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Capsaicin-Induced Apoptosis and Reduced Release of Reactive Oxygen Species in MBT-2 Murine Bladder Tumor Cells

Ji-Seon Lee, Jong-Sun Chang, Ji Youl Lee[1], and Jung-Ae Kim

College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea and [1]Department of Urology, College of Medicine, Catholic University of Korea, Seoul 137-701, Korea

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Bladder cancer is a common cancer with high risk of recurrence and mortality. Intravesicle chemotherapy after trans-urethral resection is required to prevent tumor recurrence and progression. It has been known that antioxidants enhance the antitumor effect of bacillus Calmette-Guerin (BCG), the most effective intravesical bladder cancer treatment. Capsaicin, the major pungent ingredient in genus *Capsicum*, has recently been tried as an intravesical drug for overactive bladder and it has also been shown to induce apoptotic cell death in many cancer cells. In this study, we investigated the apoptosis-inducing effect and alterations in the cellular redox state of capsaicin in MBT-2 murine bladder tumor cells. Capsaicin induced apoptotic MBT-2 cell death in a time- and dose-dependent manner. The capsaicin-induced apoptosis was blocked by the pretreatment with Z-VAD-fmk, a broad-range caspase inhibitor, or Ac-DEVD-CHO, a caspase-3 inhibitor. In addition to the caspase-3 activation, capsaicin also induced cytochrome *c* release and decrease in Bcl-2 protein expression with no changes in the level of Bax. Furthermore, capsaicin at the concentration of inducing apoptosis also markedly reduced the level of reactive oxygen species and lipid peroxidation, implying that capsaicin may enhance the antitumor effect of BCG in bladder cancer treatment. These results further suggest that capsaicin may be a valuable intravesical chemotherapeutic agent for bladder cancers.

Key words: Capsaicin, Bladder cancer, Apoptosis, Reactive oxygen species, Bcl-2, Cytochrome *c* release, Caspase-3

INTRODUCTION

Bladder cancer is a frequently diagnosed cancer in elderly people (Jemal *et al.*, 2003). Approximately 70% of the cases are superficial bladder cancers with high risk of recurrence and mortality (Lamm, 2002). Although the traditional treatment for the bladder cancer is radical cystectomy, due to the high risk of recurrence, patients with superficial bladder cancer often receive intravesical chemotherapy or immunotherapy in an attempt to prevent tumor recurrence. Instillation with bacillus Calmette-Guerin (BCG) is the most effective intravesical treatment (Akaza *et al.*, 1995; Sternberg, 1999) for bladder cancer. Recently, it has been also reported that the susceptibility of tumor cells to BCG is related to the changes in cellular levels of reactive oxygen species and thiols (Pook *et al.*, 2002).

Correspondence to: Jung-Ae Kim, College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea
Tel: 82-53-810-2816, Fax: 82-53-810-4654
E-mail: jakim@yu.ac.kr

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), the major pungent ingredient in genus *Capsicum*, has long been used in food additives and drugs (Suzuki and Iwai, 1994; Cordell and Araujo, 1993). Recently, capsaicin has been tried as an intravesical drug for overactive bladder with detrusor hyperreflexia (De Ridder *et al.*, 1997). In addition, capsaicin has been shown to inhibit growth and induce apoptosis in various transformed cell types *in vitro*, but not in normal counterpart cells (Kang *et al.*, 2003; Zhang *et al.*, 2003; Jung *et al.*, 2001; Surh, 2002). Furthermore, the compounds reportedly promote apoptosis *in vivo* as the mechanism of tumor cell elimination in animal models for carcinogenesis (Tanaka *et al.*, 2002). In addition to the proapoptotic activity, capsaicin possesses chemopreventive potential against carcinogen-induced tumorigenesis (Surh *et al.*, 1998; Surh and Lee, 1995). These observations continue to fuel the interest in capsaicin as a potential anticancer agent.

Signaling pathways associated with apoptotic effects of capsaicin in cancer cells is not fully understood and quite

controversial in terms of reactive oxygen species (ROS) production. For example, capsaicin-induced apoptosis has been correlated with the elevation of intracellular ROS production in human leukemic cells (Ito *et al.*, 2004) and HepG2 human hepatoblastoma cells (Lee *et al.*, 2004). In contrast, it also markedly reduced the basal generation of ROS and lipid peroxidation in human glioblastoma cells (Lee *at al.*, 2000). Furthermore it has been recently reported that capsaicin at low concentrations acts as a CoQ mimic by protecting against rotenone-induced ROS formation and mitochondrial membrane potential collapse whereas at higher concentrations capsaicin caused ROS formation (Galati and O'Brien, 2003).

Nevertheless, the pro-apoptotic effect and its action mechanism of capsaicin in bladder carcinoma have not been investigated yet. In the present study, we examined i) whether capsaicin induces apoptosis in a bladder tumor cell line using MBT-2 murine bladder tumor cells and ii) whether cellular ROS level is altered in capsaicin-induced apoptosis.

MATERIALS AND METHODS

Cell culture

MBT-2 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Hyclone, Utah, USA), 2.0 mg/mL sodium bicarbonate, 10 mM HEPES, 100 units/mL of penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were split in a 1:3 ratio at every other day by trypsinization.

Cell viability assay

Cell viability was assessed by the MTT assay based on the reduction of a MTT into formazan dye by active mitochondria (Mosmann, 1983). Briefly, the cells were cultured with the indicated concentration of drugs in 100 μL of RPMI 1640 medium for 48 h at an initial cell density of 5×10^3 cells/cm^2. Thereafter, 10 μL of MTT solution (5 mg MTT/mL in PBS) was added to each well of microtiter plate and incubated for 4 h. After washing, the formazan dye precipitates, the amount of which is proportional to the number of live cells, were dissolved in 200 μL of DMSO. The absorbance was read at 540 nm using an ELISA reader (Molecular Devices, USA).

DNA fragmentation detection

After incubation with or without capsaicin, the cells were collected by centrifugation at 400 g for 5 min, washed twice in PBS (pH 7.4) and resuspended in 800 μL of lysis buffer containing 5 mM Tris, 0.5% Triton X-100, 10 mM EDTA (pH 8.0). The cell suspensions were incubated on ice for 30 min, pelleted by centrifugation (13,000 g for 20 min at 4°C), and then, the supernatants were incubated with 8 μL of RNase A (10 mg/mL) for 1 h at 37°C. At the end of incubation, 8 μL of proteinase K (10 mg/mL) was added and the incubation continued for a further 1 h at 37°C. Cellular DNA was extracted with phenol: chloroform (1:1), centrifuged at 13,000 g for 10 min, added with 800 μL of isopropanol containing 0.3 M sodium acetate, and pelleted by centrifugation (13,000 g for 15 min at 4°C). The purified DNA was dissolved in loading buffer (2.5% Ficoll, 20 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol FF) and electrophoresed on a 2% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA) at 50 V and then visualized by being staining with 0.5 μg/mL ethidium bromide.

Flow cytometric analysis

To determine apoptosis, cells were stained with propidium iodide as described previously (Herrmann *et al.*, 1994). In brief, at the end of incubation, MBT-2 cells were trypsinized, washed twice with cold PBS, and fixed with 70% ethanol for 30 min at 4°C. The fixed cells were washed twice with PBS, incubated in PBS containing 50 μg/mL RNase A for 30 min at 37°C, stained with 50 μg/mL propidium iodide for 30 min, and then, filtered through a 40 μm nylon mesh filter (Falcon, USA). The cells were subjected to flow cytometric analysis using a FACS Calibur (Becton Dickinson, USA) equipped with Cell Quest program.

Measurement of caspase-3 activity

The caspase-3 activity was measured according to the manufacture's experimental instructions (Clontech, USA). In brief, the cells were harvested by centrifugation (400×g for 5 min at 4°C). After the cells were washed with PBS, they were incubated with lysis buffer for 10 min. The supernatants were collected by centrifugation at 10,000×g for 10 min at 4°C and added with equal volume of reaction buffer. Finally, the caspase-3 substrate, Ac-DEVD-pNA, was added to each sample. The caspase-3 activity was analyzed by reading the absorbance at 405 nm with an ELISA reader.

Subcellular fractionation

MBT-2 cells were washed twice with PBS, resuspended in extraction buffer (250 mM sucrose, 2 mM EDTA, 2 mM EGTA, and 20 mM Tris-HCl, pH 7.5) supplemented with 1 mM PMSF and protease inhibitor cocktail and homogenized using a glass homogenizer. The extracts were centrifuged at 1,000×g for 10 min at 4°C to remove unbroken cell, large plasma membrane pieces and nuclei. The mitochondrial fractions were pelleted by centrifugation at 10,000×g for 20 min at 4°C. This supernatants were centrifuged at 120,000×g for 1 h at 4°C and concentrated using Microcon YM-3 spin columns (Millipore).

Western blot analysis

Cell pellets were lysed for 30 min at 4°C in lysis buffer (10 mM Tris-HCl, pH 7.6, 10 mM EDTA, 150 mM NaCl, 0.1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM DTT, protease inhibitor cocktail). Lysates were centrifuged at 13,000 g for 15 min at 4°C, and supernatant proteins were separated on 14% or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membrane at 200 mA for 1 h. The membrane was blocked with 5% skim milk in TBS buffer containing 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were immunoblotted with primary antibodies (antibodies against p53, Bcl-2, Bax, cytochrome *c* and actin purchased from Santa Cruz Biotechnology Inc.) in TBST contained 5% skim milk for 2 h. After three washing with TBST, the membranes were incubated with anti-goat IgG HRP conjugated secondary antibody. After three successive washing with TBST, the proteins were developed using a chemiluminescence assay kit and exposed to ECL film (Amersham Life Science, Buckinghamshire, England).

Measurement of reactive oxygen species (ROS)

The level of cytochrome *c* reduction was measured to assess cellular release of superoxide produced in the cells. Cell medium was changed to fresh medium without FBS and treated with drugs. After treatment, the conditioned media of 1×10^5 cells were mixed with 50 μM cytochrome *c* and incubated at 37°C for 15 min. Another set of the same mixtures was reserved at 0°C for use as a blank. Incubation was terminated by placing the mixtures at 0°C. After centrifugation of blanks and incubated mixtures at 3,000×g for 5 min at 4°C, the absorbance of the incubated supernatant was read spectrophotometrically at 550 nm using unincubated blank as a reference.

Lipid peroxidation assay

The cells harvested were suspended in 1 mL of PBS and mixed with 0.2 mL of 8.1% sodium dodecylsulfate (SDS), 1.5 mL of 20% acetic acid solution (pH 3.5), and 1.5 mL of 0.8% thiobarbituric acid. The mixture was heated at 95°C for 1 h, chilled to room temperature, and extracted with 1 mL of water and 2.5 mL of *n*-butanol·pyridine mixture (15:1, v/v). The upper organic layer containing malondialdehyde produced by lipid peroxidation was measured at 532 nm. Synthetic malondialdehyde was used as an external standard, and the level of lipid peroxides was expressed as nmoles of malondialdehyde. The protein concentration was measured by the method of Bradford (Denis *et al.*, 2000).

Statistical analysis

The two-tailed Student's *t*-test was employed to assess the significance of the data. The data are presented as mean ± SEM. The level of significance was taken at $P < 0.05$.

RESULTS AND DISCUSSION

To determine capsaicin-induced apoptotic cell death in MBT-2 murine bladder cancer cells, we carried out two independent measurements using agarose gel electrophoresis and flow cytometry. Capsaicin induced DNA fragmentation in a concentration-dependent manner as depicted in Fig. 1A. Capsaicin (250 μM) also induced apoptosis in a time-related manner tested by flow cytometry by determining hypodiploid DNA content stained with PI (Bombeli *et al.*, 1997) as shown in Fig. 1B. Taken together, these results indicate that capsaicin induced apoptotic cell death in the MBT-2 cells.

In an attempt to identify the underlying signaling pathway of capsaicin-induced apoptosis, we first focused on the activation of caspase-3 which plays an important role in the formation of apoptotic bodies. Treatment of the cells with capsaicin induced caspase-3 activity in a time-dependent manner (Fig. 2A). To assess that the activation of the caspase-3 is required for the induction of apoptosis by capsaicin, we examined whether apoptotic cell death was blocked by the pretreatment with caspase inhibitors. Pretreatment of the cells with Z-VAD-fmk, the broad-spectrum caspase inhibitor, or DEVD-CHO, the caspase-3 inhibitor, significantly blocked capsaicin-induced marked reduction in cell viability measured by MTT assay (Fig. 2B) and apoptosis detected by flow cytometric analysis (Fig. 2C).

Since the release of cytochrome *c* from the mitochondria to the cytosol has been shown as the key step for caspase-3 activation (Haunstetter and Izumo, 1998; Liu *et al.*, 1996; Green and Reed, 1998), cytochrome *c* release was detected using a subcellular fractionation and a western blot analysis. As shown in Fig. 3, capsaicin caused a time-dependent decrease in mitochondrial cytochrome *c* and a concomitant increase in cytosolic cytochrome *c*.

Bcl-2 family protein is known as an upstream signal for cytochrome *c* release and subsequent caspase activation (Borner, 2003; Gross *et al.*, 1999). As shown in Fig. 4, capsaicin decreased the level of Bcl-2, an anti-apoptotic protein, with no changes of Bax expression, a pro-apoptotic protein, in whole cell lysates of MBT-2 cells. The ratio of Bax/Bcl-2 level in the capsaicin-treated cells was increased up to 3.7 times in a time-dependent manner. The similar result was also observed in hepatocarcinoma cells (Jung *et al.*, 2001). Taken together, these results suggest that capsaicin induces apoptosis through a signaling pathway of increase in the ratio of Bax/Bcl-2, cytochrome *c* release and caspase-3 activation in MBT-2 murine bladder tumor cells.

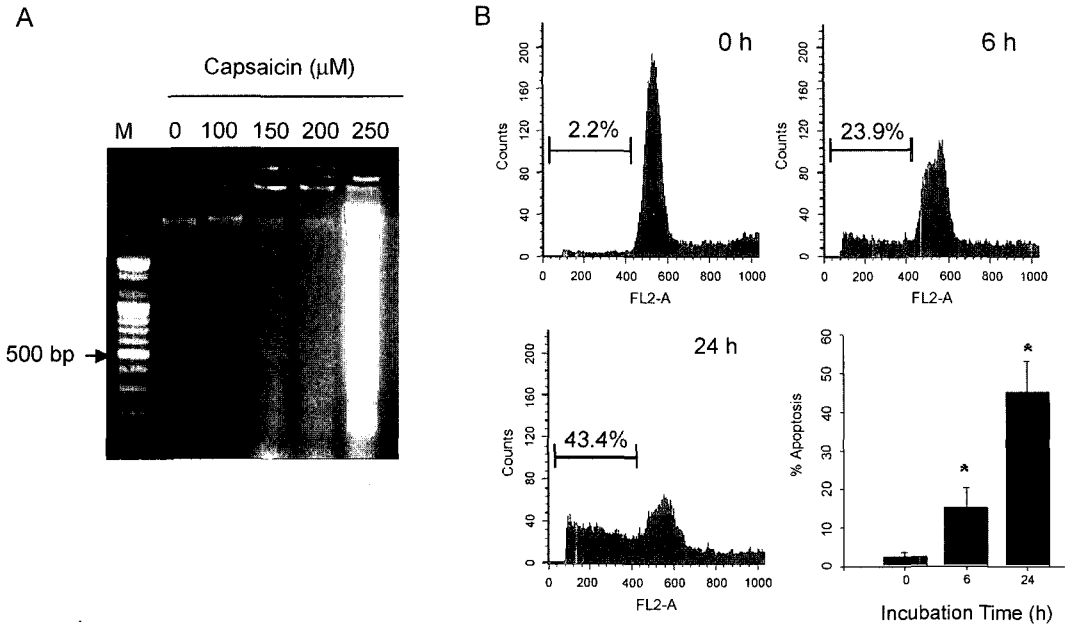

Fig. 1. Capsaicin-induced apoptosis of MBT-2 murine bladder tumor cells. In the experiments of (A) cells were treated for 24 h with or without each concentration of capsaicin. DNA was isolated from the cells and analyzed by 2% agarose gel electrophoresis. Lane M represent DNA size marker. In the experiments of (B) the cells were incubated with capsaicin (250 μM) for each designated time. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_0/G_1 peak was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs, the data represent the mean values of four replications with bars indicating SEM. *p<0.05 compared to control.

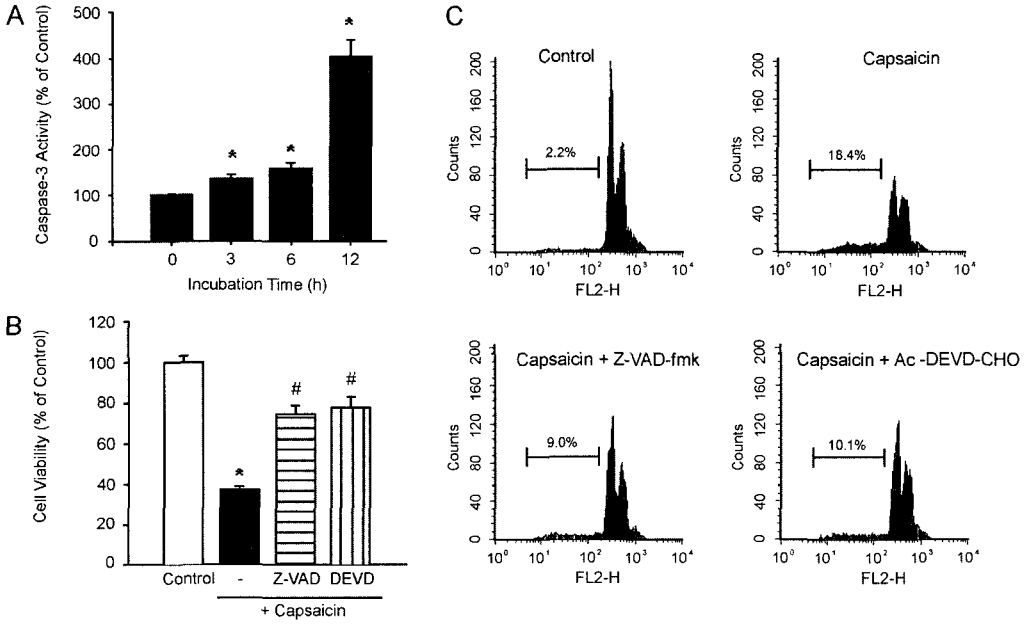

Fig. 2. Effects of caspase-3 inhibitors on the capsaicin-induced apoptotic cell death in MBT-2 cells. The data (A) show changes in caspase-3 activity as a function of time, which was measured by colorimetric assay as described in the text. In the experiments of (B and C) the cells were incubated with or without capsaicin (250 μM) for 24 h. The caspase inhibitors, Z-VAD-fmk and DEVD-CHO were given 30 min before capsaicin application. The cell viability was measured by the MTT assay and the measurement of apoptotic cells was the same as in Fig. 1. In bar graphs the data represent the mean values of four replications with bars indicating SEM. *p<0.05 compared to control. #p<0.05 compared to the group treated with capsaicin alone.

Although the results clearly demonstrate a potential for capsaicin as an anticancer agent for bladder cancers, the *possibility will be greater if capsaicin could enhance the* susceptibility of tumor cells to BCG to prevent the recur-

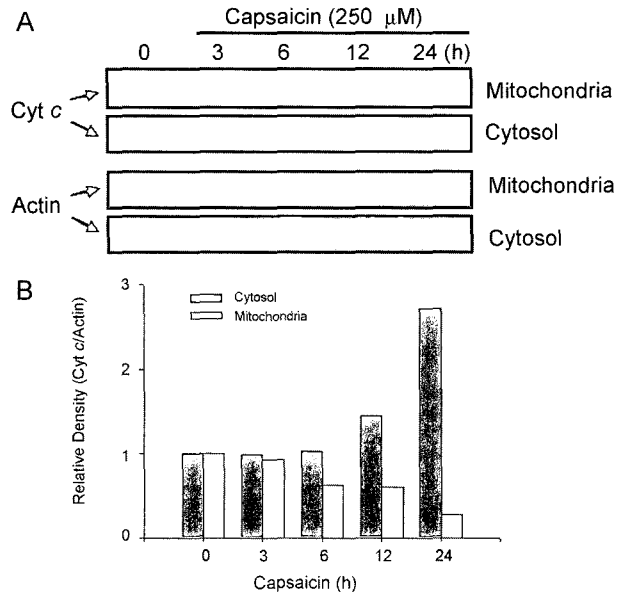

Fig. 3. Capsaicin induces cytochrome *c* release from mitochondria in MBT-2 cells. Proteins from subcellular fraction were separated on a 15% SDS-polyacrylamide gel. In the bar graph the data represent the relative intensity of the bands (cytochrome *c*/actin).

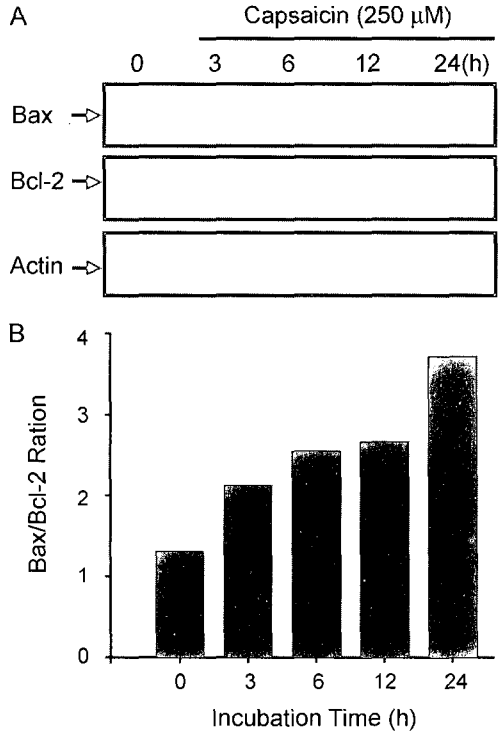

Fig. 4. The effects of capsaicin on the expression of Bcl-2 and Bax in MBT-2 cells. The cells were incubated with capsaicin (250 μM) for each designated time. Protein expression was analyzed by western blot as described in the text. The result is a representative of three independent experiments.

rence of the cancer. Since it has been reported that supplementation with an agent reducing ROS could enhance

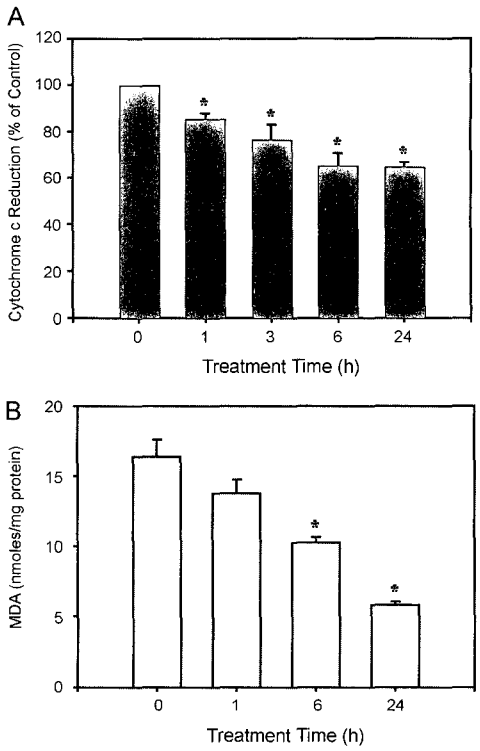

Fig. 5. Capsaicin (250 μM) reduces ROS production and lipid peroxidation in MBT-2 murine bladder tumor cells. The data (A) show changes in levels of ROS as a function of time, which was measured by cytochrome *c* reduction assay. In the experiments of (B) lipid peroxidation was expressed as malondialdehyde (MDA) concentration (nmoles/mg protein). The data represent the mean values of four replications with bars indicating SEM. *$p<0.05$ compared to control.

the antitumor effect of BCG (Pook *et al.*, 2002), we further examined whether capsaicin alters the cellular redox state. In fact, treatment of the cells with capsaicin at apoptosis-inducing concentration significantly suppressed the basal generation of ROS and lipid peroxidation as depicted in Fig. 5. The same result was also seen in capsaicin-treated glioblastoma and neuroblastoma cells (Lee *et al.*, 2000, 2002). There are reported evidences that tumor cells have increased metabolic rate and reactive ROS production (Szatrowski and Nathan, 1991) and that over-production or decreased elimination of superoxide anion provides tumor cells with a survival advantage over normal counterparts (Cerutti, 1985). Moreover, based on the previous report that hydrogen peroxide induces up-regulated expression of Bcl-2, a prototypic cell survival factor (Tu *et al.*, 1996), reduced level of ROS in this study might be responsible for the decreased expression of Bcl-2 in capsaicin-treated MBT-2 cells. More importantly, the result implies that capsaicin may enhance the antitumor effect of BCG, the most effective intravesical bladder cancer treatment.

In conclusion, capsaicin induced apoptosis through alteration of Bax/Bcl-2 ratio, cytochrome *c* release and

caspase-3 activation in MBT-2 murine bladder cancer cells. In addition, capsaicin reduced ROS production and lipid peroxidation in MBT-2 cells. These results strongly suggest that capsaicin may be a valuable intravesicle chemotherapeutic agent for bladder cancers.

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