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Potassium Cyanate Induces Apoptosis of Human Colorectal Cancer Cell via Mitochondrial Pathway

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Potassium cyanate (KOCN) is an inorganic compound and induces the carbamylation of proteins with cytotoxic effects on human cells. Although there is a potential cytotoxic molecule, the role of KOCN on the apoptosis of cancer cell is not well understood. The present study investigated the effects of KOCN on the human colorectal cancer cell line, HCT 116 cells. To understand the anti-cancer effect of KOCN on HCT 116 cells, we examined alteration of apoptosis, the intracellular Ca^{2+} concentration, the intracellular signaling pathway and generation of reactive oxygen species (ROS) in these cells treated with KOCN. The apoptosis of HCT 116 cells was induced by KOCN in a dose-dependent manner at 24 hours and 48 hours, respectively. The apoptosis was processed via the cleavage of poly ADP-ribose polymerase (PARP) and activation of caspase 3 in HCT 116 cells. KOCN induced the elevation of intracellular Ca^{2+} concentration and changed the expressions of Bcl-2 family proteins. The pro-apoptotic Bax was continuously up-regulated, and the anti-apoptotic Bcl-2 was down-regulated by KOCN. KOCN also induced the hyperpolarization of mitochondria and the generation of ROS in HCT 116 cells. Taken together, these results indicate that KOCN induces the apoptosis of HCT 116 cells by disruption of Ca^{2+} homeostasis and via mitochondrial pathway. This study provides the compound that may be used as a potent agent for the treatment of colorectal cancer.

Key Words: Potassium cyanate, Apoptosis, Colorectal cancer, Mitochondrial pathway, Ca^{2+}

INTRODUCTION

Potassium cyanate (KOCN) is an inorganic compound and is the conjugate based of cyanic acid (HOCN). Cyanic acid reacts irreversibly with the N-terminal groups of amino acid, peptides within proteins and this process is known as carbamylation (Fluckiger et al., 1981; Kraus and Kraus, 2001). Carbamylation can change the structure of proteins, and modify the activity of enzymes, cofactors, hormones and antibodies (Kuckel et al., 1993; Inoue et al., 2001). Recently, the carbamylated proteins induce cell death in various diseases, including chronic kidney disease, atherosclerosis and coronary artery disease (OK et al., 2005; Apostolov et al., 2011). These evidences indicate that KOCN induces apoptotic cell death. Apoptosis is a programmed cell death, in which mitochondria integrate various stimulators into an intrinsic death pathway (Green and Reed, 1998). Bcl-2 family members appear to regulate the commitment to proliferation or cell death by controlling the integrity of mitochondrial membrane (Cory and Adams, 2002). In addition, the functions of mitochondria are disrupted by Ca^{2+} overloading, which results in enhanced generation of reactive oxygen species (ROS). The understanding of these processes may be helpful for the development of cancer therapy. Although it has been reported that the carbamylation induced by cyanic acid mediates cell death, its use for cancer therapy has not been fully investigated.

Colorectal cancer is one of main causes of cancer-related deaths and its prevalence is increasing yearly (Rychahou et al., 2008). Colorectal cancer is a cancer caused by uncontrolled cell growth and is originated from the epithelial cells lining the colon or rectum. In intracellular signaling pathway, the signaling activities of survival-associated proteins increase, however, other pro-apoptotic proteins

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deactivate in this cancer (Markowitz and Bertagnolli, 2009). Although surgery and various chemotherapies for colorectal cancer have been developed, most response rate is still too low and these therapies have side effects with toxicity (Henriette et al., 2009). Therefore, there is a pressing need for the development of new agent for the treatment of colorectal cancer.

In the present study, we investigate the anti-proliferative effects of KOCN on the human colorectal cancer cell line, HCT 116 cells. We examined the cell death of HCT 116 cells treated with KOCN in a time- and dose-dependent manner. We also demonstrated the alteration of intracellular Ca^{2+} concentration, the intracellular signaling pathway and the generation of ROS induced by KOCN in these cells.

MATERIALS AND METHODS

Reagents

Potassium cyanate (KOCN) (Sigma Aldrich, St. Louis, MO) was dissolved in sterile distilled water to prepare the stock solutions (10 mg/ml). RPMI 1640 medium and FBS were purchased from Life Technologies, Inc. (Gaithersburg, MD). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit were purchased from BD biosciences (San Diego, CA). Anti-Bax, anti-Bcl-2, anti-PARP, anti-pro caspase 3 and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 2', 7'-dichlorofluorescein diacetate (DCFDA), Flou-3, and 3', 3'-dihexyloxacabocyanine ($DiOC_6(3)$) were purchased from Fluka Chemie GmbH (Steinheim, Switzerland).

Cell culture

HCT 116 cells were the human colorectal cancer cell line and were purchased from American Type Culture Collection (Rockville, MD, USA). These cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), and were incubated at 37℃ in 5% CO_2 incubator.

Cell apoptosis

For measurement of the apoptosis, the cells were incubated with the FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Apoptotic cells were analyzed by flow cytometry using CellQuest software and were defined as the cells in the right quadrant that stained positive for annexin V with/without PI. To analyze, 10,000 events were collected for each sample.

Intracellular Ca^{2+} concentration

The changes of intracellular Ca^{2+} concentration were determined by a fluorescent dye, fluo-3-acetoxylmethyl (AM). Cells were washed with PBS and incubated with 5 μM Fluo-3-AM for 30min at 37℃. After incubation, the cells were washed and analyzed with flow cytometry.

Alteration of mitochondrial membrane potential (MMP, ΔΨm)

Mitochondrial membrane potential was determined by the retention of the dye $DiOC_6(3)$. The cells resuspended at 1×10^6 cells in PBS and incubated with 50 nM $DiOC_6(3)$ for 30min at 37℃. After incubation, the cells were washed and analyzed with flow cytometry.

Western blotting

The cells were washed with ice-cold PBS and lysed with lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na_3VO_4, and protease inhibitors). The cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. The blots in membrane were incubated with specific antibodies against a target protein and thereafter developed using an enhanced chemiluminescence detection system. The same blot was stripped and was reprobed with anti-β actin antibody for use as an internal control. Quantification of Western blots was performed using Quantity One software (Bio-Rad Laboratory, Inc.).

ROS production

The cells were resuspended in culture medium supplemented with/without KOCN. After incubation for the indicated time, these cells were washed and were resuspended

at 1×10^7 cells/ml in prewarmed PBS, respectively. The cells were 5 μM of DCFDA to label the intracellular ROS and were incubated for 10 min at room temperature. Labeled cells were immediately observed using fluorescence-activated cell sorting (FACS) analysis (BD Biosciences).

Statistical analysis

All data were expressed as mean ± SD. Data were analyzed by Student's *t*-test using SPSS statistical software package (Version 10.0, Chicago, IL). A p values less than 0.05 was considered statistically significant.

RESULTS

KOCN induces apoptosis of HCT 116 cells in a time- and a dose-dependent manner

To determine the effect of KOCN on cell death of HCT 116 cells, we examined the apoptosis and necrosis of the

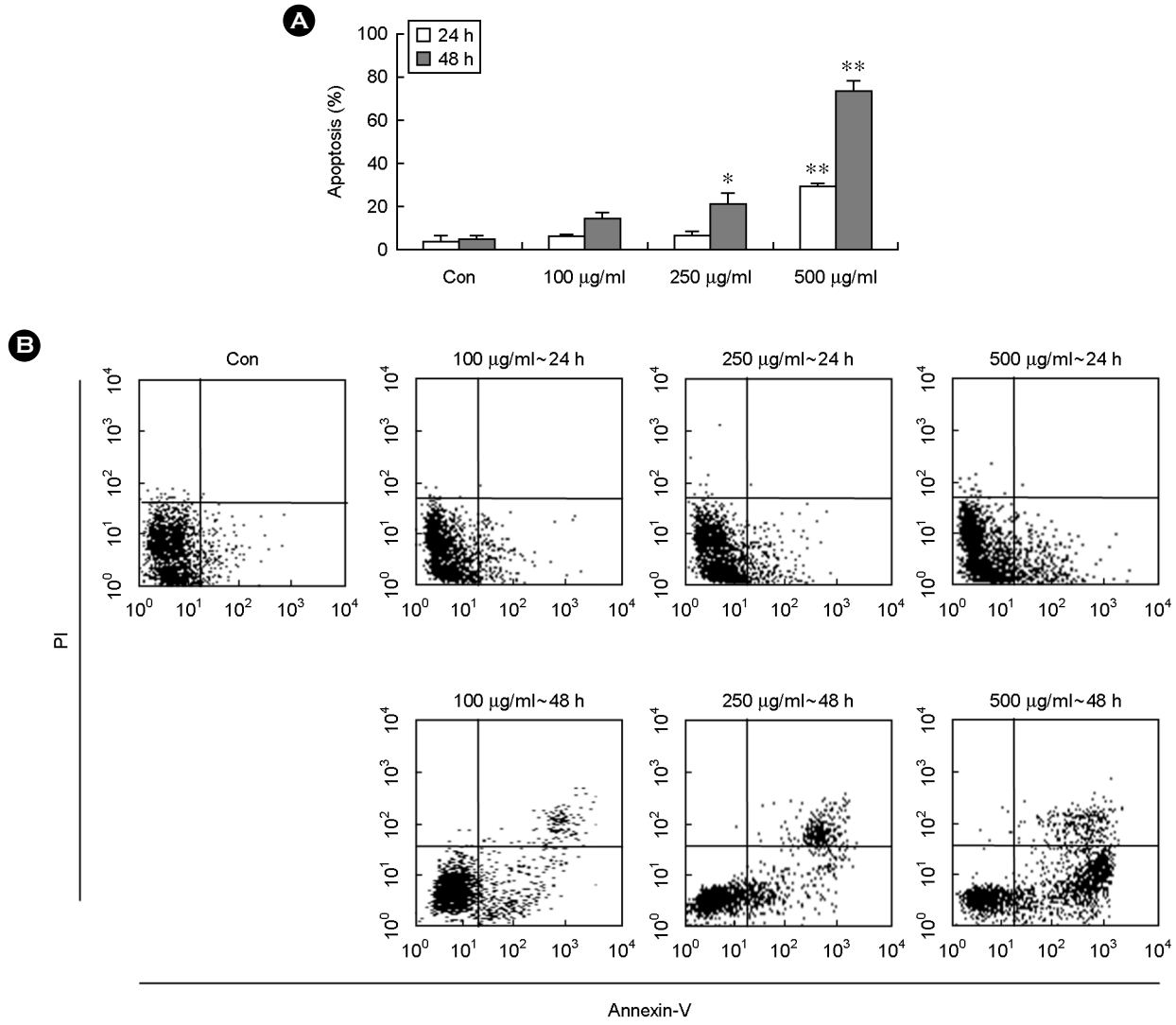

Fig. 1. The effect of KOCN on apoptosis of HCT 116 cells. (A) HCT 116 cells were incubated for 24 h and 48 h in the absence (Con) and presence of KOCN (100 μg/ml, 250 μg/ml and 500 μg/ml). The apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry as described in the materials and methods section. The percentage of apoptotic cell in total cell population was included all annexin V binding cells. Data are expressed as the means ± SD in three individual experiments. *P<0.05 and **P<0.01 were considered a significant difference between the untreated group and KOCN-treated group at same incubation time. (B) Data were displayed by dot plot and the dot plot represents the distribution of the events within the plots. The lower- and upper-right phase is the apoptotic cells.

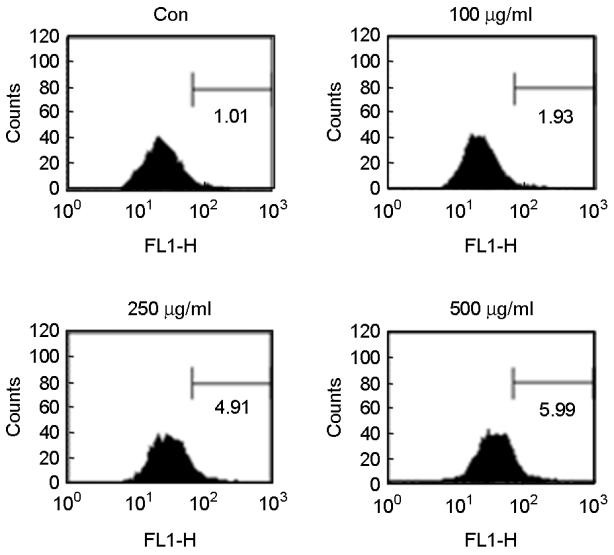

Fig. 2. The effect of KOCN on the intracellular Ca^{2+} concentration in HCT 116 cells. HCT 116 cells were incubated for 48 h in the absence (Con) and presence of KOCN (100 μg/ml, 250 μg/ml and 500 μg/ml). The intracellular Ca^{2+} concentration was determined by the fluorescent activity of Fluo-3 AM using flow cytometry as described in the materials and methods section.

cell induced by KOCN. Cell apoptosis and necrosis were assessed by FITC-conjugated annexin V and PI staining using flow cytometry. Annexin V is a marker for phosphatidylserine (PS), which exposed on plasma membrane at the initial stage of apoptosis. PI is a marker for membrane-permeability in the late-apoptosis and necrotic cells. Apoptotic cells were defined as all annexin V- positive cells. After addition of KOCN in HCT 116 cells, apoptotic cells were increased by KOCN as compared with medium alone (Fig. 1). KOCN induced the apoptosis of HCT 116 cells in a dose-dependent manner at 24 hours and 48 hours, respectively. As shown in Fig. 1A, 500 μg/ml of KOCN considerably led apoptosis of these cells at 48 hours. These results indicate that KOCN induces the apoptosis of HCT 116 cells in a time- and a dose-dependent manner.

KOCN induces the elevation of intracellular Ca^{2+} concentration in HCT 116 cells

To determine the role of Ca^{2+} signaling in KOCN-induced apoptosis, HCT 116 cells were treated with 100, 250 and 500 μg/ml for 48 hours, respectively. The intracellular Ca^{2+} concentration was measured with a calcium indicator dye, Flou-3 AM. During the period of 48 h, intracellular Ca^{2+} concentration was elevated by KOCN in a dose-dependent manner and was approximately six-fold increase at 500 μg/ml of KOCN (Fig. 2).

KOCN alters the expressions of Bcl-2 family members in HCT 116 cells

To examine the alteration of apoptosis-associated proteins, Western blot was performed to estimate the levels of Bcl-2 family members including Bax and Bcl-2. The level of β-actin was used as a reference protein for normalization. When HCT 116 cells were treated with KOCN, the level of Bax, a pro-apoptotic signal protein, increased until 48 hours incubation (Fig. 3A & 3B). In contrast, KOCN ccontinuously suppressed the level of Bcl-2, a anti-apoptotic signal protein (Fig. 3A & 3B).

KOCN induces the hyperpolarization of mitochondria and the generation of ROS in HCT 116 cells

Mitochondrial membrane potential (MMP) is an important indicator of the mitochondrial function and dysfunction (Rottenberg and Wu, 1998). After treatment of KOCN in HCT 116 cells, the process of MMP increased in a dose-dependent manner (Fig. 3C). This process is called as mitochondrial hyperpolarization that leads to the loss of MMP and represents a prerequisite for mitochondrial-mediated apoptosis (Giovannini et al., 2002).

To investigate the further mechanism of KOCN-induced apoptosis on HCT 116 cells, intracellular ROS levels were measured by DCFDA labeling of the cells. After 48 hours of KOCN treatment, ROS generation was enhanced in a dose-dependent manner (Fig. 4). As shown in Fig. 4, the exposure to KOCN for 24 hours slightly increased ROS levels (Fig. 4). These results indicate that KOCN induces the apoptosis of HCT 116 cell via mitochondrial pathway and ROS generation.

KOCN induces the cleavage of Poly (ADP-ribose) polymerase (PARP) and activation of caspase 3 in HCT 116 cells

To understand how KOCN increases the apoptosis in HCT 116 cells, we examined the protein levels of pro-apoptotic signal proteins such as PARP and procaspase 3.

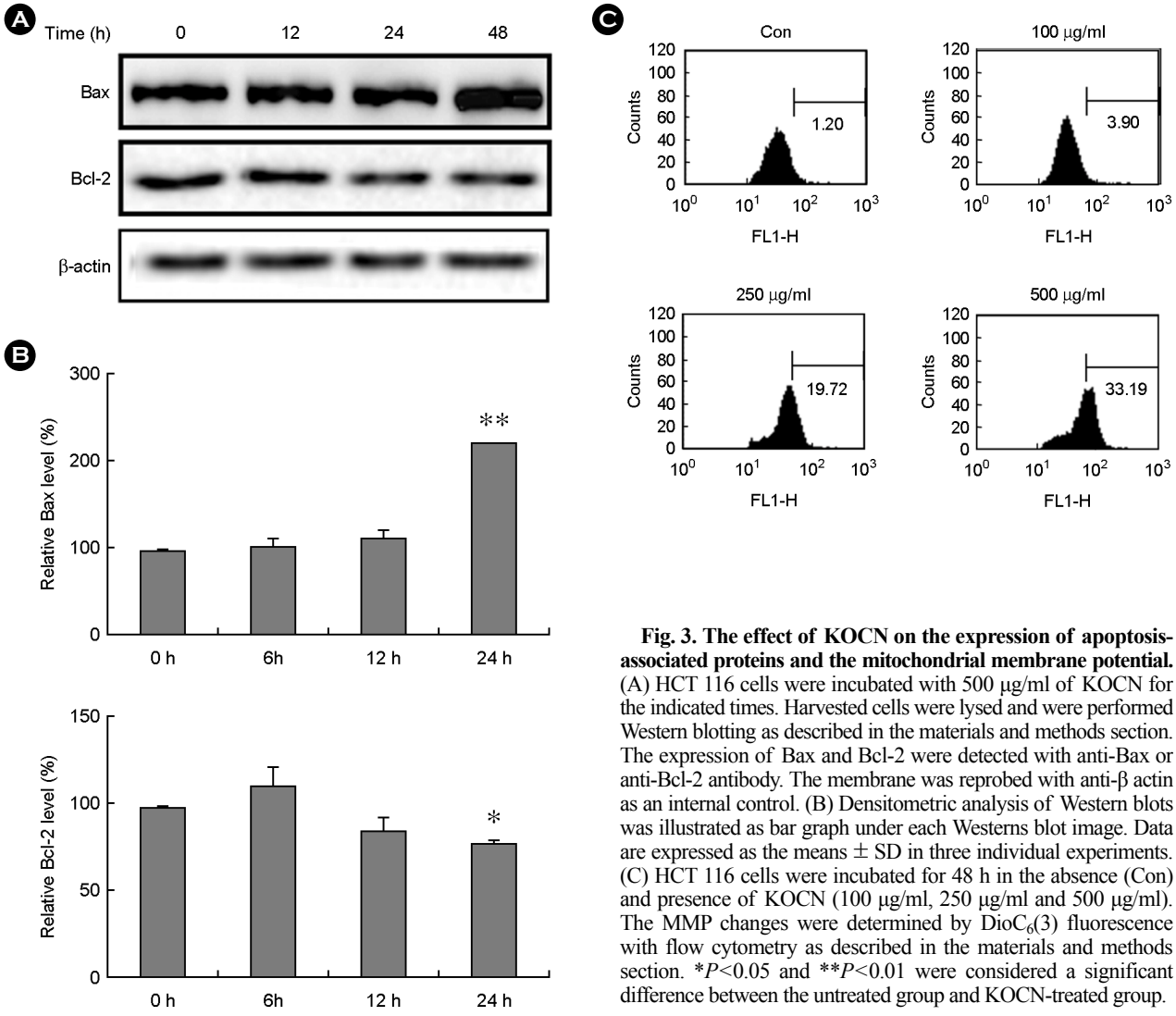

Fig. 3. The effect of KOCN on the expression of apoptosis-associated proteins and the mitochondrial membrane potential. (A) HCT 116 cells were incubated with 500 μg/ml of KOCN for the indicated times. Harvested cells were lysed and were performed Western blotting as described in the materials and methods section. The expression of Bax and Bcl-2 were detected with anti-Bax or anti-Bcl-2 antibody. The membrane was reprobed with anti-β actin as an internal control. (B) Densitometric analysis of Western blots was illustrated as bar graph under each Westerns blot image. Data are expressed as the means ± SD in three individual experiments. (C) HCT 116 cells were incubated for 48 h in the absence (Con) and presence of KOCN (100 μg/ml, 250 μg/ml and 500 μg/ml). The MMP changes were determined by $DioC_6(3)$ fluorescence with flow cytometry as described in the materials and methods section. *P<0.05 and **P<0.01 were considered a significant difference between the untreated group and KOCN-treated group.

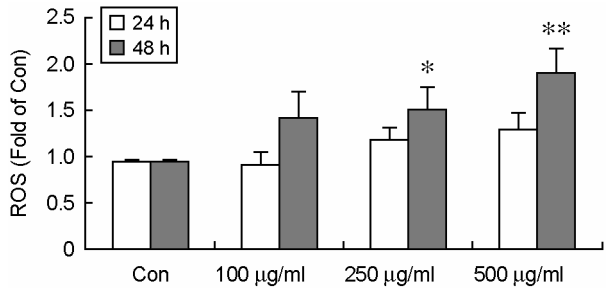

Fig. 4. The effect of KOCN on the generation of ROS in HCT 116 cells. HCT 116 cells were incubated for 24 h and 48 h in the absence (Con) and presence of KOCN (100 μg/ml, 250 μg/ml and 500 μg/ml). The ROS generation was determined by the DCFDA fluorescence with flow cytometry as described in the materials and methods section. Data are expressed as the means ± SD in three individual experiments. *P<0.05 and **P<0.01 were considered a significant difference between the untreated group and KOCN-treated group at same incubation time.

Both PARP and caspase 3 are cleaved after the initiation of apoptosis and these cleavage forms act as active molecules. After the addition of KOCN in HCT 116 cells, the cleavage form of PARP was increased and pro-caspase 3 was decreased in a time-dependent manner (Fig. 5). These data indicate that the cell apoptosis induced by KOCN is associated with the increased cleavage of PARP and procaspase 3.

DISCUSSION

Cyanate is derived from urea and normaly present in human blood plasma. However, cyanate is elevated in patients with various diseases, such as chronic kidney

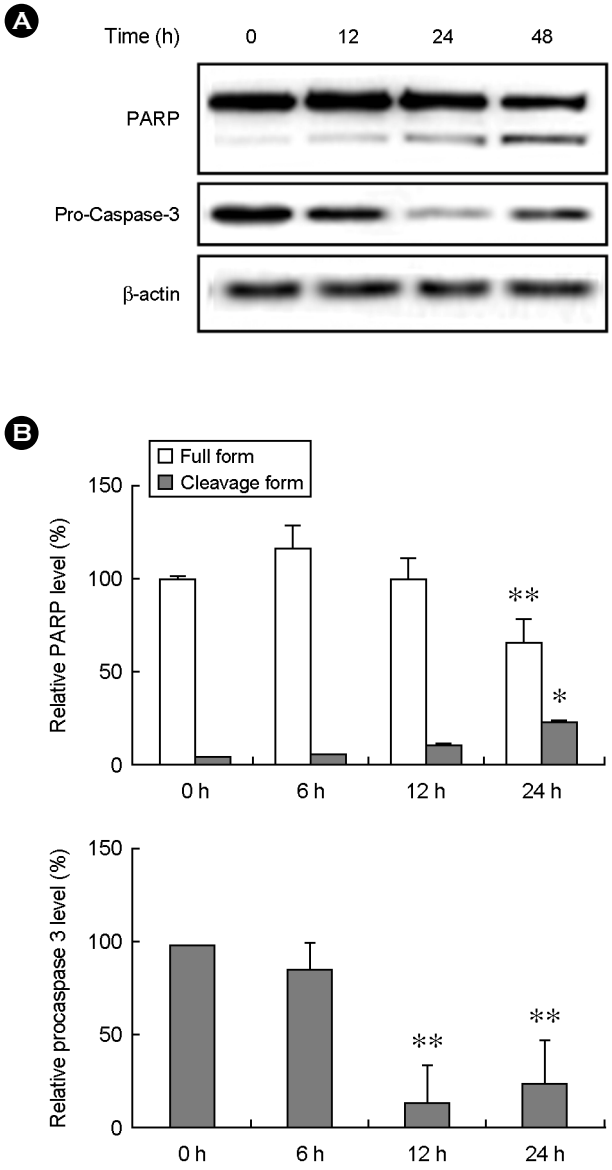

Fig. 5. The effect of KOCN on the expression of PARP and procaspase 3 proteins. (A) HCT 116 cells were incubated with 500 μg/ml of KOCN for the indicated times. Harvested cells were lysed and were performed Western blotting as described in the materials and methods section. The expression of PARP and procaspase 3 were detected with anti-PARP or anti-procaspase 3 antibody. The membrane was reprobed with anti-β actin as an internal control. (B) Densitometric analysis of Western blots was illustrated as bar graph under each Westerns blot image. Data are expressed as the means ± SD in three individual experiments. *P<0.05 and **P<0.01 were considered a significant difference between the untreated group and KOCN-treated group.

disease and atherosclerosis (Kraus and Kraus, 2001). This cyanate induces the carbamylation of proteins and these carbamylated proteins increases cell death via apoptosis (OK et al., 2005; Apostolov et al., 2011). In the present study, we focused on the cytotoxic activity of KOCN on the human colorectal cancer cell line, HCT 116 cells. We demonstrated that KOCN considerably increased the apoptosis of HCT 116 cells via intracellular Ca^{2+} signaling, mitochondrial dysfunction and ROS generation.

Apoptosis occurs by extracellular factors and induces the intrinsic death pathways. Mitochondria play a pivotal role in regulating apoptosis. In early stage of apoptosis, mitochondria membrane potential (MMP, $\Delta\Psi m$) is altered by opening of permeability transition pore (PTP), and it continuously mediates pro-apoptotic signals. MMP is important for various functions of mitochondria, such as ATP production and homeostasis of Ca^{2+} levels (Desagher and Martinou, 2000). Calcium has been recognized as ubiquitous intracellular signal responsible for a number of cellular events, such as growth, proliferation, differentiation, and survival/apoptosis (Clapham, 2007). In apoptotic pathway, calcium concentration is controlled by apoptosis-associated proteins. The anti-apoptotic Bcl-2 has been implicated in a number of cancers and involved in resistance to conventional cancer treatment. Bcl-2 has been reported to exert its inhibitory effects on apoptosis by blocking the release of cytochrome *c* and the loss of MMP, and this protein has been acting on the inhibition of cell apoptosis in cancer pathogenesis (Gross et al., 1999). In contrast to Bcl-2 protein, Bax, a pro-apoptotic protein, integrates to the outer mitochondrial memgrane and causes cytochrome *c* release. Bax also mediates Ca^{2+} fluxes and involves in sensitization of mitochondria (Zhe et al., 1999). KOCN-induced apoptosis of the HCT 116 cells causes a rise in the intracellular Ca^{2+} concentration and an alteration of apoptosis-associated protein, including the suppression of Bcl-2 expression and the increase of Bax expression (Fig. 1, Fig. 2, Fig. 3A & 3B).

MMP was increased by KOCN during the stage of apoptosis in HCT 116 cells (Fig. 3C). Hyperpolarization of mitochondria represents a prerequisite for rapid mitochondria-mediated apoptosis, and leads to the decline of MMP (Li et al., 1999). Mitochondria hyperpolarization also can hold the electron carriers of the respiratory chain in a reductive state, which generates ROS. The excess ROS production into cytosol has been associated with many forms of cell death and can act as an inducer in apoptosis

pathways. High levels of ROS mediate DNA damage or activation of the caspase pathway (Ishihama et al., 2008; Terasaka et al., 2005). Based on previous reports, the increase of ROS level in response to KOCN may be related to the induction of HCT 116 cell apoptosis (Fig. 4). Overall, the restored intracellular Ca^{2+} can serve to release apoptotic proteins from mitochondria and can generate ROS, and eventually activate apoptosis-related proteases. In apoptosis-related proteases, caspase 3 is an important molecule and act as an executioner in apoptosis. Capspase 3 is cleaved after apoptosis began, and this cleavage form acts as an active molecule. As shown in Fig. 5, KOCN diminished the expression of procaspase 4 and also increased the cleavage of PARP. PARP is found in the cells and detect the single strand DNA breaks (SSB) for the SSB repair (Yu et al., 2006; Espinoza et al., 2007). PARP is inactivated by caspase 3 cleavage during apoptosis, and the increased cleavage of PARP indicates that cleavage of procaspase 3 occurred (Orrenius et al., 2003).

In conclusion, KOCN induces the apoptosis via mitochondria-dependent pathways in the human colorectal cancer cell line, HCT 116 cells. These mitochondria-dependent pathways mediated by alteration intracellular Ca^{2+}, ROS generation and induction of apoptosis-associate proteins. These results suggest that the KOCN has a potent apoptosis-inducing activity and it may be useful for various cancer therapies.

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