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Caffeic acid and its synthetic derivative CADPE suppress tumor angiogenesis by blocking STAT3-mediated VEGF expression in human renal carcinoma cells

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Tumor angiogenesis is required for tumor development and is stimulated by angiogenic inducers like VEGF (vascular endothelial growth factor). Our previous study demonstrated that STAT3 (signal transducer and activator of transcription 3) up-regulates HIF-1α (hypoxia inducible factor-1α) protein stability and enhances HIF-1-mediated VEGF expression in hypoxic solid tumor cells, thus suggesting that the inhibition of STAT3 signaling may have clinical applications. In this study, we examined *in vitro* and *in vivo*, whether caffeic acid (CA) or its derivative CADPE [3-(3,4-dihydroxy-phenyl)-acrylic acid 2-(3,4-dihydroxy-phenyl)-ethyl ester] exert anticancer activity by targeting STAT3. It was found that CA or CADPE significantly inhibit STAT3 activity, and that this in turn down-regulates HIF-1α activity. Consequently, sequential blockade of STAT3 and HIF-1α resulted in the down-regulation of VEGF by inhibiting their recruitment to the VEGF promoter. In mice bearing a Caki-I carcinoma, both CA and CADPE retarded tumor growth and suppressed STAT3 phosphorylation, HIF-1α expression, vascularization and STAT3-inducible VEGF gene expression in tumors. Taken together, our results demonstrate that CA and CADPE are potential inhibitors of STAT3 and that they suppress tumor angiogenesis by inhibiting the activity of STAT3, the expression of HIF-1α and VEGF.

Introduction

In a previous study, we found that signal transducer and activator of transcription 3 (STAT3) plays a pivotal role in the regulation of hypoxia inducible factor-1α (HIF-1α) protein stability and that it enhances the HIF-1-mediated expression of vascular endothelial growth factor (VEGF) by interacting with HIF-1α in human renal carcinoma cells (1). These findings demonstrate that STAT3 is a critical up-regulator of hypoxia-mediated VEGF expression in solid tumor cells.

VEGF has been well established to have a crucial role in angiogenesis and tumor progression (2), and VEGF inhibition has produced promising results as a tumor anti-angiogenesis therapy in animal models and cancer patients (3). Hypoxia commonly develops within solid tumors because tumor cell proliferation is faster than the rate of blood vessel formation, and thus leads to a compromised tumor blood supply (4). Moreover, hypoxia also stimulates VEGF expression in tumors and promotes angiogenesis to meet the metabolic requirement for growth (5). Thus, factors that regulate hypoxic angiogenesis represent viable anticancer therapeutic targets. HIF-1 is one such target, as it is a key transcription factor that regulates VEGF expression. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits (6), and its biologic activity depends on the amount of HIF-1α, which is tightly regulated by oxygen tension. Under normoxic conditions, HIF-1α protein is unstable. Many studies have challenged the development of anticancer drug targeting HIF-1α which is inducible under hypoxic conditions (7).

Recently, STAT3 was found to be a direct transcriptional activator of the VEGF gene, and demonstrated to up-regulate VEGF expression in diverse human cancers (8), which suggests that STAT3 activity contributes significantly to VEGF overproduction in tumors. STAT3 is activated by phosphorylation at tyrosine residue 705 (Y705), which leads to dimer formation, its nuclear translocation, DNA binding and target gene transcription (9). Phosphorylation of STAT3 at tyrosine 705 (Y705) is critically its dimerization and nuclear translocation; both prerequisites of STAT3 activation (10). Thus, we considered that inhibiting this phosphorylation at residue 705 might reduce VEGF expression in tumor cells. During a search for inhibitors of STAT3 activity, we found that caffeic acid (CA) and its synthetic derivative, CADPE [3-(3,4-Dihydroxy-phenyl)-acrylic acid 2-(3,4-dihydroxy-phenyl)-ethyl ester], are both active. CA has been reported to possess a wide spectrum of biological effects, e.g. antioxidant activity (11–13) and anti-inflammatory properties (14,15), which are similar to the effects of many other anticancer agents.

In the present study, the observations made revealed that CA or its derivative CADPE inhibit tumor growth and angiogenesis by inhibiting the activity of STAT3, the expression of HIF-1α and VEGF in a mouse xenograft model. These findings indicate that CA or its derivative CADPE activity as an anticancer molecules and that STAT3-dependent repression of HIF-1α and VEGF may be used to control an excess angiogenesis in various solid tumor cells.

Abbreviations: CA, caffeic acid; CADPE, 3-(3,4-Dihydroxy-phenyl)-acrylic acid 2-(3,4-dihydroxy-phenyl)-ethyl ester; CAPE, caffeic acid phenetyl ester; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(aminoethylether)-tetraacetic acid; STAT3, signal transducer and activator of transcription 3; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

Materials and methods

Materials and cells

CA was kindly provided by C.H.Kim (Sungkyunkwan University, Suwon, Korea), and was re-suspended in dimethyl sulfoxide (DMSO) at a stock concentration of 500 mg/ml and stored at −20°C. CADPE was synthesized by Imagene, Seoul, Korea, re-suspended in DMSO at a concentration of 500 mg/ml and stored −20°C. The Caki-I human renal carcinoma and COS7 monkey kidney cell lines used were cultured as previously described (1).

Western blot and fraction analysis

Western blot assays were performed as previously described (1). Cells were rinsed twice and then scraped in stop buffer [10 mM Tris, pH 7.4, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), 0.1 M NaF, 0.2 M sucrose, 100 μM sodium orthovanadate and 5 mM sodium pyrophosphate and supplemented with protease inhibitors]. After centrifuging at 400*g* for 10 min at 4°C, cells were lysed in Dignam's buffer A [10 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl and 1% NP-40, supplemented with 1 mM dithiothreitol (DTT), 100 μM sodium orthovanadate and protease inhibitors]. An aliquot of lysate was removed and mixed with 4× sodium dodecyl sulfate electrophoresis buffer and used to determine total protein in lysate. The remainder was incubated for 10 min at 4°C and then centrifuged at 13 000*g* for 2 min at 4°C to pelletize nuclei. Supernatant was collected and analyzed for cytosolic proteins, and the pellet was rinsed with buffer A and recentrifuged. The nuclear pellet obtained was then re-suspended in Dignam's buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 100 μM sodium orthovanadate and protease inhibitors), vortexed and shaken at 4°C for 15 min. After 5 min of centrifugation at 13 000*g* and 4°C, nuclear proteins in supernatants were recovered and mixed with Dignam's buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1 mM DTT, 100 μM sodium orthovanadate and protease inhibitors).

Transient transfection and luciferase assay

Luciferase assay was performed as described previously (1). Cells were co-transfected with various combinations of the following constructs: specific siRNA of STAT3 contained 24 bp duplex oligoribonucleotides with the sense strand 5′-CAGCAAAGAAUCACAUGCCACUUU-3′ (named siRNA 1) and the anti-sense strand 5′-CCUGCAAGAGUCGAAUGUUCUCUAU-3′ (named siRNA 2) of the human STAT3 gene, dominant negative mutant-STAT3YF (phenylalanine was substituted for the 705 tyrosine residue of STAT3), wild-type STAT3 (250 ng of each plasmid) in association with 250 ng of human VEGF promoter reporter plasmid m67, which was inserted with an ∼2.7 kb of the 5-flanking region of the human VEGF gene.

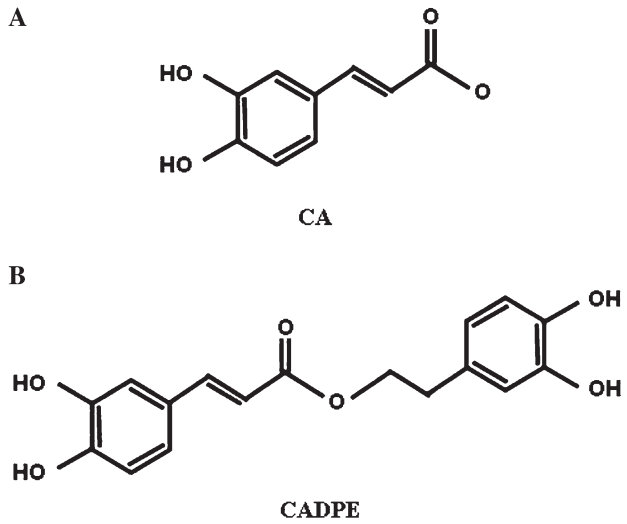

Fig. 1. Chemical structures of (**A**) CA and (**B**) CADPE.

Reverse transcriptase–polymerase chain reaction

RNA isolation and reverse transcriptase–polymerase chain reaction were performed as described previously (1). cDNA fragments were amplified by polymerase chain reaction using the following VEGF-specific primers: sense 5′-AGGAGGGCAGAATCATCACG-3′ and anti-sense 5′-CAAGGCCCACAG GGATTTTCT-3′.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitations were performed as previously described (16). Promoter-specific primers included human VEGF, 5′-AGACTCCACA GTGCATACGTG-3′ and 5′-AGTGTGTCCCTCTGACAATG-3′, which amplify 235 bp fragments flanking the STAT3-binding element and HIF-1α-binding element.

Enzyme-linked immunosorbent assay

VEGF levels in conditioned media were determined using Quantikine human VEGF Immunoassay kits (R&D Systems, Minneapolis, MN) and were normalized versus total protein content as determined using the Bradford assay.

Tube formation assay

Caki-I cells were exposed to hypoxia for 24 h with/without being pretreated with CA or CADPE for 1 h. To determine VEGF biological activities in supernatants, HUVECs were cultured for 14 h in supernatants of Caki-I cells cultured in the presence/absence of CA or CADPE under hypoxic or normoxic conditions for 24 h. Biological activities were assessed by following the formations of capillary-like networking structures produced by endothelial cells. Growth factor-depleted Matrigel was thawed at 4°C overnight. Matrigel was then distributed in 24-well plates (200 μl per well) and allowed to solidify at 37°C for at least 1 h. HUVECs were serum starved in RPMI 1640 for 1.5 h and then re-suspended in RPMI 1640. Re-suspended HUVECs were placed in 24-well plates (150 000 HUVECs per well). Additionally, all conditions were supplemented with fresh RPMI 1640 to bring the total volume in each well to 2 ml. The plates were incubated at 37°C for 14 h. Tube formation was observed, and digital pictures were captured using an Olympus digital camera (New Hyde Park, NY).

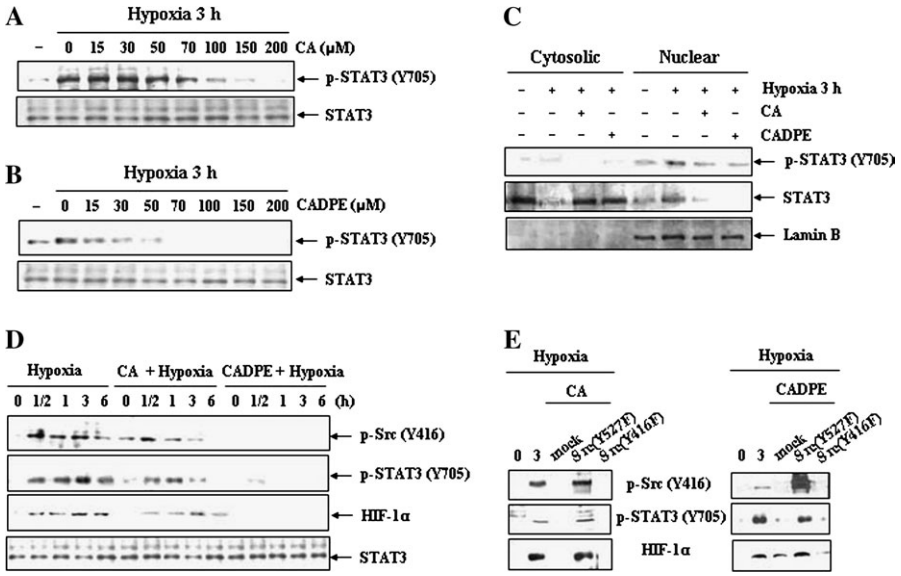

Fig. 2. Both CA and CADPE inhibited the hypoxia-induced phosphorylation of STAT3 and its translocation to the nucleus. Caki-I cells were treated with the indicated concentrations of (**A**) CA or (**B**) CADPE for 1 h before being cultured for 3 h under normoxic (20% O_2 vol/vol) or hypoxic (1% O_2 vol/vol) conditions. Phosphorylation of STAT3, STAT3 expression was analyzed by immunoblotting using mouse phospho-STAT3 (Y705) and a mouse anti-STAT3. (**C**) COS7 cells were plated onto slides at a density 1×10^6 cells per ml. Cells were then transfected with wild-type STAT3 for 24 h followed by 100 μM CA or 30 μM CADPE for 1 h and cultured for 3 h under normoxic (20% O_2 vol/vol) or hypoxic (1% O_2 vol/vol) conditions. Cells were then fractionated into cytosolic and nuclear proteins. STAT3 phosphorylation, and the expressions of STAT3 and lamin B were then analyzed by immunoblotting using mouse phospho-STAT3 (Y705), mouse anti-STAT3 and goat anti-Lamin B. (**D**) Caki-I cells were treated with 100 μM CA or 30 μM CADPE for 1 h before being cultured under normoxic (20% O_2 vol/vol) or hypoxic (1% O_2 vol/vol) conditions for the indicated times. Phosphorylations of Src and STAT3, and STAT3 expression were analyzed by immunoblotting with rabbit phospho-Src (Y416), mouse phospho-STAT3 (Y705) and mouse anti-STAT3. (**E**) Caki-I cells were plated onto slides at 1×10^6 cells per ml. The active form of Src (Y527F) or the dominant negative form of Src (Y416F) construct were transfected into cells using LipofectAMINE and cells were treated with 100 μM CA or 30 μM CADPE for 1 h. Cells were then cultured for 3 h under normoxic or hypoxic conditions. Phosphorylations of Src and STAT3, and STAT3 expression were analyzed by immunoblotting using rabbit phospho-Src (Y416), mouse phospho-STAT3 (Y705) and mouse anti-STAT3.

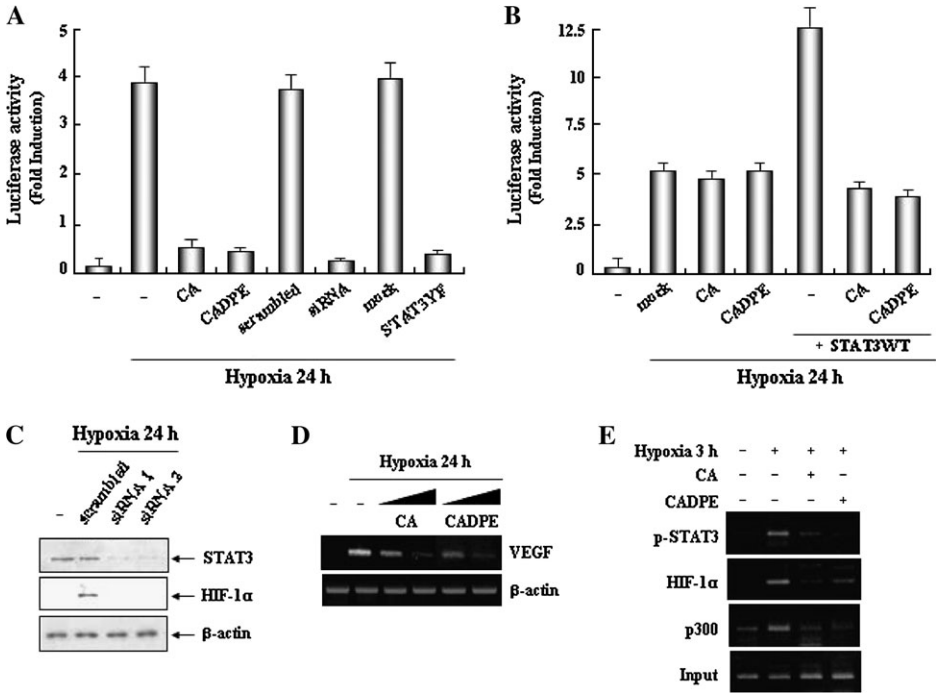

Fig. 3. CA and CADPE inhibited transcriptional activity and the recruitment of regulator to VEGF promoter in response to hypoxia. (**A**) Caki-I or (**B**) COS7 cells were seeded onto 24-well plates at a density of 4×10^4 cells per well. VEGF reporter construct was co-transfected in combination with specific siRNA of STAT3 or dominant negative STAT3 (STAT3YF) or wild-type STAT3 (STAT3WT) into cells using LipofectAMINE. Cells were then treated with 100 μM CA or 30 μM CADPE for 1 h before being cultured for 24 h under normoxic or hypoxic conditions. Cells were then lysed and luciferase assays were performed. Data shown are the means ± SDs of four independent experiments. (**C**) Caki-I cells were transfected with STAT3-specific siRNA and cultured under normoxic or hypoxic conditions for 24 h. Expressions of STAT3, HIF-1α and β-actin were analyzed by immunoblotting mouse anti-STAT3, rabbit HIF-1α and rabbit anti-β-actin antibodies. (**D**) Caki-I cells were pretreated with 50 or 100 μM CA or 15 or 30 μM CADPE for 1 h and then exposed to hypoxia for 24 h. Reverse transcriptase–polymerase chain reaction was performed using specific primers for VEGF and β-actin. (**E**) Caki-I cells were treated with 100 μM CA or 30 μM CADPE for 1 h and then cultured for 3 h under normoxic or hypoxic conditions. Cells were fixed with formaldehyde. Soluble chromatin samples were immunoprecipitated with anti-STAT3, anti-HIF-1α or anti-p300 antibodies at 4°C overnight. DNA isolated from immunoprecipitated material was then amplified by polymerase chain reaction. Promoter-specific primers included human VEGF, 5′-AGACTCCACAGTGCATACGTG-3′ and 5′-AGTGTGTCCCTCTGACAATG-3′, which amplify the 235 bp fragments flanking the STAT3-binding element, HIF-1α-binding element.

Human tumor xenografts

Male nude (BALB/cAnNCrj-nu/nu) mice were purchased from Charles River Japan (Shin-Yokohama, Japan). Animals were housed in a pathogen-free room under controlled temperature and humidity. All animal procedures were performed in accord with the procedures documented with the Seoul National University Laboratory Animal Maintenance Manual. Twenty-five, 7-week-old mice were injected with Caki-I cells (5×10^6, subcutaneous) into both flanks. Mice were then randomly assigned to one of five groups. The first group ($n = 5$; the control group) was treated with vehicle (DMSO). The second, third and fourth groups ($n = 5$) received CA (5 mg/kg, intra-peritoneal) or CADPE (5 mg/kg, intra-peritoneal) every third day for 4 weeks after tumors had reached a size of 100–150 mm^3, which occurred at ∼15 days post-Caki-I injection. Tumors were measured in two dimensions using a caliper every 2 or 3 days, and tumor volumes were calculated using $a \times b^2/2$ (where a was the width at the widest point of the tumor and b was the width perpendicular to a).

Tumor histology and CD31, p-STAT3 and HIF-1α immunostaining

On days after final CA, CADPE or vehicle injections, mice were killed and tumors were removed. Tumors were fixed in formalin and embedded in paraffin. Serial sections (6 μm thick) were cut from each paraffin block. One section was stained with hematoxylin–eosin for histologic assessment. Other sections were immunostained for p-STAT3, HIF-1α and for the endothelial cell marker CD31. Sections were deparaffinized, rehydrated through a graded alcohol series and heated in 10 mM sodium citrate (pH 6.0) for 5 min in a microwave to retrieve antigens. Non-specific sites were blocked with a solution containing 2.5% bovine serum albumin (Sigma-Aldrich, St Louis, MO) and 2% normal goat serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline (pH 7.4) for 1 h, and sections were then incubated overnight at 4°C with rabbit polyclonal anti-CD31 antibody (1:100 dilution in blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA). Negative control sections were incubated with diluent (blocking solution) in the absence of any primary antibodies. Sections were then washed and incubated with appropriate biotinylated secondary antibodies, and avidin–biotin–horseradish peroxidase complex was used to localize bound antibodies. Diaminobenzidine was used as the final chromogen. The different sections were analyzed per xenograft tumor. For histological assessment, p-STAT3-positive, HIF-1α-positive cells and CD31-positive micro vessels were identified at magnifications of ×400, ×400, and ×600, respectively, and examined using a Sony XC-77 CCD camera and a Microcomputer Imaging Device model 4 image analysis system. The expressions of HIF-1α and p-STAT3 and vessel densities were measured by counting number of immunopositive cells and vessel profiles (identified by CD31 staining) per square millimeter in each image.

Statistical analysis

All data were analyzed using Microsoft Excel 2000 software (Microsoft, Remond, WA). The Mann–Whitney *U*-test (SPSS, version 10.0; Statistical Package for the Social Sciences, Chicago, IL) was used to compare VEGF levels in culture media, numbers of p-STAT3- and HIF-1α-positive cells and vessel numbers. Tumor volumes in the untreated control and CA- or CADPE-treated groups were compared by analysis of variance followed by Duncan's multiple range test. Differences were considered statistically significant at the $P < 0.05$ level. All statistical tests were two-sided.

Results

CA and CADPE inhibited hypoxia-induced STAT3 activation and its translocation to the nucleus

Because phosphorylation of STAT3 at Tyr-705 is required for its nuclear translocation and binding to specific promoter sequences on its target genes, blocking phosphorylation at Tyr-705 provides an attractive means of inhibiting the STAT3-signaling pathway in human cancer. Thus, we investigated the activities of CA (Figure 1A) and of CADPE (Figure 1B) against STAT3 phosphorylation *in vitro* using

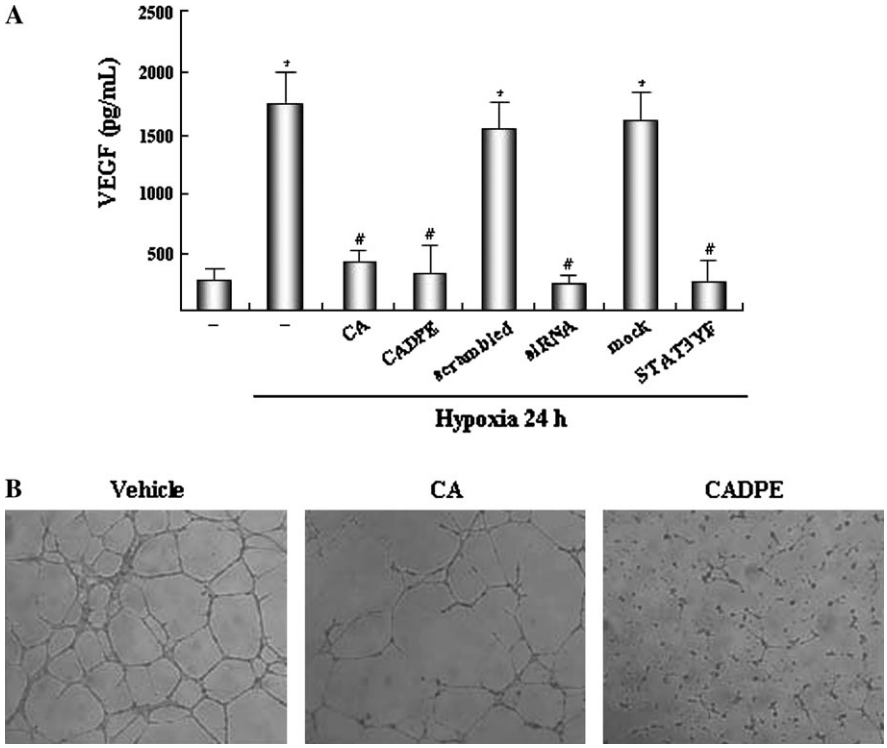

Fig. 4. CA and CADPE significantly inhibited VEGF secretion and HUVEC tube formation on Matrigel Matrix. (**A**) Caki-I cells were transfected with specific siRNAs of STAT3 or STAT3YF for 24 h or treated with 100 μM CA or 30 μM CADPE. Amounts of VEGF protein in conditioned medium from Caki-I cells that had been treated with CA or CADPE or been transfected with STAT3YF or siRNA and cultured under normoxic or hypoxic conditions for 24 h were measured using an enzyme-linked immunosorbent assay kit. VEGF concentrations were quantified in duplicate versus a series of VEGF standard samples included in the assay kit. Bars represent the means of four separate experiments and 95% confidence intervals. *Denotes significantly different relative to control supernatants from cells cultured under normoxic conditions ($P < 0.001$); # denotes significantly different relative to control supernatants from cells cultured under hypoxic conditions ($P < 0.001$). (**B**) Growth factor-depleted Matrigel was distributed in 24-well plates using precooled pipette tips and plates. HUVECs were cultured for 14 h in supernatants of Caki-I cells left unstimulated or stimulated by hypoxia with/without 100 μM CA or 30 μM CADPE for 1 h before hypoxia. Biological activities were determined by assessing the formation of capillary-like networking structures by endothelial cells after incubating plates at 37°C for 14 h. Digital pictures were captured using an Olympus digital camera (New Hyde Park, NY).

Caki-I cells. As shown in Figure 2A and B, phosphorylation of STAT3 at tyrosine 705 (Y705) increased in response to hypoxia and was decreased by CA or CADPE in a dose-dependent manner. Moreover, this inactivation of STAT3 was not caused by a decrease in its protein level. In addition, to rule out the possibility that cytotoxic effects of CA or CADPE had affected the dephosphorylation of STAT3, we used the MTT assay to evaluate cell viabilities after treating CA or CADPE at 10–300 μM (data not shown).

We next examined whether CA or CADPE reduced STAT3 translocation to the nucleus. To confirm the inhibitory effects of CA and CADPE on hypoxia-induced STAT3 translocalization, we transfected wild-type STAT3 into COS7 (STAT3-deficient cell lines) and then fractionated total cellular extracts to obtain nuclear and cytosolic proteins. Western blotting was then performed using anti-STAT3 and p-STAT3 (Y705) antibodies. As shown in Figure 2C, phosphorylated STAT3 was translocated from cytosol to the nucleus in response to hypoxia, but STAT3 remained in the cytoplasm in cells pretreated with 100 μM CA or 30 μM CADPE, despite hypoxic stimulation. In addition, we also checked lamin B expression in nuclear fractions. Taken together, our results suggest that CA or CADPE inhibit STAT3 (Y705) phosphorylation that is required for the nuclear translocation of STAT3 and also inhibit the hypoxia-induced translocation of STAT3 to the nucleus.

Disruptions of the action of STAT3 by CA or CADPE are accomplished by inhibiting Src tyrosine kinase

To identify the mechanism of STAT3 inhibition, we examined the activities of some tyrosine kinases upstream of STAT3. Src tyrosine kinase is known to activate Jak and STAT3 pathways (17), and it was found that Src tyrosine kinase-induced VEGF expression requires STAT3 in mouse 3T3 fibroblasts (8). In addition, STAT3 has been reported to regulate Src-dependent hypoxia-induced VEGF expression in pancreatic and prostate carcinomas (18). To clarify whether Src tyrosine kinase is involved in the STAT3 signaling that up-regulates HIF-1α induction under hypoxic conditions, we investigated the STAT3 signal cascade under hypoxic conditions by western blotting. Figure 2D shows that hypoxia increased the phosphorylation of Src (Y416), STAT3 (Y705) and HIF-1α induction in order. Interestingly, 100 μM CA or 30 μM CADPE significantly inhibited the Src/STAT3/HIF-1α pathway. To confirm that Src is an upstream regulator of hypoxia-induced STAT3 activation and a first target of CA or CADPE, we transfected Caki-I renal carcinoma cells with constitutive active form of Src (Y527F) or a dominant negative form of Src (Y416F) and then checked STAT3 inhibition and HIF-1α induction by CA or CADPE under hypoxic conditions. We found the recovery of STAT3 phosphorylation and HIF-1α induction in over-expressed active form of Src (Y527F) even though CA or CADPE was pretreated into cells. But, we could not detect the recovery of STAT3 phosphorylation and HIF-1α induction in over-expressed dominant negative form of Src (Y416F) (Figure 2E). These results indicate that Src tyrosine kinase is required for STAT3-mediated HIF-1α induction under hypoxic conditions, and that the disruptions of STAT3 activity by CA or CADPE are accomplished by Src tyrosine kinase inhibition.

CA or CADPE suppress VEGF transcriptional activity by inhibiting STAT3, and by inhibiting the recruitment of regulators to the VEGF promoter in response to hypoxia

In our previous study, we found that STAT3 up-regulates HIF-1-mediated VEGF expression under hypoxic conditions in renal carcinoma

cells (1). Thus, we checked whether CA or CADPE suppress VEGF expression by inhibiting STAT3. First, we performed luciferase assays in Caki-I cells using m67 (a VEGF promoter reporter plasmid), which was inserted with ~2.7 kb of the 5′ flanking region of the human VEGF gene. Hypoxia increased reporter activity (~4-fold) in Caki-I cells (Figure 3A, first two columns), but not in cells pretreated with 100 μM CA or 30 μM CADPE (Figure 3A, third and fourth columns). Next, we transfected cells with STAT3-specific siRNA and evaluated the hypoxia-induced reporter activity of VEGF promoter, which was found to be significantly reduced by the siRNA-mediated repression of STAT3 (Figure 3A, fifth and sixth columns). In addition, the reporter activity of VEGF promoter was significantly lower in cells transfected with STAT3YF (a dominant negative STAT3). To confirm that the inhibitory effects of CA or CADPE on VEGF transcriptional activity were due to STAT3 inhibition, we performed luciferase assays using COS7 cells (a STAT3-deficient cell line). As shown in Figure 3B, hypoxia induced VEGF promoter (~5-fold) reporter activity relative to normoxia (first two columns), which was attributed to HIF-1, because both HIF-1 and STAT3 have been implicated in mediating VEGF transcription (1). However, in COS7 cells, no decrease in the hypoxia-induced reporter activity of VEGF promoter was detected after treating 100 μM CA or 30 μM CADPE (Figure 3B, third and fourth columns), which contrasts with that observed in Caki-I cells (a STAT3-containing cell line). Moreover, in COS7 cells, hypoxia-induced reporter activity was further increased (~2.5-fold) by over-expressing wild-type STAT3 compared with the mock control (Figure 3B, second and fifth columns). Interestingly, 100 μM CA or 30 μM CADPE significantly suppressed the hypoxia-induced reporter activity of VEGF promoter in COS7 cells over-expressing wild-type STAT3 (Figure 3B, fifth, sixth and seventh columns), while no decreases in reporter activity by CA or CADPE were observed in the absence of STAT3. The transfection efficiency of STAT3 siRNA was checked performing western blot for STAT3 protein in Caki-I cells (Figure 3C). Interestingly, interrupting STAT3 using siRNA decreased HIF-1α protein levels during hypoxia. Reverse transcriptase–polymerase chain reaction was used to further investigate the inhibitory effect of CA or CADPE on VEGF gene expression. Caki-I cells were pretreated with/without CA or CADPE and then subjected to hypoxia. As shown in Figure 3D, CA or CADPE decreased hypoxia-induced VEGF messenger RNA expression in dose-dependent manners. Summarizing, our results suggest that the inhibitory effects of CA and CADPE on VEGF expression are due to STAT3 inhibition.

Our results show that CA or CADPE reduce the hypoxia-induced transcriptional activity of VEGF by inhibiting STAT3. Thus, to clarify whether CA or CADPE inhibit the recruitment of STAT3 on the VEGF promoter in response to hypoxia, we performed chromatin immunoprecipitation assays. As shown in Figure 3E, increased binding of STAT3 on the human VEGF promoter was observed during hypoxia. Also, bindings of HIF-1α and p300 on the human VEGF promoter increased during hypoxia. However, their bindings on the human VEGF promoters decreased in hypoxic cells pretreated with 100 μM CA or 30 μM CADPE. These results are in consistent with a previous finding (Figure 2C), namely, that CA or CADPE inhibit hypoxia-mediated STAT3 translocalization to the nucleus. Taken together, our results suggest that both CA and CADPE inhibit VEGF gene transcription by blocking STAT3 recruitment and the formation of a transcriptional unit between STAT3, HIF-1α and p300 on VEGF promoter.

CA and CADPE inhibit VEGF secretion and HUVEC tube formation on Matrigel Matrix

To examine the inhibitory effect of CA or CADPE on angiogenesis, we measured the amounts of VEGF secreted by Caki-I cells pretreated with or without CA or CADPE under hypoxic conditions. As shown in Figure 4A, VEGF secretion was significantly reduced in cells pretreated with 100 μM CA or 30 μM CADPE compared with hypoxia alone (Figure 4A, second, third and fourth columns). Also, VEGF secretion was significantly reduced by siRNA-mediated STAT3 repression (Figure 4A, fifth and sixth columns), and was significantly

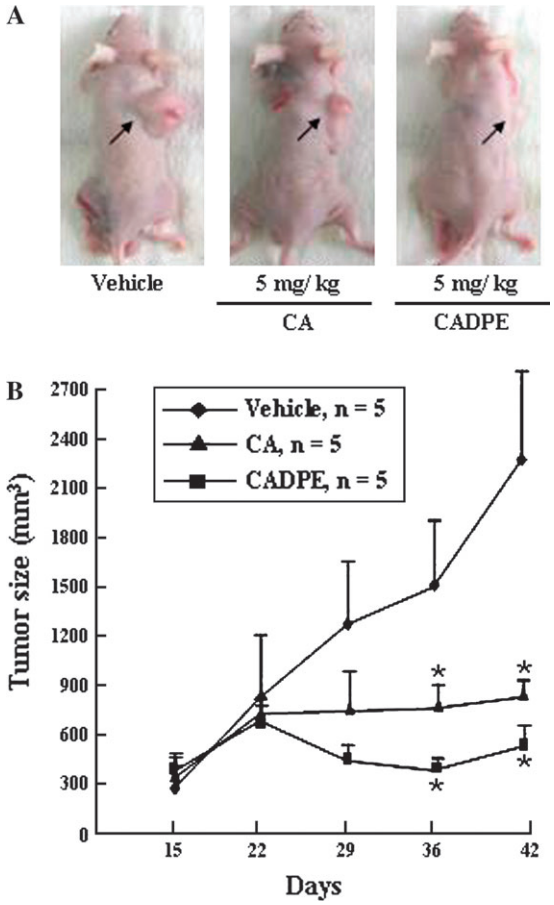

Fig. 5. The effects of CA and CADPE on xenografted human tumor growth. (**A**) Caki-I renal carcinoma cells (5×10^6) were injected subcutaneously into the flanks of male nude mice. After tumors had reached 100–150 mm^3 in size (after ~15 days), mice were treated every 3 days with intra-peritoneal injections of CA (5 mg/kg), CADPE (5 mg/kg) or vehicle (DMSO) for 4 weeks. (**B**) Differences between tumor sizes in the vehicle- and CA- or CADPE-treated groups were compared using analysis of variance. Data represent means ± SEMs; * denotes $P < 0.05$ versus the controls. Solid diamonds = vehicle, solid triangles = CA and solid squares = CADPE. Data points represent means ($n = 5$ for control; $n = 5$ for CA; $n = 5$ for CADPE).

decreased in cells transfected with STAT3YF (dominant negative of STAT3; Figure 4A, seventh and eighth columns). Next, we used a three-dimensional HUVEC tube formation assay that mimicked the angiogenic process to confirm the anti-angiogenic activities of CA or CADPE. Caki-I cells were stimulated with hypoxia for 24 h with/without 100 μM CA or 30 μM CADPE for 1 h, and then supernatants were collected. To examine the biological activities of VEGF in these supernatants, HUVECs were cultured in collected supernatants, and formations of capillary-like networking structures by endothelial cells were assessed. Tubule formation by HUVECs was found to be reduced in the presence of 100 μM CA or 30 μM CADPE (Figure 4B). These results demonstrate that both CA and CADPE inhibit hypoxia-induced VEGF secretion, and that they have anti-angiogenic activity.

The effects of CA or CADPE on the development of xenografted human tumors

Because of the observed *in vitro* effects of CA and CADPE, we investigated whether CA or CADPE inhibit angiogenesis and tumor growth by inhibiting STAT3 *in vivo*. Viable Caki-I cells (5×10^6) were injected subcutaneously into the flanks of mice, which were then

randomly assigned to one of three groups. The first group ($n = 5$) was a control group and received vehicle (DMSO) only. The second and third groups ($n = 5$) received intra-peritoneal injections of CA (5 mg/kg) or CADPE (5 mg/kg) every third day for 4 weeks, after tumors measured 100–150 mm^3 (at ~15 days). Tumors in CADPE-treated mice were visibly smaller than those in CA or vehicle-treated mice (Figure 5A). Changes in tumor size were measured and plotted as average tumor size versus time (Figure 5B). Tumor growth halted in mice treated with CA or CADPE after tumors had become established ($P < 0.05$ versus vehicle-treated group). However, body weights in the CA- or CADPE-treated groups were not reduced compared with the vehicle-treated group. These results indicate that CA or CADPE significantly inhibit tumor growth in Caki-I tumor-bearing mice.

Histopathology and immunohistochemistry of Caki-I carcinomas in nude mice

To determine whether inhibitory effects of CA or CADPE on tumor growth are associated with the suppression of angiogenesis, we examined the distribution of the endothelial marker CD31 in tumor tissues. Few or no CD31-immunopositive vessels were observed in tumor sections from CA- or CADPE-treated mice, whereas many vessels were observed in tumor sections from vehicle-treated mice (Figure 6A, top panel). As shown by our *in vitro* data, STAT3 is important in angiogenesis, and thus, we assessed the activity of STAT3 in tumor sections from vehicle- and CA- or CADPE-treated mice. Caki-I tumors from vehicle-treated mice showed many p-STAT3-immunoreactive cells (Figure 6A, middle panel), but in contrast, tumor sections from CA- or CADPE-treated mice showed few or no p-STAT3-immunoreactive cells. Interestingly, tumor sections from CA- or CADPE-treated mice showed few or no HIF-1α proteins although the tumors were hypoxic and solid (Figure 6A, bottom panel). This phenomenon is consistent with our previous report that STAT3 up-regulates HIF-1α protein stability under hypoxic conditions. We quantified numbers of p-STAT3-positive, HIF-1α-positive cells and CD31-positive vessels in tumor sections from vehicle- and CA- or CADPE-treated mice (Figure 6B). Levels of HIF-1α, p-STAT3-immunoreactive cells and of blood vessel formation were significantly lower in mice treated with CA or CADPE than in vehicle-treated mice.

To reconfirm the inhibitory effects of CA and CADPE on STAT3 in Caki-I tumors, we checked the phosphorylation of STAT3 in Caki-I tumor lysates by western blotting using anti-phospho-STAT3 (Y705) antibodies. The level of STAT3 (Y705) phosphorylation was found to be markedly lower in tumor lysates derived from CA- or CADPE-treated mice than in vehicle-treated tumors. HIF-1α expression also was lower in CA- or CADPE-treated tumors than in vehicle-treated tumors (Figure 6C). In addition, VEGF messenger RNA levels were also lower in CA- or CADPE-treated tumors than in vehicle-treated tumors (Figure 6D). Summarizing, our results suggest that STAT3 inhibition by CA or CADPE reduce HIF-1α and VEGF expression, and that this blocks tumor growth and angiogenesis in Caki-I tumors.

Discussion

This study demonstrates that CA and its derivative CADPE are potential anticancer agents that act by inhibiting STAT3 signaling. In Caki-I cells, CA and CADPE suppressed hypoxia-induced STAT3

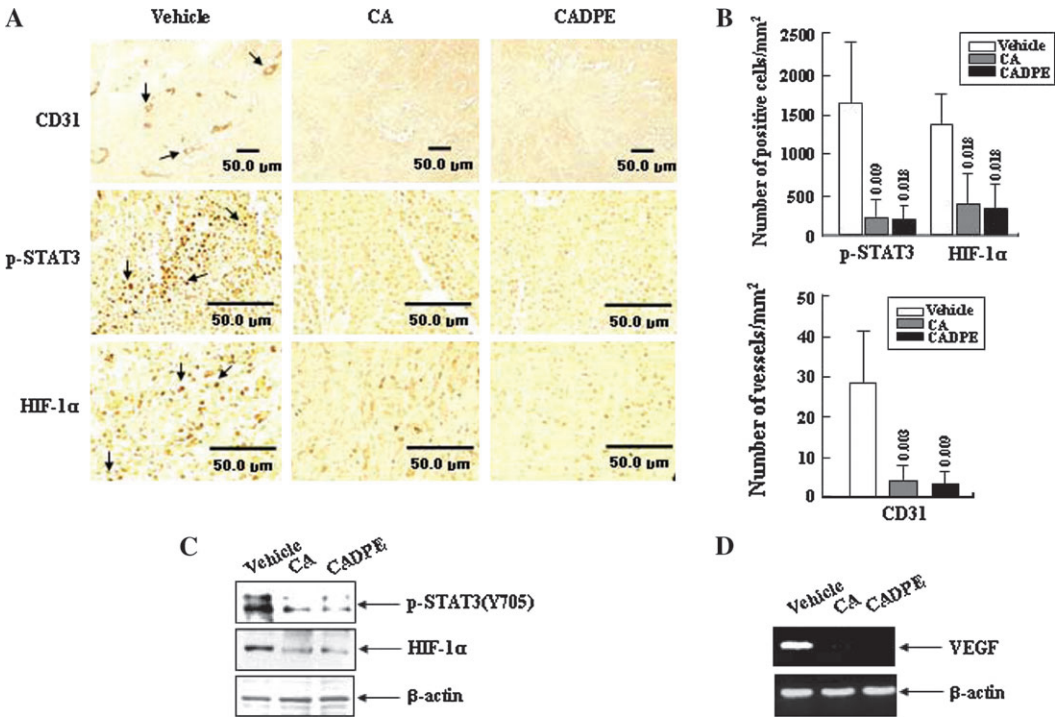

Fig. 6. Histopathology and immunohistochemistry of Caki-I tumors grown in nude mice. (**A**) Caki-I renal carcinoma cells (5×10^6) were injected subcutaneous into the flanks of male nude mice. After tumors reached 100–150 mm^3 in size (~15 days after injection), mice were administered CA (5 mg/kg, intra-peritoneal), CADPE (5 mg/kg, intra-peritoneal) or vehicle (DMSO) every 3 days for 4 weeks. Mice were then euthanized and tumors were removed, fixed in formalin and embedded in paraffin. Tumor sections were cut from paraffin blocks and processed for immunohistochemical staining with anti-CD31 antibody to detect endothelial cells, or with phospho-STAT3 antibody or rat anti-HIF-1α antibody. (**B**) Quantifications of STAT3 phosphorylation, HIF-1α expression and vascular density. Two sections per xenograft (5–10 fields per section) were subject to histological assessment (five controls, five CA and five CADPE-treated xenografts were examined). Bars represent mean and lower or upper 95% confidence intervals. Statistical significances of differences between treatment groups were compared using the Mann–Whitney *U*-test. Numbers over error bars represent *P* values of differences relative to controls. (**C**) After final treatments, mice were euthanized, tumors removed and lysates were prepared for immunoblotting. Tumor lysates from vehicle-treated mice and from CA- and CADPE-treated mice were assessed for STAT3 phosphorylation. (**D**) The messenger RNA levels of VEGF and β-actin in tumor lysates were measured by reverse transcriptase–polymerase chain reaction.

phosphorylation (at Y705), the nuclear translocation of STAT3, HIF-1α induction and finally suppressed VEGF expression. Also, in mice bearing Caki-I carcinoma, CA or CADPE retarded tumor growth and angiogenesis by inhibiting the activity of STAT3, the expression of HIF-1α and VEGF.

Although HIF-1α is an accepted target for the development of anticancer drugs, we suggest that STAT3 is superior to HIF-1α in this context of hypoxic solid tumors, because knock-down of STAT3 by specific siRNA completely suppressed the hypoxic induction of HIF-1α. Moreover, in the present study, the inhibition of STAT3 by CA or CADPE blocked HIF-1α and VEGF expression.

Because STAT3 is frequently activated in human cancer, it represents an attractive target for the development of new anticancer therapies. Recently, it was reported that targeting STAT3 blocks tumor growth induced by multiple oncogenic growth signalings (19). In terms of STAT3 signaling, the phosphorylation of STAT3 at Y705 is a decisive event. Thus, blocking STAT3 phosphorylation provides an attractive means of inhibiting STAT3 signaling in human cancer. Interestingly, we found that both CA and CADPE strongly suppress STAT3 phosphorylation at Tyr-705 and then inhibit STAT3 translocation to the nucleus. Moreover, CA or CADPE suppressed the hypoxic induction of HIF-1α through STAT3 inhibition. CA or CADPE also appeared to halt Caki-I xenografted tumor growth by blocking angiogenesis, and not via some direct cytotoxic effect. In our opinion, CA and CADPE have merits as cancer chemotherapy agents because of their low cytotoxicities. No serious toxicity was observed in any of the nude mice treated with CA or CADPE over a 6 week period, and neither CA nor CADPE affected cell viability (data not shown).

CA is a phenolic compound, and is largely found in food plants (20). CA has been extensively studied in terms of its antioxidant properties (21–24), and has also been shown to possess anti-inflammatory properties (24–26). Moreover, these antioxidant and anti-inflammatory effects extend to some caffeic acid derivatives, e.g. caffeic acid phenetyl ester (CAPE; a CA derivative originally isolated from honeybee propolis) has antioxidant properties that are similar to those of CA (27,28). In addition, CAPE is able to inhibit NF-κB activation (29) and inhibit iNOS expression (30). Moreover, recently, CA and CAPE were reported to inhibit effect angiogenesis, tumor invasion and metastasis (31,32).

Some structurally similar dihydroxy or trihydroxyphenolic acids also have antioxidant capacity (33). CA (Figure 1A) is also dihydroxyphenolic acid, and has similar properties described above. It has been reported that the number of phenolic groups in phenolic acids influences antioxidant activity (33,34). In an attempt to discover an analogue with stronger antioxidant properties than CA, we synthesized CADPE (Figure 1B) which is composed of two phenolic rings linked by an esteric bond. This work resulted in CADPE (Figure 2), which was found to have a greater STAT3 inhibiting effect than CA (CADPE inhibited STAT3 with an IC_{50} of 15–30 μM, whereas CA had an IC_{50} of 70–100 μM) (data not shown). Western blot analysis, VEGF reporter assay, HUVEC tube formation assay, enzyme-linked immunosorbent assay (VEGF secretion), Chromatin immunoprecipitation assay using 100 μM CA or 30 μM CADPE also showed that CADPE has a greater STAT3 inhibiting effect than CA *in vitro*. These findings suggest that CADPE has a greater inhibitory effect on STAT3-mediated VEGF expression than CA. In addition, our *in vivo* studies showed that CADPE (5 mg/kg) has a greater growth inhibitory effect on xenografted human tumors than CA (5 mg/kg). It has been reported that CAPE, a naturally occurring CA derivative, has a greater anticancer effect than CA at low concentration (31). In the present study, we did not compare the efficacies of CADPE and CAPE on STAT3 inhibition or VEGF expression. However, both agents have similar effects on tumor growth and are more effective than CA at low concentrations. CA is considered to be a safe material because it is one of the most frequently encountered phenolic acids in the normal diet (11). However, in the present study, neither CADPE nor CA showed any cytotoxicity in normal cells. In this respect, the anti-STAT3 activities of CA and of its derivative CADPE may be worth for further investigation with respect to their potential clinical applications in cancer therapy.

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