2′-Benzoyloxyccinnamaldehyde Induces Apoptosis in Human Carcinoma via Reactive Oxygen Species*

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Dong Cho Han, Mi-Young Lee, Ki Deok Shin, Sun Bok Jeon, Jung Min Kim, Kwang-Hee Son, Hyoung-Chin Kim, Hwan-Mook Kim, and Byoung-Mog Kwon‡

From the Korea Research Institute of Bioscience and Biotechnology, 52 Uendong Yoosunggu, Taejeon 305-600, Korea

2′-Hydroxycinnamaldehyde (HCA),1 isolated from the stem bark of Cinnamomum cassia, was reported to have an inhibitory effect on farnesyl protein transferase activity in vitro (1). The cinnamaldehydes have also been shown to have various activities such as anti-angiogenic activity (2) and to be immunomodulators (3), CDK4/cyclinD1 kinase (4), and the proliferation of several human cancer cell lines including breast, leukemia, ovarian, lung, and colon tumor cells (5). However, little is known regarding the mechanism of apoptosis induced by cinnamaldehydes.

Anti-cancer treatment using cytotoxic drugs is considered to mediate cell death by activating key elements of the apoptosis program and the cellular response (6, 7). Proteolytic enzymes (caspases) are main effectors of apoptosis. Apoptosis seems to be induced if damage exceeds the capacity of repair mechanisms. Apoptosis was initially described by its morphological characteristics including cell shrinking, membrane blebbing, chromatin condensation, and nuclear fragmentation (8). Defects in apoptotic pathways are considered to contribute to a number of human diseases ranging from neurodegenerative disorders to malignancy. The notion that apoptosis might influence the malignant phenotype goes back to the early 1970s. Subsequent studies reveal high frequency of apoptosis in spontaneously regressing tumors and in tumors treated with cytotoxic anti-cancer agents.

Reactive oxygen species (ROS) are involved in the induction of apoptosis but are not the common mediator (9–11). ROS are involved in apoptosis induced by transforming growth factor-β (12) and by p53 (13, 14). Oxidative radical stress-induced apoptosis is partly suppressed by Bcl-2 (15). Bcl-2 may inhibit pro-oxidant-induced mitochondrial change and then prevent the formation of ROS. Cell death caused by abnormal redox was postulated to be one of the major causes of several diseases such as Parkinson’s disease.

For protection against increased level of ROS, cells possess several anti-oxidants or reductants that maintain the intracellular redox environment in a highly reduced state (11). Glutathione (GSH) is a major cellular reductant found in all eucaryotic cells (16). Cellular H2O2 and other peroxides are eliminated by seleno-enzyme GSH peroxidase-catalyzed reduction with GSH as substrate. The dosage of the redox signal is an important factor in the modulation of cell functions (17). Indeed, the cell cycle is arrested when cells are exposed to moderate levels of oxidative radical stress (18), whereas a very low dose of ROS induces mitogenic effects to stimulate cell proliferation. The mode of cell death is also shifted from apoptosis to necrosis, corresponding to increases in the level of oxidative radical stress (19).

The tumor suppressor p53 is known to be an important regulator for cell cycle progression and apoptotic cell death. The p53 tumor suppressor gene is inactivated by point mutation in approximately 50% of all human cancers. Such a high frequency of mutation in p53 indicates that loss of p53 function is a very important step in tumor progression. It is involved in the regulation of apoptosis induced by Bax (20), CD95 or DR5 (21), ROS (14), and cytochrome c (22). The p53 induces apoptosis through a three-step process: 1) the transcriptional induc-
tion of redox-related genes; 2) the formation of ROS; and 3) the oxidative degradation of mitochondrial components (14). Because p53 has been shown to be involved in apoptosis and >50% of human cancer has mutated p53, it is important for pharmacological drugs to be able to induce apoptosis in a p53-independent pathway.

Our study has provided a mechanism involving 2′-benzoyloxyconnamaldehyde (BMA) or HCA-induced apoptosis in p53-mutated cancer cell lines. The observed apoptotic activity of HCA and BCA was associated with ROS generation. This means that ROS can induce apoptosis in a p53-independent pathway. Furthermore, BCA significantly blocked tumor growth in a nude mouse assay, demonstrating BCA as a good drug candidate for cancer therapy.

EXPERIMENTAL PROCEDURES

Materials—All of the chemicals used in this experiments were purchased from Sigma. HCA and BCA were prepared by the reported method, and Me2SO was the solvent for the drug stock and dilution. Polyclonal anti-EGFR was purchased from Upstate Biotechnology. Antibodies against MAPK and phospho-MAPK, cleaved-caspase-3, cleaved-PARP, and PARP were purchased from Cell Signaling. PY-20, annexin V-FITC, anti-Bcl-X, anti-Fas ligand, and Z-YAD-fmk were purchased from BD Biosciences. Anti-Bcl-2 antibody was purchased from Santa Cruz Biotechnology and BD Biosciences.

Cell Culture—The cell lines used were obtained originally from ATCC. SW620 (human colon cancer cell line) and MDA-MD-231 (human breast cancer cell line) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and washed three times with lysis buffer. Cell cultures were maintained at 37 °C under a humidified atmosphere of 5% CO2 in an incubator. Exponentially growing cells were exposed to HCA or BCA at the indicated concentrations for different lengths of time.

Cell Proliferation Assays—Cells (5,000 cells) were seeded into 96-well plates in RPMI 1640 medium or Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. Collected cells were centrifuged for 3 min at room temperature, washed twice with phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4-7H2O, 0.24 g of KH2PO4, pH 7.2) containing 1% fetal bovine serum. Cell cultures were maintained at 37 °C under a humidified atmosphere of 5% CO2 in an incubator. Exponentially growing cells were exposed to HCA or BCA at the indicated concentrations for different lengths of time.

Cell Cycle Analysis—To analyze the DNA content by flow cytometry, cells were trypsinized from the culture flask. After centrifugation at 300 × g for 5 min at room temperature, the supernatant was removed. The cells were then washed twice with phosphate-buffered saline solution and fixed with 3 ml of ice cold 70% ethanol overnight. Fixed cells were harvested by centrifugation at 300 × g for 3 min at room temperature and washed twice with phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4-7H2O, 0.24 g of KH2PO4, pH 7.2) containing 1% fetal bovine serum. Collected cells were suspended in phosphate-buffered saline (100 μl of 106 cells/ml) and treated with 100 μg/ml RNase A at 37 °C for 30 min. Propidium iodide was added to a final concentration of 50 μg/ml for DNA staining, and 20,000 fixed cells were analyzed on a FACScalibur (BD Biosciences). Cell cycle distribution was analyzed using the Modfit program (BD Biosciences).

Detection of DNA Fragmentation—MDA-MB-231 cells were treated with 10 μM or 30 μM HCA or BCA for 48 h. Cells were harvested and resuspended with 0.5 ml of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 30 mM NaH2PO4, 50 mM NaF, 0.5 mM Na2VO4, 2 mM phenylmethylsulfonyl fluoride, and 1% aprotinin). After incubation on ice for 30 min, genomic DNA was extracted by removing proteins with phenol, precipitated with ethanol and sodium acetate, and separated on 1.5% agarose gel. DNA in the gel was then transferred to a nitrocellulose membrane and photographed under UV light.

Annexin V-FITC Staining—To analyze the apoptosis by flow cytometry, cells were collected 24 h after BCA or HCA treatment, washed twice with phosphate-buffered saline, and resuspended in 1× binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2) at a concentration of 1 × 106 cells/ml. 100 μl of cells were transferred to a 5-ml culture tube and 5 μl of annexin V-FITC and 10 μl of propidium iodide and mixed and incubated for 15 min at room temperature in the dark. 400 μl of 1× binding buffer was added into each tube, and stained cells were analyzed by flow cytometry.

Western Blotting and Immunoprecipitation—A 20-μg protein was resolved by 7.5 or 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Roche Applied Science). Membrane was blocked with 5% nonfat dry milk or TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). The primary antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA) or from Transduction Laboratories (Lexington, KY) or from BD Biosciences (San Diego, CA). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG from Jackson Immunolothics. The antibodies were used at dilution recommended by the manufacturers. Membrane was incubated with primary antibody for 2 h at room temperature, washed five times with TBS-T, and visualized with chemiluminescence peroxidase reagents (Roche Applied Science). For immunoprecipitation, 200 μg of lysates were incubated with primary antibody for 2 h at 4 °C in rotatory shaker and then 40 μl of protein A/G-agarose beads were added. After 1 h, beads containing lysates were centrifuged and washed three times with lysis buffer. Bead-bound proteins were resolved by SDS-PAGE and blotted using specific antibody.

Nude Mouse Xenograft Assay—For the evaluation of BCA for anti-tumor activity in vivo, SW620 human colon adenocarcinoma cells (3 × 105 cells/mouse) were implanted subcutaneously into the right flank of nude mice on day 0. Compound was dissolved in 0.5% Tween 80 and was administered intraperitoneally for 18 days or orally for 25 days at a concentration of 30 or 100 mg/kg/day. Tumor volumes were estimated as length (mm) × width (mm) × height (mm)/2. To determine the toxicity of the compound, the body weight of tumor-bearing animals was measured. On day 18 or 25, the mice were sacrificed and the tumors were weighed.

RESULTS

Inhibition of Tumor Cell Growth—To determine whether connamaldehyde inhibits cancer cell proliferation, MDA-MB-231 and SW620, both of which have mutated p53, were treated with HCA or BCA at different concentrations (0-100 μM) for 48 h (Fig. 1, A and B). HCA exhibited a dose-dependent inhibition of cell growth in a broad range of concentrations, and the IC50 value of HCA for in vitro growth inhibition was ~10 μM. BCA was synthesized as an analogue of HCA, and it showed similar activity as HCA with the exception of low toxicity in beagle dog. One of the characteristic features of HCA or BCA treated cells was rounding of cell morphology and detachment from culture plate. Therefore, for the further studies, cells were collected both from the attached and the suspended cells.

To determine the effect of BCA on the cell cycle, FACS analysis was performed using human cancer cell line SW620 and MDA-MB-231. The concentration of BCA was 10 μM. The cells were harvested at 6, 12, 24, and 48 h after treatment and analyzed with a FACScalibur. As shown in Fig. 1, C and D, BCA treatment of SW620 cells for 12 h increases in DNA content to tetraploid, indicating cell cycle arrest at G2/M phase. In addition, significant fractions of cells were detected from subdiploid range, suggesting apoptosis of treated cells. Similarly, HUSMC was treated with 10 μM BCA and analyzed using FACScalibur shown in Fig. 2. BCA showed no effect on the cell cycle profile of the HUSMC, the normal human cell line. In addition, BCA could not affect the cell cycle distribution of mouse fibroblast NIH3T3 cells (data not shown). These results suggest that BCA has tumor selectivity for inducing cell cycle arrest at G2/M phase.

Cinnamaldehyde Induces Apoptosis in MDA-MB-231 and SW620 Cancer Cells—Because of subdiploid population from HCA- or BCA-treated cells, we tested whether HCA or BCA can induce chromosomal DNA fragmentation. As shown in Fig. 3, both HCA and BCA caused chromosomal degradation in MDA-

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MB-231 and SW620 cells. PARP is involved in DNA repair in response to environmental stress and one of the apoptosis markers. As shown in Fig. 3, PARP degradation was induced by HCA and BCA from MDA-MB-231 and SW620 cells. These results suggested that HCA and BCA induced apoptosis of MDA-MB-231 and SW620 cells in a p53-independent manner.

To distinguish between necrosis and apoptosis, FACS analysis was carried out after staining cells with annexin V-FITC and propidium iodide. As shown in Fig. 4, 30 μM HCA induced apoptosis from >40% MDA-MB-231 cells. Similarly, 30 μM BCA induced apoptosis from >35% SW620 cells. 30 μM BCA caused significant necrosis of MDA-MB231 cells compared with HCA, and this necrosis was not detected from SW620. These results imply that HCA and BCA have different efficiencies on inducing apoptosis of MDA-MB-231 and SW620 cells.

To study BCA-induced apoptosis in detail, we monitored time-course response of cells after BCA treatment. 0.5 h after BCA treatment, MDA-MB-231 cells lost spreading, becoming round in morphology (Fig. 5A). 12 h after the treatment, caspase-3 was activated and PARP was degraded (Fig. 5B and C).

To examine further the effect of caspase-3 activation on BCA-induced apoptosis in the MDA-MB-231 cells, we used the pan-caspase inhibitor Z-VAD-fmk to determine whether
Caspase-3 activation is linked to apoptosis after BCA treatment. As expected, pretreatment of cells with Z-VAD-fmk could block BCA-induced chromosomal DNA fragmentation (Fig. 6A), caspase-3 activation (Fig. 6B), and PARP degradation (Fig. 6C). These results suggest that BCA may induce apoptosis through caspase-3 activation.

**BCA Induced Sustained EGF Receptor and p38 Kinase Activation**—To characterize mechanisms of BCA-induced apoptosis in MDA-MB-231 cells, we analyzed the profile of tyrosine phosphorylation of proteins after cells were treated with 30 μM BCA (Fig. 7A). Interestingly, we found that tyrosine phosphorylation of an ~170-kDa protein was enhanced 0.5 h after treatment of BCA and reached maximum 12 h after treatment. MDA-MB-231 has been known to highly express EGF receptor (23). To test whether highly phosphorylated protein is the EGF receptor, the EGF receptor was immunoprecipitated by using EGF

![Figure 2: Representative cell cycle distribution of HUSMC cells in the presence and absence of BCA.](image)  
A and B, HUSMC cells were treated with 10 μM BCA (B) or with 0.1% Me2SO (DMSO) (A) for 6, 12, 24, and 48 h and harvested, fixed, and stained with propidium iodide. 20,000 stained cells then were subjected to FACScalibur analysis to determine the distribution of cells.

![Figure 3: Degradation of chromosomal DNA and PARP were induced by HCA and BCA.](image)  
MDA-MB-231 (A) or SW620 (B) cells were treated with different concentrations of HCA or BCA or with vehicle solvent (0.1% Me2SO (DMSO)) for 48 h, and fragmentation of chromosomal DNA was monitored as described under “Experimental Procedures.” The same lysates were resolved by SDS-PAGE and immunoblotted with anti-PARP antibody (C).
receptor antibody, resolved by SDS-PAGE, and blotted using PY-20 antibody (Fig. 7, B and C). As shown in Fig. 7B, tyrosine phosphorylation of EGF receptor was enhanced 0.5 h after treatment of BCA and further maintained until 48 h after treatment. This is unusual case because EGF receptor was activated for 1 h after EGF treatment and quickly returned basal level. This prolonged activation of EGF receptor might be involved BCA-induced apoptosis.

Because BCA induced sustained activation of EGF receptor, we tested the effect of BCA on the activity of ERK1/2, JNK, and p38 MAPK using antibodies specifically recognizing the active phosphorylated forms (Fig. 7, D–F). p38 kinase was activated 0.5 h after treatment of BCA and sustained its activation for 24 h (Fig. 7E). However, we could not detect any significant increase of activation of ERK1/2. This is unexpected because ERK1/2 is well known downstream of EGF receptor signaling. The lack of activation of ERK1/2 by EGF receptor was postulated to be due to oncogenic K-Ras (23, 24). In addition, we could not see activation of JNK by treatment of BCA.

To examine the importance of EGF receptor signaling for the induction of apoptosis by BCA, we used AG1478 to inhibit EGF receptor. When cells were pretreated with the inhibitor, we could not detect any enhancement of survival of BCA-treated cells (Fig. 8). This result was further confirmed by using a cell line, B104-1-1, expressing constitutive ErbB-2, an EGF receptor family member. When cell cycle profiles of B104-1-1 or NIH3T3 cell lines were examined after treatment of Me2SO or BCA, we could not detect any change of profile of cell cycle (data not shown). These results suggest that EGF receptor may not be involved in BCA-induced apoptosis and cell cycle arrest at G2/M phase.

When cells were treated with BCA, they became round in morphology within 1 h and detached from the bottom of culture dishes (Fig. 5). It was reported that cell detachment triggers p38 kinase-dependent overexpression of Fas ligand (25). This suggests the possibility that BCA may induce apoptosis through p38 activation and overexpression of Fas ligand. However, we could not detect any overexpression of Fas ligand after BCA treatment (data not shown). Furthermore, when cells were pretreated with SB203580, a p38 kinase inhibitor, we could not observe any enhancement of survival of BCA-treated cells (Fig. 8). These results implied that BCA-induced apoptosis is not through p38 pathway. Interestingly, pretreatment of cells with N-acetyl-cysteine (NAC) efficiently blocked BCA-induced apoptosis (Fig. 8). This finding suggests that oxidative stress may be involved in BCA-induced apoptosis.

Cinnamaldehyde-induced Apoptosis Was Caused by Elevation of ROS Content—ROS are constantly generated under normal conditions as a consequence of aerobic metabolism state (9–11). Under normal conditions, anti-oxidant system minimizes the adverse effect caused by ROS. When ROS overwhelm the biological defense of the cell, the result is oxidative stress. Potentiation of stress kinase is a common feature of transformed cells by ROS. Therefore, we measured ROS production...
in tumor cells in the absence or presence of BCA. As shown in Fig. 9A, the ROS production was elevated in tumor cells by treatment of BCA and HCA (data not shown) and was inhibited by pretreatment of cells with glutathione. In addition, BCA-induced caspase-3 activation and PARP degradation were also abolished by the pretreatment of cells with glutathione. Furthermore, BCA-induced EGFR activation was abolished by anti-oxidants, suggesting that EGFR is downstream of ROS. These results support that BCA induced apoptosis of tumor cell through the elevation of ROS contents.

GSH has dual roles in the regulation of cellular responses to xenobiotics. In addition to scavenging ROS as a substrate of glutathione peroxidase, it can also neutralize drugs via the reaction between the thiol group of GSH and the reactive functional groups such as an α,β-unsaturated group of substrate. This would prevent BCA action and could also account for the changes seen in the presence of NAC or GSH. Therefore, we incubated BCA with NAC or GSH for 1 h and analyzed its mixture using reverse-phase high pressure liquid chromatography. Even though BCA and NAC or GSH were incubated for 1 h, we could not observe any changes of their retention times and integration of its peaks, supporting that BCA and NAC or

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**Fig. 5.** Time courses of morphology and cleavage of caspase-3 and PARP. A, MDA-MB-231 cells were treated with 30 μM BCA for different times, and lysates were prepared with RIPA buffer. Before making lysates, cell morphology was imaged by Nikon fluorescence microscope. Lysates were resolved by SDS-PAGE and immunoblotted with anti-cleavage-specific caspase-3 (B) or anti-cleavage-specific PARP (C).

**Fig. 6.** BCA-induced chromosomal DNA degradation, caspase-3 activation, and PARP degradation was blocked by Z-VAD-fmk, a broad caspase inhibitor. A–C, MDA-MB-231 cells were treated with Z-VAD-fmk (40 μM). After 30 min, 30 μM BCA were treated and fragmentation of chromosomal DNA was monitored as described under “Experimental Procedures.” The same lysates were resolved by SDS-PAGE and immunoblotted with anti-cleaved caspase-3 or anti-PARP antibody. DMSO, Me₂SO.
GSH were not covalently conjugated (data not shown). This result can exclude the possibility that BCA was inactivated by NAC or GSH through chemical reaction.

Oxidative Stress Mediated Down-regulation of Bcl-2—Because inhibition of apoptosis by anti-apoptotic Bcl-2 and Bcl-xL is associated with a protection against ROS (15), we tested the HCA and BCA effect on Bcl-2 and Bcl-xL. As shown in Fig. 10, amounts of Bcl-2 and Bcl-xL were decreased by either HCA or BCA treatment. These results suggest that down-regulation of Bcl-2 and Bcl-xL by HCA or BCA is correlated with the induction of the cell death in breast and colon cancer cell lines.

In Vivo Effect of BCA on Tumor Cell Growth—SW620 tumor xenograft model of nude mice was used to investigate inhibitory activity of BCA on tumor growth. SW620 cells were implanted subcutaneously into the right flank of a nude mouse on day 0, and the compound was intraperitoneally administered at a concentration of 30 or 100 mg/kg/day. To determine the toxicity of the compound, the body weight of tumor-bearing animals was measured. On day 18, the mice were sacrificed and the tumors were weighed. On 18 days after implantation, tumor volume was decreased by 88% compared with control mice (Fig. 11A).

Because BCA is a derivative of cinnamaldehyde, an ingredient of food, we examined the anti-tumor activity of BCA when orally administrated. The BCA was given daily using an orogastric feeding tube with 0.5% Tween 80 as a control. As shown in Fig. 11B, daily oral administration of BCA (100 mg/kg) significantly reduced tumor growth (41.1%, p < 0.001). However, body weight loss was not observed in mice that were administrated with BCA and implanted with SW620 cells. This result

GSH were not covalently conjugated (data not shown). This result can exclude the possibility that BCA was inactivated by NAC or GSH through chemical reaction.
suggests that BCA is an effective anti-cancer agent without any detectable toxicity.

**DISCUSSION**

Cinnamaldehyde, isolated from edible source, has been known to have anti-tumor activity. However, the mechanism is still in a debate. In this paper, we demonstrate that cinnamaldehydes, HCA, and its analogue BCA induce apoptosis of cancer cells through elevation of ROS and caspase-3 activation. When BCA was administrated intraperitoneally or orally into nude mouse, it inhibited tumor growth without any detectable weight change. These results suggest that BCA is an effective anti-cancer agent without any detectable acute toxicity. In the case of adriamycin, tumor growth was more efficiently inhibited than that by BCA. However, adriamycin-treated mice lost body weight, suggesting its toxicity (data not shown).

We used natural form of cinnamaldehyde (HCA) and its synthetic derivative (BCA) to characterize its anti-cancer activity. Surprisingly, HCA or BCA activated EGF receptor that...
has been known as a mitogenic component (Fig. 7). The activation of the EGF receptor tyrosine kinase in turn stimulated the MAPK. In most target cells, these events resulted in proliferation. However, BCA-induced EGF receptor activation did not induce cell proliferation. In contrast, it caused cell cycle arrest and apoptosis (Figs. 1, 3, and 4). There are several reports that activation of EGF receptor causes cell death (26–29). Studies of A431 and MDA-MB-468 suggested that treatment of EGF inhibits cell proliferation and induces apoptosis. The mechanism of EGF-induced apoptosis is not well characterized. In our case, the activation of EGF receptor may not be the cause of BCA-induced apoptosis per se. Pretreatment of cancer cells with inhibitor of EGF receptor, AG1478, could not block BCA-induced apoptosis.

Treatment of cells with BCA activates p38 kinase that is known to regulate cell cycle and apoptosis. However, pretreatment of cells with SB203580 could not rescue BCA-induced cell cycle arrest and apoptosis (Fig. 8). Fas ligand was reported to be overexpressed in a p38 kinase-dependent manner when cells were detached (25). However, we could not detect any overexpression of Fas ligand after BCA treatment (data not shown). These results imply that BCA-induced apoptosis is independent of p38 kinase.

How does BCA activate EGF receptor? Currently, we do not know how BCA activates EGF receptor. One possibility is that BCA activates the upstream regulator of EGF receptor. Recently, growing evidences have indicated that cellular ROS plays an essential role not only in cell survival but also in cellular signaling system (11). When cells are stimulated with ROS, signals are transferred through the same signaling pathways as those triggered by growth factors (30–32). H2O2 activates EGF receptor, MAPKs, protein kinase C, and other kinases. The level of ROS in MDA-MB-231 cells was increased by treatment of BCA, and pretreatment of cells with glutathione one abolished BCA-induced ROS (Fig. 9). Pretreatment of glutathione blocked not only ROS but also phosphorylation of EGF receptor. These findings indicate that BCA-induced ROS causes the activation of EGF receptor. The reduced cellular environment is maintained primarily by glutathione, which is 5–10 μM inside of the cell (11). The life span of ROS within the cell is very short because they are quenched by cellular antioxidants or because the ROS rapidly oxidize cellular macromolecules.

Cell detachment has been shown to cause down-regulation of anti-apoptotic molecule Bcl-xL (25, 33). The Bcl-2 family proteins were involved in positive and negative regulation of apoptosis (34, 35). Among them, Bcl-2 and Bcl-xL were negative regulators of apoptosis, preventing cells from suicide. As shown in Fig. 10, the amounts of Bcl-2 and Bcl-xL were significantly down-regulated by BCA or HCA. ROS formation came mainly from the mitochondria and NADPH oxidase. Bcl-2, in addition to its role to maintain permeability of outer mitochondrial membrane, might act as an anti-oxidant partner to block a putative ROS-mediated step in the cascade of apoptosis (36). Overexpression of Bcl-2 shifted the cellular redox potential to a more reduced state (37). These results imply that HCA and BCA may use mitochondria to amplify death signals.

How does BCA induce ROS accumulation? It is hard to guess what is the target molecule(s) of HCA or BCA. ROS are generated in cells by several pathways. Approximately 1–2% of electrons in the mitochondria respiratory chain were leaks to generate O2 in reaction mediated by coenzyme Q and ubiquinone and its complexes (9). Thus, mitochondria are believed to be a major site of ROS production in vivo. Another site of ROS production was Rac1-dependent plasma membrane NADPH oxidase (38). To identify the source of ROS, diphenyliodonium, an NADPH oxidase inhibitor, was treated with BCA. However, we could not see any increase of cell viability by diphenyl iodonium treatment (data not shown). This result implies that BCA-induced ROS may come from mitochondria. Therefore, it is possible that down-regulation of Bcl-2 and Bcl-xL may affect...
mitochondrial integrity, changing it to leak more electrons to produce more ROS.

In summary, the cinnamaldehyde (HCA and BCA) exerts anti-tumor effects by inducing G2/M phase arrest and apoptosis through accumulation of ROS and activation of caspase-3 in a p53-independent manner. The anti-tumor activity shown in cell culture and animal model supports its promise as an anti-tumor compound.

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