Withaferin A induces apoptosis through the generation of thiol oxidation in human head and neck cancer cells

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Abstract. Withaferin A is a steroidal lactone purified from the Indian medicinal plant, Withania somnifera. Withaferin A has been shown to inhibit the proliferation, metastasis, invasion and angiogenesis of cancer cells. In the present study, we investigated whether withaferin A induces apoptosis in the human head and neck cancer cells, AMC-HN4. Withaferin A markedly increased the sub-G1 cell population and the cleavage of poly(ADP-ribose) polymerase (PARP), which are markers of apoptosis. Pan-caspase inhibitor, z-VAD-fmk (z-VAD), markedly inhibited the withaferin A-induced apoptosis. However, the withaferin A-induced increase in the expression of COX-2 was not affected by treatment with z-VAD. Furthermore, withaferin A upregulated cyclooxygenase-2 (COX-2) expression. The COX-2 inhibitor, NS-398, reduced the withaferin A-induced production of prostaglandin E₂. However, treatment with NS-398 did not affect the sub-G1 population and the cleavage of PARP. In addition, the withaferin A-induced apoptosis was independent of reactive oxygen species production. Thiol donors [N-acetylcysteine (NAC) and dithiothreitol (DTT)] reversed withaferin A-induced apoptosis. Therefore, our data suggest that withaferin A induces apoptosis through the mechanism of thiol oxidation in head and neck carcinoma cells.

Introduction

Withaferin A is a steroidal lactone purified from the Indian medicinal plant, Withania somnifera and has been shown to exert anticancer effects in different types of cancer cells, such as prostate cancer (1), myeloid leukemia (2), breast cancer (3) and renal cancer (4) cells. The mechanisms responsible for the withaferin A-mediated anticancer effects are multiple. For example, withaferin A has been shown to induce apoptosis through the upregulation of intracellular reactive oxygen species (ROS) generation (5,6), prostate apoptosis response-4 (Par-4) induction (1), p38 MAP kinase activation (7), Akt inactivation (8) and the upregulation of endoplasmic reticulum (ER) stress (4). It has been reported that withaferin A induces apoptosis in head and neck carcinoma cells and inhibits Akt activation (9). However, the cellular and molecular mechanisms underlying withaferin A-induced apoptosis in head and neck carcinoma cells are not yet fully understood.

Cyclooxygenase (COX) converts arachidonic acid into prostaglandin (PG)G₂, which is then reduced to PGH₂, a is precursor of other prostanoids. COX is divided into 2 isoforms. COX-1 is constitutively expressed in the majority of tissues and has physiological functions, whereas COX-2 is induced by inflammatory cytokines (10), the mutation of oncogenes (11) and tumor promoters (12). COX-2 is overexpressed in multiple types of cancer, such as pancreatic (13), colon (14), cervical (15), renal (16) and head and neck (17) cancer. The overexpression of COX-2 has been shown to be associated with the promotion of angiogenesis, invasion and proliferation, and the inhibition of apoptosis. Therefore, the downregulation of COX-2 expression and activity enhances apoptosis (18,19). Furthermore, Limami et al (20) reported that the attenuation of COX-2 expression and COX-2 downregulation by siRNA enhanced apoptosis in ursolic acid-treated colorectal cancer cells. Therefore, the upregulation of COX-2 expression by anticancer drugs may promote resistance to apoptosis; thus, the downregulation of COX-2 expression and activity may enhance susceptibility to apoptosis.

In the present study, we investigated whether the withaferin A-induced COX-2 upregulation is involved in resistance to apoptosis in the human head and neck carcinoma cells, AMC-HN4.

Materials and methods

Cells and materials. The human head and neck cancer cells, AMC-HN4, were obtained from the Asan Medical Center (Seoul, Korea). The cells were cultured in Dulbecco’s modified Eagle’s medium that contained 10% fetal bovine serum, 20 mM HEPES buffer and 100 mg/ml gentamicin. Withaferin A was purchased from Biomol Research Laboratories, Inc. (Plymouth, PA).

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Meeting, PA, USA). z-VAD-fmk (a pan-caspase inhibitor) and NS-398 (a COX-2 specific inhibitor) were purchased from Calbiochem (San Diego, CA, USA). Anti-poly(ADP-ribose) polymerase (PARP) antibodies (sc-25780) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-actin antibody (A5441) was obtained from Sigma (St. Louis, MO, USA). Anti-COX-2 (Cat#160106) antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). N-acetylcyesteine (NAC), dithiothreitol (DTT) and all the other chemicals were obtained from Sigma.

Flow cytometric analysis and cell morphology. Approximately 1x10^6 cells were suspended in 100 µl PBS, and 200 µl 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS and resuspended in 250 µl 1,12% sodium citrate buffer (pH 8.4) together with 12.5 µg RNase. Incubation was carried out at 37°C for 30 min. Cellular DNA was then stained by applying 250 µl propidium iodide (50 µg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) on a FACScan flow cytometer (E5464; Becton-Dickinson, Franklin Lakes, NJ, USA) for relative DNA content based on red fluorescence. Cell morphology was analyzed using a light microscope (Zeiss Axiovert 200M; Carl Zeiss, Göttingen, Germany).

Western blot analysis. The cells were collected, washed with cold PBS and lysed on ice in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na,VO_4, and 1 mM NaF) containing protease inhibitors (100 µM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 2 mM EDTA). The lysates were centrifuged at 13,000 x g for 15 min at 4°C and the supernatant fractions were collected. Proteins were separated by SDS-PAGE and transferred onto an immobilon-P membrane (Amersham, Uppsala, Sweden). Specific proteins were detected using enhanced chemiluminescence.

Prostaglandin E_2 (PGE_2) assay. PGE_2 levels in the culture medium were assayed using an enzymemunoassay kit following the manufacturer's instructions (Cayman Chemical Co.). The assay is based on the competition between peroxidase (or alkaline phosphate)-conjugated tracer PGE_2 and PGE_2 in the medium for a limited number of PGE_2-specific Abs. The amount of remaining tracer PGE_2 was determined by the addition of substrates for peroxidase (or alkaline phosphatase). OD values were determined at 405 (or 450) nm, as previously described (21).

Measurement of ROS production. The intracellular accumulation of ROS was determined using the fluorescent probe, 2,7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). H_2DCFDA is commonly used to measure ROS generation (22). The AMC-HN4 cells were pre-treated with 200 µM trolox and 500 µM MnTBAP for 30 min, and the cells were then incubated with 3 µM withaferin A for 30 min. The cells were stained with the fluorescent dye, H_2DCFDA, for an additional 10 min. Subsequently, the cells were trypsinized and resuspended in PBS, and fluorescence was measured at specific time intervals with a flow cytometer (Becton-Dickinson) or was detected using a fluorescence microscope (Zeiss, Goettingen, Germany).

Statistical analysis. The data were analyzed using one-way ANOVA and post-hoc comparisons (Student-Newman-Keuls test) using the Statistical Package for Social Sciences 22.0 software (SPSS Inc., Chicago, IL, USA).

Results

Withaferin A induces apoptosis and COX-2 expression in the human head and neck carcinoma cells, AMC-HN4. We have previously reported that withaferin A induces apoptosis in human renal carcinoma Caki cells (23) and human leukemia U937 cells (8). In this study, to determine whether withaferin A induces apoptosis, AMC-HN4 cells were treated with the indicated concentrations of withaferin A for 24 h. FACS analysis for the measurement of DNA content and western blot analysis for the detection of the cleavage of PARP, a substrate of caspase-3, were performed. Withaferin A markedly increased the sub-G1 population and the cleavage of PARP in a dose- and time-dependent manner (Fig. 1). COX-2-overexpressing cancer cells are resistant to anticancer drug-mediated apoptosis (24). Therefore, we examined whether withaferin A increases COX-2 expression in the AMC-HN4 cells. As shown in Fig. 1, withaferin A increased COX-2 expression in a dose- and time-dependent manner.

Subsequently, we investigated whether the withaferin A-induced expression of COX-2 is a consequence of apoptosis. Treatment with the pan-caspase inhibitor, z-VAD-fmk (z-VAD), markedly inhibited the withaferin A-induced increase in the sub-G1 population and the cleavage of PARP (Fig. 2). However, the withaferin A-induced increase in the expression COX-2 was not affected by treatment with z-VAD. Therefore, these data indicate that the withaferin A-mediated COX-2 expression is not a consequence of apoptosis.

Withaferin A-induced COX-2 expression has no effect on apoptosis. Previous studies have reported that the downregulation of COX-2 expression and the inhibition of PGE_2 production enhances anticancer drug-mediated apoptosis (19,20,22,23,25). Therefore, we examined whether the inhibition of PGE_2 production increases withaferin A-mediated apoptosis. NS-398, a COX-2 inhibitor, markedly blocked the withaferin A-mediated production of PGE_2 (Fig. 3A). However, the withaferin A-induced increase in the sub-G1 population and the cleavage of PARP were not affected by NS-398 treatment (Fig. 3B). These data indicate that the withaferin A-induced expression of COX-2 is not associated with apoptosis in the head and neck carcinoma cells, AMC-HN4.

Withaferin A-mediated apoptosis is independent of ROS signaling. Withaferin A has been shown to increase ROS production, and ROS is involved in apoptosis (5). Therefore, we investigated whether withaferin A increases intracellular ROS levels in AMC-HN4 cells. Withaferin A markedly increased intracellular ROS production (Fig. 4A). Subsequently, we wished to determine whether ROS are involved in withaferin A-induced apoptosis. The ROS scavengers, trolox and MnTBAP, inhibited withaferin A-mediated ROS production (Fig. 4A); however, the sub-G1 population and the cleavage of PARP were not affected (Fig. 4B). Furthermore, the induction of COX-2 expression by withaferin A was independent of...
ROS production (Fig. 4B). These data indicate that ROS is not associated with withaferin A-mediated apoptosis.

**Withaferin A-mediated apoptosis is reversed by thiol donors.** Previous studies have reported that excess amounts of thiol donors block the effects of withaferin A (26,27). To determine whether thiol donors inhibit withaferin A-induced apoptosis, AMC-HN4 cells were treated with NAC and DTT. Both thiol donors markedly inhibited morphological changes in the withaferin A-treated cells (Fig. 5A). Furthermore, NAC and DTT blocked the increase in the sub-G1 population and the cleavage of PARP (Fig. 5B). These data suggest that the mechanism of thiol oxidation is important for withaferin A-mediated apoptosis.

**Discussion**

In the present study, we demonstrated that withaferin A induced apoptosis in the human head and neck carcinoma cells, AMC-HN4. Withaferin A increased COX-2 expres-
Figure 3. Upregulation of cyclooxygenase-2 (COX-2) expression is not associated with withaferin A-induced apoptosis in AMC-HN4 cells. (A and B) AMC-HN4 cells were treated with the indicated concentrations of NS-398 for 30 min prior to treatment with withaferin A (3 µM) for 24 h. (A) PGE₂ production was determined using an enzyme immunoassay kit. (B) The sub-G1 population was analyzed by fluorescence activated cell sorting (FACS) (left panel). Equal amounts of cell lysates (60 µg) were separated by gel electrophoresis and analyzed by western blotting for PARP and COX-2 protein. (B) Actin served as a control for protein loading (right panel). *p<0.05 compared to treatment with withaferin A alone.

Figure 4. Reactive oxygen species (ROS) production is not involved in withaferin A-mediated apoptosis and the upregulation of cyclooxygenase-2 (COX-2) expression in AMC-HN4 cells. (A) AMC-HN4 cells were treated with trolox (200 M) and MnTBAP (500 µM) for 30 min prior to treatment with withaferin A (3 µM) for 30 min and then stained with the fluorescent dye, H₂DCFDA, for an additional 10 min. Fluorescence was immediately assayed by flow cytometry. (B) AMC-HN4 cells were treated with trolox (200 µM) and MnTBAP (500 µM) for 30 min prior to treatment with withaferin A (3 µM) for 24 h. The sub-G1 population was analyzed by fluorescence activated cell sorting (FACS) (left panel). Equal amounts of cell lysates (60 µg) were separated by gel electrophoresis and analyzed by western blot analysis for PARP and COX-2 protein. Actin served as a control for protein loading (right panel).
sion and PGE\(_2\) production, but PGE\(_2\) was not associated with apoptosis. Furthermore, the upregulation of intracellular ROS had no effect on apoptosis. Thiol donors only markedly inhibited withaferin A-mediated apoptosis. Therefore, our results suggest that thiol oxidation plays an important role in withaferin A-mediated apoptosis.

COX-2 was overexpressed and the levels of PG, such as PGE\(_2\), were increased in the cancer cells. Previous studies have reported that COX-2 induces proliferation, angiogenesis, migration and invasion, and inhibits apoptosis. A selective COX-2 inhibitor (celecoxib) has been shown to induce apoptosis in prostate carcinoma (25), colon carcinoma (28), cholangiocarcinoma (29), pancreatic carcinoma and melanoma (30) cells. Furthermore, the overexpression of COX-2 reduces apoptosis. Sun et al (31) reported that COX-2 overexpression inhibits the release of cytochrome c and caspase activation in colon carcinoma cells treated with COX-2 inhibitor and 5-fluorouracil. By contrast, the overexpression of COX-2 in osteosarcoma cells has been shown to decrease cell viability (32). Xu et al (32) reported that apoptosis by COX-2 overexpression is independent of PGE\(_2\), whereas the inhibition of ROS production reduces apoptosis in osteosarcoma cells. In head and neck carcinoma, celecoxib and sulindac have been shown to reduce proliferation and induce apoptosis (33). Furthermore, the inhibition of COX-2 enhances sensitivity to anticancer drugs, such as doxorubicin, vincristine, cisplatin, bleomycin and 5-fluorouracil (33). Therefore, we hypothesized that the withaferin A-induced expression of COX-2 and PGE\(_2\) production enhances resistance to apoptosis. However, although NS-398 (a COX-2 inhibitor) markedly blocked withaferin A-mediated PGE\(_2\) production, apoptosis was not affected (Fig. 3B). Therefore, the role of COX-2 in anticancer effects is dependent on cell type and cell conditions.

Withaferin A exerts pro-apoptotic (4-8), anti-proliferative (34), anti-angiogenic (35), and anti-invasive (36) effects through multiple mechanisms. Among these, the upregulation of intracellular ROS is important for withaferin A-mediated apoptosis (37). We also detected withaferin A-mediated ROS production in AMC-HN4 cells (Fig. 4A). ROS scavengers (trolox and MnTBAP) reduced ROS production in the withaferin A-treated cells, whereas the sub-G1 population and the cleavage of PARP were not affected (Fig. 4B). Thiol oxidation is important for the function of withaferin A. Withaferin A inhibits I\(\kappa\)B kinase-\(\beta\) activity, and DTT reverses the inhibitory effects (27). Furthermore, withaferin A-mediated apoptosis is reversed by DTT in erythromyelogenous leukemia cells (26). In this study, the thiol donors, DTT and NAC, markedly blocked withaferin A-mediated apoptosis (Fig. 5B). Withaferin A has \(\alpha,\beta\)-unsaturated ketone moiety in the A ring, which reacts with protein thiol nucleophiles (38). Therefore, withaferin A may target cysteine residues of proteins, such as kinases, phosphatases and chaperons. Therefore, further studies are required to identify the target proteins of withaferin A in head and neck carcinoma cells.

Taken together, our results suggest that the withaferin A-mediated apoptosis is independent of COX-2 expression and ROS production. Thiol oxidation is an important mechanisms of withaferin A-induced apoptosis in head and neck carcinoma cells.

References


