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Sodium 4-Phenylbutyrate Induces Apoptosis of Human Lung Carcinoma Cells Through Activating JNK Pathway

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Abstract Sodium 4-phenylbutyrate (PB) has been used in the therapy of urea cycle defects for many years. Recently, it has been shown to cause cellular differentiation, growth arrest, and apoptosis in certain malignancies. We have analyzed the effects of PB on human lung carcinoma cells. PB has distinct patterns of effects on different lung carcinoma cells, inducing apoptosis in NCI-H460 and NCI-H1792 cells, causing G1 arrest in A549 and SK-LU-1 cells, but having no effect on a non-transformed bronchial epithelial cell line HBE4-E6/E7. We investigated the role of MAP kinase family members, extracellular signal-regulated kinase (ERK), JNK, and p38 mitogen-activated protein kinase (MAPK), as well as other important cell survival signaling molecules in PB-induced apoptosis. We observed activation of JNK and ERK by PB in the lung cancer cells. JNK was activated only in the two apoptotic cells, whereas ERK was activated in both the apoptotic and the growth-arrested cells, demonstrating a correlation between apoptosis and activation of JNK in response to PB. Both JNK inhibitor and JNK RNA interference (RNAi) inhibited PB-induced apoptosis, whereas MEK inhibitor did not, supporting that apoptosis induced by PB is through activation of JNK. De novo protein synthesis is required for the PB-induced JNK activation and induction of apoptosis. However, the production of known upstream activators of JNK, namely Fas/Fas ligand, tumor necrosis factor (TNF)-α, TNF-β, and TRAIL, are not altered by PB treatment. Therefore, PB activates JNK through an unidentified and cell type-specific mechanism. Understanding of this mechanism is of therapeutic value in treating cancer patients with PB. J. Cell. Biochem. 93: 819–829, 2004.

Key words: sodium 4-phenylbutyrate; JNK; apoptosis; lung carcinoma; RNA interference

Sodium 4-phenylbutyrate (PB), an aromatic fatty acid, has been used as a scavenger of glutamine in urea cycle defects for many years [Trock et al., 1990]. Recently, PB has been studied for the treatment of certain malignancies and has been shown to cause cellular differentiation, growth arrest, and apoptosis in myeloid leukemia cells, prostate cancers, and others [Carducci et al., 1996; Mandal et al., 1997; Higashi, 1999]. However, the molecular mechanisms underlying the PB and its analogs-induced apoptosis and growth arrest have not been well understood. A number of explanations have been suggested, including causing changes in bcl-2 family protein expression, increasing caspase activity, sensitizing Fas-Fas ligand interaction, and having effects on mitochondrial membrane function [Hague et al., 1997; Amin et al., 2001; Chopin et al., 2002]. In addition, histone deacetylase inhibition, novel protein/DNA interactions, and alterations in the expression of a myriad of cellular genes, such as c-myc, K-ras, p53, p21, p16, and others, have also been suggested as causative [Janson et al., 1997; Alexandrov et al., 1998; Bernhard et al., 1999; Turner et al., 2002].

Proteins comprising the mitogen-activated protein kinase (MAPK) family constitute important mediators of signal transduction processes

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that serve to coordinate cellular responses [Cartee et al., 2000]. The different MAPKs, which include the extracellular signal-regulated kinase (ERK), JNK, and p38, are members of separate modules and are regulated by distinct extracellular stimuli. The best characterized MAPK pathway is the Ras/Raf/MEK cascade leading to the activation of ERK1/2 in response to receptor tyrosine kinase activation to provide proliferation or differentiation signals [Chao and Yang, 2001; Husain et al., 2001; Boldt et al., 2002]. JNK and p38 MAPK are key mediators of stress signals and inflammatory responses evoked by a variety of agents such as tumor necrosis factor, UV- and irradiation, heat shock, osmotic stress, and inflammatory cytokines [Chuang et al., 2000; Gekle et al., 2000; Greenberg et al., 2002]. Recently, considerable attention has been focused on the potential role of these kinases in apoptotic signaling. Both JNK and ERK have been shown to be involved in the induction of differentiation and apoptosis [Amin et al., 2001].

To gain insights into the mechanisms of apoptosis induced by PB, we analyzed the regulation and function of MAPK signaling pathways in human lung carcinoma cells in response to PB. We found that PB induced dose-dependent apoptosis in some of the human lung carcinoma cells, but growth arrest in others. Among the MAPKs, PB induced only the activation of ERK and JNK, and only the JNK activation is associated with the induction of apoptosis. Inhibition of JNK alleviated PB-induced apoptosis. In conclusion, these data suggest that JNK activation is a key step in PB-induced apoptosis of cancer cells.

MATERIALS AND METHODS

Reagents

PB was kindly provided by Dr. David Rishikof (Pulmonary center, Boston University Medical Center). 4′, -Diamidino-2-phenylindole (DAPI) was purchased from Sigma (St. Louis, MO). Anti-JNK1/2, ERK1/2, p38MAPK polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MEK1/2 inhibitor (PD98059), JNK inhibitor (SP600125), and p38 MAPK inhibitor (SB 203580) were purchased from Calbiochem (San Diego, CA). Anti-phospho-ERK (Ser) and anti-phospho p38 MAPK rabbit polyclonal antibodies were purchased from New England Biolabs, Inc. (Beverly, MA). Anti-phospho JNK rabbit polyclonal antibodies were purchased from Promega (Madison, WI). The enhanced chemiluminescene (ECL) system for Western analysis was purchased from Amersham (Alington Heights, IL).

Cell Cultures

Human lung adenocarcinoma cells, NCI-H1792, A549, and SK-LU-1 and human lung large cell carcinoma NCI-H460 cells, were purchased from American Type Cell Culture Collection (ATCC, Rockville, MD), and were cultured in DMEM medium supplemented with 10% fetal calf serum. HBE4-E6/E7 (human papilloma virus 16 (HPV-16) E6/E7 immortalized normal lung bronchus epithelial cells) was purchased from ATCC and cultured in Keratinocyte-Serum Free Medium (GIBCO BRL, Gaithersburg, MD) supplemented with 0.05 mg/ml bovine pituitary extract, 5 ng/ml recombinant human epithelial growth factor, and 10 ng/ml cholera toxin.

Cell Death Assay

The MTT assay was performed essentially as described by Furukawa et al. [1991]. Briefly, cells (200 μl/well) were grown in 96-well dishes and then treated with different drugs for 24 h. At the end of incubation, 20 μl MTT (5 mg/ml in phosphate-buffered saline, Sigma (St. Louis, MO)) was added into each well and incubated for 2 h. The formazan crystal was dissolved with 100 μl DMSO and the optical density (OD) was read with Dias Microplate Well reader (Dynatech Medical Products Ltds, UK) at a wavelength of 540 nm (OD540). The OD values of the drug-treated groups were divided by the OD values of untreated controls and presented as percentage of cell survival. All concentrations of drugs were tested in four replicate wells and each experiment was performed at least three times.

DNA Extraction and Electrophoresis

Cells were lysed in protein lysis buffer containing 0.5% Triton X-100, 10 mM EDTA (pH 8.0), 10 mM Tris (pH 7.6). The cell lysates were kept on ice for 1 h and then was extracted twice with an equal volume of a mixture of chloroform/phenol, followed by one time extraction with chloroform, and precipitated overnight in ethanol. DNA electrophoresis was carried out in 1.5% agarose gels with TAE buffer (40 mM Tris base, 40 mM acetate, and 1 mM EDTA).

Flowcytometry Analysis

Cells were cultured in 6-well plates and treated with different drugs. All cells, floating and adherent, were collected, washed with PBS, resuspended in 70% ice-cold ethanol, and fixed at 4°C for 20 min. The cell pellet was resuspended in a solution containing 69 μM propidium iodide and 0.05 mg/ml RNase A in PBS, then incubated at room temperature for 30 min in the dark and analyzed with a Becton Dickinson FACScan flowcytometer using the Cell Quest software (Becton-Dickison, Franklin Lakes, NJ).

Western Blotting

Cells were washed with PBS and lysed for 30 min at 4°C in lysis buffer (50 mM, Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM PMSF, 1 mM orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Proteins were resolved by 7.5% SDS–PAGE. The protein was transferred onto a nitrocellulose filter, blocked in TBST (TBS containing 0.05% Tween 20) and 5% non-fat milk for 1 h at room temperature. Filters were incubated with primary antibody (1:1,000 dilution) overnight at 4°C, followed by 1 h incubation with horseradish peroxidase-conjugated secondary antibody (1:2,800 dilution). The proteins were detected by ECL detection system (Amersham, Piscataway, NJ).

Plasmid Construction

pRETRO-SUPER RNA interference (RNAi) constructs were cloned as described previously [Brummelkamp et al., 2002]. The sense strand of inserted oligonucleotide for JNK1 was: 5′-gatccccAACCTATAGGCTCAGGAGCttcaagagaGCTCCTGAGCCTATAGGTTtttttggaaa-3′, and for JNK2 was 5′-gatccccGCCGTCCTTTTCAGAACCAttcaagagaTGGTTCTGAAAAGGACGGCtttttggaaa-3′. The 19-nt of target sequences are indicated in capital letters in the oligonucleotide sequence.

Cell Transfection and Retroviral Infection

NCI-H1792 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Ecotropic retroviral supernatants were produced by transfection of packaging cells using calcium-phosphate precipitation. Forty-eight hours after transfection, the cell culture medium was filtered through a 0.45 μm filter, and the virus containing supernatant was used for the infection of cells after addition of 4 μg/ml polybrene. Cells were infected for at least 6 h and allowed to recover for 24 h with fresh medium. Infected cells were selected with puromycin (2 μg/ml) (Invitrogen, Carlsbad, California) for 48 h.

RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Gibco BRL), following the manufacturer's instructions. Two grams of total RNA were converted to cDNA with the Superscript™ Preamplification Kit (Gibco BRL, Gaithersburg, MD), following the manufacturer's instructions. The PCR primers to amplify Fas Ligand are: forward, 5′-AGCTACCTGGGGCAGTGTTCAAT-3′; reverse, 5′-CTCTTCACATGGCAGCCCAGAGTT-3′. The actin primers are: forward, 5′-ACAATGAGCTGCGTGTGGCT-3′; reverse, 5′-TCTCCTTAATGTCACGCACGA-3′. The PCR reactions were set up as described previously [Archacki et al., 2003].

RESULTS

PB Induces Apoptosis in Some Lung Carcinoma Cells, But G1 Arrest in Others

To analyze the effect of PB on human lung cancer cells, three human lung adenocarcinoma cell lines, A549, SK-LU-1, and NCI-H1792, one human lung larger cell carcinoma cell line NCI-H460, and one immortalized but not transformed human lung bronchial epithelial cell line HBE4-E6/E7 were treated with PB for 36 h. Cell numbers of all treated cancer cell lines were reduced by PB treatment. PB, however, had no apparent effect on the relative normal HBE control cells (Fig. 1A,B). Flowcytometric analysis revealed that 3 mM (48 h) and 10 mM (24 h) PB induced apoptosis in NCI-H460 and NCI-H1792 cells ($P < 0.01$, < 0.001, < 0.001, < 0.001, respectively), but growth arrest in A549 and SK-LU-1 cells ($P < 0.05$, < 0.05, < 0.01, < 0.01, respectively) (Fig. 1C,D). We then used H460 cells to investigate the dose-dependence and time-course of PB-induced apoptosis. Cell apoptosis was detected by DNA laddering analysis (Fig. 2A) and PARP cleavage analysis (Fig. 2B,C). The effective concentration of PB for induction of apoptosis is in the millimolar range, which is consistent with its effective concentration in other systems [Carducci et al., 2001; Svechnikova et al., 2003].

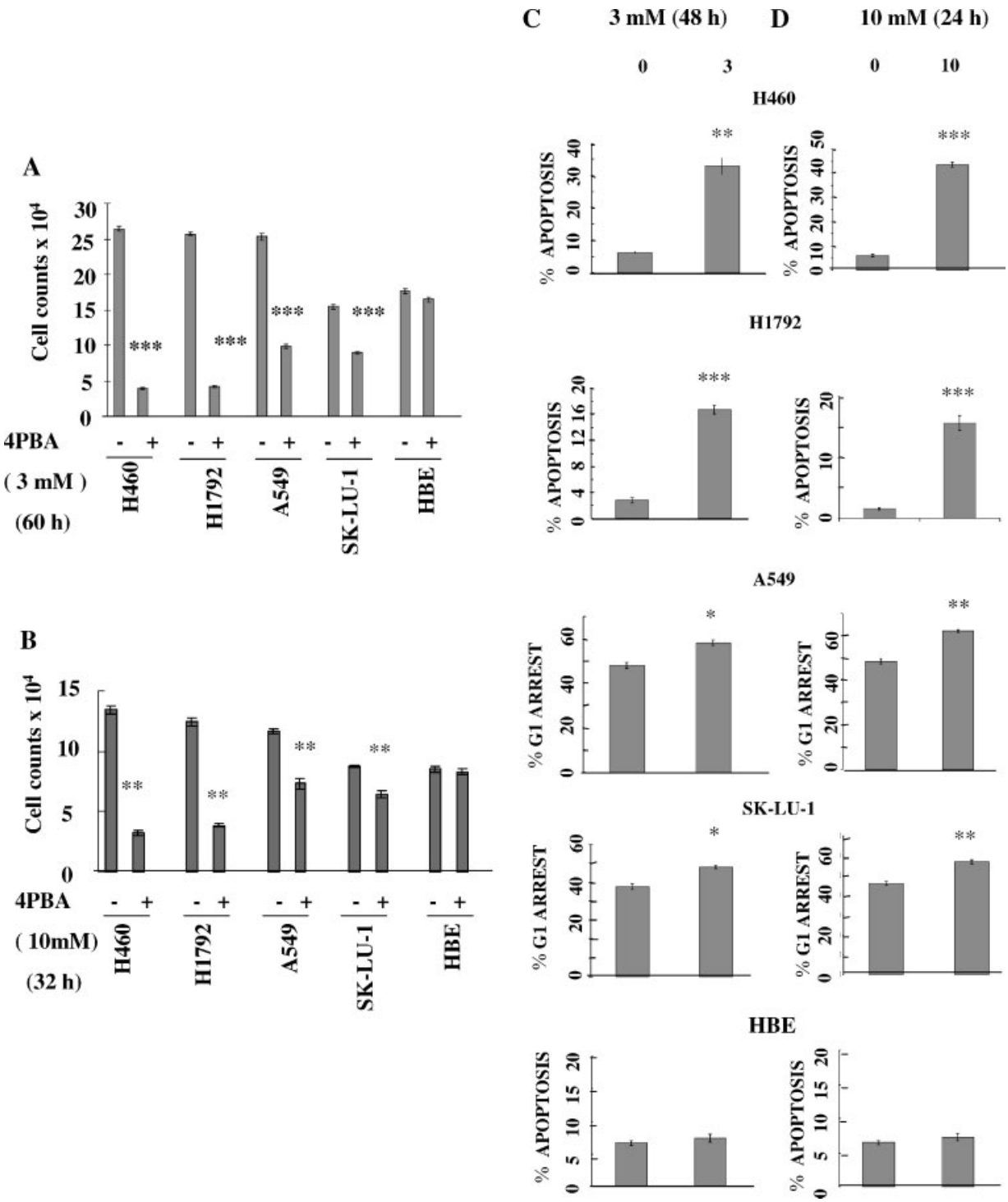

Fig. 1. Growth arrest and apoptosis induced by PB in lung carcinoma cells. **A**, **B**: H460, H1792, A549, SK-LU-1, and HBE cells (5.0×10^4) were incubated in DMEM medium for 8 h and then either treated with (+) or without (−) 3 mM PB for 60 h or 10 mM PB for 32 h, as indicated. Cells were trypsinized, washed with PBS and the cell numbers were counted. Lung cancer cell growth was inhibited by 3 mM PB ($P < 0.001$, < 0.001, 0.001, and < 0.001, respectively) and 10 mM PB ($P < 0.01$, < 0.01, < 0.01, and < 0.01, respectively). **C**: Cells were plated in DMEM for 24 h, and then were treated either without drug or with 3 mM PB for additional 48 h, or 10 mM PB for additional 24 h. Cells were then fixed with 70% ethanol, stained with 69 μM propidium iodide, and analyzed with FACS to determine the percentage of cells in different phases of cell cycle. The percentage of apoptotic cells ($P < 0.01$, < 0.001, < 0.001, < 0.001, respectively) and the G1 arrested cells ($P < 0.05$, < 0.05, < 0.01, < 0.01, respectively) were summarized and presented as histograms. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

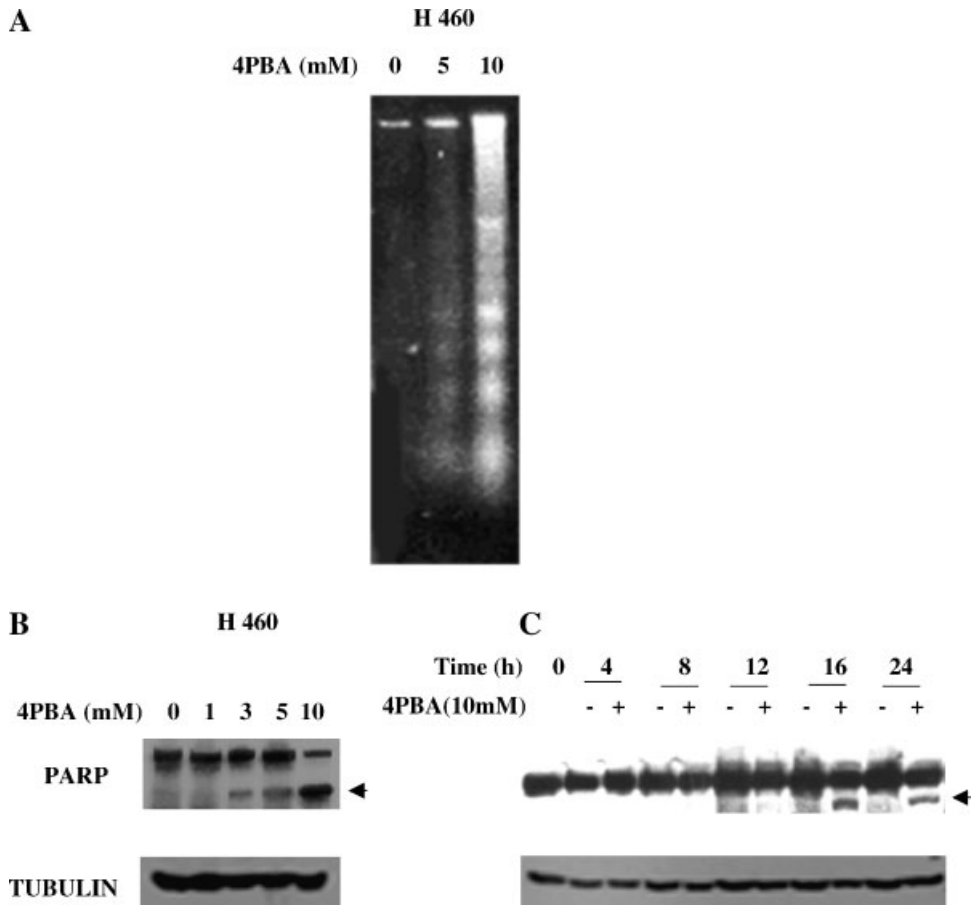

Fig. 2. PB induced apoptosis in H460 cells in time- and dose-dependent manner. **A**: DNA analysis of PB-induced apoptosis. H460 cells were cultured for 24 h then treated with 5 or 10 mM of PB for 16 h, as indicated. DNA from these cells was analyzed by electrophoresis. **B**, **C**: PARP cleavage analysis of PB-induced apoptosis. H460 cells were treated with 1, 3, 5, or 10 mM PB for 24 h (B), or treated with 10 mM PB for 4, 8, 16, 24 h (C), as indicated. Cells were then harvested, cell lysates were resolved by SDS–PAGE. PARP cleavage was assessed by immunoblotting analysis using an anti-PARP monoclonal antibody (**upper panel**). Anti-tubulin antibody was used to show equal protein loading (**lower panel**). The PARP cleavage products are indicated with arrows.

In summary, PB has distinct patterns of effects on human lung cells, inducing cell death in certain tumor cells, inhibiting cell proliferation of others, and having little effect on non-transformed cells.

Activation of JNK by PB Is Associated with Apoptosis

MAPK signaling pathway has been shown to be activated in response to certain cellular stresses. To investigate the roles of ERK, JNK, and p38 MAPK in PB-induced apoptosis, we analyzed the phosphorylation/activation of these signaling proteins in response to PB treatment. The JNK phosphorylation was increased after 12 h of PB treatment and remained to be activated during the remaining period. However, it was only activated in the apoptotic H460 and H1792 cells but not in the growth-arrested A549 and SK-LU-1 cells (Fig. 3A,B). The phosphorylation of ERK protein was increased with similar kinetics in both apoptotic and growth-arrested cells (Fig. 3C). The phosphorylation of p38 MAPK protein, on the other hand, was not affected by PB treatment (data not shown).

We also analyzed phosphorylation of other important signaling proteins such as FAK, Akt, and Src. However, no phosphorylation change was found for these proteins after PB treatment, suggesting that phosphorylation of theses proteins do not play a role in PB-induced apoptosis or growth-arrest (data not shown).

JNK Activation Is Essential for PB-Induced Apoptosis

To address the function of JNK and ERK activation in PB-induced apoptosis, we exam-

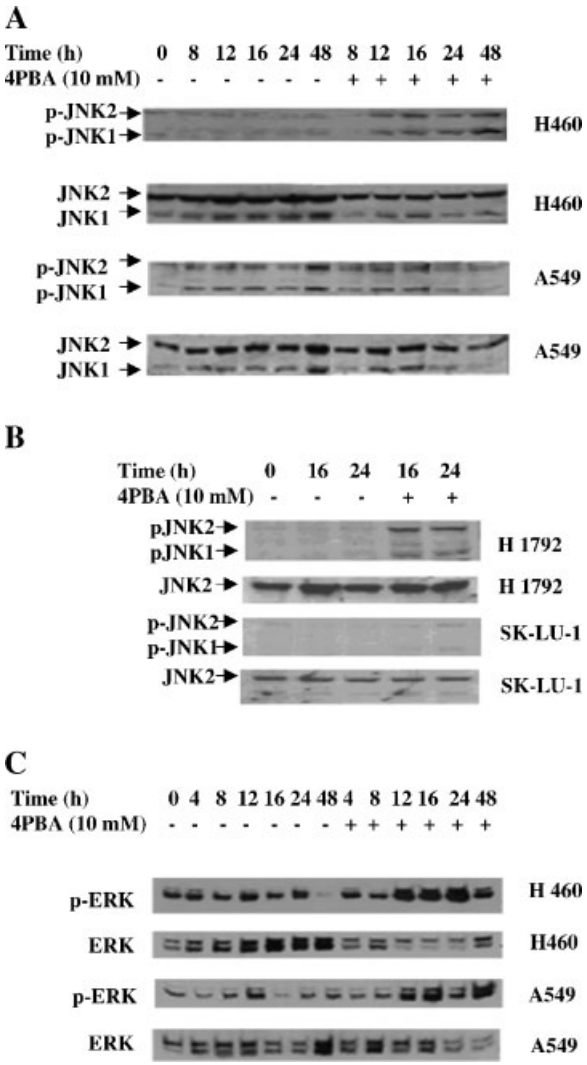

Fig. 3. Activation of ERK and JNK by PB. **A**, **B**: H460 and A549 cells were treated with 10 mM PB for 8, 12, 16, 24, 48 h; H1792 and SK-LU-1 cells were treated with 10 mM PB for 16, 24 h, as indicated. Cells were lysed and the cell lysates were resolved by SDS–PAGE. JNK activation was detected by Western blot analysis using anti-phospho-JNK antibody (p-JNK). Anti-JNK antibody was used to show equal protein loading (JNK). **C**: H460 and A549 cells were treated with 10 mM PB for 4, 8, 12, 16, 24, 48 h, as indicated. ERK activation was detected by Western blot analysis using anti-phospho-ERK antibody (p-ERK). Anti-ERK antibody was used to show equal protein loading (ERK).

ined the effects of inhibiting the ERK and JNK pathways by their specific inhibitors. H460 cells were pretreated with 15 μM of JNK inhibitor for 30 min before addition of PB. Although apoptotic effects of PB can be seen at as low as 3 mM, we used 15 mM PB to induce cell apoptosis to obtain better apoptotic effects. JNK inhibitor (SP600125) was then used to alleviate apoptotic effects of PB. Treatment of cells with JNK inhibitor markedly suppressed the apoptotic morphology changes induced by PB (data not shown). DNA analysis, PARP cleavage analysis, and MTT assay ($P < 0.01$) all confirmed the inhibitory effect of JNK inhibitor on cell death induced by PB (Fig. 4–C). On the other hand, the pretreatment of the cells with MEK inhibitor, PD98059, did not inhibit apoptosis induced by PB (Fig. 4D).

To further demonstrate the importance of JNK in PB-induced apoptosis, we inhibited gene expression of JNK in NCI-H1792 cells using small RNAi technology. JNK RNAi oligonucleotides were cloned into a retroviral vector pRETRO-SUPER. Virus were produced from package cells and used to infect NCI-H1792 cells. Infected cells were selected and then treated with PB. The JNK protein level was significantly reduced in the infected cells (Fig. 4E). Inhibition of JNK expression by RNAi also reduced cell apoptosis induced by PB (Fig. 4E). Therefore, JNK is the key mediator of PB-induced apoptosis.

New Protein Synthesis Is Required for PB-Induced Apoptosis

Phosphorylation of JNK occurred 12 h after PB treatment, instead of an immediate response, suggesting that it may not be a primary response of PB treatment. To further understand the mechanism of PB-induced apoptosis, particularly the upstream events that activate JNK, we examined the requirement of new protein synthesis in PB-induced apoptosis. Both cell death, as indicated by PARP cleavage, and JNK activation/phosphorylation were inhibited by 1 μg/ml protein synthesis inhibitor Cyclohexamide, suggesting that new protein synthesis is required for induction of cell death, as well as JNK activation (Fig. 5). We then analyzed possible induction of death signaling proteins FasL/FAS, TNF/TNFR, TRAF, and TRAIL in response to PB treatment. The level of most of these death-signaling proteins did not change after PB treatment (Fig. 6A–C). The level of TNFR1 appeared to be cell cycle-dependent (Fig. 6A). This cell cycle-dependent regulation of TNFR1 was abolished by PB treatment (Fig. 6A). Nonetheless, none of these death signaling proteins was induced by PB treatment. Therefore, induction of death signaling proteins did not seem to have a role in activating JNK and in inducing cell death.

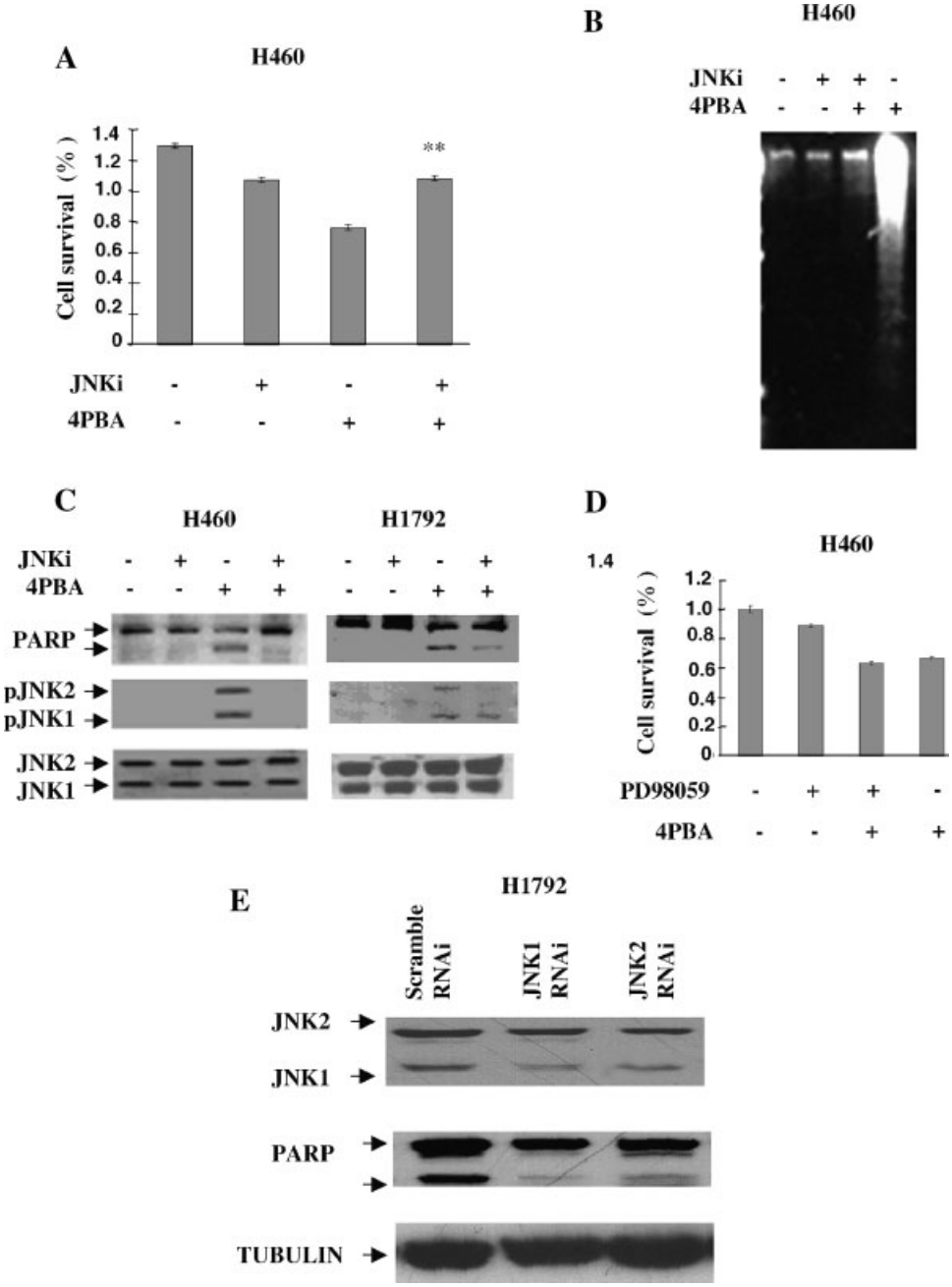

Fig. 4. Effect of JNK inhibitor on PB-induced apoptosis. **A**: MTT assay of cell survival. H460 cells were pretreated with DMSO or with 15 μM JNK inhibitor (JNKi) for 30 min. PB (15 mM) were then added to the culture and the cells were incubated for 24 h, as indicated. The cell survivals were then analyzed by MTT assay ($P < 0.01$). Data indicates mean of triplicates of a representative experiment. Experiments were repeated at least three times. **B**: DNA analysis of PB-induced apoptosis. H460 cells were treated the same as described in (A), and DNA from these cells were isolated and analyzed by gel electrophoresis. **C**: PARP cleavage analysis of PB-induced apoptosis. H460 cells and H1792 cells were treated the same as described in (A). Cleavage of the caspase substrate PARP were detected by Western blot analysis. JNK activation was detected by anti-phospho-JNK antibody on Western blot. **D**: Effect of MEK inhibitor on apoptosis induced by PB. H460 cells were pretreated without (−) or with 50 mM MEK inhibitor PD98059 (+) for 30 min before addition of PB (+), as indicated. MTT assay was then performed according to the Materials and Methods. **E**: H1792 cells were infected with pRetrosuper/JNK1, pRetrosuper/JNK2 small RNA interferences (RNAi), or scramble RNA interference retrovirus. The cells were then selected with puromycin for 48 h. The selected cells were then treated with 15 mM PB for overnight. Cell lysates were analyzed for the level of JNK and the cell death induced by PB, as indicated by PARP cleavage, by Western blot analysis using the antibodies as indicated. Anti-tubulin antibody was used to show equal protein loading.

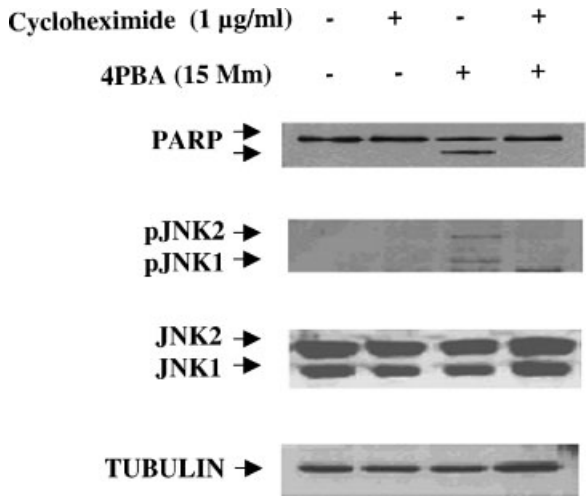

Fig. 5. New protein synthesis is required for PB-induced apoptosis. H460 cells were pretreated with 1 μg/ml cycloheximide for 12 h before addition of 0 (−) or 15 mM PB (+) for 24 h, as indicated. Cells were lysed, separated by SDS–PAGE, and immunoblotted with anti-PARP, anti-phospho-JNK, anti-JNK, or anti-tubulin antibody.

DISCUSSION

PB has been used successfully in the treatment of urea cycle defects. PB binds to glutamine and works by promoting an alternative nitrogen excretion pathway in people with urea cycle defects. PB is naturally synthesized in the intestinal tract of animals and humans, and therefore, is of relatively low toxicity. PB has been shown to be well tolerated when used in treatment of urea disorders in doses that may also inhibit histone deacetylases (HDACs) [Svechnikova et al., 2003]. Recently, PB has been studied as a drug for the treatment of certain malignancies [Carducci et al., 1996; Yu et al., 1999]. We have analyzed the effects of PB on human lung carcinoma cells. PB has distinct patterns of effects on different lung carcinoma cells, inducing apoptosis in NCI-H460 and NCI-H1792 cells, causing G1 arrest in A549 and SK-LU-1 cells, but having little effect on a non-transformed bronchial epithelial cell line HBE4-E6/E7. In vitro, PB significantly inhibited growth of A549 and SK-LU-1 cells and induced apoptosis of H460 and H1792 cells after 48 h treatment. Svechnikova reported that treatment with 10 mM of PB for 24 h (or 5 mM for 48 h) significantly inhibited Hep3B cell growth in vitro. Because of low toxicity and well-tolerated characteristics, phenylbutyrate is under investigation in phase I and II clinical trails as an anti-cancer drug, despite of its weaker anti-tumor activity in vitro, as compared to, for example, hydroxamic acids trichostain A [Svechnikova et al., 2003]. Therefore, PB is an effective anti-tumor agent and displays low toxicity, which provides the rationale for the initiation of clinical trials of PB in tumor.

The mode of action of PB is generally believed to be transcriptional. It alters chromatin structure and modulates transcription via the inhibition of histone deacetylase activity [Sawa et al., 2001]. Although the inhibition of histone deacetylase is known to be associated with regulation of critical cell cycle regulators, such as cyclin D, p21 (CIP1/WAF1), and p27 (KIP1) [Chen et al., 2003], the molecular pathways through which PB and its analogs mediate the anti-apoptotic effects have not been fully elucidated.

JNK has also been shown to affect terminal differentiation in some cells. Activation of ERK correlated with proliferation and initial differentiation; whereas activation of JNK and p38 likely played a role in later stage of cell differentiation or in cell apoptosis. Phosphorylation and activation of downstream transcription factor c-Jun by JNK likely play a central role in differentiation and apoptosis [Wisdom et al., 1999].

Function of JNK in cell death has well been recognized. Injurious stimuli including UV light, H_2O_2, ceramide, or TNF-α all activate JNK, while inhibition of JNKK (SEK1), JNK, or its target c-Jun can prevent apoptosis. JNK is activated by many microtubule-interfering agents, including Taxotere and paclitaxel, and has been implicated in mediating their cytotoxic effects [Wang and Wieder, 2004].

In this study, we analyzed the effects of PB on the activities of the MAPK family members and studied their relevance to the PB-induced cell death in human lung carcinoma cells. We observed a close correlation between PB-induced activation of JNK and cell apoptosis. We further demonstrated that induction of JNK activation is essential for PB-induced apoptosis. We also observed an induction of ERK activation in response to PB treatment, but its activation does not seem to either correlate or contribute to the PB-induced apoptosis.

ERK activity has been shown to decrease in response to butyrate treatment and the down-regulation of ERK seems to be associated with cell differentiation [Ding et al., 2001]. We,

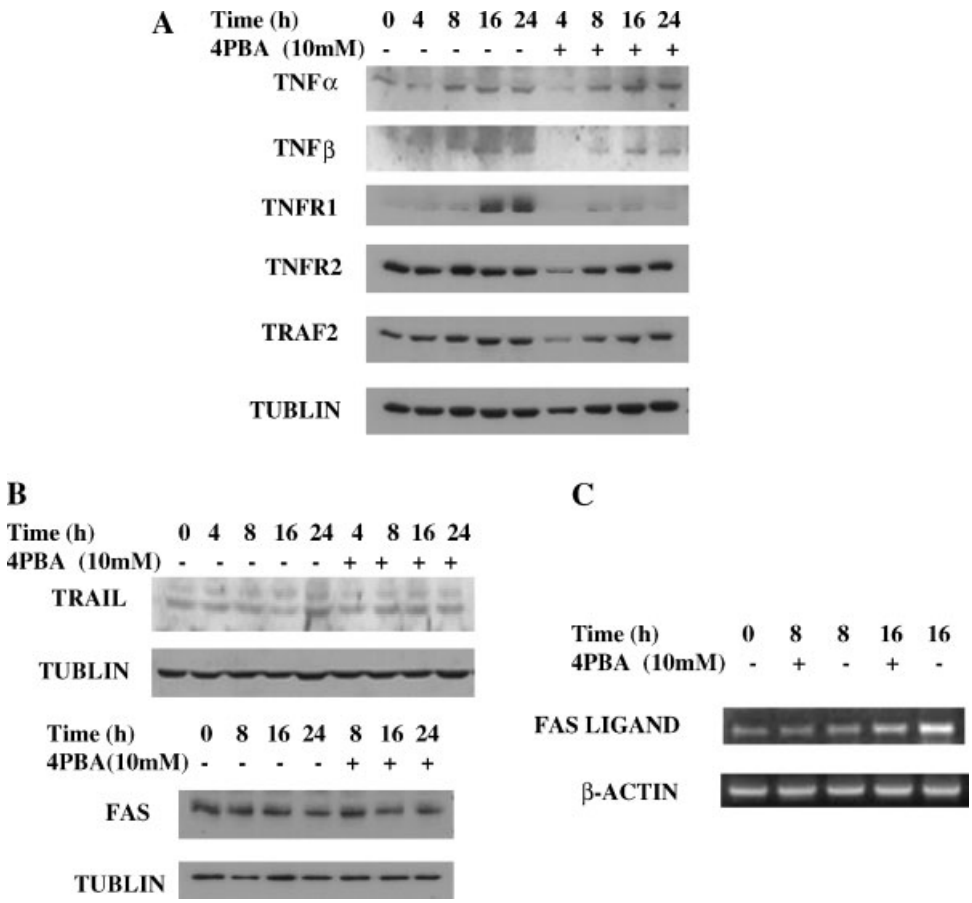

Fig. 6. Effect of PB on the expressions of TNF family genes TNF-α, TNF-β, TNFR1, TNFR2, TRAIL, TRAF, Fas, and Fas ligand. **A**, **B**: Western blot analysis of protein levels of TNF-α, TNF-β, TNFR1, TNFR2, TRAIL, TRAF, Fas, and Fas ligand. H460 cells were treated with 10 mM PB (+) for different time periods as indicated. The lysates were resolved by SDS–PAGE and analyzed by immunoblotting with anti-TNF-α, TNF-β, TNFR1, TNFR2, TRAIL, TRAF, or Fas antibody. Anti-tubulin antibody was used to show the protein loading level. **C**: RT-PCR analysis for FAS ligand expression in H460 cells treated with PB. H460 cells were treated with 10 mM PB (+) for 8 or 16 h as indicated. Total RNA was isolated. RT-PCR and gel electrophoresis were performed to estimate the RNA level of Fas ligand.

however, observed an increase of ERK activation in PB-treated human lung cancer cells. It is not clear what the basis for this difference is. One possible explanation is that the regulation of MAPKs by butyrate is cell-type specific. In this regard, it is intriguing to note that PB induces JNK activation and apoptosis in only a subset of human lung carcinoma cells, namely the H460 and H1792 cells, but not in A549, SK-LU-1, or the non-transformed HBE cells. We have noticed some interesting differences between the two groups of cells. Both H460 and H1792 are more aggressive-type of tumor cells, comparing to the other three types of cells. They form larger aggregates in suspension culture and form larger colonies in soft agar (unpublished observation). It is not clear how general this correlation is at present. More data is needed to make a general conclusion. Lotan et al. reported that the level of galectin-1 mRNA increased in MDA-886LN cells treated by butyrate, suggesting a role for butyrate in transcriptional regulation of galectin-1 expression. Galectin-1 is a beta-galactoside-binding protein thought to be important for cell–cell interactions [Gillenwater et al., 1998]. It may suggest that some kind of cell–cell interactions is required for the activation of JNK by PB.

The mechanism by which PB activates JNK remains to be understood. Although cytokines and growth factors act via specific receptors, the mechanisms used by stresses to activate JNK MAPKs are currently unknown [Basu and Kolesnick, 1998; Higashi, 1999]. It has been suggested that receptor clustering and internalization could play a role [Rosette and Karin,

1996]. Indeed, the exposure of cells to UV-radiation or osmotic shock promoted clustering and internalization of cell-surface receptors for tumor necrosis factor (TNF), epidermal growth factor (EGF), and interleukin-1 (IL-1) in the absence of their ligands [Rosette and Karin, 1996]. Several lines of evidence indicate that Rho family GTPase mediate the activation JNK caused by receptor tyrosine kinases [Coso et al., 1995; Minden et al., 1995]. The activation of JNK by cytokine receptors appears to be mediated by the TRAF group of adapter proteins. Activation of TNF receptor leads to recruitment of TRAF2, which is needed for JNK activation; the IL-1 receptor recruits TRAF6 to activate JNK [Liu et al., 1996; Yeh et al., 1997; Lomaga et al., 1999]. FAS, a member of the TNF receptor family, also activates JNK. FAS may engage in two independent pathways that induce cell death: one pathway via FADD/caspase-8/2 and the other via Daxx/JNK activation [Zornig et al., 2001]. It has been reported that butyrate increased Fas and Fas ligand levels and induced apoptosis in MCF-7 cells [Chopin et al., 2002]. Although our data demonstrated that new protein synthesis is required for the PB-induced apoptosis, we did not observe any change in the levels of Fas/Fas ligand, TNF, or TRAIL in response to PB treatment. The expression of TNFR1, the receptor for TNF, appeared to be cell cycle-dependent. There was an induction of TNFR1 expression between 8 and 16 h after the cells were plated, suggesting that TNFR1 expression may be cell cycle-dependent. This induction of TNFR1 expression was inhibited by PB treatment. The mechanisms of the regulation of TNFR1 expression and its inhibition by PB are not clear at present. However, induction of cell death signaling proteins does not seem to be the mechanism for PB-induced cell death, because none of them was induced by PB treatment. Therefore, there appear to be other unidentified proteins whose de novo synthesis is required for PB-induced JNK activation and apoptosis.

PB is now entering clinical trials in combination with other agents to demonstrate potentially synergistic effects. One combination involves PB and 13-*cis*-retinoic acid. PB is also in trial with 5-azacytidine, a DNA methyltransferase inhibitor [Carducci et al., 2001]. Identification of molecules and pathways that PB utilizes to induce apoptosis of cancer cells is of therapeutic value in classification of cancer and treatment of cancer patients with PB or PB in combination with other agents.

In conclusion, PB, an HDAC inhibitor with low toxicity and selectivity toward cancer cells, may provide a novel therapeutic strategy for cancers refractory to traditional anti-tumor agents. Moreover, our data are the first genetic evidences to suggest that PB-induced apoptosis is JNK signaling pathway-dependent.

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