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RESEARCH PAPER

β-Carotene-induced apoptosis is mediated with loss of Ku proteins in gastric cancer AGS cells

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Abstract High dietary intakes and high blood levels of β-carotene are associated with a decreased incidence of various cancers. The anticancer effect of β-carotene is related to its pro-oxidant activity. DNA repair Ku proteins, as a heterodimer of Ku70 and Ku80, play a crucial role in DNA double-strand break repair. Reductions in Ku70/80 contribute to apoptosis. Previously, we showed that reactive oxygen species (ROS) activate caspase-3 which induces degradation of Ku proteins. In the present study, we investigated the mechanism of β-carotene-induced apoptosis of gastric cancer AGS cells by determining cell viability, DNA fragmentation, apoptotic indices (increases in cytochrome c and Bax, decrease in Bcl-2), ROS levels, mitochondrial membrane potential, caspase-3 activity, Ku70/80 levels, and Ku-DNA-binding activity of the cells treated with or without antioxidant *N*-acetyl cysteine and caspase-3 inhibitor z-DEVED-fmk. As a result, β-carotene induced apoptosis (decrease in cell viability, increases in DNA fragmentation and apoptotic indices) and caspase-3 activation, but decreased Ku70/80 levels and Ku-DNA-binding activity. β-Carotene-induced alterations (increase in caspase-3 activity, decrease in Ku proteins) and apoptosis were inhibited by *N*-acetyl cysteine and z-DEVED-fmk. Increment of intracellular and mitochondrial ROS levels and loss of mitochondrial membrane potential were suppressed by *N*-acetyl cysteine, but not by z-DEVED-fmk in β-carotene-treated cells. Therefore, β-carotene-induced increases in ROS and caspase-3 activity may lead to reduction of Ku70/80 levels, which results in apoptosis in gastric cancer cells. Loss of Ku proteins might be the underlying mechanism for β-carotene-induced apoptosis in gastric cancer cells.

Keywords Apoptosis · β-Carotene · Caspase-3 · Ku proteins · Reactive oxygen species

Abbreviations

ROS	Reactive oxygen species
PARP	Poly [ADP-ribose] polymerase
NAC	*N*-acetyl cysteine
z-DEVED-fmk	z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F
MMP	Mitochondrial membrane potential

Introduction

Apoptosis is induced by a wide variety of extracellular and intracellular signals, including reactive oxygen species (ROS). ROS induce apoptosis by damaging cellular components such as DNA. The process is characterized by a series of distinct morphological changes in cell structure (Haunstetter and Izumo 2000; Saraste and Pulkki 2000; Mates and Sanchez-Jimenez 2000). ROS-induced DNA damage is an important cellular mechanism in carcinogenesis (Thompson 1995). During apoptosis, various apoptotic proteins and caspases are activated. ROS have been reported to play important roles in caspase activation and in the initiation and execution phases of apoptosis (Matsura et al. 1999; Mizutani et al. 2002; Takuma et al. 2002; Emanuele et al. 2002). ROS induce the

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overexpression of Bax protein, which modulates the mitochondrial membrane potential and triggers cytochrome c release. Cytosolic cytochrome c induces caspase-3 activity, triggering the destruction of specific proteins, DNA fragmentation, and thereby apoptosis (Chaube et al. 2014). Activated caspase-3 cleaves numerous key cellular proteins, including poly [ADP-ribose] polymerase (PARP) (Nicholson et al. 1995; Esposti et al. 1999; Gross et al. 1999). However, caspase-3 might cleave other important proteins to induce apoptosis.

The Ku proteins are heterodimeric proteins composed of Ku70 (70 kDa) and Ku80 (80 kDa). Ku proteins are the DNA-binding regulatory subunits of DNA-dependent protein kinase (Bliss and Lane 1997; Featherstone and Jackson 1999). Binding of the Ku heterodimer to double-strand DNA breaks stimulates DNA repair, which might affect apoptosis or cell proliferation (Woo et al. 1998; Liu et al. 1998). In our previous study, oxidative stress decreased nuclear Ku70/80 levels, which induced apoptosis in pancreatic acinar AR42 J cells (Song et al. 2003). The study showed that ROS activate caspase-3, which may induce degradation of Ku proteins. Reduction of Ku proteins may decrease the repair process for the damaged DNA and induces apoptotic cell death. Lee et al. (2005) have demonstrated that justicidin A induces apoptosis in human colorectal cancer cells by reducing Ku70 levels. These studies indicate that a reduction in Ku70/80 levels plays a critical role in cell death mechanisms.

β-Carotene is a red–orange pigment and naturally derived carotenoid, which is abundant in many vegetables and fruits. It has been reported to induce apoptosis in cancer cells, including leukemia, adenocarcinoma, and melanoma cells (Palozza et al. 2001, 2003a). Studies demonstrated that the anticancer effect of β-carotene derives from its pro-oxidant activity (Palozza et al. 2002; Guruvayoorappan and Kuttan 2007), anti-oxidant action (Mishra et al. 2013), regulation of cell differentiation (Lim et al. 2014), or cell cycle arrest (Gloria et al. 2014). At high concentrations, β-carotene acts as a pro-apoptotic agent in adenocarcinoma cells by increasing the levels of intracellular ROS (Palozza 2005). β-Carotene-induced apoptosis is associated with ROS production in HL-60 human leukemia cells (Palozza et al. 2003a, b). In Molt four cells, treatment with β-carotene increased intracellular ROS level, which then triggered caspase activation (Prasad et al. 2006). However, the mechanism by which ROS induce apoptosis in β-carotene-treated cancer cells remains unclear.

The present study investigated whether β-carotene decreases Ku70/80 levels and apoptosis in gastric cancer AGS cells by activating caspase-3 and promoting apoptotic indices (increase in cytochrome c and Bax, decrease in Bcl-2) in a ROS-dependent manner. Cells were treated with or without the antioxidant *N*-acetyl cysteine (NAC) and the caspase-3 inhibitor z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (z-DEVED-fmk). In addition, changes in the mitochondrial membrane potential (MMP) were assessed in AGS cells treated with β-carotene because mitochondria are considered the main source of internal ROS in cells (Chang et al. 2013). Both cellular levels and cell surface expression levels of Ku70 and Ku80 were determined by Western blotting and flow cytometry.

Materials and methods

Cell culture

A human gastric cancer cell line, AGS (gastric adenocarcinoma; ATCC CRL 1739), was purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in complete medium consisting of RPMI 1640 supplemented with 10 % fetal bovine serum, 2 mM glutamine, 100 μ/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA).

Experimental protocol

The cells were plated at 1.5×10^5 cells/well in a 12-well culture plate and cultured overnight. β-Carotene was dissolved in tetrahydrofuran and freshly prepared before each experiment (Jang et al. 2009a). The cells were treated with β-carotene (final concentration of 100 μM) for 30 min (to assess intracellular and mitochondrial ROS levels and MMP) or 12 h (to determine cell viability, DNA fragmentation, caspase-3 activity, cellular and cell surface expression levels of Ku proteins, apoptotic indices, and Ku-DNA-binding activity). Before the main experiments, the cells were cultured with β-carotene (final concentration of 20, 50, or 100 μM) for various periods (0, 4, 8, or 12 h) to determine the appropriate concentration of β-carotene and time point for assessing the effect of NAC or z-DEVED-fmk on β-carotene-induced alterations. In other sets of experiments, the cells were pretreated with or without NAC (2 mM) or z-DEVED-fmk (5 μM; Calbiochem, San Diego, CA, USA) for 2 h before treatment with β-carotene (100 μM). The concentrations of NAC and z-DEVED-fmk were adapted from our previous studies (Song et al. 2003; Choi et al. 2012).

Determination of cell viability

Viable cell numbers were determined by direct counting using a hemocytometer and the trypan blue exclusion test (0.2 % trypan blue; Sigma).

Assessment of DNA fragmentation

DNA fragmentation was determined by measuring the amount of oligonucleosome-bound DNA in the cell lysate. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. The nucleosomes were quantified using a sandwich ELISA (Cell Death Detection ELISA^{PLUS} kit; Boehringer Mannheim GmbH, Ingelheim, Germany).

Preparation of whole-cell extracts and nuclear extracts

The cells were scraped in phosphate-buffered saline (PBS) and pelleted by centrifugation at $300\times g$ for 5 min. The cell pellets were suspended in lysis buffer containing 10 mM Tris, pH 7.4, 15 mM NaCl, 1 % NP-40, and a commercial protease inhibitor complex (Complete; Roche, Mannheim, Germany) and lysed by passage through a 1-mL syringe with several rapid strokes. The mixture was then incubated on ice for 30 min and centrifuged at $15{,}000\times g$ for 10 min. The supernatants were collected and used as whole-cell extracts. For the extraction of nuclei, the cells were extracted in buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.5 mM $MgCl_2$, 0.2 % Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The nuclear pellet was resuspended on ice in nuclear extraction buffer containing 20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25 % glycerol, 1 mM DTT, and 0.5 mM PMSF and then centrifuged. The supernatants were used as nuclear extracts.

Western blot analysis for cytochrome C, Bcl-2, and Bax

Whole-cell extracts (100–200 µg) were loaded in each lane, separated by 8–12 % SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred to nitrocellulose membranes (Amersham, Inc., Arlington Heights, IL, USA) by electroblotting. The transfer of protein was verified through reversible staining with Ponceau S. Membranes were blocked in 3 % nonfat dry milk, and proteins were detected with antibodies for cytochrome c, Bcl-2, Bax, and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibodies were diluted in Tris-buffered saline with Tween 20 (TBST) containing 3 % nonfat dry milk and incubated with the membranes at 4 °C overnight. The membranes were washed with TBS-T, and primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies, which were visualized with an ECL detection system (sc-2048; Santa Cruz Biotechnology). Actin served as a loading control.

Measurement of intracellular ROS

The cells were loaded with 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, CA, USA) for 30 min, washed, and scraped into 1 mL of PBS. The fluorescence of 2′,7′-dichlorofluorescein (DCF) was analyzed using a fluorescence multi-well plate reader (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) at excitation and emission wavelengths of 485 and 530 nm, respectively (Kim et al. 2014a). The amount of ROS trapped in the cells was expressed as the relative increase over the ROS level in cells cultured in the absence of β-carotene, which was set at 100.

Measurement of caspase-3 activity

Caspase-3 activity was determined using a fluorometric immunosorbent enzyme assay (Roche, Indianapolis, IN, USA). The cells were harvested, washed three times with PBS, and suspended in ice-cold buffer containing 10 mM DTT. The mixture was briefly vortexed and incubated for 1 min in an ice bath. After centrifugation ($16{,}000\times g$ for 1 min), the supernatant was used directly for the determination of enzyme activity. The cell lysate (100 µL) was incubated at 37 °C with the specific caspase-3 substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethyl-coumarin (Ac-DEVDAFC) for 2 h. Caspase-3 activity is proportional to the amount of fluorochrome (amidofluorocoumarin) formed from Ac-DEVDAFC. The amounts of released AFC were measured with a fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 400 and 505 nm, respectively (Ito et al. 1999).

Electrophoretic mobility shift assay (EMSA) for the assessment of Ku-DNA-binding activity

Nuclear extracts (10 µg) were incubated with the ^{32}P-labeled double-strand oligonucleotide 5′-GGGCCAAGAATCTTAGCAGTTTCGGG-3′ in buffer containing 12 % glycerol, 12 mm HEPES (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 25 mM KCl, 5 mM $MgCl_2$, and 0.04 µg/mL poly[d(I-C)] at room temperature for 30 min. The extracts were then subjected to electrophoretic separation at room temperature and 30 mA on a nondenaturing 5 % acrylamide gel using 0.5 × Tris borate/EDTA buffer. The gels were dried at 80 °C for 1 h and exposed to radiography film for 6–18 h at −70 °C with an intensifying screen (Lim et al. 2012).

Fluorescence detection of mitochondrial ROS

MitoSOX Red is a mitochondria-targeted form of dihydroethidium that is relatively specific for superoxide. Oxidation of MitoSOX Red generates the DNA-binding red fluorophore ethidium bromide. 4′,6-Diamidino-2-phenylindole (DAPI; 359-nm excitation/461-nm emission) was used to label nuclei (invitrogen). The cells were loaded with MitoSOX Red (5 μM; Molecular Probes, Eugene, OR, USA) and DAPI (1 μM) for 10 min, washed, and mounted with mounting solution (Sigma). Fluorescence images of ROS were observed at excitation and emission wavelengths of 514 and 585 nm, respectively, using a laser scanning confocal microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany).

Flow cytometric analysis of the mitochondrial membrane potential

Disruption of the MMP can be measured with cationic lipophilic fluorochromes such as MitoTracker Red (MR) (Palozza et al. 2003a). These probes diffuse passively across the plasma membrane and accumulate in the negatively charged mitochondrial matrix. The extent of dye uptake depends on the size of the MMP; dissipation of the MMP decreases cell-associated fluorescence, which can be detected with flow cytometry. To assess changes in the MMP, 1×10^6 cells were incubated with 100 nM MR for 30 min at 37 °C in the dark. Samples were then immediately analyzed on a FACSCalibur flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA).

Flow cytometric analysis of cell surface expression levels of Ku proteins

The cells were detached by treatment with 0.25 % trypsin/1 mM EDTA and then washed twice with PBS containing 2 % FBS. The cells were incubated with anti-Ku70 or anti-Ku80 antibody at room temperature for 30 min and then washed three times with PBS containing 2 % FBS. The cells were incubated with PE-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature for 30 min, washed three times with PBS containing 2 % FBS, and analyzed on a FACSCalibur flow cytometer (Becton–Dickinson). The results were shown as histograms and mean fluorescence intensities.

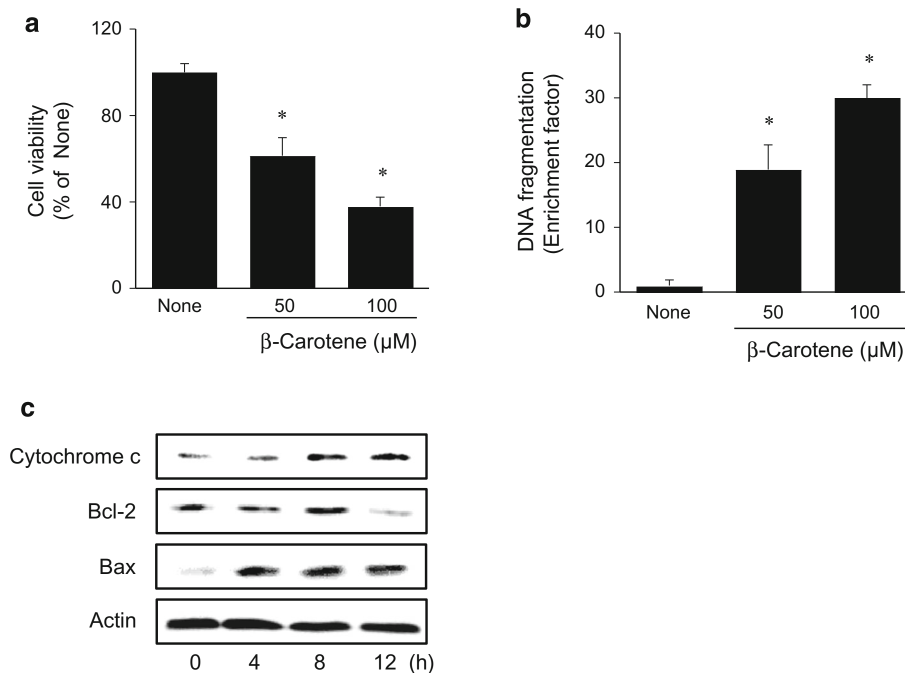

Fig. 1 β-Carotene induces apoptosis in AGS cells. The cells were cultured with β-carotene (final concentration of 100 μM) for 12 h (**a**, **b**) or for various periods (0, 4, 8, 12 h) (**c**). **a** Cell viability was determined by counting the number of viable cells. The viability of cells treated without β-carotene (none) was set at 100 %. The results are expressed as the mean ± SEM of four separate experiments. *$p < 0.05$ versus cells treated without β-carotene (none). **b** DNA fragmentation was detected by measuring the amount of oligonucleosome-bound DNA in the cell lysate. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. DNA fragmentation in cells treated without β-carotene (none) was set at 1. The results are expressed as the mean ± SEM of four separate experiments. *$p < 0.05$ versus cells treated without β-carotene (none). **c** Apoptotic indices (protein levels of cytochrome c, Bcl-2, and Bax) were assessed with Western blot analysis. Actin served as a loading control

Statistical analysis

Results were expressed as the mean ± SEM of four separate experiments. Analysis of variance followed by the Newman-Keul's post hoc test was used for statistical analysis. $p < 0.05$ was considered statistically significant.

Results

Effect of β-carotene on apoptosis in AGS cells

To examine whether β-carotene induces apoptosis in AGS cells, cell viability, nucleosome-bound DNA as an index of DNA fragmentation, and apoptotic indices (levels of cytochrome c, Bcl-2, and Bax) were assessed. At 12 h of culture, β-carotene decreased cell viability and increased DNA fragmentation in a dose-dependent manner (Fig. 1a, b). β-Carotene increased cytochrome c and Bax levels, but decreased Bcl-2 levels in gastric cancer AGS cells in a time-dependent manner (Fig. 1c).

Effect of β-carotene on intracellular ROS levels, caspase-3 activity, Ku70/80 levels, and Ku-DNA-binding activity in AGS cells

β-Carotene increased intracellular ROS levels (Fig. 2a, determined at 30 min) and caspase-3 activity (Fig. 2b, determined at 12 h) in a dose-dependent manner. Because a reduction in Ku protein might be a downstream event in the caspase-activating apoptotic pathway, Ku70/80 levels and Ku-DNA-binding activity were assessed with Western blot analysis and EMSA, respectively. β-Carotene, at final concentration of 100 μM, reduced the levels of Ku70 and Ku80 as well as the Ku-DNA-binding activity in a time-dependent manner (Fig. 2c, d). These results suggest that β-carotene induces oxidative stress, activates caspase-3, and reduces Ku protein levels, which might decrease the repair of oxidative DNA damage.

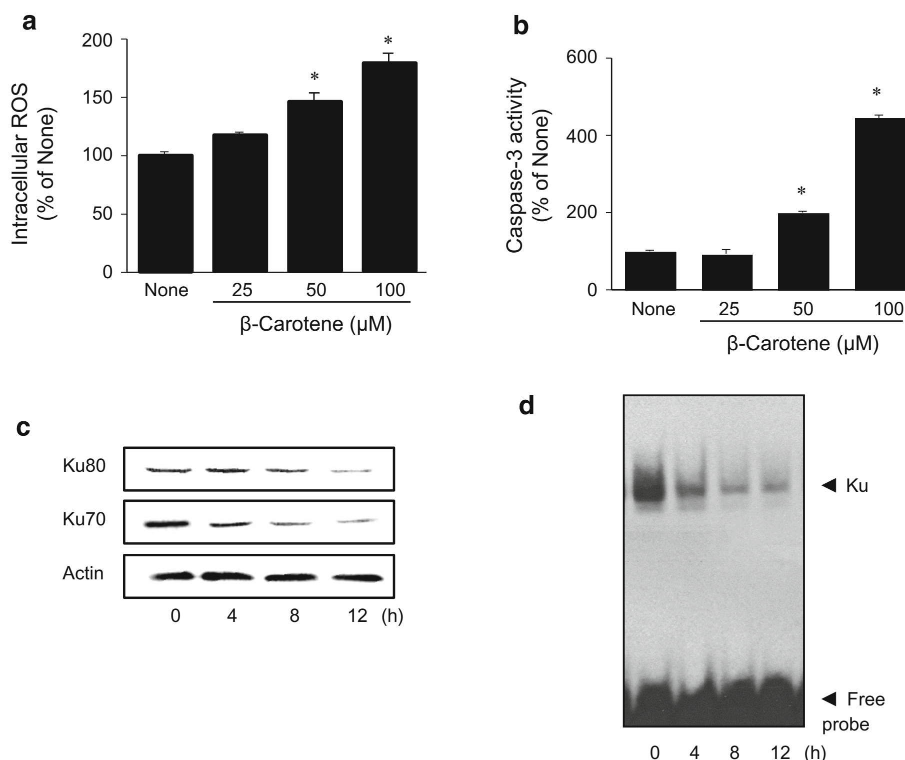

Fig. 2 β-Carotene induces increases in intracellular ROS levels and caspase-3 activity, and decreases in Ku70/80 levels and Ku-DNA-binding activity in AGS cells. The cells were cultured with β-carotene (final concentration of 100 μM) for 12 h (**a**, **b**) or for various periods (0, 4, 8, 12 h) (**c**, **d**). **a** Intracellular ROS levels were determined by measuring DCF fluorescence with a fluorescence multi-well plate reader. The results are expressed as the mean ± SEM of four separate experiments. *$p < 0.05$ versus cells treated without β-carotene (none). **b** Caspase-3 activity was determined by measuring the cleavage of the fluorescent peptide substrate DEVDAFC. Caspase-3 activity in cells treated without β-carotene (none) was set at 100 %. The results are expressed as the mean ± SEM of four separate experiments. *$p < 0.05$ versus cells treated without β-carotene (none). **c** The levels of Ku70 and Ku80 in total cell lysates were determined with Western blot analysis. Actin served as a loading control. **d** Ku-DNA-binding activity in nuclear extracts was determined with EMSA

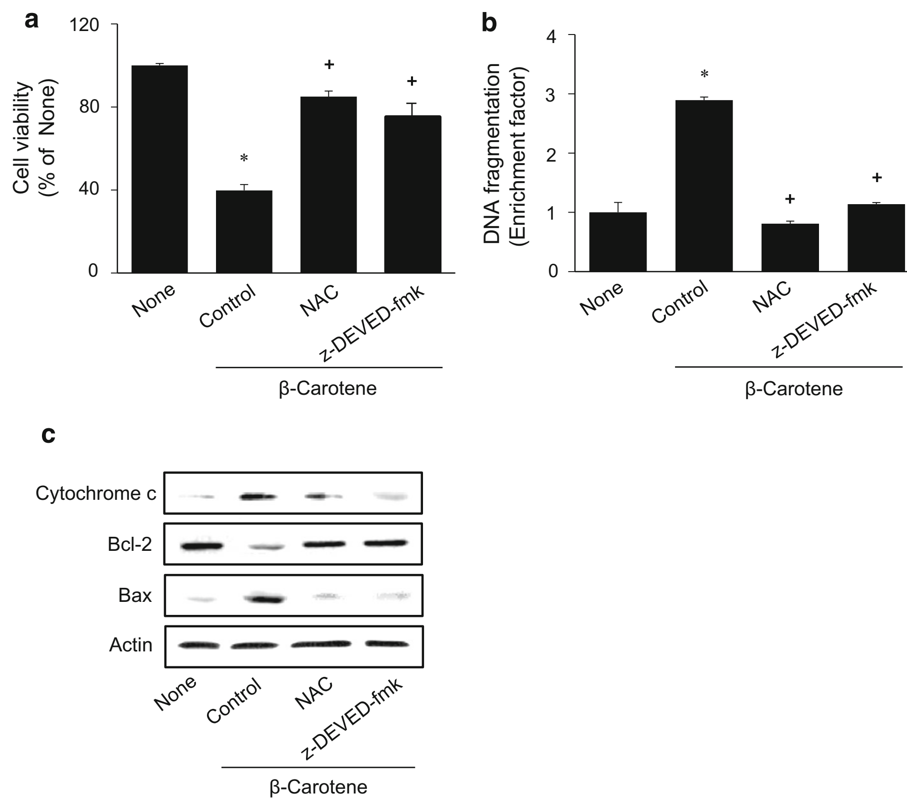

Fig. 3 Effect of NAC and z-DEVED-fmk on β-carotene-induced apoptosis in AGS cells. The cells were pretreated with NAC (2 mM) or z-DEVED-fmk (5 µM) for 2 h and then cultured with β-carotene (100 µM) for 12 h (**a**–**c**). **a** Cell viability was determined by counting the number of viable cells. The viability of cells treated without β-carotene (none) was set at 100 %. The results are expressed as the mean ± SEM of four separate experiments. $*p < 0.05$ versus cells treated without β-carotene (none); $+p < 0.05$ versus cells treated with β-carotene alone (control). **b** DNA fragmentation was detected by measuring the amount of oligonucleosome-bound DNA in the cell lysate. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. DNA fragmentation in cells treated without β-carotene (none) was set at 1. The results are expressed as the mean ± SEM of four separate experiments. $*p < 0.05$ versus cells treated without β-carotene (none); $+p < 0.05$ versus cells treated with β-carotene alone (control). **c** Apoptotic indices (protein levels of cytochrome c, Bcl-2, and Bax) were assessed with Western blot analysis. Actin served as a loading control

Effect of NAC and z-DEVED-fmk on β-carotene-induced apoptosis in AGS cells

To investigate the involvement of ROS and caspase-3 in β-carotene-induced apoptosis, the cells were pretreated with an antioxidant (NAC) or a caspase-3 inhibitor (z-DEVED-fmk) for 2 h and then treated with β-carotene (100 µM) for 12 h. In cells treated with NAC or z-DEVED-fmk, the β-carotene-induced decrease in cell viability and increase in DNA fragmentation were attenuated (Fig. 3a, b). As shown in Fig. 3c, the β-carotene-induced increase in apoptotic indices (increase in cytochrome c and Bax, decrease in Bcl-2) was suppressed by treatment with NAC and z-DEVED-fmk. These results indicate that oxidative stress and caspase-3 activation contribute to β-carotene-induced apoptosis in AGS cells.

Effect of NAC and z-DEVED-fmk on the β-carotene-induced alterations of intracellular ROS levels, caspase-3 activity, Ku70/80 levels, and Ku-DNA-binding activity in AGS cells

To determine the involvement of ROS and caspase-3 in the β-carotene-induced downregulation of Ku proteins, cells were pretreated with NAC or z-DEVED-fmk for 2 h and then treated with β-carotene for 12 h. As shown in Fig. 4a, the β-carotene-induced increase in intracellular ROS levels was inhibited by NAC, but not by z-DEVED-fmk. Therefore, ROS generation might be upstream of caspase-3 activation in β-carotene-treated cells.

The β-carotene-induced increase in caspase-3 activity was inhibited by NAC and z-DEVED-fmk (Fig. 4b). In β-carotene-treated cells, treatment with NAC and z-DEVED-fmk restored Ku70/80 levels and Ku-DNA-binding activity

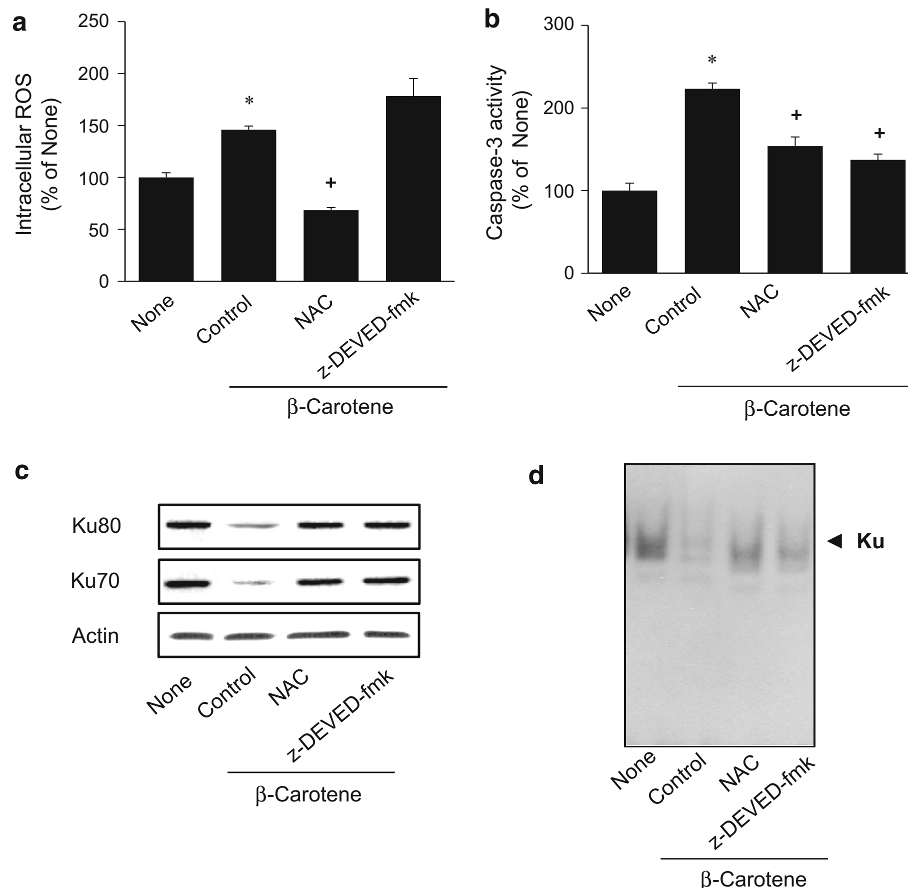

Fig. 4 Effect of NAC and z-DEVED-fmk on the β-carotene-induced alterations of intracellular ROS levels, caspase-3 activity, Ku70/80 levels, and Ku-DNA-binding activity in AGS cells. The cells were pretreated with NAC (2 mM) or z-DEVED-fmk (5 μM) for 2 h and then cultured with β-carotene (100 μM) for 30 min **a** and 12 h (**b**–**d**). **a** Intracellular ROS levels were assessed by measuring DCF fluorescence. The relative fluorescence intensity of cells treated without β-carotene (none) was set at 100 %. The results are expressed as the mean ± SEM of four separate experiments. $*p < 0.05$ versus cells treated without β-carotene (none); $+p < 0.05$ versus cells treated with β-carotene alone (control). **b** Caspase-3 activity was determined by measuring the cleavage of the fluorescent peptide substrate DEVDAFC. Caspase-3 activity in cells treated without β-carotene (none) was set at 100 %. The results are expressed as the mean ± SEM of four separate experiments. $*p < 0.05$ versus cells treated without β-carotene (none); $+p < 0.05$ versus cells treated with β-carotene alone (control). **c** The levels of Ku70 and Ku80 in total cell lysates were determined with Western blot analysis. Actin served as a loading control. **d** Ku-DNA-binding activity in nuclear extracts was determined with EMSA. None, cells treated without β-carotene; control, cells treated with β-carotene alone

(Fig. 4c, d). These results indicate that ROS mediate caspase-3 activation, which might decrease Ku70/80 levels and Ku-DNA-binding activity in β-carotene-treated cells.

Effect of NAC and z-DEVED-fmk on the β-carotene-induced increase in mitochondrial ROS levels and loss of the mitochondrial membrane potential in AGS cells

β-Carotene increased mitochondrial ROS levels, which were determined by measuring MitoSOX Red signals (Fig. 5a, right panel), and promoted loss of the MMP (Fig. 5b). Because MMP loss reflects mitochondrial dysfunction, the increase in mitochondrial ROS might be caused by β-carotene-induced mitochondrial damage. NAC suppressed the increase in mitochondrial ROS levels and the loss of the MMP in β-carotene-treated cells. However, z-DEVED-fmk had no effect on reduction of MMP caused by β-carotene (Fig. 5b). These results indicate that β-carotene promotes MMP loss and mitochondrial ROS generation in AGS cells. ROS might mediate the β-carotene-induced loss of the MMP in AGS cells. Staining of nuclei with DAPI was not changed by treatment of NAC (Fig. 5a, left panel).

Effect of NAC and z-DEVED-fmk on the β-carotene-induced decrease in cell surface expression of Ku proteins in AGS cells

Several studies showed that Ku70/80 has been detected on the plasma membrane of a wide range of different human

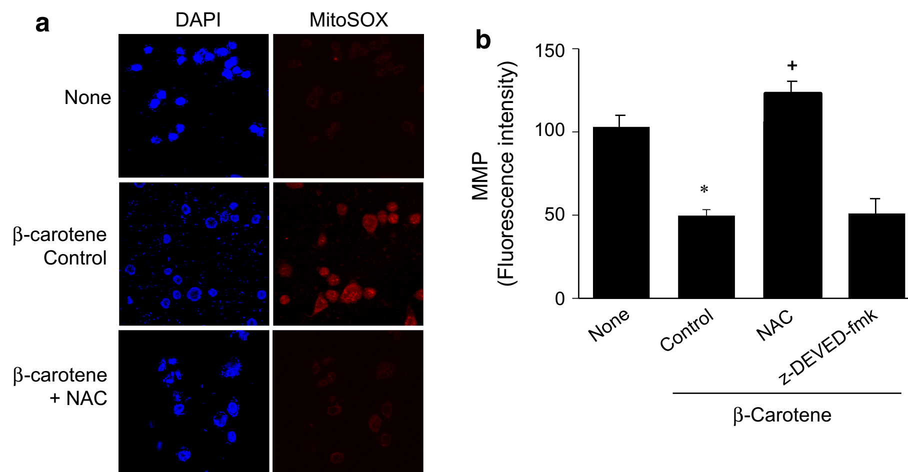

Fig. 5 Effect of NAC and z-DEVED-fmk on β-carotene-induced increase in mitochondrial ROS levels, and MMP loss in AGS cells. The cells were pretreated with NAC (2 mM) or z-DEVED-fmk (5 μM) for 2 h and then cultured with β-carotene (100 μM) for 30 min. **a** Mitochondrial ROS levels were determined by measuring MitoSOX Red signals. Nuclei were stained with DAPI. **b** MMP was analyzed with flow cytometry. The relative fluorescence intensity of the cells treated without β-carotene (none) was set at 100 %. The results are expressed as the mean ± SEM of four separate experiments. *$p < 0.05$ versus cells treated without β-carotene; +$p < 0.05$ versus control cells treated with β-carotene alone

tumor-derived cell lines such as myeloma, leukemia, and cervix carcinoma (Fransson and Borrebaeck 2006; Muller et al. 2005). Ku70/80 levels in the cell surface of the transformed cells are involved in adhesion, migration, and invasion (Muller et al. 2005; Tai et al. 2002). To examine whether β-carotene alters levels of Ku proteins in cell membrane, their expression levels in cell membrane were determined by flow cytometric analysis. As shown in Fig. 6a and b, β-carotene induced reduction of Ku70/80 levels in cell membrane. Treatment of NAC and z-DEVED-fmk suppressed β-carotene-induced decrease in Ku70/80 levels in cell membrane. The results were in parallel with decreased cellular levels of Ku70/80 in β-carotene-treated cells, which was restored by treatment with NAC and z-DEVED-fmk in AGS cells (Fig. 4c).

Discussion

Recent studies show that β-carotene inhibits tumorigenesis by regulating cell differentiation via the upregulation of vimentin, peripherin, and neurofilament (Lim et al. 2014), and suppresses invasion and metastasis by decreasing hypoxia-inducible factor-1α (Kim et al. 2014b) in neuroblastoma. β-Carotene inhibits cell proliferation, arrests the cell cycle in different phases, and increases apoptosis in breast cancer cells (Gloria et al. 2014). β-Carotene acts as antioxidant or pro-oxidant, depending on the redox potential of the biological environment in which it acts (Palozza 2005; Bendich 2004; El-Agamey and Lowe 2004). At low concentrations (< 5 μM), the carotenoid may serve as an antioxidant, while at relatively high concentrations and/or in the presence of a chronic oxidative stress (for instance, smoke or cancer tissues), it may behave as a pro-oxidant, consuming endogenous antioxidants and inducing DNA oxidative damage (Palozza 2005). Cui et al. (2007) reported that β-carotene acts as pro-oxidant to increase intracellular ROS, which may be responsible for mitochondrial dysfunction and cytochrome C release. ROS production plays important roles in the regulation of β-carotene-induced cancer cell apoptosis.

It is evident that high levels of intracellular ROS are sufficient to trigger cell death, suggesting that ROS are biochemical mediators of apoptosis (Pastorino and Hoek 2000). In the present study, β-carotene increased intracellular and mitochondrial ROS levels and promoted MMP loss, an index of mitochondrial damage.

Uncoupling of the mitochondrial respiratory chain leads to increases in ROS, and ROS might induce MMP loss (Poot and Pierce 1999). Siems et al. (2005) have suggested that carotenoid breakdown products increase oxidative stress by impairing mitochondrial function in isolated rat liver mitochondria. Several studies have shown that MMP loss leads to the release of cytochrome c from the mitochondria, which occurs as an early event in apoptosis, and finally cell death (Kim et al. 2003; Kirsch et al. 1999). Consistent with these studies, the present study showed that β-carotene induced cytochrome c release and DNA fragmentation and that NAC blocked β-carotene-induced apoptosis and MMP loss. Thus, mitochondrial oxidative stress might be involved in β-carotene-mediated apoptosis in gastric cancer AGS cells.

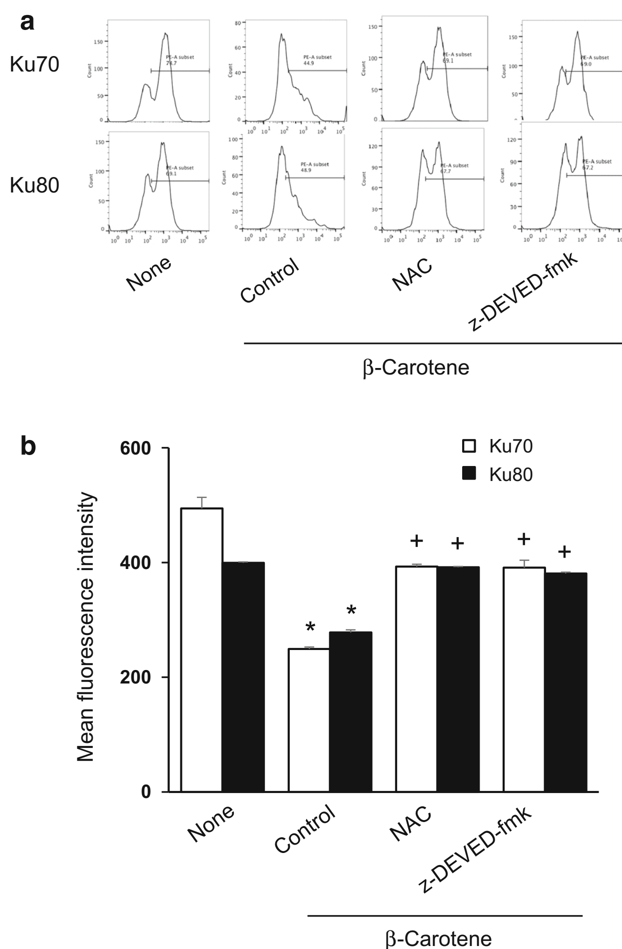

Fig. 6 Effect of NAC and z-DEVED-fmk on β-carotene-induced decrease in cell surface expression levels of Ku proteins in AGS cells. The cells were pretreated with NAC (2 mM) or z-DEVED-fmk (5 μM) for 2 h and then cultured with β-carotene (100 μM) for 12 h. The expression of Ku70/80 in cell membrane was analyzed by flow cytometry. **a** Representative histograms of Ku70/80 expression in cell membrane are shown. **b** Mean fluorescence intensity for Ku70/80 expression in cell membrane was analyzed. The results are expressed as the mean ± SEM of four separate experiments. $*p < 0.05$ versus corresponding cells treated without β-carotene; $+p < 0.05$ versus corresponding control cells treated with β-carotene alone

In addition, we found that β-carotene-induced apoptosis is mediated with increased ROS levels and caspase-3 activity. Treatment with z-DEVED-fmk and NAC suppressed β-carotene-induced cell death. These results indicate that the β-carotene-induced increase in ROS activates caspase-3 and then triggers apoptosis in gastric cancer AGS cells. The results are supported by previous studies, showing that ROS activates caspase-3 in human cancer cells and astrocytes (Matsura et al. 1999; Mizutani et al. 2002; Takuma et al. 2002; Emanuele et al. 2002).

The absence of Ku70/80 has been reported to impair DNA double-strand break repair (Gu et al. 1997; Lees-Miller et al. 1995; Taccioli et al. 1994). In addition, two Ku-null mutant cell lines, $Ku70^{-/-}$ and $Ku80^{-/-}$, are highly sensitive to anticancer drugs, when compared to wild-type cells (Kim et al. 1999). We previously reported that ROS-induced apoptosis occurred in parallel with the loss of nuclear Ku proteins and that a caspase-3 inhibitor prevented the ROS-induced loss of nuclear Ku proteins and cell death in pancreatic acinar AR42 J cells (Song et al. 2003). Here, we demonstrated that β-carotene decreased Ku proteins and Ku-DNA-binding activity and that the effect was inhibited by the antioxidant NAC and the caspase-3 inhibitor z-DEVED-fmk. Therefore, a reduction in Ku proteins might be a downstream event in the caspase-3-activating apoptotic pathway induced by ROS.

As possible mechanisms for anticancer effect of β-carotene, pro-oxidant activity (Palozza et al. 2002; Guruvayoorappan and Kuttan 2007), anti-oxidant action (Di Tomo et al. 2012; Mishra et al. 2013), and cell cycle regulation (Lim et al. 2014; Gloria et al. 2014) have been demonstrated. Di Tomo et al. (2012) reported that low concentration of β-carotene (2.5 μM) decreased ROS generation in TNF-α-treated human umbilical vein endothelial cells. Gloria et al. (2014) showed that β-carotene and lycopene (0.5–10 μM) induce cell cycle arrest, growth inhibition, and apoptosis in human breast cancer cells. This study suggested that carotenoids are potential agents for biological interference with cancer.

In addition, β-carotene (5–20 μM) reduces cell growth and induces neuronal cell differentiation by increasing phosphorylation of extracellular signal-regulated kinases in neuroblastome cells (Lee et al. 2013). β-Carotene inhibits neuroblastoma cell invasion and metastasis in vitro (5–10 μM) and in vivo (2 mg/kg b.w. twice a week for 8 weeks) (Kim et al. 2014b). Pham et al. (2013) reported that lower expression of β-carotene 15,15′-monooxygenase (BCMO1), which converts β-carotene to retinaldehyde, is associated with higher invasiveness of colon cancer cells and increased expression of matrix metalloproteinase (MMP) 7 and MMP 28. β-Carotene (10 μM) showed antitumor effect by increasing BCMO1 expression and reducing invasiveness with a decrease in expression of MMP in cancer cells. β-carotene inhibited cell proliferation by stimulating Gα2-coupled receptor-mediated signaling which involves cAMP and PKA in squamous carcinoma cells (Al-Wadei and Schuller 2009). Ataxia-telangiectasia-mutated (ATM) activates a variety of multiple signaling pathways including DNA repair and apoptosis. Jang et al. (2009b) found that nuclear loss of ATM is involved in induction of apoptosis in β-carotene-treated gastric cancer cells.

Several studies assessed cellular uptake of β-carotene by HPLC. Treatment of β-carotene in the media increased intracellular concentrations of β-carotene in a concentration-dependent manner (Eicker et al. 2003; Rodríguez et al.

2005; Briviba et al. 2001). For instance, human colon carcinoma cells were cultured in the media containing β-carotene (30 μM). After 3 days, intracellular levels of β-carotene and retinol were 22 pmol/10^6 cells and 45 pmol/10^6 cells, respectively (Briviba et al. 2001). Even though cellular uptake of β-carotene may be dependent on cell types and culture time, we could presume that β-carotene (100 μM) treatment to the medium may increase intracellular levels of β-carotene (about 70 pmol/10^6 cells) and retinol (about 150 pmol/10^6 cells), based on the previous study (Briviba et al. 2001). Further study should be performed to determine the intracellular levels of β-carotene and retinol after treatment of β-carotene in various types of cells and different culture conditions.

In conclusion, β-carotene reduces Ku70/80 levels and Ku-DNA-binding activity by increasing intracellular ROS levels and promoting caspase-3 activation, thereby inducing apoptosis in gastric cancer cells. ROS-mediated loss of Ku proteins might be the underlying mechanism for β-carotene-induced apoptosis in gastric cancer AGS cells.

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Conflict of interest The authors declare that they have no conflict of interest.

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