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Sodium orthovanadate potentiates EGCG-induced apoptosis that is dependent on the ERK pathway

Yun-Jung Choi,[a,1] Sung-Yup Lim,[b,1] Ju-Hyung Woo,[a] Young-Ho Kim,[a] Young Kyu Kwon,[b] Seong-Il Suh,[c] Sung-Hee Lee,[a] Wha-Youl Choi,[d] Jong-Guk Kim,[d] In-Seon Lee,[e] Jong-Wook Park,[a] and Taeg Kyu Kwon[a,*]

[a] *Department of Immunology, School of Medicine, Keimyung University, 194 DongSan-Dong Jung-Gu, Taegu 700-712, South Korea*
[b] *Departments of Prescription and Physiology, College of Oriental Medicine, Kyungsan University, South Korea*
[c] *Department of Microbiology, School of Medicine, Keimyung University, Taegu, South Korea*
[d] *Department of Microbiology, College of Natural Sciences, Kyungpook University, Taegu, South Korea*
[e] *Department of Food Science and Technology, Keimyung University, Taegu, South Korea*

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Abstract

Epigallocatechin-3-gallate (EGCG) is a potent chemopreventive agent in many test systems and has been shown to inhibit tumor promotion and induce apoptosis. In the present study, we determined the effect of vanadate, a potent inhibitor of tyrosine phosphatase, on EGCG-induced apoptosis. Investigation of the mechanism of EGCG or vanadate-induced apoptosis revealed induction of caspase 3 activity and cleavage of phospholipase-γ1 (PLC-γ1). Furthermore, vanadate potentiated EGCG-induced apoptosis by mitogen-activated protein kinase (MAPK) signaling pathway. Treatment with EGCG plus vanadate for 24 h produced morphological features of apoptosis and DNA fragmentation in U937 cells. This was associated with cytochrome *c* release, caspase 3 activation, and PLC-γ1 degradation. EGCG plus vanadate activates multiple signal transduction pathways involved in coordinating cellular responses to stress. We demonstrate a requirement for extracellular signal-regulated protein kinase (ERK), a member of the mitogen-activated protein kinase family in EGCG plus vanadate-induced apoptosis in U937 cells. Elevated ERK activity that contributed to apoptosis by EGCG plus vanadate was supported by PD98059 and U0126, chemical inhibitor of MEK/ERK signaling pathway, prevented apoptosis. Taken together, our finding suggests that ERK activation plays an active role in mediating EGCG plus vanadate-induced apoptosis of U937 cells and functions upstream of caspase activation to initiate the apoptotic signal.

Keywords: EGCG; Vanadate; U937 cells; Apoptosis; Caspase 3; ERK

Programmed cell death, or apoptosis, is a normal physiological process that occurs during embryonic development, as well as in the maintenance of tissue homeostasis, and elimination of unwanted or damaged cells from multicellular organisms [1,2]. The aberrant regulation of apoptosis has been observed in many forms of human disease such as neuronal disease, autoimmune disease, AIDS, and cancer [2]. Many therapeutic agents eliminate tumor cells by inducing apoptotic cell death [2]. Therefore, understanding the mechanism of apoptosis has important implications in the prevention and treatment of many diseases.

Tea is one of the most popular beverages in the world and the possible beneficial health effects have received a great deal of attention. Polyphenols are the most significant group of tea components, especially the catechin group of flavonols. The major tea catechins are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EG), (+)-gallocathechin, and (+)-catechin. EGCG has been considered to be a major constituent of tea [3–5]. These polyphenol components are known to have antioxidative activities due to their radical scavenging and metal chelating functions as well as anti-mutagenic activities

* Corresponding author. Fax: +82-53-250-7074.
E-mail address: kwontk@dsmc.or.kr (T.K. Kwon).
[1] Contributed equally to this work.

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[6–8]. Recently, Lin and Lin [9] reported that EGCG could prevent the binding of NFκB to the inducible nitric oxide synthase (iNOS) promoter, thereby inhibiting the induction of iNOS transcription. It has been suggested that EGCG may play a role in preventing carcinogenesis and anti-inflammation. However, conflicting results regarding its anti-cancer effect have been reported. Anti-apoptotic effects of EGCG include inhibition of AP-1 transcription activity and AP-1 DNA binding activity in JB6 cells by arsenite [10]. However, Islam et al. [11] reported induction of apoptosis by EGCG treatment via activation of caspase 3 on human chondrosarcoma cells. The purpose of the present study was to characterize further the biological consequences of EGCG or EGCG plus protein tyrosine phosphatase inhibitor (sodium orthovanadate) treatment in monocytic leukemia U937 cells. We found that these compounds induce apoptosis in human monoblastic leukemia U937 cells. In particular, the relationship between cytochrome *c* release, caspase 3 activity, and expression levels of IAP family proteins was analyzed. Our results indicate that vanadate can markedly potentiate EGCG-induced apoptosis in U937 cells, in addition, EGCG plus vanadate triggers cytochrome *c* release as an early event preceding caspase activation. The present study sought to examine the roles of the MAPK signaling pathways in regulating ECGC plus vanadate-induced apoptosis in U937 cells. Although ERK and p38 were found to be activated in response to EGCG plus vanadate treatment, only ERK activity appears to be involved in regulating cell survival. We provide evidence that ERK is important in mediating EGCG plus vanadate-induced apoptosis through a caspase 3 activity-dependent mechanism.

Materials and methods

Cell cultures. Human leukemia U937 cells were obtained from the American Type Culture Collection (ATCC: Rockville, MD, USA). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium, containing 10% fetal calf serum (FCS), 20 mM Hepes buffer, and 100 μg/ml gentamycin. They were cultured at 37 °C in a humidified chamber containing 5% CO_2. EGCG was directly added to cell cultures at the indicated concentrations.

Drugs and materials. Anti-cIAP1, anti-cIAP2, and anti-HSP70 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-p38 antibodies were purchased from New England Biolabs (Beverly, MA). Antibodies against the following proteins were purchased from the indicated suppliers: cytochrome *c* from PharMingen (San Diego, CA), actin from sigma (St. Louis, MO), and XIAP from R&D systems (Minneapolis, MN). Sodium orthovanadate was purchased from Biomol (Plymouth Meeting, PA, USA). The MEK1/2 inhibitor, PD98059, U0126, and the p38 inhibitor (SB203580) were obtained from CalBiochem (San Diego, CA). EGCG and other chemicals were purchased from Sigma.

Western blotting. Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4 °C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Cell count and flow cytometry analysis. Cell counts were performed using a hemocytometer. Approximately 1×10^6 U937 cells were suspended in 100 μl PBS and 200 μl of 95% ethanol was added while vortexing. The cells were incubated at 4 °C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg RNase. Incubation was continued at 37 °C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

DNA fragmentation assay. After treatment with drugs, U937 cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10,000*g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml of ethidium bromide.

Caspase 3 activity assay. To evaluate caspase 3 activity, cell lysates were prepared after their respective treatment with EGCG or sodium orthovanadate. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl of reaction buffer (1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, and 10% glycerol) containing the caspase 3 substrate (DEVD–pNA) at 5 μM. Lysates were incubated at 37 °C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Analysis of cytochrome c release. Cells (2×10^6) were harvested, washed once with ice-cold phosphate-buffered saline, and gently lysed for 2 min in 80 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris–HCl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Lysates were centrifuged at 12,000*g* at 4 °C for 10 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). The resulting cytosolic fractions were used for Western blot analysis with an anti-cytochrome *c* antibody.

Results

Apoptosis can be induced in cells by treatment with EGCG and sodium orthovanadate

Apoptotic cell death is characterized by chromatin condensation, membrane blebbing, intranucleosomal fragmentation of DNA, and apoptotic body formation [1]. Another feature of apoptotic cell death is the activation of caspase 3 protease in response to death-inducing stimuli resulting in the cleavage of PLC-γ1 [12,13]. To determine whether treatment with EGCG-induced apoptosis was associated with the activation of the caspase 3 and cleavage of PLC-γ1, we determined the cleavage of PLC-γ1 and caspase 3 level and activity in U937 cells that had been exposed to various concentrations of EGCG. Caspase 3 is activated by proteolytic processing of the 32 kDa form into two

smaller subunits. Activity of caspase 3 during EGCG-induced apoptosis was measured by a decrease in the proenzyme level using Western blot analysis and a proteolytic activity with a chromogenic substrate. As shown in Fig. 1A, treatment with EGCG resulted in a decrease in levels of caspase 3 in U937 cells exposed to 150–200 μM EGCG for 24 h. Caspase 3 represents one of the key proteases known to be responsible for the cleavage of poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase C-δ (PKC-δ), and other substrates [14–16]. Among the downstream targets of activated caspase 3 in vivo, PLC-γ1 has recently been shown to be cleaved into a 60 kDa fragment [12]. Subsequent Western blotting demonstrated proteolytic cleavage of PLC-γ1 in U937 cells after 24 h of 150 μM EGCG. The cleavage of PLC-γ1 was dose-dependent in U937 cells. To further investigate and quantitate the proteolytic activity of caspase 3, we performed an in vitro assay based on the proteolytic cleavage of DEVD–pNA by caspase 3 into the chromophore *p*-nitroanilide (pNA). U937 cells showed a 3-fold increase in DEVD–pNA cleavage after 24 h exposure to 150 μM EGCG.

Functional stimulation by sodium orthovanadate is accompanied by increase of tyrosine phosphorylation of proteins due to the stimulation of a kinase or more likely, to the inhibition of tyrosine phosphatase activity [17–20]. In order to understand the mechanism of action of vanadate in the induction of apoptosis, we determined the cleavage of PLC-γ1 and caspase 3 level and activity in U937 cells that had been exposed to various concentrations of sodium orthovanadate. As shown in Fig. 1B, treatment with sodium orthovanadate resulted in a decrease in levels of pro-caspase 3 in U937 cells exposed to 50–75 μM sodium orthovanadate for 24 h. When cells were treated for 24 h with high concentration of sodium orthovanadate, activity of caspase 3 and the cleavage of PLC-γ1 increased in a dose-dependent manner.

Sodium orthovanadate enhances EGCG-induced caspase 3 activity

To elucidate whether cotreatment of the vanadate and EGCG had any effect on cell viability and apoptosis induction, U937 cells were treated for 24 h with 10 μM EGCG, 25 μM sodium orthovanadate, and cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate. At the cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate, significant loss of viability was detected during the 24 h of treatment. However, after treatment with 10 μM EGCG and 25 μM sodium orthovanadate for 24 h, 89% and 91% of the cells survived in culture, respectively (Fig. 2A). We have previously shown that 10 μM EGCG and 25 μM sodium orthovanadate treated cells did not cause activation of caspase 3 and cleavage of PLC-γ1 (Figs. 1A and B). To elucidate whether reduction of cell viability was caused by apoptosis, three established criteria were subsequently used to assess apoptosis in our system. First, apoptosis was determined in the U937 cells using FACS analysis demonstrating hypodiploid DNA. As shown in Fig. 2B, cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate in U937 cells resulted in a markedly increased accumula-

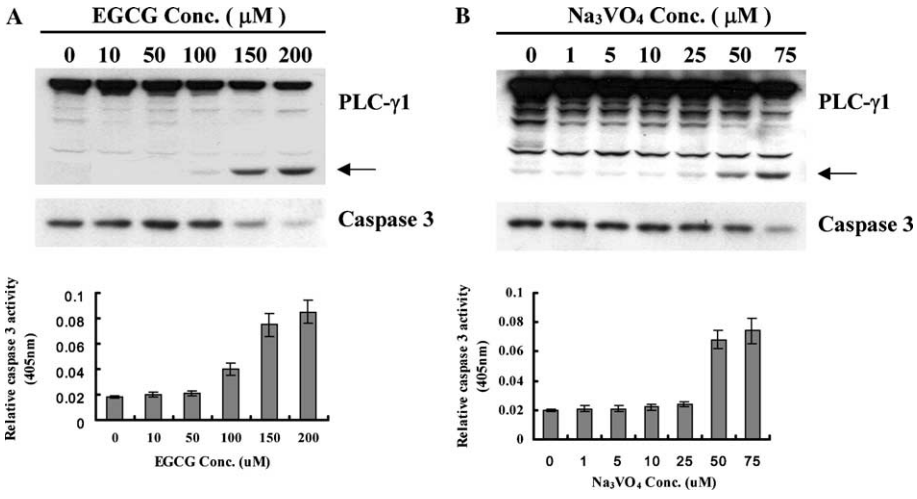

Fig. 1. Effect of EGCG and sodium orthovanadate on caspase-specific cleavage of PLC-γ1 and caspase 3 activity. (A) Cells were treated with the indicated concentrations of EGCG. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for caspase 3 and PLC-γ1. The proteolytic cleavage of PLC-γ1 is indicated by arrow. Enzymatic activities of caspase 3 were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (DEVD–pNA) in a 100 μl assay buffer for 2 h at 37 °C. The release of chromophore *p*-nitroanilide (pNA) was monitored spectrophotometrically (405 nm). Data are mean values from three independent experiments and bars represent standard deviations. (B) U937 cells were treated with sodium orthvanadate for 24 h and harvested in lysis buffer. Western blot for caspase 3 and PLC-γ1, and caspase 3 activity were determined as described above.

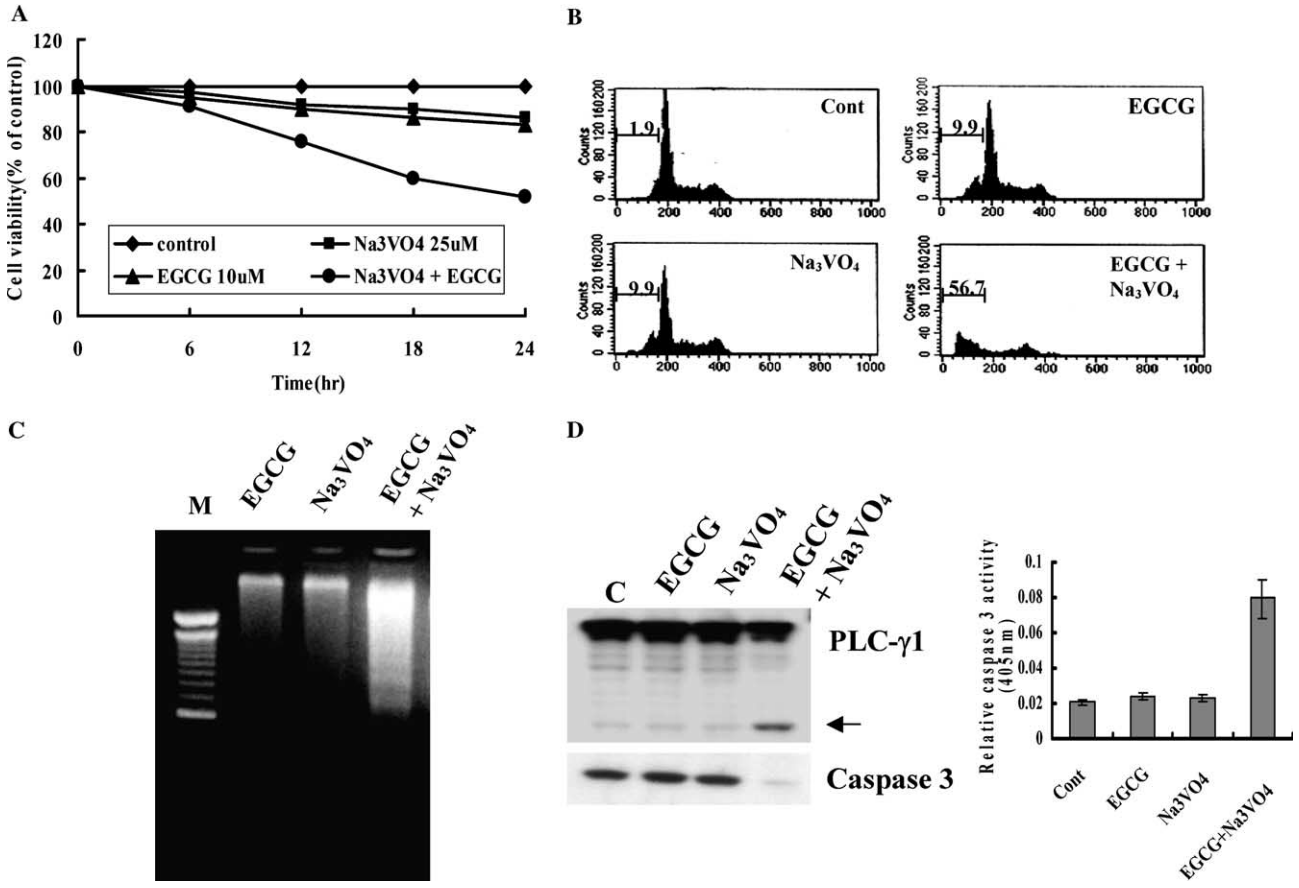

Fig. 2. EGCG plus vanadate induces apoptosis in U937 cells. (A) U937 cells were treated with vehicle (control), 10 μM EGCG, 25 μM sodium orthovanadate, and 10 μM EGCG plus 25 μM sodium orthovanadate for various time periods. Cell death was determined by using trypan blue exclusion. (B) FACS analysis of apoptotic cells. Cells were treated for 24 h with vehicle (cont), 10 μM EGCG, 25 μM sodium orthovanadate, and 10 μM EGCG plus 25 μM sodium orthovanadate and then evaluated for DNA content after propidium iodide staining. (C) Fragmentations of genomic DNA in cells were treated for 24 h with 10 μM EGCG, 25 μM sodium orthovanadate, and 10 μM EGCG plus 25 μM sodium orthovanadate. Fragmented DNA was extracted and analyzed on 2% agarose gel. (D) U937 cells were treated for 24 h with vehicle (cont), 10 μM EGCG, 25 μM sodium orthovanadate, and 10 μM EGCG plus 25 μM sodium orthovanadate and then harvested in lysis buffer. Equal amounts of soluble lysates (40 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against PLC-γ1 and caspase 3. The proteolytic cleavage of PLC-γ1 is indicated by arrow. Caspase 3 activity was determined as described in Fig. 1A.

tion of sub-G1 phase. Second, we analyzed DNA fragmentation, which is another hallmark of apoptosis. Following agarose gel electrophoresis of U937 cells treated with 10 μM EGCG and 25 μM sodium orthovanadate for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed. In contrast, DNA fragmentation in U937 cells treated with EGCG alone or orthovanadate alone was significantly reduced (Fig. 2C). In addition, because cells undergoing apoptosis execute the death program by activating caspases and cleavage of PLC-γ1, we analyzed whether cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate gave rise to the activation of caspase 3, a key executioner of apoptosis. The Western blot analyzed expression levels of pro-caspase 3 and cleavage of PLC-γ1. As shown in Fig. 2D, exposure to EGCG and vanadate led to a disappearance of the 32-kDa precursor, accompanied by a concomitant revealed cleavage of PLC-γ1. Taken together, these results indicate that cotreatment of the vanadate and EGCG potentiates to induce apoptosis in U937 cells.

Caspases mediate cotreatment of the vanadate and EGCG-induced apoptosis

To address the significance of caspase activation in cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate-induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk (benzyloxycarbony-Val-Ala-Asp-fluoromethyl ketone). Cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate strongly stimulated caspase 3 protease activities, but z-VAD-fmk pretreated cells abolished EGCG plus vanadate-induced caspase 3 activities (Fig. 3A). Furthermore, cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate in U937 cells generated a 60 kDa cleavage product of PLC-γ1. However, z-VAD-fmk pretreated cells significantly inhibited a 60 kDa cleavage product of

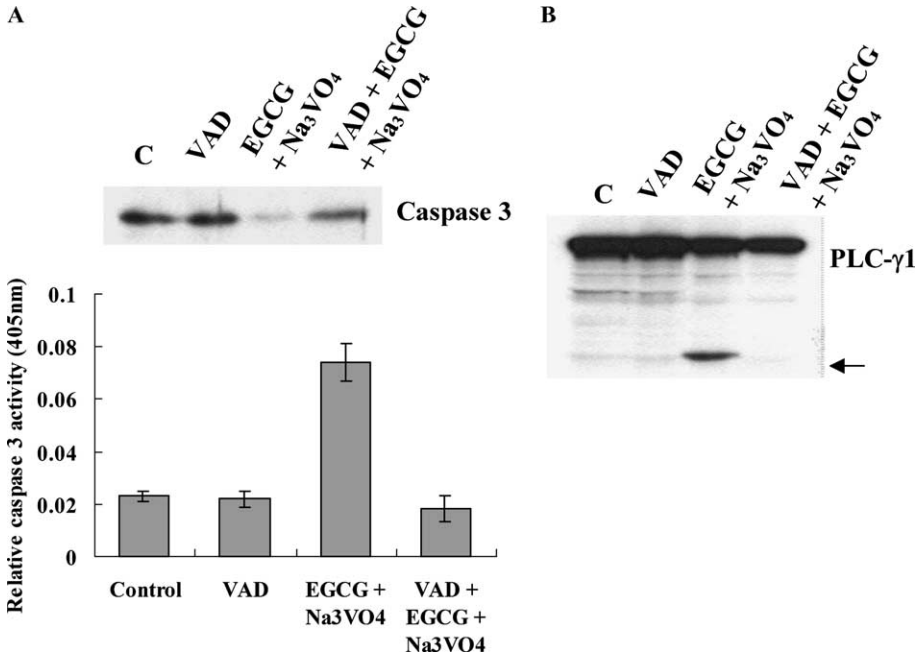

Fig. 3. Caspase-mediated apoptosis induced by EGCG plus vanadate. (A) Effects of z-VAD-fmk on EGCG plus vanadate-induced caspase 3 activation. U937 cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with EGCG plus vanadate. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for caspase 3. Caspase activity was determined as described in Fig. 1. Data are mean values from three independent experiments and bars represent standard deviations. (B) Effects of z-VAD-fmk on cleavage of PLD-γ1. U937 cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with EGCG plus vanadate. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for PLC-γ1. The proteolytic cleavage of PLC-γ1 is indicated by arrow.

PLC-γ1 (Fig. 3B). These data clearly indicate that cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate-induced apoptosis is associated with caspase activation.

Modulation of Bcl-2, IAP protein families, and cytochrome c release and MAPK activation in cotreatment of EGCG and sodium orthovanadate-induced apoptosis in U937 cells

We also examined whether cotreatment of EGCG and sodium orthovanadate induces cell death by modulating the expression of Bcl-2 family members, which ultimately determine the cell's response to apoptotic stimuli. Treatment of U937 cells with cotreatment of 10 μM EGCG and 25 μM sodium orthovanadate that are sufficient to induce apoptosis did not significantly alter the expression of Bcl-2 and Bax proteins after 24 h (Fig. 4A). To determine whether the activity of caspase was associated with the levels of caspase inhibitors in cotreatment of EGCG and sodium orthovanadate-induced apoptosis, we determined the expression levels of IAP family proteins in U937 cells that had been exposed to EGCG alone, sodium orthovanadate alone, and cotreatment of EGCG and sodium orthovanadate. As shown in Fig. 4B, cotreatment of EGCG and sodium orthovanadate resulted in a decrease in levels of cIAP1 and survivin, but not XIAP and cIAP2 in U937 cells exposed for 24 h.

There is accumulating evidence to suggest that the mitochondria play an essential role in many forms of apoptosis by releasing apoptogenic factors, such as cytochrome *c* and apoptosis-inducing factor (AIF). Cytochrome *c* normally resides in mitochondria, but is released into cytoplasm following exposure of cells to certain stresses. In the cytoplasm it directly activates caspases by binding to Apaf-1 in the presence of ATP [21]. To examine the release of cytochrome *c* in cotreatment of EGCG and sodium orthovanadate-treated U937 cells, we conducted Western blot analysis with cytosolic fractions. As shown in Fig. 4C, cytochrome *c* levels in the cytoplasm increased in response to cotreatment of EGCG and sodium orthovanadate, and this was correlated with activity of caspase 3 and cleavage of PLC-γ1.

The MAPK signaling pathway has been shown to be activated in response to certain cellular stresses [22,23]. To investigate whether cotreatment of EGCG and sodium orthovanadate led to MAPK activation, lysates obtained from vehicle alone, EGCG, vanadate, and EGCG plus vanadate treated cells were subjected to Western blot analysis using an anti-phospho-MAPK antibody to detect phosphorylated (and therefore activated) MAPK. The same blots were subsequently stripped and reprobed with an antibody that recognizes MAPK to verify equal amounts of the protein in the various samples. As shown in Fig. 4D, cotreatment of EGCG and vanadate resulted in significant apoptosis

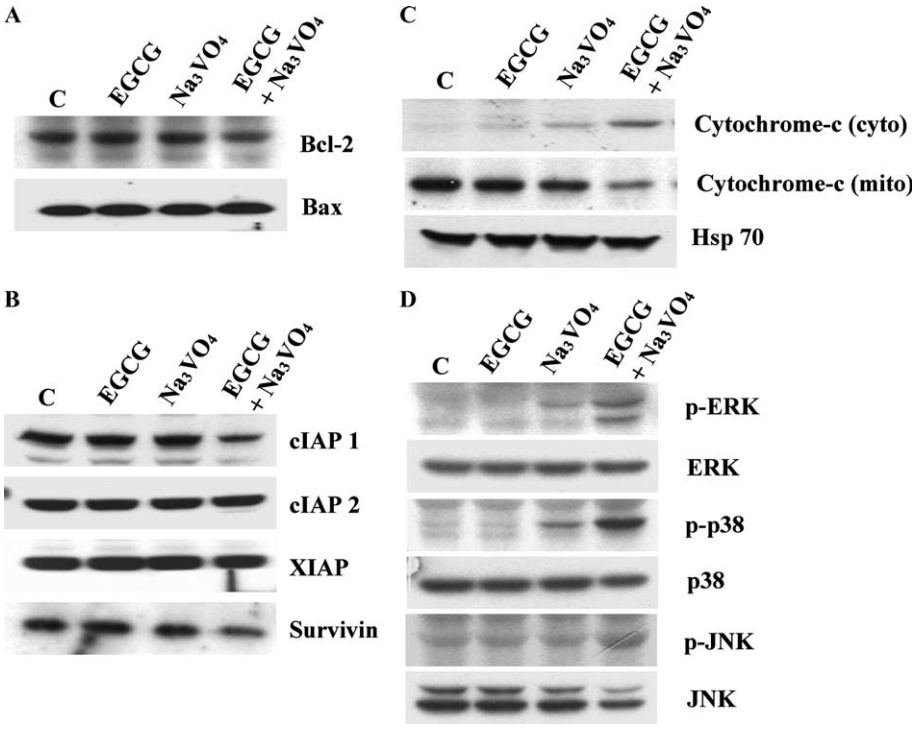

Fig. 4. The expression levels of apoptosis related proteins and release of cytochrome *c* in U937 cells by treatment with EGCG and vanadate. (A) U937 cells were treated with vehicle (cont), 10 μM EGCG, 25 μM sodium orthovanadate, and 10 μM EGCG plus 25 μM sodium orthovanadate. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bcl-2 and anti-Bax). A representative study is shown; two additional experiments yielded similar results. (B) Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-cIAP1, anti-cIAP2, anti-XIAP, and anti-survivin). All other conditions were as in (A). A representative study is shown; two additional experiments yielded similar results. (C) Cytosolic extracts were prepared as described under Materials and methods. Thirty μg of cytosolic protein was resolved on 12% SDS–PAGE, then transferred to nitrocellulose, and probed with specific anti-cytochrome *c* antibody, or with anti-Hsp70 antibody to serve as control for the loading of protein level. All other conditions were as in (A). (D) Equal amounts of soluble lysates (40 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against phospho-ERK, phospho-p38, and phospho-JNK. The lower panel shows the same blot stripped and reprobed with ERK, p38, and JNK antibodies as an internal control of the protein contents per lane. All other conditions were as in (A).

induction and led to strong activation of ERK and p38 kinase, but not JNK. Activation became apparent at about 20 h following treatment with EGCG plus vanadate. However, EGCG alone did not activate any type of MAPK signaling, but vanadate weakly induced ERK and p38 kinase.

MEK1/2 inhibitor blocks EGCG plus vanadate-induced apoptosis

To evaluate the role of the MAPK pathway in the induction of apoptosis, U937 cells were pretreated with specific inhibitors. Specific inhibitor MEK1/2, PD98059, has been developed, which is highly selective in their inhibition of the ERK pathway [24]. To elucidate whether ERK activation is required for cotreatment of EGCG and vanadate-induced apoptosis, U937 cells were pretreated with various concentrations of PD98059 for 30 min prior to addition of EGCG plus vanadate. As shown in Fig. 5A, apoptotic population was significantly attenuated by treatment with PD98059 in the present of the EGCG plus vanadate. To further confirm that activation of ERK plays an important role in cotreatment of EGCG and vanadate-induced apoptosis, we analyze caspase 3 activity, expression levels of procaspase 3, and cleavage of PLC-γ1 in MEK inhibitor treated cells. As shown in Fig. 5B, PLC-γ1 cleavage and activity of caspase 3 were significantly inhibited by the presence of PD98059. To confirm that the apoptosis-inducing effect of cotreatment of EGCG and vanadate was related to its ability to activate ERK, another MEK specific inhibitor, U0126, was used to assess suppression of apoptosis. U0126 revealed similar results to that of PD98059 (data not shown). To determine whether cytochrome *c* release occurred in response to MEK inhibitor treatment, U937 cells were treated with EGCG and vanadate in the presence or absence of PD98059. As shown in Fig. 5C, cytochrome *c* levels in the cytoplasm were markedly inhibited in the presence of the MEK inhibitor. These findings indicate that ERK acts upstream of cytochrome *c* release to exert its apoptotic influence on EGCG and vanadate treated cells. Thus, activation of the ERK pathway participates in the induction of apoptosis by cotreatment of EGCG and

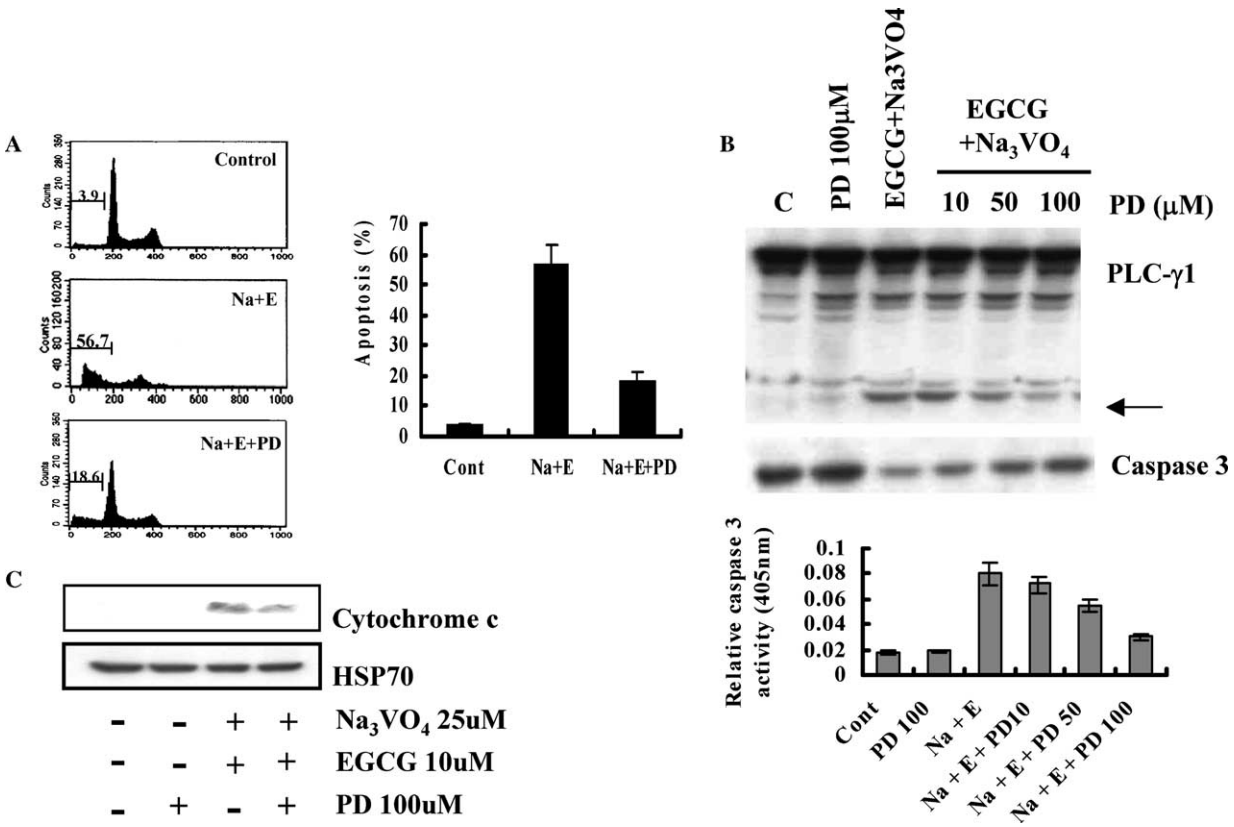

Fig. 5. MEKK1/2 inhibitor blocks EGCG plus vanadate-induced apoptosis. (A) FACS analysis of apoptotic cells. Cells were treated for 24 h with vehicle (cont), 10 μM EGCG plus 25 μM sodium orthovanadate, and 100 μM PD98059 combined with 10 μM EGCG plus 25 μM vanadate, and then evaluated for DNA content after propidium iodide staining. The fraction of apoptotic cells as indicated (right panel). Data are mean values obtained from three independent experiments and bars represent standard deviations. (B) Equal amounts of soluble lysates (40 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against PLC-γ1 and caspase 3. The proteolytic cleavage of PLC-γ1 is indicated by arrow. Caspase activity was determined as described in Fig. 1. Data are mean values from three independent experiments and bars represent standard deviations. (C) Thirty micrograms of cytosolic protein was resolved on 12% SDS–PAGE, then transferred to nitrocellulose, and probed with specific anti-cytochrome *c* antibody, or with anti-Hsp70 antibody to serve as control for the loading of protein level.

vanadate. p38 activity increases upon cotreatment with EGCG plus vanadate in U937 cells, but not JNK. To evaluate the role of the p38 kinase pathway in cotreatment of EGCG and vanadate-induced apoptosis, we used the pharmacologic agent SB203580, which acts as specific inhibitor of p38 kinase [25]. As shown in Fig. 6, PLC-γ1 cleavage and activity of caspase 3 were not inhibited by the presence of SB203580. Taken together, these results indicate that ERK pathway plays a role in regulating EGCG plus vanadate-induced apoptosis of U937 cells but not p38 kinase.

Discussion

EGCG has been reported to exert a variety of biological effects including antioxidant, anti-proliferative, and cancer chemopreventive activity [6–8]. Although EGCG seems to have a wide range of potential targets, the underlying mechanisms of apoptosis induction are not well understood. Recently, contradictory results were reported on the induction of apoptosis by EGCG. Anti-apoptotic effects of EGCG include inhibition of AP-1 transcription activity in JB6 cells by arsenite and 6-hydroxydopamine-induced apoptosis in PC12 cells [10,26]. However, several studies have reported that EGCG led to both growth inhibition and the induction of apoptosis [11,27,28]. However, the mechanism of apoptosis induction by EGCG is poorly understood. In this study, we evaluated the possibility that EGCG induces apoptotic cell death in U937 cells. EGCG-induced apoptosis is mediated by caspase 3 activation. Furthermore, sodium orthovanadate, protein tyrosine phosphatase inhibitor, potentiates EGCG-induced apoptosis in U937 cells by activation of caspase 3 activity, down-regulation of cIAP1, and activation of ERK signal pathway.

It is generally accepted that protein tyrosine kinases (PTKs) and protein tyrosine phosphatase maintain the tyrosine phosphorylation of cellular proteins in homeostasis [19,20]. Since the process of tyrosine phosphorylation and dephosphorylation is a major component of the intracellular response to external stimuli during signal transduction, such a protein modification may play an important role in the mechanism of apoptosis.

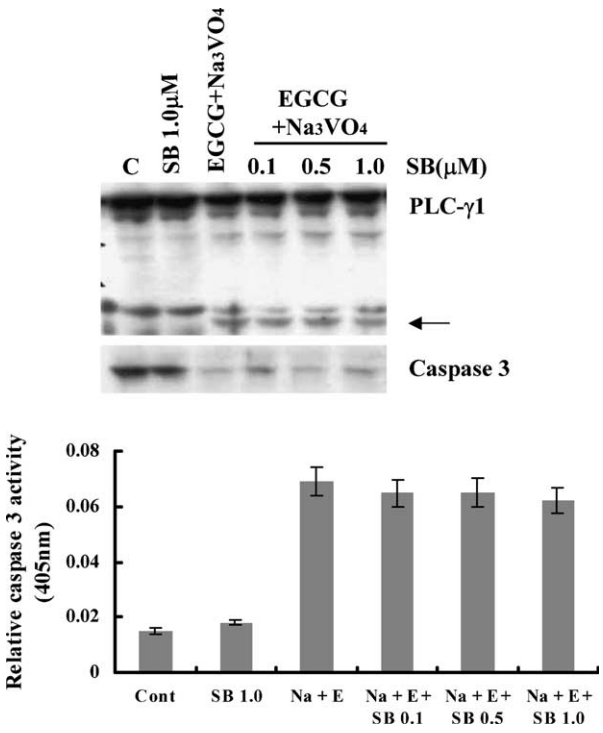

Fig. 6. Roles for p38 in regulating EGCG plus vanadate-induced apoptosis in U937 cells. U937 cells were treated with vehicle, 1 μM SB203580, 10 μM EGCG plus 25 μM vanadate, and indicated concentrations of SB203580 combined with 10 μM EGCG plus 25 μM vanadate and then harvested in lysis buffer. Equal amounts of soluble lysates (40μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against PLC-γ1 and caspase 3. The proteolytic cleavage of PLC-γ1 is indicated by arrow. Caspase activity was determined as described in Fig. 1. Data are mean values from three independent experiments and bars represent standard deviations.

Since the discovery of Bcl-2 as an anti-apoptotic protein, several theories concerning Bcl-2 anti-apoptotic mechanism have been proposed [29–31]. Bcl-2 anti-apoptotic function may be explained by their ability to control several key steps of apoptosis signaling. Bcl-2 can form ion channels in biological membranes [32,33]. This ion channel activity of Bcl-2 may control apoptosis by influencing the permeability of intracellular membranes and cytochrome *c* release from mitochondria [32,33]. The release of cytochrome *c* induces the activation of caspase 3 and fragmentation of DNA. Our data showed that the release of cytochrome *c* from mitochondria in U937 cells preceded caspase 3 activation by EGCG plus vanadate. The other reason for caspase 3 activation in EGCG plus vanadate treated U937 cells may be decreased IAP expression. Human IAP proteins, including XIAP, c-IAP1, c-IAP2, NAIP, and survivin, are characterized by the presence of one to three copies of a 70 amino acid motif, the BIR domain, which bears homology to sequences found in the baculovirus IAP proteins [34]. IAPs have been reported to inhibit apoptosis due to their function as direct inhibitors of activated effector caspases, caspase 3 and caspase 7. Furthermore, cIAP1 and cIAP2 are also able to inhibit cytochrome *c*-induced activation of caspase 9 [34–36].

The importance of MAP kinase signaling pathway in regulating apoptosis during conditions of stress has been widely investigated. Many such studies have supported the general view that activation of ERK pathway delivers a survival signal that counteracts pro-apoptotic effects associated with JNK and p38 activation [37–41]. However, in the present study, we have provided evidence that activation of ERK is important for the induction of cotreatment of EGCG and vanadate-induced apoptosis in U937 cells. Cotreatment of EGCG and vanadate resulted in high and sustained activation of ERK in these cells. PD98059, the specific inhibitor of MEK1/2, which has little effect on other kinase, including the other members of the MAPK family, was used to block activation of p42/44 MAPK [42]. PD98059 was able to block cytotoxicity, caspase 3 activation, PLC-γ1 cleavage, and cytochrome *c* release, suggesting that MAPK activation is upstream of caspase activation and is critical for apoptosis induction. We also determined that cotreatment of EGCG and vanadate does not profoundly affect JNK phosphorylation, suggesting that this pathway is unlikely to be involved in EGCG plus vanadate-induced apoptosis. Likewise, although we also showed that p38 was activated in response to EGCG plus vanadate, its inhibition failed to alter the cellular outcome. Thus, of the three MAPKs, only ERK appears to play a major role in influencing the induction of apoptosis by EGCG plus vanadate.

In summary, our studies demonstrate that cotreatment of EGCG and vanadate on U937 cells induces cytochrome *c* release which activates pro-caspase 3 and DNA fragmentation. Moreover, EGCG and vanadate mediated apoptosis is dependent on caspase 3 activity, and does result in a down-regulation of cIAP1 and survivin. ERK is important in mediating EGCG plus vanadate-induced apoptosis through a caspase 3 activity-dependent mechanism. In view of accumulating evidence that naturally occurring agents such as green tea polyphenols may be an important determinant of clinical response in cancer, further efforts to explore this therapeutic strategy appear warranted.

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