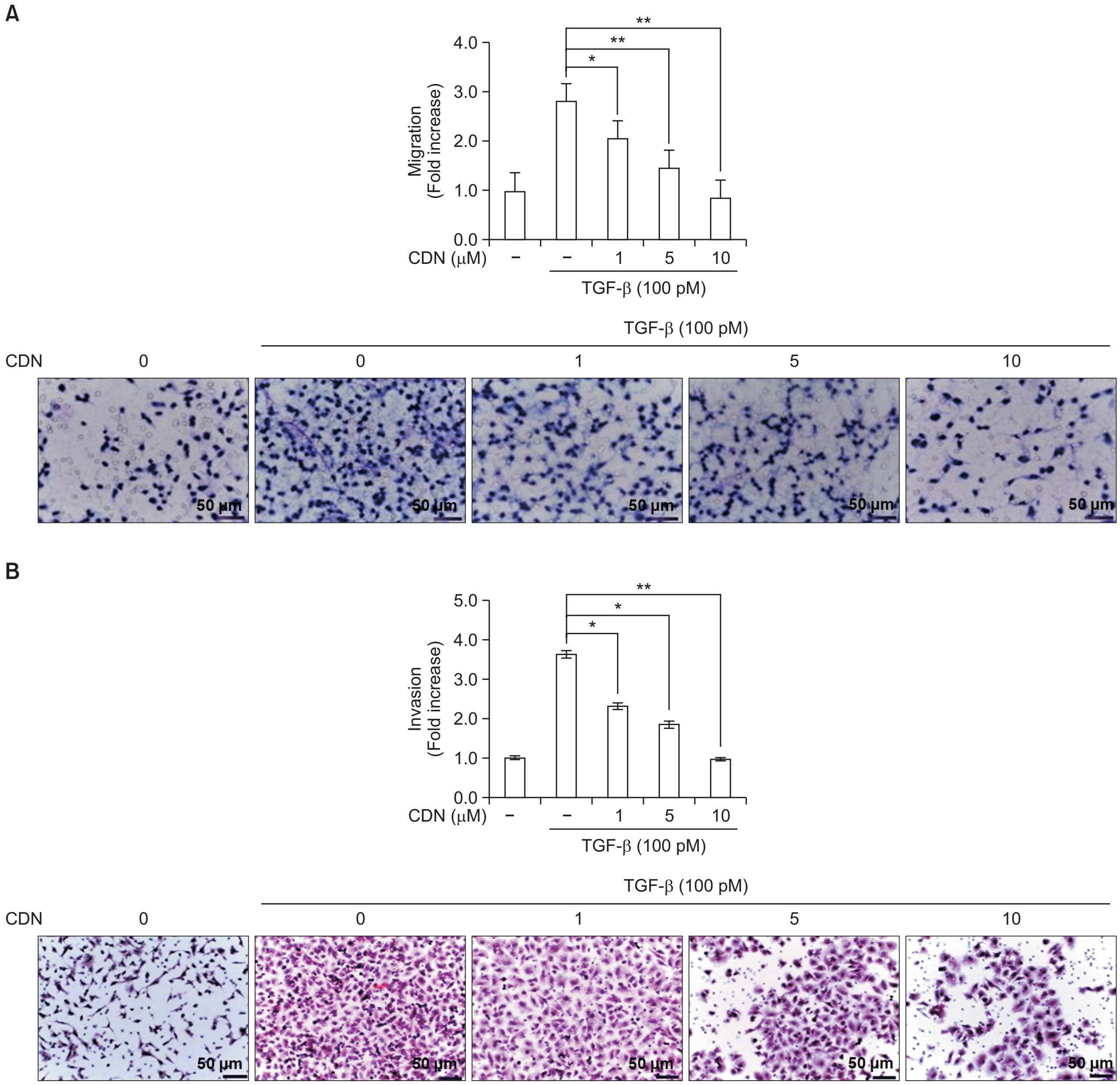

Fig. 2. CDN inhibited migration and invasion by TGF-β-induced EMT in A549 cells. (A) Effects of CDN on TGF- β1-induced cell migration. For the migration assay, lower-chamber transwells were coated with fibronectin (10 μg/ml). (B) Effects of CDN on TGF-β1-induced cell invasion. For the invasion assay, lower-chamber transwells were coated with matrigel (0.5 μg/ml). The data represent the means ± S.D. of three wells. *$p<0.05$, **$p<0.01$ compared with TGF- β1-treated group.

CDN suppressed JNK activation in TGF-β1-induced EMT of A549 lung cancer cell lines

In another previous report, we showed that JNK is activated in TGF-β1-induced EMT, and thus leads to N-cadherin expression (Park *et al.*, 2013b). So, we investigated, in the present study, whether CDN suppresses TGF-β1-induced JNK activation. According to our results, TGF-β1 treatment induced phosphorylation of JNK (active JNK), and CDN dose-dependently suppressed JNK activation (Fig. 3A). This was confirmed by confocal microscopy (Fig. 3B).

CDN restored PP2A expression in TGF-β1-induced EMT of A549 lung cancer cell lines

PP2A dephosphorylated phospho-JNK (active form) to dephosphorylated JNK (inactive form) (Huang *et al.*, 2009). Thus we examined whether PP2A is involved in CDN's effects on EMT. To that end, PP2A expression was reduced in TGF-β1-induced EMT (Fig. 4A). The results showed that CDN restored PP2A expression in TGF-β1-induced EMT (Fig. 4A). Confocal microscopy data confirmed this observation (Fig. 4B).

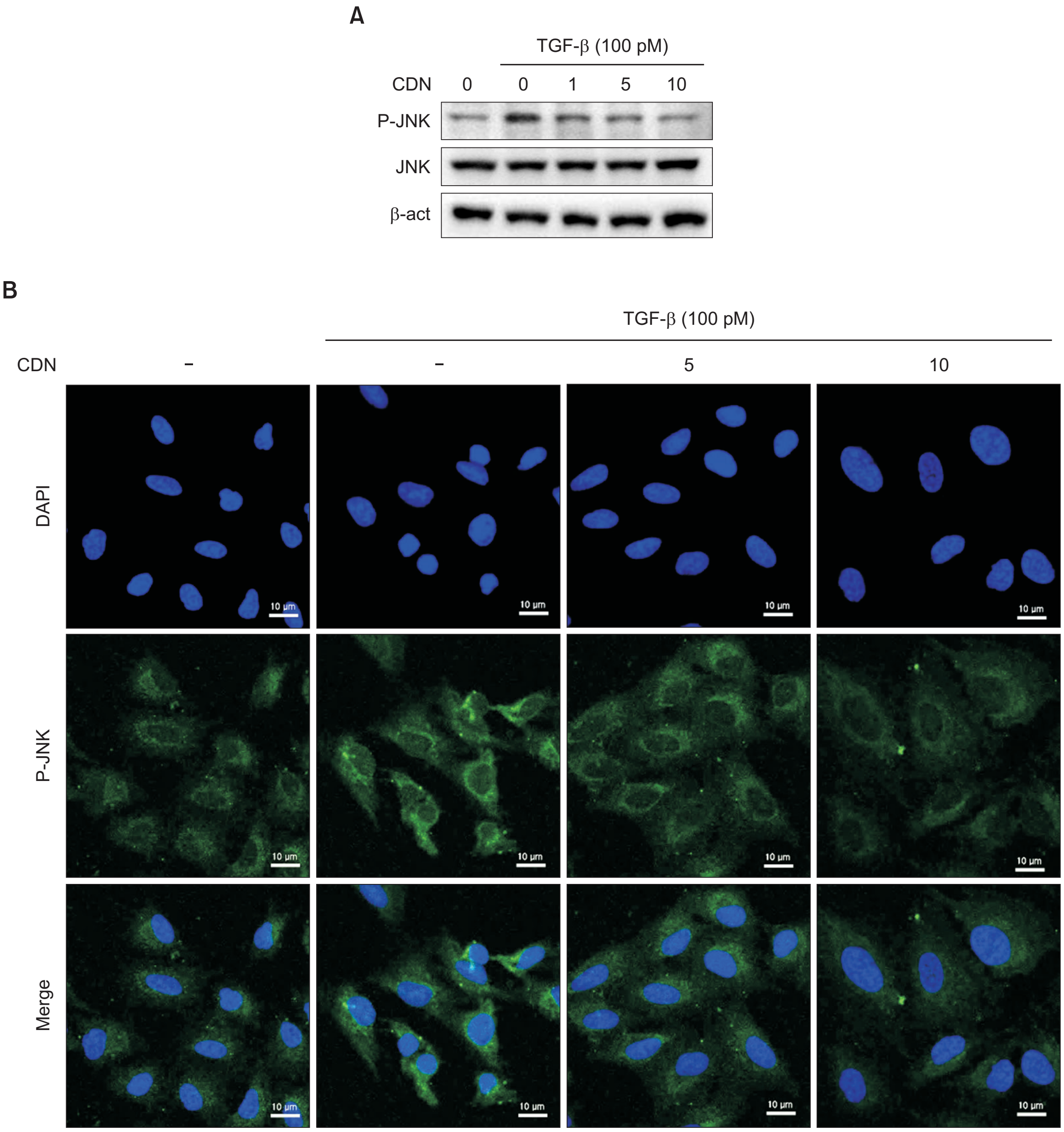

Fig. 3. CDN inhibited TGF-β-induced phosphorylation of JNK in A549 cells. (A) Immunoblot analysis of phosphor-JNK and JNK in A549 cells treated with CDN (1, 5 and 10 μM) and TGF-β1 (100 nM) for 48 h. β-Actin was used here as an internal control. (B) Confocal microscopic examination of phosphor-JNK (60× magnification).

DISCUSSION

EMT is an important early step in metastasis of cancer cells (Kalluri and Weinberg, 2009; Sheen *et al.*, 2013; Ryoo *et al.*, 2014). EMT is also implicated in the phenotype of cancer stem cells (Blick *et al.*, 2010), therefore understanding and controlling EMT is essential to the prevention of metastasis.

Fig. 1 and 2 show that CDN inhibits TGF-β1-induced expression of N-cadherin and migration and invasion of A549 cancer cells. Fig. 3 shows that TGF-β1 induced JNK activation, which is inhibited by CDN. Previous research has demonstrated that TGF-β1-induced JNK activation is dependent on Tgase-2 (Park *et al.*, 2013b). These results suggest that CDN might suppress N-cadherin expression, migration and invasion via Tgase-2 inhibition-based mechanism (Park *et al.*, 2013a; Park *et al.*, 2014a; Park *et al.*, 2014b).

Recently, several reports have indicated that JNK activation is involved in EMT (Wang *et al.*, 2010; Park *et al.*, 2013b; Li *et al.*, 2014). JNK is activated by MAP kinase pathway or by PP2A reduction (Yamashita *et al.*, 2008; Park *et al.*, 2013b). Reduction of PP2A expression by Tgase-2 contributes to JNK activation, since PP2A plays a key role in inactivating JNK (Park *et al.*, 2013b). Correspondingly, in the present study, CDN restored PP2A expression in TGF-β1-treated A549 cancer cells (Fig. 4).

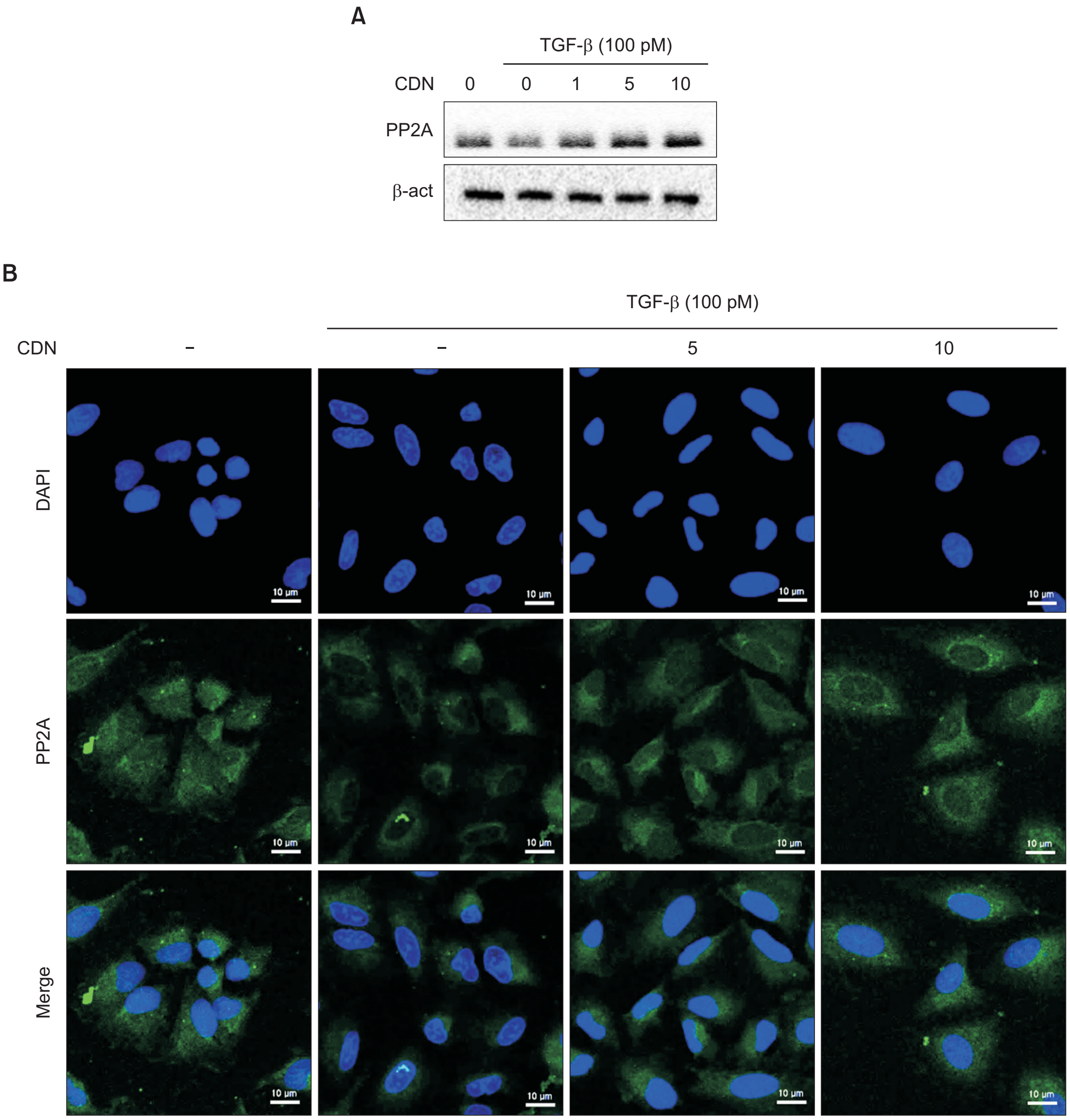

Fig. 4. CDN induced PP2A expression reduced by TGF-β1 in A549 cells. (A) Immunoblot analysis of PP2A in A549 cells treated with CDN (1, 5 and 10 μM) and TGF-β1 (100 nM) for 48 h. β-Actin was used here as an internal control. (B) Confocal microscopic examination of PP2A (60× magnification).

Based on these results, Tgase-2 inhibition should be added to other proposed mechanism of action of CDN including mTOR, NF-κB, and WNT pathways responsible for its diverse biological activities (Park *et al*., 2013a; Park *et al*., 2013c; He *et al*., 2014; Tang *et al*., 2014). It should be noted that phosphorylation and dephosphorylation are key steps in several of the suggested mechanism of action of CDN, including the previously mentioned pathways. We speculated that CDN-induced PP2A restoration via Tgase-2 inhibition lead to the suppression of several key kinases in mTOR, NF-κB, and WNT pathway. However, it is still unclear how Tgase-2 inhibition leads to PP2A restoration.

Recently, special attention has been centered on drugs that restore or activate PP2A in cancer cells (Saddoughi *et al*., 2013; Neviani and Perrotti, 2014). FTY720 activated PP2A via binding to protein SET, a PP2A inhibitory protein (Saddoughi *et al*., 2013), and a dithiolethione compound inhibited AKT signaling in human breast and lung cancer cells by increasing PP2A activity (Switzer *et al*., 2009). According to the present research findings, CDN could be considered as a PP2A-restoration compound.

In the present study, we found that CDN inhibited N-cadherin expression and restored PP2A expression via Tgase-2 inhibition, which suppresses TGF-β1-induced EMT that is critical to metastasis. Tgase-2 inhibition might be new way of restoring PP2A, and CDN appears to have potential as an antimetastatic compound.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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