Carnosic acid induces apoptosis through inactivation of Src/STAT3 signaling pathway in human renal carcinoma Caki cells

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Abstract. Carnosic acid (CA), the major bioactive compound of Rosmarinus officinalis L., has been reported to possess anti-inflammatory and anticancer activities. However, the molecular mechanisms underlying the anticancer effects of CA remain poorly understood. In the present study, we investigated that CA significantly reduced the viability of human renal carcinoma Caki cells. CA-induced apoptosis was connected with the cleavage of caspase-9, -7 and -3, and that of PARP. Moreover, CA increased the expression of pro-apoptotic protein Bax and diminished the expression of anti-apoptotic protein Bcl-2 and Bcl-xL, thereby releasing cytochrome c into the cytosol. Treatment with CA in Caki cells also induced the expression of p53 and its target gene product, p27, through down-regulation of Murine double minute-2 (Mdm2). Furthermore, CA generated reactive oxygen species (ROS), and pretreatment with ROS scavenger N-acetyl cysteine (NAC) abrogated CA-induced cleavage of PARP and expression of p53. One of the key oncogenic signals is mediated through signal transducer and activator of transcription-3 (STAT3), which promotes abnormal cell proliferation. Incubation of cells with CA markedly diminished the phosphorylation of STAT3 and its upstream, Src, and reduced the expression of STAT3 responsive gene products, such as D-series of cyclins and survivin. Taken together, the present study revealed that CA induced apoptosis in Caki cells by induction of p53 and suppression of STAT3 signaling.

Introduction

Renal cell carcinoma (RCC) represents the most common malignant tumor of the kidney. The overall incidence of RCC increases significantly from the age of 50 to 70 years (1,2). With high degree of heterogeneity, RCC exhibits poor prognosis because of increased rate of metastasis and resistance to conventional chemotherapy and radiotherapy (3). Therefore, alternative novel therapeutic agents are needed for effective treatment of RCC. Since the evasion from apoptosis is one of the hallmarks of cancer, it has long been attempted to develop anticancer drugs that can selectively induce apoptosis in cancer cells (4). A wide variety of natural products, especially phytochemicals, have been shown to suppress cell growth, modulate cell differentiation and induce cancer cell death (5).

Apoptosis, a physiological process of forcing unwanted cells to commit suicide, is characterized by cell shrinkage, plasma membrane blebbing, DNA fragmentation and the formation of apoptotic bodies (6). Biochemical mechanisms of apoptosis involve two different pathways, one is the intrinsic or the mitochondria-mediated pathway, and the other is the extrinsic or death receptor (DR)-mediated pathway (7). The intrinsic pathway involves disruption of mitochondrial membrane potential and the release of cytochrome c from the mitochondrial inter-membrane space into the cytosol, resulting in the activation of caspase-9, -7 and -3 through the proteolytic cleavage of respective pro-caspases (8). The mitochondrial membrane potential is regulated by proteins of the B cell lymphoma-2 (Bcl-2) family (9). These include anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL), pro-apoptotic multidomain protein (Bak and Bax), and BH-3-only pro-apoptotic proteins (Bad, Bid and Bim) (10). Alteration in cellular redox status shifts the balance between the expression of anti-apoptotic and pro-apoptotic proteins, thereby leading to cell fate decision (11). A critical determinant of cell fate decision is the intracellular accumulation of reactive oxygen species (ROS), which are generated as by-products of cellular metabolism. High levels of ROS may lead to cell death through mitochondrial collapse (12).

The extrinsic pathway, on the contrary, is activated by death-inducing signal molecules of tumor necrosis factor...
(TNF) family, such as FasL, TNF and TNF-related apoptosis inducing ligand (TRAIL) (13), which bind to various plasma membrane-bound DRs (14). Upon stimulation of an appropriate apoptotic signal, the activation of DRs leads to the formation of the death-inducing signaling complex (DISC) and the activation of caspase-8 and -10, which in turn activates the rest of downstream caspases, such as caspase-3, thereby inducing apoptosis (15). A number of plant polyphenols have been reported to induce apoptosis via the intrinsic or extrinsic pathway in various cancer cells (16).

Signal transducer and activator of transcription-3 (STAT3) plays a decisive role in regulating cell growth, survival, angiogenesis and immune escape of tumor cells (17). The blockade of STAT3 signaling caused induction of apoptosis, inhibition of cell proliferation, suppression of angiogenesis and stimulation of immune responses (18-21). In cancer cells, STAT3 becomes constitutively active through the phosphorylation by upstream Src family kinases or Janus-activated kinases (Jaks) (19). Since the STAT3-regulated gene products, such as Bcl-xL, Bcl-2, survivin, c-Myc and D-series of cyclins are involved in enhanced cell proliferation, selective inhibition of STAT3 signaling can suppress proliferation and induce apoptosis in cancer cells (19). Previous studies have shown that suppression of STAT3 signaling inhibits the growth of various cancer cells including those of the stomach, liver, head and neck, skin, and lung (22). STAT3, a signal mediator of various pro-inflammatory cytokines and growth factors acting constitutively in an inflammatory tumor microenvironment, is an important molecular target of various anticancer drugs (23).

Rosmarinus officinalis L., generally known as rosemary, has a long-standing reputation for improving memory and has been used as a symbol of reminiscence in Europe (24). In addition, leaves of the plants have been used as a means to weight loss due to its ability to inhibit lipid absorption activities in the digestive system (25). Major bioactive components of the plant include polyphenolics, such as carnosic acid (CA), carnosol, rosmarinic acid and ursolic acid (26-29). CA (Fig. 1A) has a wide range of biological activities, including antioxidant, anticancer, anti-inflammatory, anti-adipogenic and neuroprotective effects (30-34). The present study investigated the underlying molecular mechanisms of anticancer effects of CA in human renal carcinoma (Caki) cells. We found that CA induced anti-proliferative and apoptotic effects in Caki cells through mitochondria-dependent caspase activation and the interference with STAT3 signaling pathway via the generation of ROS.

Materials and methods

Materials. CA (purity 99%) and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against cleaved caspase-9, -7, -3, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bcl-xL, Bax, cytochrome c, STAT3, p-STAT3 (Y705), p-STAT3 (S727), Src, p-Src, cyclin D1, D2, and D3 and survivin were from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibody against each of p53, murine double minute-2 (Mdm2), p27, and horse-raddish peroxidase-conjugated secondary antibodies were from Sigma Chemical Co. (St. Louis, MO, USA). The 2,7′ dichlorofluorescin diacetate (DCF-DA) was from Invitrogen (Carlsbad, CA, USA). Hank's balanced salt solution (HBSS) was from the Meditech (Herndon, VA, USA).

Cell culture and treatment. Caki cells were obtained from Dr T.K. Kwon (Keimyung University, Korea) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin) at 37°C in a humidified incubator containing 5% CO₂ and 95% air. In all the experiments, cells were seeded at 1x10⁵ cells/well and incubated with CA at 50-60% confluence. All chemicals were dissolved in ethanol and the final ethanol concentration was <0.1%.

Cell viability assay. The cell growth effect was measured by the MTT assay. Cells (2x10⁵) were incubated in triplicate in a 96-well plate in presence or absence of CA in a final volume of 100 µl for different time intervals at 37°C. Thereafter, 10 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h. Medium was removed, formazan was dissolved in DMSO and absorbance at 550 nm was measured by using microplate reader (Tecan Trading AG, Männedorf, Switzerland). Cell viability was described as the relative percentage of control.

Annexin V staining. Annexin V staining was performed using fluorescein isothiocyanate (FITC)-Annexin V staining kit (BD Biosciences, San Jose, CA, USA) following the manufacturer’s instructions. Briefly, CA-treated cells were washed with PBS and resuspended in binding buffer containing Annexin V and propidium iodide (PI). Fluorescence intensity was measured using flow cytometry (BD Biosciences).

Western blot analysis. Cells were harvested and lysed with RIPA buffer, and the resulting protein samples were quantified by using bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amount of protein extracts were denatured by boiling at 100°C for 5 min in sample buffer. The proteins were separated on 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 buffer (TBS-T) (10 mM Tris, 150 mM NaCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature. The membranes were washed 3 times for 10 min each with TBS-T buffer and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. The membranes were washed 3 times for 10 min each with TBS-T buffer. Immunoblot membranes were incubated with Super-signal pico-chemiluminescent substrate or dura-luminol substrate (Thermo Scientific, Waltham, MA, USA) according to manufacturer’s instruction and visualized with imagequant™ LAS 4000 (Fujifilm Life Science, Tokyo, Japan).

Measurement of reactive oxygen species (ROS) accumulation. Cells were treated with CA in the presence or absence of NAC for 24 h and then loaded with 25 µM of 2′7′-dichlorofluorescin diacetate (DCF-DA). After incubation for 30 min at 37°C in a 5% CO₂ incubator, cells were washed twice with HBSS.
solution, suspended in the complete media and were examined under a fluorescence microscope to detect the intracellular ROS. Fluorescence of oxidized DCF was also measured at an excitation wave length of 480 nm and emission wavelength of 525 nm using flow cytometry.

Electrophoretic mobility gel shift assay (EMSA). The nuclear extract was prepared from cells incubated with or without CA. The STAT3 oligonucleotide probe 5'-AGC TTC ATT TCC CGT AAA TCC CTA-3' (Biomedic, Korea) was labeled with [γ-32P]-ATP using T4 polynucleotide kinase. The EMSA was performed according to the protocol described earlier (35).

Statistical analysis. When necessary, data were expressed as mean ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student’s t-test and p-value <0.05 was considered to indicate a statistically significant difference.

Results

CA induces apoptosis in Caki cells. In the present study, the effect of CA on the viability of Caki cells was first examined by the MTT assay. As shown in Fig. 1B, treatment of Caki cells with CA significantly reduced the cell viability in a

Figure 1. Cytotoxic effect of carnosic acid (CA) in Caki cells. (A) Chemical structure of CA. (B) Caki cells were treated with indicated concentrations of CA for 24, 48 or 72 h. Cell viability was determined by the MTT assay. Values are expressed as means ± SD. *p<0.001, compared to control. (C) Cells were treated with indicated concentrations of CA for 24 h and cell morphology was analyzed by microscopy (x200). (D) The apoptotic index (%) was determined by flow cytometry upon treatment of cells with CA (20, 50 or 100 µM) for 24 h and staining with Annexin V and PI. (E) This graph shows statistical analysis of apoptosis. Data are representative of three independent experiments. *p<0.05, **p<0.001, compared to control.
CA induces apoptosis through the mitochondrial pathway in Caki cells. Caspases are crucial components of the apoptosis pathway. The mitochondrial and caspase pathways are interconnected. Release of cytochrome c into the cytosol activates the caspase adaptor Apaf-1 and procaspase-9, which activates executioner caspases (caspase-3, -6 and -7) (36). In the present study, incubation of Caki cells with CA (20, 50 or 100 µM) induced activation of caspase-9, -7 and -3 and the cleavage of PARP (Fig. 2A), indicating the involvement of mitochondria in CA-induced apoptosis. Since Bcl-2 family proteins regulate the mitochondrial membrane integrity, the effect of CA on the expression of Bcl-2 family proteins was then examined. The present study revealed that CA not only downregulated the expression of anti-apoptotic protein Bcl-2 and Bcl-xL, but also increased the expression of apoptosis inducing protein Bax, thereby releasing cytochrome c into the cytosol (Fig. 2B). As shown in Fig. 2C, incubation of cells with CA increased expression of p53 and diminished protein level of its cytosolic repressor protein Mdm2 in a concentration-dependent manner. p53 induces the cell cycle arrest through transcriptional activation of its cell cycle regulatory gene product, p27. We examined whether CA treatment has an effect on p27 expression in Caki cells. As a result, expression of p27 is increased by CA treatment in according to p53 protein levels.

Involvement of ROS in CA-induced apoptosis in Caki cells. Since the accumulation of intracellular ROS can induce cell death, the effect of CA on ROS generation was examined. Treatment of cells with CA (20, 50, 100 µM) for 24 h generated ROS as revealed by the immunofluorescence analysis after DCF-DA staining (Fig. 3A) as well as by the FACS analysis (Fig. 3B). Pretreatment of cells with NAC abolished CA-induced ROS accumulation (Fig. 3C and D). Quantification and statistical analysis of CA-induced ROS generation are presented in Fig. 3E.
CA attenuates the activation of STAT3 signaling pathway. Since STAT3 plays a key role in cell proliferation through transcriptional activation of pro-survival genes, we examined the effect of CA on the expression of cell proliferation markers, which are transcriptionally regulated by STAT3. The present study showed that CA diminished the constitutive phosphorylation at both Y705 and S727 residues of STAT3 (Fig. 4A). CA also reduced the constitutive STAT3 DNA-binding activity (Fig. 4B). In addition, CA treatment suppressed the expression of STAT3-regulated cell proliferative gene products, such as c-Myc, survivin and D-series of cyclins (Fig. 4C). STAT3 is known to be phosphorylated by upstream kinase, such as Src. Since CA was found to suppress STAT3 activation, the effect of CA on the activation of upstream signaling proteins of STAT3 were checked. As shown in Fig. 4D, CA treatment resulted in the suppression of Src phosphorylation in concentration-dependent manner in Caki cells.

Role of ROS in CA-induced inhibition of STAT3 signaling and induction of apoptosis in Caki cells. We next examined the role of CA-induced ROS generation in blocking STAT3 signaling and induction of apoptosis in Caki cells. Treatment with CA in the absence or presence of NAC revealed that inhibition of ROS generation abrogated the inhibitory effect
of CA on the phosphorylation of STAT3 and Src (Fig. 5A). To investigate the possible mechanism underlying the induction of apoptosis by CA via ROS generation, we also examined the cleavage of caspase-3 and PARP and expression of p53. As shown Fig. 5B and 5C, activation of p53, the cleavage of PARP and apoptosis induction through ROS generation was abolished by pretreatment of NAC in CA-treated Caki cells. These findings suggest that ROS play critical roles in CA-induced apoptosis in Caki cells.

Discussion

Cancer is a leading cause of death worldwide (37). Benign tumor is primarily removed by surgery and the residual cancer cells are eradicated by chemotherapy and/or radiotherapy. However, the chemotherapy regimens frequently cause various side effects, such as considerable death of healthy cells and drug tolerance. Thus, agents that specifically target tumor cells are ideal for cancer therapies. Based on this requirement, natural therapies, which take advantage of the abundant and vast resources of natural compounds derived from medicinal plants, are being developed to overcome the side effects of chemotherapeutic agents. The present study investigated the effect of CA, a major ingredient of medicinal herb *Rosmarinus officinalis* L., against human renal cancer cells and elucidated its mechanism of action.

Apoptosis is typically composed of two main pathways: the extrinsic pathway that mediates signals via death receptors, and the intrinsic pathway that involves mitochondrial dysfunction (38). The extrinsic pathway is provoked through the binding of cytokine ligands, such as FasL, TNF and TRAIL to the plasma membrane receptors (14). The interaction between ligands and receptors result in the formation of DISCs, which activate caspase-8 and release the DISC into the cytoplasm (39). Caspase-8 can activate caspase-3 directly or via release of cytochrome c mediated by mitochondria. The second pathway is the intrinsic pathway, which was triggered with a variety of stress stimuli, such as DNA damage and the actions of some oncoproteins (40). Signaling of this pathway is accompanied by the alteration in the expression of Bcl-2 family proteins, decreased mitochondrial membrane potentials, and the release of cytochrome c from the mitochondria to cytoplasm (41). The release of cytochrome c and regulation of Bcl-2 family proteins are critical processes in the mitochondria-mediated apoptosis (41,42). In addition, the Bcl-2 family proteins play a central function in inducing apoptosis and involve members with either pro- or anti-apoptotic activity (43).
Our study demonstrated that cleaved PARP and procaspase-9, -7 and -3, accompanied by decrease in the expression of anti-apoptotic proteins, such as Bcl-xL and Bcl-2, increase the expression of pro-apoptotic protein Bax in CA-treated Caki cells. These findings indicated that CA-induced apoptosis could partly be mediated through mitochondria-mediated apoptotic pathway. Cross-talk between the death-receptor and mitochondria-mediated pathway is achieved by Bid protein, which is altered by caspase-8. The death signal induced in the extrinsic pathway, for example, via DR4 and DR5, can lead to the disruption of mitochondrial membrane integrity (8,44). The signal cascade is associated with the ability of caspase-8 to proteolytically cleave Bid into truncated Bid (tBid). The pro-apoptotic protein tBid, a member of the Bcl-2 family, transfers to the mitochondria where it allows other proteins of the same family to combine with the outer mitochondrial membrane to release cytochrome c. We found that incubation of cells with CA upregulated the expression of DR4 and DR5 and increased the proteolytic cleavage of caspase-8 in Caki cells (data not shown). Taken together, the induction of apoptosis by CA involves both the extrinsic and the intrinsic pathways.

Tumor suppressor p53 is another important factor that affects the cellular response to drugs, while exert effects on growth inhibition and apoptosis induction (45). The p53 is sequestered in cytoplasm by binding with its inhibitory protein Mdm2, which possesses ubiquitin ligase property and plays an important role in p53 turnover. Thus, a decrease in Mdm2 level leads to stabilization of p53. In response to DNA damage, p53 is phosphorylated, dissociated from Mdm2 and translocated to the nucleus. Within the nucleus, phosphorylated p53 functions as a transcription factor that regulates transcrip-
tional activation of genes involved in cell cycle regulation and apoptosis (46,47). The p53-dependent apoptosis is associated with the caspase cleavage and the expression of pro-apoptotic proteins, such as Bax (48). Moreover, p53 induces the cell cycle arrest through transcriptional activation of its several cell cycle regulatory gene products, such as p21 and p27. Thus, inhibiting Mdm2 expression and concomitantly elevating expression of p53. Taken together, these findings suggest that CA may cause apoptosis in a p53-dependent manner.

Since the activation of p53 occurs in response to oxidative stress (49), the effect of CA on ROS generation in Caki cells was examined. ROS, including free radicals such as superoxide, hydroxyl radicals (OH), and the non-radical H₂O₂ are generated through multiple sources in the cells, such as the electron transport chain in mitochondria, ionizing radiations and enzymes (e.g., phagocytic and non-phagocytic NADPH oxidases, lipoxygenases and cyclooxygenases) producing superoxide anions. Moreover, ROS is known to instigate the apoptotic cascade by causing damage to many cellular components including proteins, lipids, nucleic acid, and the mitochondria itself, which in turn facilitates the release of apoptogenic factors (50,51). The present study showed that incubation with CA significantly increased the ROS level, which was scavenged by pretreatment of cells with NAC. Furthermore, NAC treatment rescued cells from CA-induced apoptosis by blocking the cleavage of PARP and the expression of p53. Thus, CA-induced ROS generation may activate p53, resulting in the downregulation of Bcl-2 and induction of Bax expression, thereby leading to the activation of caspases and induction of apoptosis.

Constitutive STAT3 activation has been related to a variety of tumors including breast, prostate and colon cancer (52). Recent studies have indicated that the STAT3 signaling pathway is closely associated with cell proliferation, differentiation and evasion of apoptosis (53,54). The activation of STAT3 signaling involves phosphorylation of Y705 and S727 residues followed by dimerization and nuclear translocation, and subsequently DNA binding for the transcriptional activation of its target genes (55). Since CA abolished the expression of Bcl-2 and Bcl-xL, which are regulated by STAT3, the effect of CA on the activation of STAT3 signaling in Caki cells was examined. The present study revealed that CA blunted the constitutive phosphorylation of STAT3 and reduced the expression of its target genes D-series of cyclins, survivin and c-Myc. Intracellular kinases, such as Src, have been shown to phosphorylate STAT3. Thus, the inhibitory effect of CA on the
phosphorylation of Src suggests that the compound interferes with STAT3 signaling pathway.

In conclusion, the present study demonstrates for the first time that CA induces both the extrinsic and intrinsic pathways of apoptosis in Caki cells through the generation of ROS and inactivation of STAT3 signaling (Fig. 6).

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