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Protein Phosphatase 1 Activation and Alternative Splicing of Bcl-X and Mcl-1 by EGCG + Ibuprofen

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Abstract Epigallocatechin-3-gallate (EGCG) and ibuprofen synergistically act to suppress proliferation and enhance apoptosis of prostate cancer cell lines, PC-3 and LNCaP. The purpose of this study was to investigate the mechanism of underlying this synergism. Most interestingly, EGCG + ibuprofen treatment in PC-3 cells resulted in altering the ratio of the splice variants of Bcl-X and Mcl-1, downregulating the mRNA levels of anti-apoptotic Bcl-X(L) and Mcl-1(L) with a concomitant increase in the mRNA levels of pro-apoptotic Bcl-X(s) and Mcl-1(s). However, there were no apparent changes in splicing variants in either ibuprofen or EGCG treated cells. Induction of alternative splicing was correlated with increased activity of protein phosphatase 1 (PP1) in EGCG + ibuprofen-treated cells, since pretreatment with calyculin A and tautomycin blocked EGCG + ibuprofen-induced alternative splicing in PC-3 cells in contrast to pretreatment with okadaic acid. On the other hand, EGCG + ibuprofen treatment in LNCaP cells did not alter splicing variants of Bcl-X and Mcl-1, despite the increase in protein phosphatase activity. In both cell lines, EGCG + ibuprofen inhibited cell proliferation synergistically. Taken together, this study demonstrate for the first time that EGCG + ibuprofen upregulated PP1 activity, which in turn induced alternative splicing of Bcl-X and Mcl-1 in a cell-type specific manner. Our study also demonstrates that the activation of PP1 contributes to the alternative splicing of Mcl-1. J. Cell. Biochem. 104: 1491–1499, 2008.

Key words: prostate cancer; EGCG; ibuprofen; alternative splicing; Bcl-2 protein family; protein phosphatase

Bcl-2 family proteins are key regulators of apoptosis, either inhibiting or promoting it [Cory and Adams, 2002]. They include the anti-apoptotic family members Bcl-2, Bcl-X(L), Mcl-1(L), and Bfl-1, and the pro-apoptotic members Bax, Bcl-X(s), Mcl-1(s), Bak, Bik, and Bid. By physical interaction with each other, the Bcl-2 family proteins are capable of forming a complex network of homo- and heterodimers via molecular regions named Bcl-2 homology (BH) domains (BH1, BH2, BH3, BH4). These physical interactions play important roles in the net effects of pro- and anti-apoptotic members of the family.

Alternative splicing of primary transcripts is now widely accepted to constitute the major mechanism for generating protein diversity and almost every member of the Bcl-2 family exists as multiple splice variants, adding a further layer of complexity to the control of apoptosis [Akgul et al., 2004]. Alternative splicing of Bcl-X gives rise to two transcripts coding for either a long (Bcl-X(L)) or short (Bcl-X(S)) form of the protein [Akgul et al., 2004]. Bcl-X(L) contains all four BH domains plus a C-terminal transmembrane domain, which is responsible for its mitochondrial localization. This isoform inhibits cell death. On the other hand, Bcl-X(S) contains BH3 and BH4 domains, and antagonizes the anti-apoptotic action of both Bcl-2 and Bcl-X(L) [Akgul et al., 2004]. Previously, activation of protein phosphatase 1 (PP1) by ceramide contributed to the alternative splicing of Bcl-X in lung cancer cells [Chalfant et al., 2002].

Another Bcl-2 family member, Mcl-1 also gives rise to two splicing variants, Mcl-1(L) which is anti-apoptotic and Mcl-1(S) which is pro-apoptotic [Bae et al., 2000]. The full-length

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Mcl-1(L) shares sequence homology with Bcl-2 in its BH1, 2, and 3 domains, and possesses a C-terminal membrane anchor domain that targets it to mitochondria [Bae et al., 2000]. The short splicing variant (Mcl-1(S)) of Mcl-1 contains BH3 domain only and has the pro-apoptotic characteristic of BH3-only protein. Mcl-1(S) specifically dimerizes with the long form of Mcl-1(L) and antagonize the anti-apoptotic function of Mcl-1(L) [Bae et al., 2000]. The mechanism of Mcl-1 alternative splicing has not been elucidated. Thus, these findings suggest that alternative splicing of apoptosis-regulating proteins can decide cell fate of life or death by modulating relative amounts of two isoforms with opposing functions.

Epigallocatechin-3-gallate (EGCG) is the most abundant component of epicatechins in green tea leaves and has recently been studied intensively as a potential anti-carcinogenic and anti-angiogenic agent [Cao and Cao, 1999; Kuroda and Hara, 1999; Adhami et al., 2003]. Numerous studies have demonstrated that EGCG treatment of prostate carcinoma cells, both hormone-sensitive LNCaP cells and hormone-insensitive DU-145 and PC-3 cells, results in the induction of apoptosis [Paschka et al., 1998]. EGCG treatment of androgen-sensitive LNCaP and androgen-insensitive DU-145 resulted in G0/G1 cell cycle arrest and induction of apoptosis (40–50% of cells) in a dose- and time-dependent manner (40–80 μg/ml and 48 h) [Gupta et al., 2000].

In addition, an increasing number of studies have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) are effective in the prevention and treatment of many common cancers including prostate cancer [Norrish et al., 1998; Sabichi and Lippman, 2003; Sabichi et al., 2003]. Ibuprofen, a non-selective NSAID, also effectively reduced cell proliferation and induced apoptosis in vitro studies (LNCaP and DU-145 cells) [Andrews et al., 2002], as well as enhanced the radiation response of prostate cancer cells in vitro and in vivo xenograft models [Palayoor et al., 1998].

Previously, we determined that EGCG + ibuprofen synergistically act to promote death in DU-145 cells via the generation of ceramide and concomitant oxidative stress [Kim and Chung, 2007]. In this study, we investigate synergistic action of EGCG and ibuprofen in PC-3 and LNCaP prostate cancer cell lines. Further, we investigate the hypothesis that their synergistic action is mediated via protein phosphatase activation and alternative splicing of Bcl-X and Mcl-1.

MATERIALS AND METHODS

Reagents

EGCG and ibuprofen were purchased from Sigma (St. Louis, MO). Calyculin A, okadaic acid, and tautomycin were purchased from EMD Biosciences (La Jolla, CA). Antibodies against PP1 α and catalytic subunit of protein phosphatase 2A (PP2A) were from Chemicon and BD Transduction.

Cell Culture

PC-3 and LNCaP-FGC cell lines were obtained from ATCC (Manassas, VA) and were maintained in culture medium (CM) containing F12K-Kaighn's modification (Invitrogen/Gibco, Carlsbad, CA) (PC-3 cells) or in RPMI-1640 (ATCC) (LNCaP cells) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate and 100 unit/ml penicillin G sodium. All cultures were maintained in a humidified 5% CO_2 incubator at 37°C, and routinely passed when 80–90% confluent.

WST Assay for Cell Proliferation

WST (Roche, Indianapolis, IN) is a water-soluble tetrazolium salt that is cleaved to formazan only in viable cells. The formazan dye formed is quantified using ELISA microplate reader (El_x808_{IU} UltraMicroplate Reader, Bio-Tek Instruments, Inc., Winooski, VT) and directly related to number of viable cells present in a well. Cells were seeded at 5,000 cells (PC-3) and 10,000 cells (LNCaP) per well in 96-well plates and treated with none, EGCG at 20 or 40 μg/ml, ibuprofen at 1 mM (LNCaP cells) or at 2 mM (PC-3 cells), or in combination of EGCG at 20 or 40 μg/ml + ibuprofen at 2 mM for 3 days (PC-3 cells) or at 1 mM for 2 days (LNCaP cells), followed by WST assay according to manufacturer's protocol (Roche). Standard curve was drawn from the WST value of known number of cells and cell number of each WST value was calculated from the standard curve equation. The experiment was done in triplicate wells per treatment and repeated at least three times.

RNA Isolation and RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) accord-

ing to the manufacturer's instructions. Total RNA isolated from 300,000 cells was reverse transcribed to cDNA using oligo-dT and random primers (Promega). The cDNA was amplified by PCR using primers specific for each Bcl-2 family protein. Primer sequences for Bcl-X [Chalfant et al., 2002] and Bfl-1 [Ko et al., 2003] were adapted from previous publications. Sequences are shown in Table I. The amplified products were visualized on 1% agarose gels by ethidium bromide staining. Quantitation of band density was done using UN-SCAN-IT gel 6.1 software (Silk Scientific, Inc., Orem, UT). When pretreated with phosphatase inhibitors, inhibitors were added to cells 1 h prior to EGCG + ibuprofen treatment. RNA was prepared at 6 h later for RT-PCR analysis of Bcl-X and Mcl-1. PCR was repeated at least three times using cDNAs prepared at two separate times.

Ser/Thr Phosphatase Activity Assay

The Ser/Thr phosphatase activity was determined by using a non-radioactive, malachite green-based Ser/Thr phosphatase assay kit (Upstate Biotechnology, Inc., Charlottesville, VG) according to the manufacturer's instructions and Chen et al. [2005]. In brief, cells were treated with EGCG 40 μg/ml + ibuprofen 1 mM (LNCaP) or EGCG 40 μg/ml + ibuprofen 2 mM (PC-3) for 0, 2, 4, or 6 h. At each time point, cells were scraped and lysed by sonication in lysis buffer (20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0 with protease inhibitor cocktail (Santa Cruz Biotechnology)). PC-3 (5 μg) or LNCaP cell extract was incubated with 175 μM of the phosphopeptide substrate (K-R-pT-I-R-R) in the phosphatase assay buffer (20 mM MOPS, pH 7.5, 60 mM 2-mercaptoethanol, 0.1 M NaCl, and 0.1 mg/ml BSA) with a total volume of 25 μl. After 10 min incubation, the malachite green solution was added to terminate reaction and allowed to stand for 15 min to permit color development. Absorption at 630 nm was measured in a microplate reader (El_x808_{IU} UltraMicroplate Reader, Bio-Tek Instruments, Inc.). Experiments were repeated at least twice using lysates prepared at two different times with the same treatment.

Immunoblot Analysis

Whole cell lysates (25 μg of protein) were prepared as described in Ser/Thr phosphatase activity assay, separated on a 10% SDS polyacrylamide gel under reducing conditions and then transferred to a PVDF membrane. The membrane was blocked for 1 h at room temperature (RT) in T-PBS, pH 7.5 (PBS with 0.1% Tween-20) with 5% non-fat dry milk. After washing, the blot was incubated with primary antibodies for 1 h at RT. The blot was washed three times in T-PBS and incubated with HRP-coupled secondary antibody (Pierce) for 1 h at RT. After extensive washing, the bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate System (Pierce). The resulting chemiluminescence was visualized by a UVP BioImaging System (UVP, LLC, Upland, CA) equipped with a CCD camera. Biotinylated protein standard (BioRad, Hercules, CA) and streptavidin-HRP conjugates were used to estimate protein MW on the blot. After stripping, the blot was reprobed with β-actin antibody to determine the equal loading of proteins. Experiments were repeated at least twice using lysates prepared at two different times with the same treatment.

Statistical Analysis

The average percentage of apoptosis is expressed as mean ± standard deviation (SD). The significance values between the control and treated groups were calculated by one-way analysis of variance (ANOVA) using InStat software (Graphpad, San Diege, CA), and *P*-values less than 0.05 were taken as significant in all the experiments.

TABLE I. Primer Sequences of Bcl-2 Family of Proteins

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)	PCR size (in bp)
Bcl-X	GAGGCAGGCGACGAGTTTGAA	TGGGAGGGTAGAGTGGATGGT	460 (L); 271 (S)
Mcl-1	CTCGGTACCTTCGGGAGCAGGC	CCAGCAGCACATTCCTGATGCC	452 (L); 206 (S)
Bcl-2	TTCTTTGAGTTCGGTGGGGTC	TGCATATTTGTTTGGGGCAGG	303
Bfl-1	AGCTCAAGACTTTGCTCTCCACC	TGGAGTGTCCTTTCTGGTCAACAG	577 (L); 633 (S)
Bax	ATGCGTCCACCAAGAAGC	GTGAGTGAGGCGGTGAGC	400
Bak	TTACCGCCATCAGCAGGAA	GGTAGCCGAAGCCCAGAAG	290
β-Actin	TGTGATGGTGGGAATGGGTCAG	TTTGATGTCACGCACGATTTCC	514

RESULTS

Regulation of Alternative Splicing of Bcl-X and Mcl-1 by EGCG + Ibuprofen Treatment

We investigated the expression of Bcl-2 family of proteins by RT-PCR analysis in EGCG + ibuprofen-treated PC-3 cells. We observed a decrease in the mRNA expression of anti-apoptotic proteins, Bcl-2 and Bfl-1 in EGCG + ibuprofen treated cells, but no apparent changes of mRNA expression of pro-apoptotic proteins, Bak and Bax proteins (Fig. 1b). On the other hand, treatment with EGCG or ibuprofen alone modestly decreased Bcl-2 expression, while it had no significant effect on the mRNA expression of other Bcl-2 family of proteins (Fig. 1a).

Most interestingly, EGCG + ibuprofen treatment induced alternative splicing of Bcl-X and Mcl-1 (Fig. 2b). In the case of Bcl-X, there was a increase in the ratio of Bcl-X(S)/Bcl-X(L) from 1 (at 0 h) to 3.0 (at 16 h), and in the case of Mcl-1, the ratio of Mcl-1(S)/Mcl-1(L) was also increased from 1 (at 0 h) to 10.5 (at 16 h) in EGCG + ibuprofen treated cells (Fig. 2c). However, there were no apparent changes in splicing variants in either ibuprofen or EGCG singly treated cells (Fig. 2a).

Activation of Ser/Thr Phosphatase Activity by EGCG + Ibuprofen Treatment

A previous study showed that ceramide-induced alternative splicing of Bcl-X was dependent on PP1 activity [Chalfant et al., 2002]. To establish whether PP1 was involved

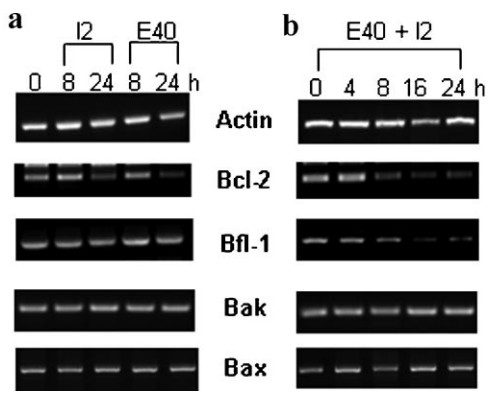

Fig. 1. RT-PCR analysis of Bcl-2 family of protein expression in PC-3 cells. Cells were treated with (**a**) ibuprofen 2 mM (I2), EGCG 40 μg/ml (E40), or (**b**) both (E40 + I2) for the indicated times and harvested for total RNA preparation and cDNA synthesis. RT-PCR analysis of Bcl-2 family of proteins was done as described in Section ''Materials and Methods.'' The RT-PCR results are representative of at least three separate experiments.

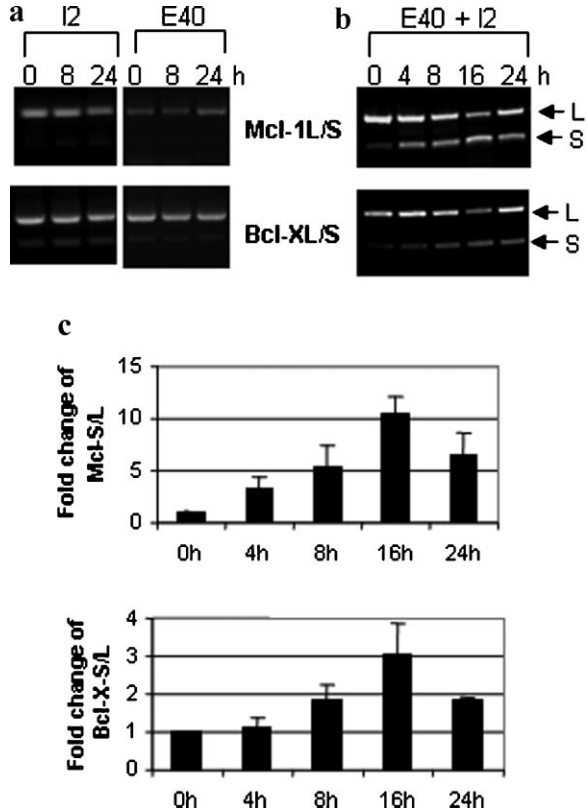

Fig. 2. Regulation of alternative splicing by EGCG + ibuprofen treatment. PC-3 cells were treated with ibuprofen 2 mM (I2), EGCG 40 μg/ml (E40), or both (E40 + I2) for the indicated times. **a**: RT-PCR of Mcl-1 and Bcl-X. Arrows indicate long splicing form (L) or short splicing form (S). **b**: The graphs depict the ratio of Bcl-X(S) mRNA to Bcl-X(L) mRNA or Mcl-1(S) mRNA to Mcl-1(L) mRNA as determined by densitometry of RT-PCR bands stained with ethidium bromide by Un-SCAN-IT gel digitalization software. The fold change were calculated when ratio at 0 h set at 1. Data are expressed as the mean. Bars are SDs. The RT-PCR results are representative of at least three separate experiments.

in EGCG + ibuprofen-induced alternative splicing of Bcl-X and Mcl-1, we pretreated PC-3 cells for 1 h with various phosphatase inhibitors with different specificity. Tautomycin, calyculin A, and okadaic acid exhibit distinct specificity in protein phosphatase inhibition. Tautomycin is a highly specific PP1 inhibitor [Mitsuhashi et al., 2003]. Okadaic acid at low doses ($\leq$100 nM) is selective for PP2A [Gupta et al., 1997]. Calyculin A lacks selectivity between PP1 and PP2A. As shown in Figure 3a, these inhibitors had differential effects on the alternative splicing of Bcl-X and Mcl-1. Tautomycin at 100 nM (lane 3) and calyculin at 10 nM (lane 5) abrogated the EGCG + ibuprofen-induced alternative splicing, whereas okadaic acid at 100 nM (lane 7) did not. Since PP1 represents a common target for calyculin A and tautomycin, these findings

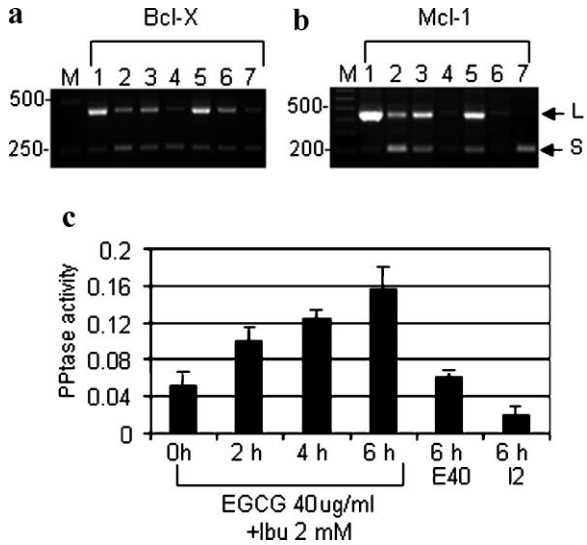

Fig. 3. **a**: Inhibition of alternative splicing by protein phosphatase inhibitors. PC-3 cells were pretreated with none (lane 2), tautomycin (TM) at 100 and 500 nM (lanes 3 and 4), calyculin (CA) at 10 and 25 nM (lanes 5 and 6), or okadaic acid (OA) at 100 nM (lane 7) for 1 h prior to the addition of EGCG at 40 μg/ml + ibuprofen at 2 mM (lanes 2–7). Vehicle-treated cells served as control (lane 1). Cells were further incubated for 6 h, followed by total RNA harvest and cDNA preparation. Splicing of Bcl-X and Mcl-1 were determined by RT-PCR. **b**: Increase of Ser/Thr phosphatase activity by EGCG + ibuprofen treatment. PC-3 cells were treated with EGCG + ibuprofen for 0, 2, 4, or 6 h, with EGCG 40 μg/ml (E40) or ibuprofen 2 mM (I2) for 6 h, and harvested for lysate preparation as described in Section ''Materials and Methods.'' Ser/Thr phosphatase activity was measured using phosphopeptide substrate (K-R-pT-I-R-R) in a 96-well plate. The RT-PCR results are representative of at least three separate experiments.

suggest that increased PP1 activity induces alternative splicing in EGCG + ibuprofen-treated PC-3 cells.

Next, we measured Ser/Thr phosphatase activity in PC-3 cell lysates treated with EGCG + ibuprofen. As shown in Figure 3b, EGCG + ibuprofen treatment increased Ser/Thr phosphatase activity in a time-dependent manner. However, EGCG at 40 μg/ml alone had no effect on phosphatase activity, while ibuprofen at 2 mM alone decreased phosphatase activity by 50%.

Regulation of Protein Phosphatases by EGCG + Ibuprofen Treatment

To further gain insights of regulation of protein phosphatase activity, we investigated protein levels of protein phosphatases in EGCG + ibuprofen-treated cells (Fig. 4). Interestingly, EGCG + ibuprofen treatment increased PP1-α protein level, while EGCG at 40 μg/ml or

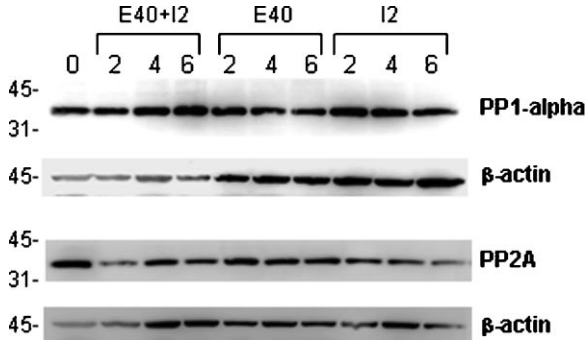

Fig. 4. Regulation of PP1 and PP2A proteins by EGCG + ibuprofen treatment. Cell lysates were prepared from EGCG at 40 μg/ml (E40), ibuprofen at 2 mM (I2), or EGCG at 40 μg/ml + ibuprofen at 2 mM (E40 + I2)-treated PC-3 cells at the indicated times. Immunoblotting was performed using antibodies specific for PP1-α or PP2A. The same blots were stripped and reprobed with β-actin to confirm equal loading of proteins. Each blot is a representative from two independent blots of two independents experiments with similar results.

ibuprofen at 2 mM alone did not. On the contrary, protein level of PP2A catalytic subunit was not significantly affected by EGCG + ibuprofen treatment in PC-3 cells. β-Actin served as a loading control.

Alternative Splicing of Bcl-X and Mcl-1 in LNCaP Cells

We also investigated the alternative splicing of Bcl-X and Mcl-1 in LNCaP cells treated with EGCG + ibuprofen (Fig. 5a). LNCaP cells express Bcl-X(L) and Mcl-1(L) constitutively (Fig. 5a). However, EGCG + ibuprofen treatment had no effect on the alternative splicing of these anti-apoptotic proteins, or on the level of their expression. Nevertheless, EGCG + ibuprofen increased the activity of protein phosphatase in LNCaP cells in a time-dependent manner (Fig. 5b).

Synergistic Effect of EGCG + Ibuprofen on the Growth Inhibition of PC-3 Prostate Cancer Cells In Vitro

We determined proliferation of PC-3 and LNCaP cells treated with vehicle alone, ibuprofen, EGCG, or EGCG + ibuprofen. In PC-3 cells (Fig. 6a), addition of ibuprofen 2 mM had synergistic growth inhibitory effect on EGCG at 20 or 40 μg/ml, decreasing cell number by 80% and by 90% compared to 20% growth inhibition by EGCG alone, respectively.

In LNCaP cells (Fig. 6b), treatment with EGCG at 40 μg/ml alone reduced cell numbers by 10%, compared to that of control cells. On the

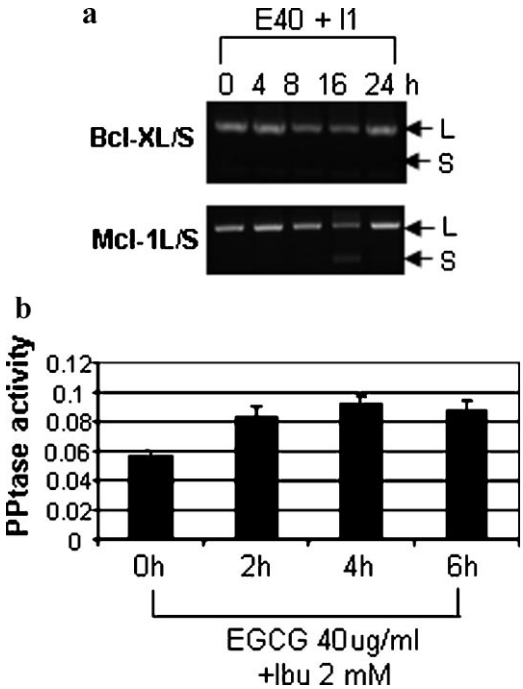

Fig. 5. No effect on alternative splicing in LNCaP cells despite of increase in protein phosphatase activity. **a**: RT-PCR of Mcl-1 and Bcl-X. LNCaP cells were treated with ibuprofen 1 mM (I1), EGCG 40 μg/ml (E40), or both (E40 + I1) for the indicated times and analyzed for alternative splicing of Bcl-X and Mcl-1. The RT-PCR results are representative of at least three separate experiments. **b**: Increase of Ser/Thr phosphatase activity by EGCG + ibuprofen treatment. LNCaP cells were treated with EGCG + ibuprofen for 0, 2, 4, or 6 h and assayed for protein phosphatase activity as described in Section ''Materials and Methods.''

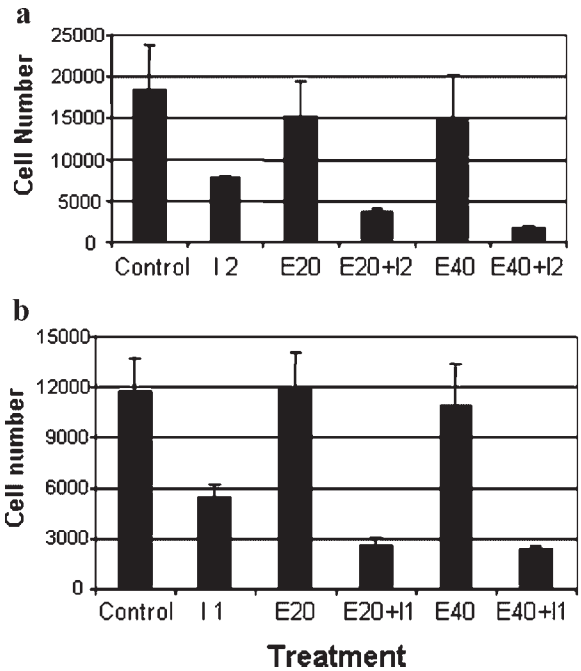

Fig. 6. EGCG + ibuprofen synergistically inhibited cell proliferation of PC-3 (**a**) and LNCaP (**b**) cells. Cells were seeded at 5,000 cells per well in 96-well plates and treated with the indicated doses of ibuprofen, EGCG, or both. I1, ibuprofen 1 mM; I2, ibuprofen 2 mM; E20, EGCG 20 μg/ml; E40, EGCG 40 μg/ml; E20 + I1 (I2), EGCG 20 μg/ml + ibuprofen 1 mM (2 mM); E40 + I1 (I2), EGCG 40 μg/ml + ibuprofen 1 mM (2 mM). WST was performed on Day 3 (PC-3) or on Day 2 (LNCaP). The data represent the means from three independent experiments, each performed in triplicate; bars, SD.

other hand, the combination of EGCG (40 μg/ml) + ibuprofen (1 mM) (EGCG + ibuprofen) augmented the growth inhibitory effect of ibuprofen or EGCG alone, resulting in 80% growth inhibition.

We also analyzed cell death of PC-3 cells by DNA histogram assays in which apoptotic cells appeared in the sub-G_0 population (Table II). As shown in Table II, synergistic effect of ibuprofen on EGCG-mediated cell death was observed. While control cells showed 3.98% death, EGCG + ibuprofen treatment increased cell death to 24.6% on Day 3 of treatment, compared to 13.42% with ibuprofen 2 mM alone and 8.16% with EGCG 40μg/ml alone (average% apoptosis ± SD). Taken together, these results clearly demonstrated that ibuprofen + EGCG exerted synergistic growth inhibitory effect on PC-3 and LNCaP prostate cancer cells, which was in part due to cell death induced by EGCG + ibuprofen treatment.

DISCUSSION

We previously reported that the combination of EGCG and ibuprofen (EGCG + ibuprofen) elicited synergistic effect on cell death in DU-145 prostate cancer cells via oxidative stress and MAPK activation [Kim and Chung, 2007]. In the present study, we determined that EGCG + ibuprofen treatment induced synergistic inhibition of cell proliferation and upregulated protein phosphatase activity which in turn induced alternative splicing of Bcl-X and Mcl-1 in a cell-type specific manner. In PC-3 cells, EGCG + ibuprofen treatment induced alternative splicing of Bcl-X and Mcl-1 which depended on protein phosphatase activity. In LNCaP cells, however, EGCG + ibuprofen treatment had no effect on Bcl-X and Mcl-1 splicing, despite the increase of protein phosphatase activity. In addition, EGCG + ibuprofen treatment downregulated mRNA expression of Bcl-2 and Bfl-1 in PC-3 cells. We speculate that

TABLE II. Effect of EGCG and Ibuprofen, Alone or in Combination, on PC-3 Cells

PC-3	% Apoptosis on Day 3			
Experiment	1	2	3	Average% apoptosis ± SD
Control	4.01	3.85	4.08	3.98 ± 0.12
Ibu 2 mM	12.86	13.46	13.95	13.42 ± 0.55[a]
EGCG 40 μg/ml	7.61	10.13	6.75	8.16 ± 1.76[b]
EGCG 20 + Ibu 2	12.72	14.63	14.97	14.1 ± 1.21[c]
EGCG 40 + Ibu 2	22.92	26.03	25.0	24.6 ± 1.58[d]

EGCG + ibuprofen synergistically induce apoptosis in PC-3 cells. Percentage of cells present in sub-G_0 population of DNA histogram analysis were considered apoptotic and shown here. The data are from three independent experiments.
[a]$P < 0.001$ vs. control.
[b]$P < 0.05$ vs. control.
[c]$P < 0.001$ vs. E40 μg/ml and vs. control, $P > 0.05$ vs. Ibu 2 mM.
[d]$P < 0.001$ vs. Ibu 2 mM and vs. EGCG 40 μg/ml.

increased protein phosphatase activity played a central role in the synergistic inhibition of cell growth and induction of apoptosis by EGCG + ibuprofen in PC-3 and LNCaP cells. To our knowledge, this study provides the first evidence of EGCG and ibuprofen upregulating protein phosphatase activity and inducing alternative splicing. In this case, EGCG + ibuprofen reduced the expression of the cell survival factors Bcl-X(L) and Mcl-1(L) and increased the expression of the pro-apoptotic factors Bcl-X(S) and Mcl-1(S).

This study showed that EGCG + ibuprofen upregulated Ser/Thr protein phosphatase (PPtase) activity. Moreover, when pretreated with PPtase inhibitors, tautomycin and calyculin A, EGCG + ibuprofen treatment was no longer able to induce alternative splicing of Bcl-X and Mcl-1. PP1 is a common target for calyculin A and tautomycin. It should also be noted that EGCG + ibuprofen increased protein level of PP1-α isoform, but not that of PP2A in PC-3 cells. On the other hand, treatment with EGCG or ibuprofen alone did not increase PP1 protein level. Thus, these results strongly suggest that EGCG + ibuprofen upregulated specifically PP1 activity in part via its protein level increase, which in turn caused alternative splicing. Ceramide is an inducer of PP1 activity in A549 lung carcinoma cells [Chalfant et al., 2002]. We previously showed that treatment with EGCG + ibuprofen, but not with EGCG or ibuprofen alone, induced apoptosis in part via ceramide synthesis in DU-145 prostate cancer cells [Kim and Chung, 2007]. It is thus likely that treatment with EGCG + ibuprofen increased PP1 activity indirectly via second messenger generation such as ceramide in PC-3 cells. Further study is needed to determine ceramide levels in EGCG + ibuprofen-treated PC-3 cells.

Studies showed that dephosphorylation of SR splicing factors by PP1 was involved in the regulation of alternative splicing of Bcl-X in ceramide-treated lung carcinoma cells [Chalfant et al., 2001; Chalfant et al., 2002]. Although the mechanism of alternative splicing of Mcl-1 has not been elucidated, our study suggests that PP1 activity was involved in Mcl-1 alternative splicing. We thus speculate that EGCG + ibuprofen treatment induced alternative splicing via activation of PP1 and dephosphorylation of SR splicing factors in PC-3 cells.

We further showed that EGCG + ibuprofen synergistically inhibited cell proliferation of LNCaP cells and upregulated protein phosphatase activity. However, EGCG + ibuprofen did not induce alternative splicing of Bcl-X and Mcl-1 in LNCaP cells. Increase of protein phosphatase activity (unpublished observation, M.H. Kim) was also observed in EGCG + ibuprofen-treated DU-145 cells without inducing alternative splicing of Bcl-X [Kim and Chung, 2007]. DU-145 cells do not express Mcl-1 [Kim and Chung, 2007].

PP1 regulates an enormous variety of cellular functions through the interaction of its catalytic subunits with over 50 regulatory (bona fide or putative) subunits in a mutually exclusive manner [Cohen, 2002]. Although PP1 exhibits universal distribution in almost all cell types, cell-type specific expression of regulatory subunits gives PP1 distinct substrate specificities, restricted subcellular locations and diverse regulation in a cell-type specific manner [Cohen, 2002]. PP1 activity is not only present in the cytosol, but is also located in the nucleus

[Kuret et al., 1986] where it is likely to regulate nuclear processes including pre-mRNA splicing [Mermoud et al., 1994; Misteli and Spector, 1996]. Several nuclear factors are found to be associated with PP1 in the nucleus [Cohen, 2002]. They are the polypyrimidine tract-binding protein-associated splicing factor (PSF) [Hirano et al., 1996], nuclear inhibitor of PP1 (NIPP1) [Van Eynde et al., 1995] and p99 [Kreivi et al., 1997], all of which inhibit PP1 activity. To date, no study has been reported on the expression of these factors in PC-3 and LNCaP cells. Thus, it is possible that the cell-type specific expression of any of these PP1-inhibitory factors might inhibit the dephosphorylation of SR factors by PP1 in LNCaP cells, preventing PP1-mediated alternative splicing of Bcl-X and Mcl-1.

Recent studies have highlighted a major role of serine/threonine protein phosphorylation in apoptosis regulation [Garcia et al., 2003]. Reversible phosphorylation and dephosphorylation by kinases and phosphatases are indeed the major mechanisms that directly regulate the activity of both anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family [Ruvolo et al., 2001]. In particular, the cell death inducing function of Bad is inactivated by serine phosphorylation at Ser-112 and Ser-136 which retain Bad in the cytoplasm by the interaction with 14-3-3 protein [Zha et al., 1996], whereas phosphorylation at Ser-155 triggers dissociation of Bad from Bcl-XL [Tan et al., 2000]. A recent study showed that PP1-α dephosphorylated Bad on Ser-112 and Ser-136 prior to induce apoptosis in response to IL-2 deprivation [Ayllon et al., 2001]. The expression of Bad and its role in prostate cancer apoptosis have been reported [Yamaguchi et al., 2005; Aziz et al., 2006]. Thus, Bad dephosphorylation by the increased PP1 activity could be another mechanism of EGCG + ibuprofen-induced cell death.

In summary, we believe that the increase of PP1 activity plays a central role in EGCG + ibuprofen-induced cell death regardless of cell types, but the cell-type specific regulation of PP1 activity mediates apoptotic pathway in a cell-type specific manner. In both DU-145 and LNCaP cells, EGCG + ibuprofen caused synergistic inhibition of cell proliferation and increases PP1 activity. The increased PP1 activity resulted in the induction of alternative splicing of Bcl-X and Mcl-1 in PC-3 cells but not in LNCaP cells. Further study is needed to determine Bad dephosphorylation in EGCG + ibuprofen-treated PC-3 and LNCaP cells. Nevertheless, we demonstrated for the first time that EGCG + ibuprofen upregulated protein phosphatase activity, which in turn induced alternative splicing of Bcl-X and Mcl-1 in a cell type specific manner, increasing pro-apoptotic short forms of Bcl-X(S) and Mcl-1(S) in PC-3 cells. We also showed that PP1 played a role in Mcl-1 alternative splicing.

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