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p38 MAPK Activation, JNK Inhibition, Neoplastic Growth Inhibition, and Increased Gap Junction Communication in Human Lung Carcinoma and *Ras*-Transformed Cells by 4-Phenyl-3-Butenoic Acid

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ABSTRACT

Human lung neoplasms frequently express mutations that down-regulate expression of various tumor suppressor molecules, including mitogen-activated protein kinases such as p38 MAPK. Conversely, activation of p38 MAPK in tumor cells results in cancer cell cycle inhibition or apoptosis initiated by chemotherapeutic agents such as retinoids or cisplatin, and is therefore an attractive approach for experimental anti-tumor therapies. We now report that 4-phenyl-3-butenoic acid (PBA), an experimental compound that reverses the transformed phenotype at non-cytotoxic concentrations, activates p38 MAPK in tumorigenic cells at concentrations and treatment times that correlate with decreased cell growth and increased cell-cell communication. H2009 human lung carcinoma cells and *ras*-transformed rat liver epithelial cells treated with PBA showed increased activation of p38 MAPK and its downstream effectors which occurred after 4 h and lasted beyond 48 h. Untransformed plasmid control cells showed low activation of p38 MAPK compared to *ras*-transformed and H2009 carcinoma cells, which correlates with the reduced effect of PBA on untransformed cell growth. The p38 MAPK inhibitor, SB203580, negated PBA's activation of p38 MAPK downstream effectors. PBA also increased cell–cell communication and connexin 43 phosphorylation in *ras*-transformed cells, which were prevented by SB203580. In addition, PBA decreased activation of JNK, which is upregulated in many cancers. Taken together, these results suggest that PBA exerts its growth regulatory effect in tumorigenic cells by concomitant up-regulation of p38 MAPK activity, altered connexin 43 expression, and down-regulation of JNK activity. PBA may therefore be an effective therapeutic agent in human cancers that exhibit down-regulated p38 MAPK activity and/or activated JNK and altered cell–cell communication. J. Cell. Biochem. 113: 269–281, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: p38 MAPK; CONNEXIN; JNK; SB203580

p38 MAPKs are stress-activated members of the mitogen-activated protein kinase (MAPKs) family that play important roles in the control of cell proliferation in a wide variety of cell types [Raingeaud et al., 1995; Wang et al., 1997; Lewis et al., 1998; Schaeffer and Weber, 1999; Pearson et al., 2001; Engelberg, 2004]. p38 MAPK transmits signals from the cell membrane to the nucleus in response to oxidative or osmotic stress, cytokines, and radiation [Zarubin and Han, 2005; Dhillon et al., 2007]. Activation of p38

Abbreviations used: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; IAA, iodoacetamide; MAPK, mitogen-activated protein kinase; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate; PAM, peptidylglycine alpha-monooxygenase; PBA, 4-phenyl-3-butenoic acid; PBA-Me, 4-phenyl-3-butenoic acid methyl ester; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate.

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MAPK via phosphorylation activates the transcription factors ATF-2 and HSP-27 [Rouse et al., 1994; Cobb and Goldsmith, 1995; Raingeaud et al., 1995], among others [Cohen, 1997; Yang et al., 1999], and other kinases such as MAPKAPK-2 [Kyriakis and Avruch, 2001; Kumar et al., 2003]. Inhibition of tumorigenesis by p38 MAPK activation has been shown to occur in mice that express mutant Erb2 or H-Ras [Bulavin et al., 2004]. p38 MAPK activation also correlated with increased apoptosis [Cao et al., 2004; Liberto et al., 2004; Lowe et al., 2005], and p38 MAPK has been described as a tumor suppressor [Timofeev et al., 2005; Bradham and McClay, 2006; Dhillon et al., 2007]. In addition, regulation of p38 MAPK has been correlated with changes in phosphorylation of the gap junction protein, connexin 43, and the kinase may directly phosphorylate this protein [Lee et al., 2004; Ogawa et al., 2004; Aranvindakshan and Cyr, 2005]. Thus, there appears to be a link between p38 MAPK activity, connexin phosphorylation, and neoplastic transformation.

Lung cancer is the leading cause of cancer death for both men and women, with over 200,000 new cases occurring in the United States in 2010 [American Cancer Society, 2010]. Five-year survival rates for lung cancer are below 10% [Brognard and Dennis, 2002]. Current therapies for lung cancer are poorly effective and there is a need to identify additional therapeutic agents. Human lung neoplasms frequently express mutations in *ras*-genes and inactivation or deletion of p53 and other tumor suppressor genes [Mitsudomi et al., 1992; Ruch et al., 1998]. Clinical trials of inhibitors of the EGF receptor, which activates the Ras pathway, showed some therapeutic effectiveness in a small subset of individuals [Levitzki, 2003; Comis, 2005; Cho et al., 2007; Wong et al., 2010]. However, these anti-tumor agents were not effective in patients with *ras*-mutations or defects in downstream effectors [Levitzki, 2003]. Thus, additional agents are needed to treat this common and deadly form of cancer.

PBA is an irreversible turnover-dependent inhibitor of peptidylglycine-alpha-monooxygenase (PAM) in vitro [Katopodis and May, 1990; Katopodis et al., 1990], with anti-inflammatory effects in vivo mediated by a non-COX inhibitory pathway [Bauer et al., 2007]. PBA decreased lung cancer cell proliferation, presumably by inhibiting the synthesis of amidated growth factors [Iwai et al., 1999]. We previously demonstrated that PBA inhibited the growth of *ras*-transformed WB-F344 cells and up-regulated gap junction-mediated cell–cell communication and connexin 43 [Sunman et al., 2004]. The WB-F344 cells are a diploid rat liver epithelial cell line that exhibits contact inhibition of growth and is non-tumorigenic [Tsao et al., 1984]. These cells also exhibit high levels of gap junction cell–cell communication and connexin 43 expression [Matesic et al., 1994]. A highly tumorigenic and poorly communicating derivative of the WB-F344 cell line known as WB-*ras* was generated by stable retroviral transduction with the v-Ha-*Ras* oncogene [De Feijter et al., 1990]. Gap junction-medicated cell–cell communication is involved in cellular growth control and differentiation, and can inhibit tumorigenesis [Trosko and Ruch, 1998]. We now report that PBA activates p38 MAPK in both *ras*-transformed WB and human lung carcinoma cells at concentrations and treatment times that correlate with decreased cell growth and altered connexin expression. The methylated derivative of PBA, PBA-Me, also inhibits growth and activates p38 MAPK, but at a fivefold lower concentration. PBA's effects on p38 MAPK and cell–cell communication in *ras*-transformed cells were prevented by a specific p38 MAPK inhibitor and suggest a link between p38 MAPK activation and cell–cell communication. In addition, PBA inhibited activation of another stress-activated MAPK, JNK, which is over-activated in selected cancers [Antonyak et al., 2002; Rennefahrt et al., 2004]. These studies underscore the therapeutic potential of PBA and PBA-Me in lung and other cancers that have reduced cell–cell communication and p38 MAPK activity, and/or increased JNK activity.

MATERIALS AND METHODS

MATERIALS

WB-*neo* and WB-r*as* cells were derived from WB-F344 rat liver epithelial cells [De Feijter et al., 1990] and were obtained from Dr. James Trosko at Michigan State University. H2009 human lung carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Alpha Modification of Eagle's medium was purchased from Mediatech (Herndon, VA). RPMI medium, L-glutamine, trypsin, and phosphate-buffered saline (PBS), were from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA). PBA, Lucifer Yellow-CH fluorescent dye, phenylmethylsulfonyl fluoride (PMSF), iodoacetamide (IAA), protease inhibitor cocktail, G418, trypan blue solution, and Ponceau Red solution were from Sigma Chemical Co. (St. Louis, MO). Connexin43 monoclonal antibody (MAb3086) was obtained from Chemicon International (Temecula, CA). Phospho-p38 MAP kinase (Thr180/Tyr182) polyclonal antibody, p38 MAP kinase polyclonal antibody, phospho-ATF2 (Thr71) polyclonal antibody, phospho-HSP27(Ser82) polyclonal antibody, JNK polyclonal antibody, phospho-JNK (Thr183/Tyr185) polyclonal antibody, phospho-MAPKAPK-2 (Thr334) polyclonal antibody, Akt polyclonal antibody, phospho-Akt (Ser473) polyclonal antibody, cdc2 polyclonal antibody, phospho-cdc2 (Thr161) polyclonal antibody, CDK2 polyclonal antibody, phospho-CDK2 (Thr160) polyclonal antibody, phospho-PKC pan (βII ser660) polyclonal antibody, β-actin polyclonal antibody, and anti-rabbit IgG alkaline phosphatase-conjugated antibody were from Cell Signaling Technology (Beverly, MA). Tween-20, TRIS–HCl, *DC* Protein Assay, SDS, nonfat dry milk, 25× alkaline phosphatase color development buffer, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT), protein molecular mass standards, and all electrophoresis and transfer buffer components were from Bio-Rad (Hercules, CA). LumiPhos chemiluminescence reagent was from Thermo Scientific (Rockford, IL). Biotin-(GT) anti-mouse IgG antibody and alkaline phosphatase-conjugated streptavidin were from MP Biomedicals, LLC (Irvine, CA). Dieldrin was from Accustandard (New Haven, CT). Polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA). SB203580, p38 MAPK inhibitor III and TdT-FragEL DNA fragmentation detection kit were obtained from EMD Biosciences (La Jolla, CA).

PBA-methyl ester (PBA-Me) was synthesized as follows: 10 g of PBA was dissolved in 100 ml HPLC-grade methanol acidified with 10 drops of concentrated HCl. The reaction mixture was heated to reflux and allowed to proceed overnight. The reaction was then quenched via the addition of 100 ml water and the pH adjusted to 7.0

using NaOH. The solution was then extracted four times with ethyl acetate, and the organic layer was dried over magnesium sulfate, filtered, and evaporated under reduced pressure to yield 9.2 g (85%) of the final product. PBA was re-purified from the Sigma stock by re-crystallization. All other chemicals, reagents, and solvents used were of analytical grade.

CELL CULTURE

WB-*neo* and WB-r*as* rat liver epithelial cells were subcloned from single cells to obtain WB-*neo*3 and WB-r*as*1 lines, and were used between passages 5 and 18. Cells were grown in alpha Modification of Eagle's Medium supplemented with 2 mM/L L-glutamine and 5% FBS. G418 antibiotic was diluted 1:2 in PBS and added to the cell growth media at a concentration of 500 μM, but was omitted for experiments. H2009 human lung carcinoma cells were grown in RPMI medium supplemented with 2 mM/L L-glutamine and 10% FBS. Confluent cells were subcultured by trypsinization and plated at 5–25% confluence during each passage. Cells were incubated in an atmosphere of 5% CO_2 at 37°C.

CELL GROWTH ASSAY

Cells were plated at 5–10% confluence in 2 ml of media on 35 mm dishes and allowed to acclimate for 24 h. Cells were then treated with vehicle or drug dissolved in sterile H_2O (PBA, 0.1 mg/ml = 613 μM) with or without 2 μM SB203580 and incubated at 37°C for the duration of the experiment. Cells were washed once with PBS, treated with 0.5 ml of trypsin until cells no longer adhered to the dish, quenched with 1.5 ml of media, and cells in solution were counted using a hemocytometer.

APOPTOSIS ASSAY

H2009 cells plated in 35 mm dishes were treated with vehicle or 0.1 mg/ml PBA twice (at $t = 0$ and 24 h). Apoptotic cells were fixed with 4% formaldehyde in PBS and stained using a TdT-FragEL DNA fragmentation detection kit according to the manufacturer's protocol for fixed cell preparations.

PREPARATION OF MEMBRANE-ENRICHED/ALKALI-RESISTANT FRACTION FOR WESTERN BLOT ANALYSIS

Cells were grown in 15 ml of media with vehicle or drug(s) in 75 cm^2 flasks to 90–100% confluence. Media was removed and the cells were washed with PBS, then 0.375 ml of TRIS–IAA buffer (10 mM TRIS (pH 7.5), 10 mM IAA, and 1 mM PMSF) was swirled over the cells and 0.55 ml of 40 mM NaOH was added. The cells were then scraped and transferred to microcentrifuge tubes on ice. Each sample was sonicated (two 15 s pulses at 35% maximum power with a Bronson Cell Disruptor 185 Sonicator, allowing 1–2 min between pulses). Samples were centrifuged at 14,000 × g for 30 min at 4°C. The supernatants were discarded and the pellets were washed with 1 ml of TRIS–PMSF buffer (10 mM TRIS (pH 7.5), and 1 mM PMSF). Samples were centrifuged at 14,000 × g for 15 min at 4°C and the supernatants were again removed. Each pellet was re-suspended in 75 μl of TRIS/PMSF buffer and three 5 μl aliquots were removed for total protein assay using the Bio-Rad DC protein assay. The remaining sample was frozen in liquid nitrogen and stored at −20°C.

WESTERN BLOT ANALYSIS OF CONNEXIN 43

Membrane-enriched/alkali-resistant protein samples were loaded onto 1 mm, 10 well, 12.5% acrylamide gels and run at 60 V until the samples had passed through the stacking gel, then at 120–150 V. Proteins were transferred onto PVDF membranes at 20 V overnight in the presence of 0.05% SDS. PVDF membranes were washed in water and stained with Ponceau Red solution for 2–3 min. Membranes were incubated for 1 h in block buffer (4% nonfat dry milk, 40 mM TRIS, pH 7.5, and 0.1% Tween-20) and overnight with anti-connexin43 monoclonal antibody (2 μl/10 ml block buffer) with shaking at 4°C. Membranes were washed with block buffer and incubated at room temperature for 1 h with a secondary antibody (anti-mouse biotinylated antibody 25 μl/10 ml block buffer) on a shaker, followed by washing and incubation for 1 h at room temperature with alkaline phosphatase-conjugated streptavidin (diluted 1:400 in block buffer containing 0.5 M NaCl). After washing, bands were visualized using BCIP/NBT.

WESTERN BLOT ANALYSIS OF SIGNALING PATHWAY PROTEINS

Cells were grown to 80–90% confluence in 25 cm^2 flasks, washed with 10 ml of PBS and extracted with 250 μl 2% SDS, 1 mM PMSF, and 1:100 dilution of protease inhibitor cocktail. Lysed cells were scraped, transferred to microcentrifuge tubes, and sonicated for two, 15 s pulses at room temperature. Protein concentrations were determined by Bio-Rad DC assay and proteins were separated on 12.5% acrylamide SDS gels and transferred to PVDF membranes overnight at 20 V or 1 h at 180 V. Membranes were stained with Ponceau Red, then incubated in block buffer for 1–2 h. P38 MAPK, phospho-p38 MAPK, phospho-HSP-27, phospho-ATF-2, JNK, phospho-JNK, Akt, phospho-Akt, cdc2, phospho-cdc2, CDK2, phosphoCDK2, phospho-PKC, or β-actin polyclonal antibodies (1 μl/ml block buffer) were incubated separately with blots in block buffer overnight at 4°C. Immunopositive bands were detected using alkaline phosphatase-linked anti-rabbit secondary antibody and development with BCIP/NBT as substrates, or Lumi-Phos where noted. Blots were scanned on an HPscanjet 4400C scanner and band densities determined using UN-SCAN-IT software (version 5.1) from Silk Scientific, Inc. (Orem, UT). Two to five replicate blots were analyzed for each experiment.

FLUORESCENCE DYE TRANSFER ASSAY

To quantify gap junction-mediated cell–cell communication, a fluorescence dye transfer assay was performed, modified slightly from that previously described [Jou et al., 1993]. Cells were grown to 90–100% confluence in 35 mm culture dishes and treated with vehicle or drug as indicated. Two dishes in each group were treated with 10 μM dieldrin, a gap junction inhibitor, for 30 min prior to assay. Dishes were then washed once with Ca^{2+}/Mg^{2+} PBS and twice with PBS. One milliliter of Lucifer Yellow dye (0.5 mg/ml in PBS) was added to each dish and six to eight score lines were made in the cell monolayer with a surgical blade. Dishes were kept in the dark for 2 min, then washed three times with PBS and once with Ca^{2+}/Mg^{2+} PBS. Cells were fixed with 1.5 ml of 4% paraformaldehyde for 30 min, and then washed again with PBS. Fluorescence was observed using a Leitz microscope with a 10× objective lens. Several randomly selected fields on each dish were digitally

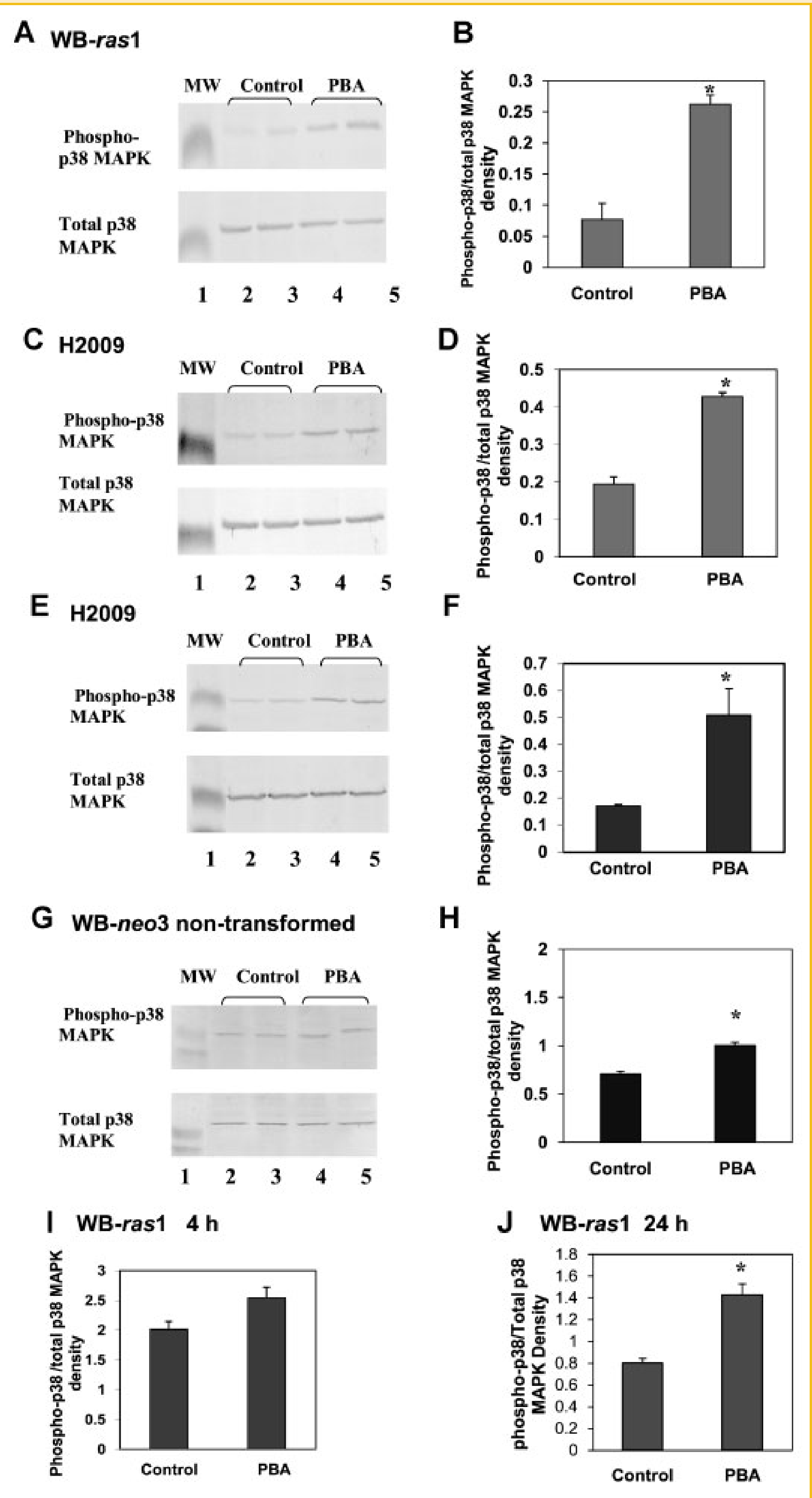

Fig. 1. Effect of PBA on activated p38 MAPK (Thr180/Tyr182 phosphorylation) content in: WB-*ras*1 (A), H2009 (C and E), or WB-*neo*3(G) cells. Cells were treated with 0.1 mg/ml PBA for 48 h (A, C G) or 14 days (E) and extracted for total protein for Western blot analysis as described in Materials and Methods. Samples from vehicle control (lanes 2 and 3) or PBA-treated (lanes 4 and 5) replicate cultures are shown on each blot. Identical blots were incubated with phospho-p38 MAPK (upper panels) or p38 MAPK (lower panels) antibodies. (MW = 41 kD pre-stained molecular weight marker). The denstometric quantification of bands in scanned blots (panels B, D, F, and H) are presented as the mean ± SD, and are representative of six (WB-*ras*1), three (H2009, 48 h), one (H2009 14d), and two (WB-*neo*3) independent experiments (asterisks indicate $P < 0.05$ compared to vehicle control). Panels I and J show the effects of 0.1 mg/ml PBA on activated p38 MAPK in WB-*ras*1 cells following 4 h (I) and 24 h (J) treatment. Values are the mean ± SD, $P < 0.05$ compared to vehicle control. Phospho-p38 MAPK antibodies used are specific for Thr180/Tyr182.

photographed and the number of fluorescent cells adjacent to score lines was counted in a defined unit area. The number of communicating cells was determined by subtracting the average number of fluorescent cells per unit area in the dieldrin-treated dishes (non-communicating cells) from the number of fluorescent cells per unit area in vehicle and drug-treated dishes.

STATISTICAL ANALYSES

Data are presented as the mean ± the standard deviation (SD). One-way analysis of variance (ANOVA) was used to test for significance between repeated measures. Tukey's post hoc test was used following one-way ANOVA to determine significant differences within a group. $P < 0.05$ was considered statistically significant in all calculations. Statistical analyses were performed using Statistix for Windows v8.1.

RESULTS

PBA ACTIVATES p38 MAPK IN *Ras*-TRANSFORMED EPITHELIAL AND HUMAN LUNG CARCINOMA CELLS

Treatment of WB-*ras*1 cells with 0.1 mg/ml PBA for 48 h increased phosphorylation of p38 MAPK on key activation sites, Thr180/Tyr182 (Fig. 1A). Lanes 4 and 5 (separately treated, replicate samples) of Figure 1A shows increased immunoreactive band density of phospho-p38 MAPK (top panel), compared to vehicle-treated control lanes 2 and 3 (also separately treated, replicate samples). PBA did not substantially alter total p38 MAPK content in the cells (Fig. 1A lower panel). Densitometric scans of blots (Fig. 1B) revealed a threefold increase in the density of phospho-p38 MAPK (normalized to total p38 MAPK density) in PBA-treated WB-*ras*1 cells compared to vehicle-treated control. PBA similarly increased p38 MAPK phosphorylation in H2009 human lung tumor cells (Fig. 1C, top panel, lanes 4 and 5 compared to vehicle-treated control lanes 2 and 3), and had no effect on total p38 MAPK levels (Fig. 1C, bottom panel, lanes 2–5). Densitometric scans revealed a ~2.5-fold increase in phospho-p38 MAPK in PBA-treated H2009 cells (Fig. 1D). This increased p38 MAPK phosphorylation by PBA was sustained for as long as 14 days post-treatment (Fig. 1E, top panel, PBA-treated lanes 4 and 5 compared to vehicle-treated control lanes 2 and 3 and quantification in F). In non-transformed plasmid control WB-*neo*3 cells, a substantially smaller increase in p38 MAPK phosphorylation was observed after treatment with PBA at 0.1 mg/ml for 48 h (Fig. 1G,H) compared to WB-*ras*1 cells treated over the same time (Fig. 1A,B).

No change in p38 MAPK phosphorylation occurred in WB-*ras*1 cells treated for 2 h (not shown). A small increase was seen at 4 h which was not significant (Fig. 1I, $P > 0.05$), while at 24 h treatment, a ~1.7-fold increase in p38 MAPK phosphorylation was observed (Fig. 1J, $P < 0.05$). Similarly, treatment of H2009 cells for 4 h showed no significant change in p38 MAPK phosphorylation (not shown). In the absence of PBA, basal levels of activated p38 MAPK were lower in WB-*ras*1 and H2009 cells compared to the non-transformed WB-*neo*3 cells (Fig. 2).

PBA INCREASES PHOSPHORYLATION OF THE p38 MAPK DOWNSTREAM EFFECTORS, HSP-27 AND ATF-2, IN *Ras*-TRANSFORMED EPITHELIAL AND HUMAN LUNG CARCINOMA CELLS

Treatment of WB-*ras*1 and H2009 cells with 0.1 mg/ml PBA for 48 h increased the phosphorylation of HSP-27 and ATF-2 at key activation sites by approximately twofold to sixfold in identical samples (Fig. 3A,B, compare lanes 4 and 5 with vehicle control lanes 2 and 3 in the top and third panels and quantification in B, C, E, and F). Treatment of H2009 cells with PBA (0.1 mg/ml PBA for 48 h) also significantly increased phosphorylation of MAPKAPK-2, a substrate for p38 MAPK and HSP-27 kinase (8.6 ± 0.13 for PBA-treated cells versus 0.3 ± 0.16 for vehicle-treated cells in relative density units, $P < 0.01$). PBA had no significant increase on phosphorylation of HSP-27 in WB-*ras*1 cells treated with 0.1 mg/ml PBA for 2 h or 6 h, or in H2009 cells treated for 4 h ($P > 0.05$, not shown).

PBA-ME ALSO INCREASES p38 MAPK PHOSPHORYLATION IN H2009 HUMAN CARCINOMA AND WB-*ras*1 CELLS

The methylated form of PBA, PBA-Me, also increased the phosphorylation of p38 MAPK in H2009 cells at tenfold lower concentration (10 μg/ml) than the parent compound. (Fig. 4A, compare lanes 4 and 5 with vehicle control lanes 2 and 3) and this effect was further increased at 20 μg/ml PBA-Me

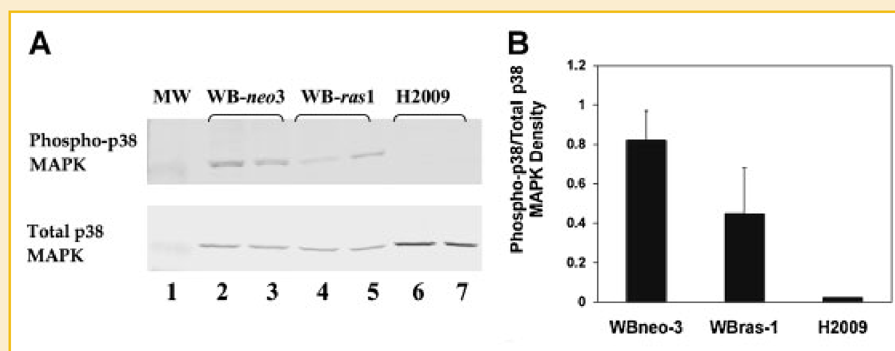

Fig. 2. Basal levels of activated p38 MAPK (Thr180/Tyr182) in WB-*neo*3 (lanes 2 and 3), WB-*ras*1 (lanes 4 and 5), and H2009 (lanes 6 and 7) cells (panel A) and quantification by densitometric scanning of band (panel B). Values are the mean ± SD (MW = 41 kD pre-stained molecular weight marker). Phospho-p38 MAPK antibodies used are specific for Thr180/Tyr182.

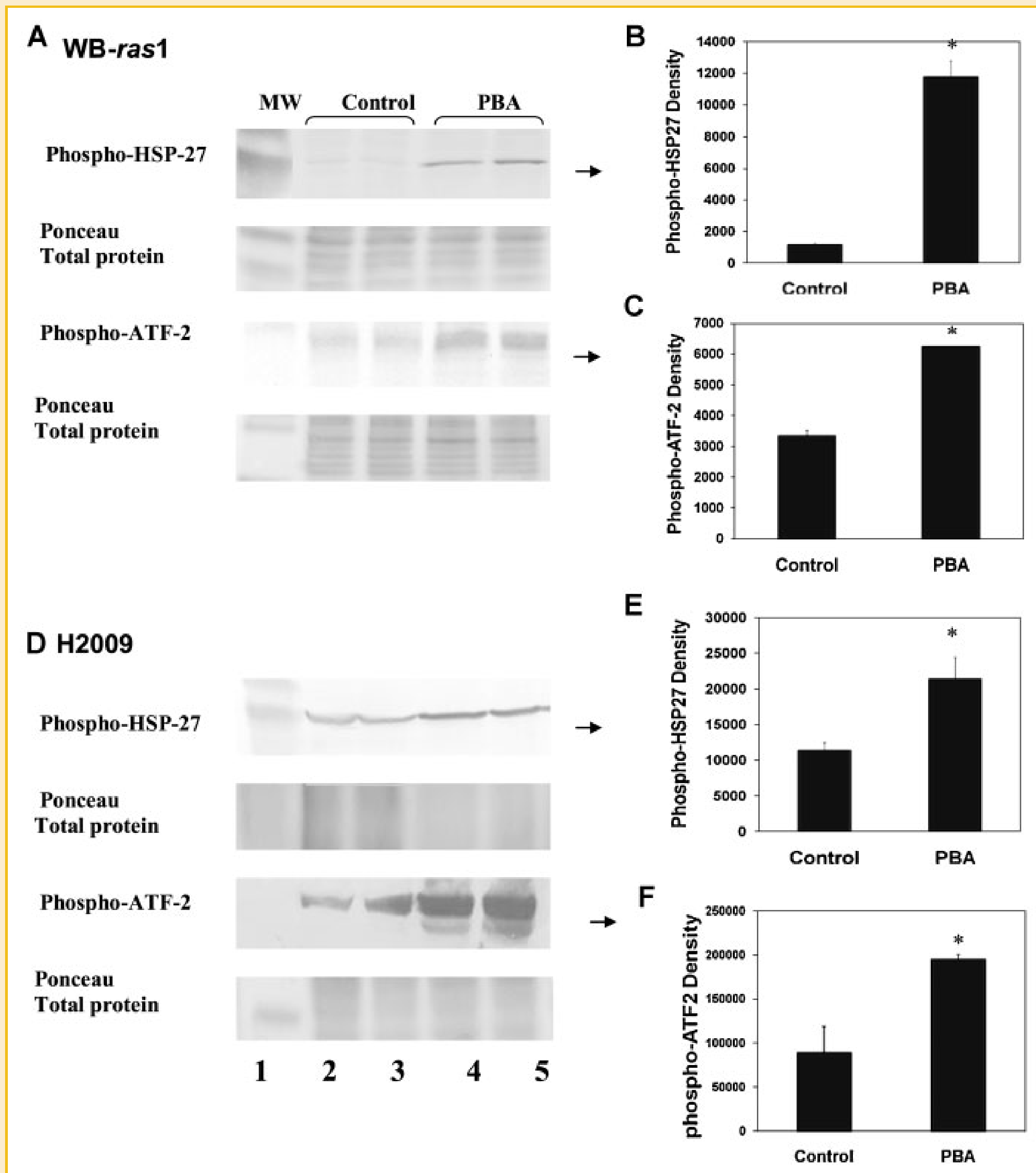

Fig. 3. Effect of PBA on activation of p38 MAPK downstream effectors, HSP-27 and ATF-2, in WB-*ras*1 (A) and H2009 (D) cells. Cells were treated with 0.1 mg/ml PBA for 48 h and total proteins were extracted as described in Materials and Methods. Samples from vehicle control (lanes 2 and 3) or PBA-treated (lanes 4 and 5) replicate cultures are shown on each blot. (MW = 41 kD pre-stained molecular weight marker). The densitometric quantification of bands in scanned blots (panels B, C, E, F) are presented as the mean ± SD, ($P < 0.05$ compared to vehicle control cells) and representative of at least two independent experiments. Phospho-HSP-27 and ATF-2 antibodies used are specific for Ser82 and Thr71, respectively.

(Fig. 4A, lanes 6 and 7 with lanes 4 and 5, and quantification in Fig. 4B). Similarly, 20 µg/ml PBA-Me stimulated p38 MAPK and HSP-27 phosphorylation in WB-*ras*1 cells by more than twofold (data not shown).

THE p38 MAPK INHIBITOR, SB203580, NEGATES THE EFFECT OF PBA ON THE p38 MAPK DOWNSTREAM EFFECTOR, HSP-27

The p38 MAPK active site inhibitor, SB203580, inhibits the actions of the kinase on downstream effectors such as HSP-27. When WB-*ras*1 cells were treated with 2 µM SB203580, basal levels of p38 MAPK phosphorylation (Fig. 5A, top panel, lanes 2, 3, 6, and 7) were detected and the stimulation of p38 MAPK phosphorylation by PBA was not substantially affected (Fig. 5A, top panel, lanes 8 and 9 compared to lanes 6 and 7, quantification on Fig. 5B). This was expected, since the inhibitor does not affect phosphorylation at the regulatory Thr180/Tyr182 activation sites. However, SB203580 greatly reduced PBA-stimulated phosphorylation of the p38 MAPK downstream target, HSP-27 (Fig. 5A, 4th panel from the top, lanes 8

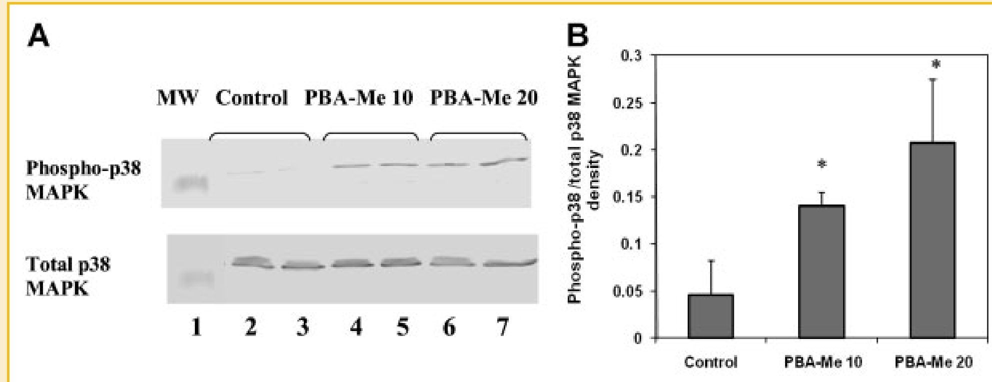

Fig. 4. Effect of PBA-Me on activated p38 MAPK content in WB-*ras*1 cells. The cells were treated with PBA-Me (0.01 mg/ml or 0.02 mg/ml dissolved in DMSO; 61.2 or 122.4 μM final concentrations) for 72 h, then extracted for Western blot analysis of activated p38 MAPK or total p38 MAPK (panel A). Densitometric quantification of bands is shown in panel B and represent the mean ± SD ($P < 0.05$ compared to control).

and 9 compared to lanes 4 and 5 and quantification in Fig. 5C). β-actin content and Ponceau staining indicate equivalent protein loading of the blots. SB203580 also reduced PBA-stimulated phosphorylation of HSP-27 in H2009 cells. PBA-stimulated phosphorylation of HSP-27 was also reduced in WB-*ras*1 cells by treatment with another p38 MAPK-specific inhibitor, p38 MAPK inhibitor III (data not shown).

THE p38 MAPK INHIBITOR, SB203580, REDUCES PBA-INDUCED P2 PHOSPHORYLATION OF CONNEXIN 43 AND PBA-ENHANCED CELL–CELL COMMUNICATION

PBA increased the content of the connexin 43-P_2 phosphoform relative to levels of the P_0 (non-phosphorylated) and P_1 phosphoforms in WB-*ras*1 cells (Fig. 6A, B), as we previously reported [Sunman et al., 2004]. SB203580 prevented the increase in connexin

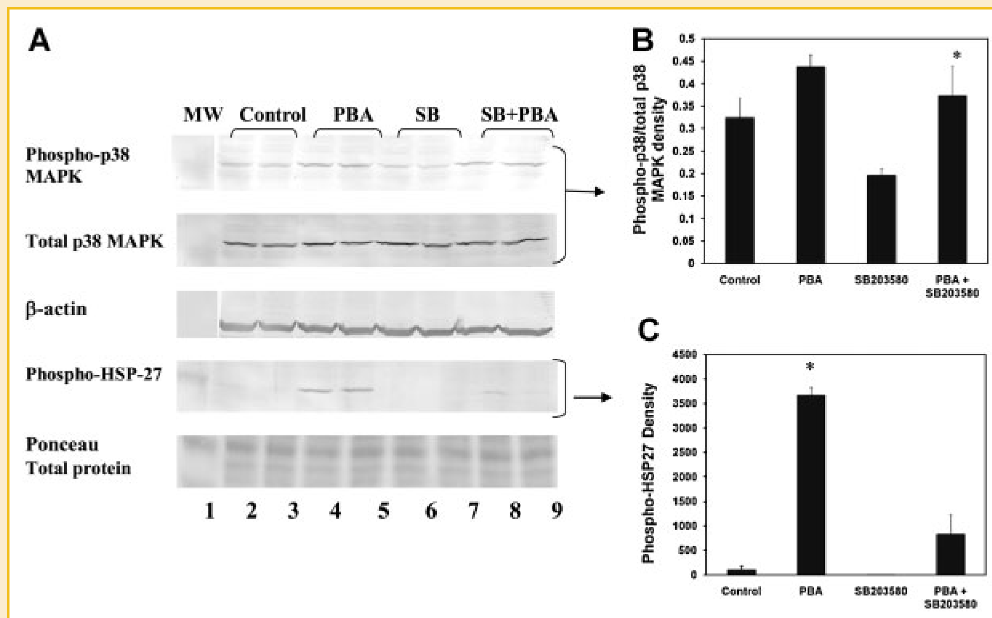

Fig. 5. Effect of the p38 MAPK inhibitor, SB203580, on activation of the p38 MAPK downstream effector, HSP-27. WB-*ras*1 cells were treated with PBA (0.1 mg/ml) and/or SB203580 (2 μM) or with vehicle (control lanes) for 48 h, then extracted for Western blot analyses. as described in Materials and Methods. (A) Two duplicate vehicle-control lanes (lanes 2 and 3), 0.1 mg/ml PBA-treated lanes (lanes 4 and 5), 2 μM SB203580 (lanes 6 and 7), or 0.1 mg/ml PBA + SB203580 (lanes 8 and 9) are shown on each blot. Paired lanes represent samples from two separately treated cultures. Three separate blots were used to identify phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, and phospho-HSP-27 (Ser 82) as indicated; the β-actin blot is a re-probe of the phospho-p38 MAPK blot. (B) and (C) Densitometric quantification of bands in scanned blots indicated to the left are presented as the mean ± SD (asterisks indicate $P < 0.05$ compared to SB203580 only in (B) and $P < 0.01$ compared to control in (C)).

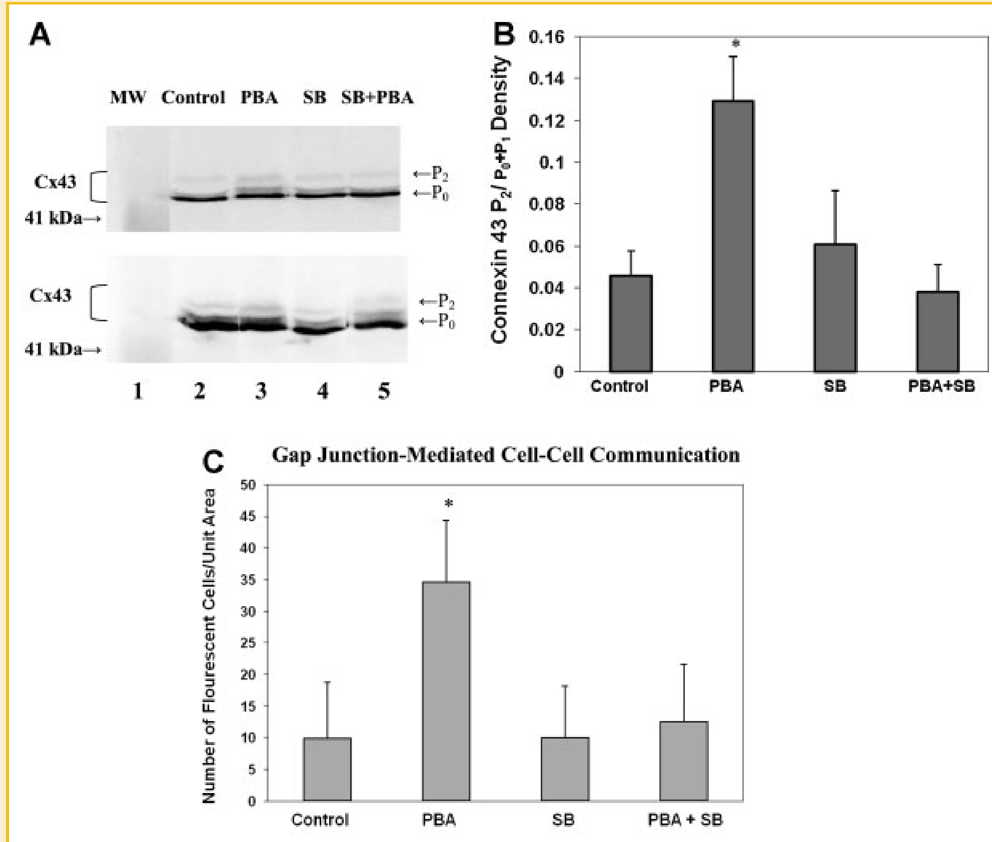

Fig. 6. Effect of PBA and/or the p38 MAPK inhibitor, SB203580, on phosphorylation of connexin 43 and gap junction-mediated cell–cell communication. In panel A, WB-*ras*1 cells from two independent experiments were treated with vehicle (control), 0.1 mg/ml PBA, 2 μM SB203580, or 0.1 mg/ml PBA + 2 μM SB203580 for 48 h, then extracts were prepared and used for Western blotting of connexin 43. In panel B, the connexin 43-P_2 bands were quantified by densitometry and are shown as the mean ± SD. In panel C, cell–cell communication in similarly treated cultures was determined by dye-transfer (mean ± SD, n = 11–13 replicate cultures. Asterisks indicate statistical significance $P < 0.05$ compared to control).

43-P_2 and had no effect on the basal levels of this phosphoform (Fig. 6A,B). PBA also increased cell–cell communication approximately 3.5-fold in these cells, but this increase was negated by SB203580 (Fig. 6C). SB203580 had no effect on the basal level of cell–cell communication in these cells. Treatment of H2009 cells with PBA (0.1 or 0.2 mg/ml for 48 h or 5 days) did not increase cell–cell communication or connexin 43 P_2 content. However, a threefold increase in the amount of P_0 connexin 43 was observed (3.1 ± 0.4 for vehicle-treated cells versus 9.3 ± 0.6 for 0.1 mg/ml PBA-treated cells at 5 days in relative density units, $P < 0.01$).

PBA INHIBITS GROWTH OF *Ras*-TRANSFORMED AND HUMAN LUNG CARCINOMA CELLS

We previously demonstrated that 0.1 mg/ml PBA inhibited the growth of WB-*ras*1 cells and that PBA-Me was inhibitory at ~10-fold lower concentration [Sunman et al., 2004]. As seen in Figure 7A, PBA at 0.1 mg/ml also inhibited the growth of H2009 cells over 14 days. Growth was significantly inhibited as early as 2 days of treatment (day 3 of growth). PBA also increased apoptosis in these cultures treated for 2 days with 0.1 mg/ml from 5.5 ± 0.5% apoptotic cells in control dishes to 8.1 ± 0.6% apoptotic cells in PBA-treated dishes ($P < 0.05$). SB203580 alone also inhibited growth of WB-*ras*1 cells (Fig. 7B) and H2009 cells (not shown) and was therefore not efficacious in preventing PBA's inhibitory effect on cell growth.

PBA DECREASES ACTIVATION OF JNK

Treatment of WB-*ras*1 cells with 0.1 mg/ml PBA for 48 h decreased phosphorylation of JNK on key activation sites (Thr183/Tyr185) by ~2-fold (Fig. 8A,B). PBA (0.1 mg/ml PBA for 48 h) similarly decreased JNK phosphorylation in H2009 human lung tumor cells (Fig. 8C, D) No significant effect on JNK Thr183/Tyr185 phosphorylation was seen in WB-*ras*1 or H2009 cells treated for 4 h (not shown).

PBA HAS NO EFFECT ON ACTIVATION OF Akt, cdc2, CDK2, PKC, OR p44/42 MAPK

We previously demonstrated that PBA had no effect on activation of the p42/44 MAPK pathway after 4 h, 48 h, or 5 days treatment [Sunman et al., 2004]. To further test the specificity of PBA for the p38 MAPK pathway, we monitored activation of other signaling pathways. PBA had no effect on the activation of Akt, cdc2, CDK2, or PKC (Fig. 9). Densitometric evaluations revealed no differences between controls and PBA-treated cells ($P > 0.1$; data not shown).

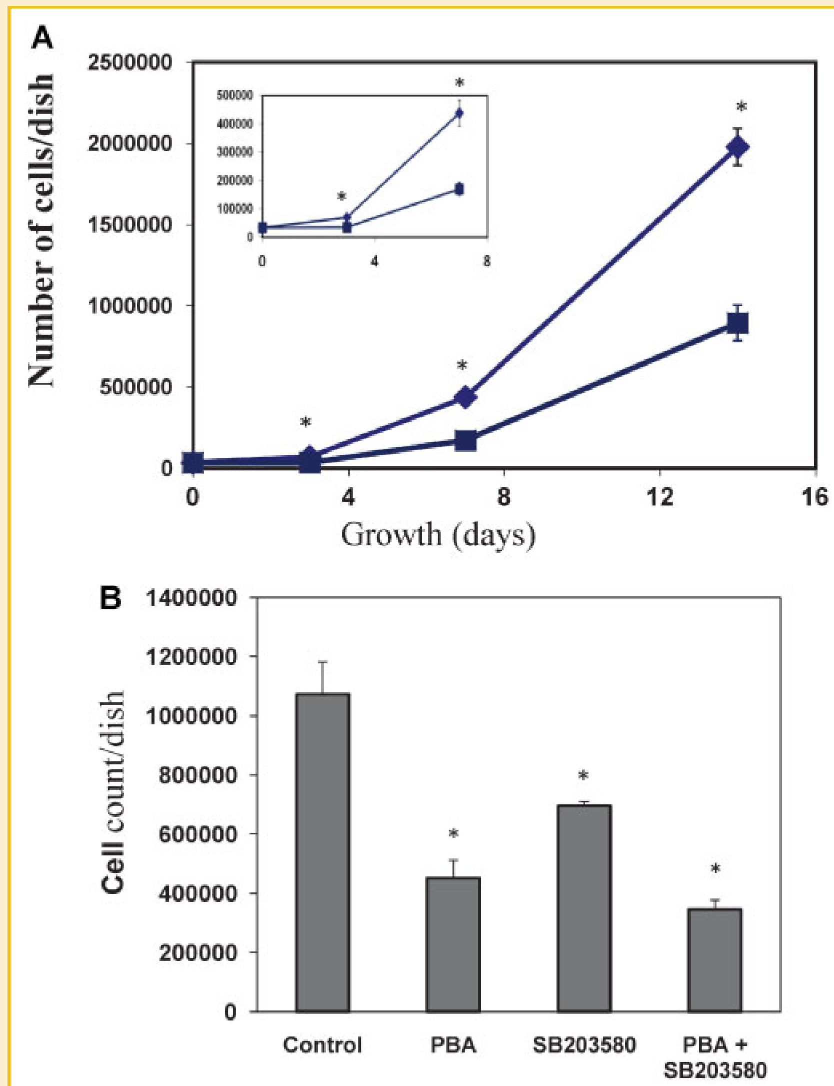

Fig. 7. Effect of PBA on H2009 anchorage-dependent cell growth. (A) Cells were grown on 35 mm^2 culture dishes over 14 days in the presence (■) or absence (vehicle-treated) (◆) of 0.1 mg/ml PBA and counted using a hemocytometer on days 0, 3, 7, and 14. Cells were plated on day 0 and treated on day 1, yielding treatment times of 2, 6, and 13 days. Inset shows only data plotted to 7 days to show effects at shorter times. Data are presented as the mean ± SD (n = 3); asterisks indicate $P < 0.01$ versus control for all time points. (B) Effect of PBA and/or SB203580 on the growth of WB-*ras*1 cells. The cells were treated with vehicle, 0.1 mg/ml PBA and/or 2 μM SB203580 for 48 h. (mean ± SD; asterisks indicate $P < 0.05$ vs. control).

DISCUSSION

The results presented above demonstrate that PBA and PBA-Me increased phosphorylation of p38 MAPK on Thr180/Tyr182 in both *ras*-transformed epithelial cells and H2009 human lung carcinoma cells. Furthermore, PBA increased phosphorylation on activation sites of HSP-27, MAPKAPK-2, and ATF-2, which are downstream effectors of p38 MAPK. Increased phosphorylation of p38 MAPK on Thr180/Tyr182 and increased phosphorylation of downstream effectors HSP-27, MAPKAPK-2, and ATF-2 are known indicators of activation of the p38 MAPK signaling pathway [Rouse et al., 1994; Cobb and Goldsmith, 1995; Raingeaud et al., 1995]. However, in our experiments, increased activation of p38 MAPK and downstream effectors was only observed with treatment durations of greater then 4 h (Fig. 1I). The time-course of these effects suggests that PBA did not directly activate the prototypic p38 MAPK signaling cascade since this would have occurred within minutes. We therefore hypothesize that PBA and PBA-Me activate p38 MAPK in *ras*-transformed and human lung carcinoma cells by an alternative mechanism. In support of this, we found no effect of PBA on activation of MKK3/MKK6, which are upstream kinases that phosphorylate p38 MAPK [Enslen et al., 1998; Brancho et al., 2003], or MLK-3, an upstream kinase of MKK3/MKK6 in the p38 MAPK signaling cascade (unpublished observations). However, MKK4 was also shown to be capable of activating p38 MAPK in vivo [Brancho et al., 2003; Dhillon et al., 2007]. In addition, a MAPKK-independent mechanism of p38 MAPK activation has been reported that involves the TAK1-binding protein, TAB1, [Ge et al., 2002]. Alternatively,

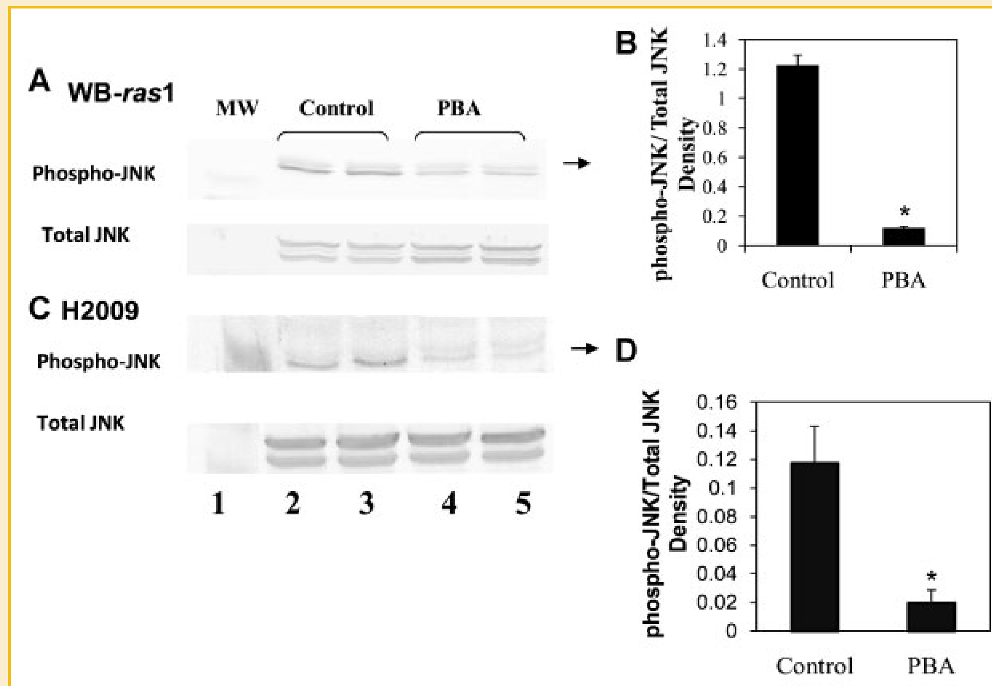

Fig. 8. Effect of PBA on JNK activation in WB-*ras*1 (A) and H2009 (C) cells. Cells were treated with 0.1 mg/ml PBA for 48 h, then extracted for Western blotting of phospho-JNK or total JNK. Quantification by densitometric scanning of bands is shown in panels B and D. Values are the mean ± SD; asterisks indicate $P < 0.05$ versus control. Phospho-JNK antibodies used are specific for Thr183/Tyr185.

PBA-induced inhibition of a p38 MAPK phosphatase, Wip1, [Bulavin et al., 2004] that acts on Thr 180 and/or Tyr 182 could result in the observed enhanced p38 MAPK activation. Other possible mechanisms include metabolic, structural, or gene expression changes in the cells that alter p38 MAPK activation over time which would correlate with PBA-enhanced phosphorylation of p38 MAPK (Fig. 1E, F) and effects on cell growth that occurred over 2–14 days (Fig. 7A). PBA also decreased phosphorylation of JNK, which correlated in time with p38 MAPK activation. This suggests a link between these two pathways following PBA treatment, that may occur via crosstalk mechanisms proposed by Wagner and Nebreda [2009].

There are four isoforms of p38 MAPK: alpha, beta, gamma, and delta [Kumar et al., 2003]. HSP-27 activation can be mediated by the alpha or beta isoforms, whereas all four isoforms can activate ATF-2. Our data suggest the alpha and beta isoforms are affected by PBA and PBA-Me, but the results do not allow a definitive answer.

Specificity of PBA for the two related stress-activated MAPK signaling pathways, p38 MAPK and JNK is demonstrated by its lack of effect on activation of enzymes in other key signaling pathways, as shown in Figure 9. PBA also showed a higher degree of activation of p38 MAPK in WB-*ras*1 cells compared to the plasmid control WB-*neo*3 cells (Fig. 1), which correlates with its greater growth inhibition in WB-*ras*1 cells versus WB-*neo*3 cells [Sunman et al., 2004]. The higher level of endogenous p38 MAPK activation in WB-*neo*3 cells compared to WB-*ras*1 may explain why PBA is less effective in up-regulating p38 MAPK in the non-transformed cells compared to the *ras*-transformed cells.

Our data demonstrate an approximately threefold increase in p38 MAPK phosphorylation at concentrations of PBA or PBA-Me that caused significant decreases in cell growth [Sunman et al., 2004] and Figure 7. While the Western blot signal for phospho-p38 MAPK cannot be correlated directly with kinase activity, it suggests that a moderate change in phosphorylation of p38 MAPK correlates with a large reduction in neoplastic cell growth. Timofeev et al. [2005] also noted suppression of in vivo tumorigenesis that was related to modest changes in p38 MAPK activity. HSP-27 and MAPKAPK-2 activation by PBA, on the other hand, were as high as sixfold and eightfold greater than controls, respectively, suggesting amplification of downstream signals or differences in the balance of upstream kinases and phosphatases acting on p38 MAPK compared to its downstream effectors. Furthermore, PBA-enhanced phosphorylation of p38 MAPK (Fig. 1E, F) and its effects on cell growth were observed as long as 14 days following treatment (Fig. 7A). This suggests that PBA and PBA-Me may be effective anti-tumor agents with a long duration of action despite the lack of more dramatic changes in activation of p38 MAPK. The approximately twofold increase in apoptosis elicited by PBA suggests cell death contributes to this growth inhibition.

Reduced gap junction-mediated cell–cell communication, as seen in WB-*ras*1 cells [De Feijter et al., 1990], is a phenotypic characteristic of many neoplastic cells that allows them to avoid the growth regulatory influences of adjacent cells [Yamasaki and Naus, 1996; Trosko and Ruch, 1998]. Restoration of gap junction-mediated communication in such cells often decreases their growth and tumorigenicity. The results of our present and previous

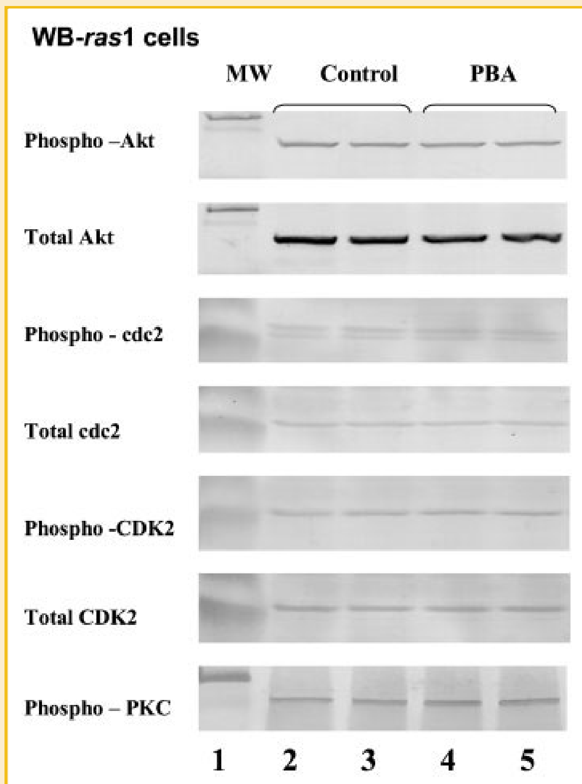

Fig. 9. Effects of PBA on the content of activated Akt, cdc2, CDK2, and PKC in WB-*ras*1 cells. The cells were treated with 0.1 mg/ml PBA for 48 h (pAkt/Akt) or 24 h and total proteins were extracted for Western blot analysis.

experiments [Sunman et al., 2004] demonstrate that PBA strongly increases gap junction-mediated communication between WB-*ras*1 cells. A key finding of our present study is that treatment of cells with the p38 MAPK specific inhibitor, SB203580, prevented this enhanced cell–cell communication and also reduced connexin43 P_2 formation (Fig. 6). The P_2 phosphoform has been associated with high levels of gap junction communication and the occurrence of large gap junction plaques in WB cells [Musil and Goodenough, 1991; Matesic et al., 1994]. These results suggest PBA stimulates p38 MAPK or a downstream effector to phosphorylate connexin43 to the P_2 phosphoform which increases gap junction-mediated communication. Whether p38 MAPK can directly phosphorylate connexin 43 in WB-*ras*1 cells, as seen in other cells [Lee et al., 2004; Ogawa et al., 2004], remains to be determined. While H2009 cells are also deficient in cell–cell communication, PBA did not increase cell–cell communication in these cells at 48 h or 5-day treatments. This correlated with a lack of increased connexin 43 P_2 phosphorylation and suggests gap junction regulation and connexin 43 phosphorylation pathways are different in H2009 and WB-*ras*1 cells. Additionally, H2009 cells do not appear to be good indicators of SB203580 actions on cell–cell communication.

Lee et al. [2004] reported that SB203580 treatment increased connexin 43 phosphorylation in similar H-*ras*-transformed rat liver epithelial cells and this was correlated with increased gap junction communication. This result conflicts with our observation that SB203580 prevented PBA-enhanced cell–cell communication and P_2 phosphorylation in WB-*ras*1 cells. However, Lee et al. used substantially higher concentrations (5–20 μM) of the inhibitor and shorter incubation times (1 h), which may account for the differences in results.

Inhibition of p38 MAPK, and concomitant activation of JNK, play a critical role in *ras*-induced transformation that is independent of Raf activation [Pruitt et al., 2002]. Conversely, activation of p38 MAPK resulted in cancer cell cycle inhibition or apoptosis initiated by retinoids, cisplatin, and other chemotherapeutic agents [Iyoda et al., 2003; Losa et al., 2003; Olson and Hallahan, 2004]. In addition, Iyoda et al. [2003] reported that increased p38 MAPK activity in hepatocarcinoma cells transfected with a MKK6 mutant gene decreased the growth of these cells. Hui et al. [2007] demonstrated that in chemical-induced liver cancer development, mice carrying a liver-specific deletion of p38α showed enhanced hepatocyte proliferation and tumor growth that correlated with activation of the JNK signaling pathway, and provided further evidence suggesting that p38α may suppress cancer cell proliferation by antagonizing the JNK pathway. Our data demonstrate that PBA and PBA-Me activate p38 MAPK, suppress neoplastic cell growth, inhibit JNK, and enhance gap junction communication and thus may be a similarly effective therapeutic agent for cancers with down-regulated p38 MAPK and/or over-activated JNK. The increased gap junction communication may in turn enhance other forms of cancer therapy including bystander cytotoxicity of radiation and chemotherapeutic agents [Prise and O'Sullivan, 2009].

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