Published in final edited form as:
J Cell Biochem. 2012 January ; 113(1): 269–281. doi:10.1002/jcb.23353.

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

Diane F. Matesic[1,*], **Tatyana S. Sidorova**[1], **Timothy J. Burns**[1], **Allison M. Bell**[1], **Paul L. Tran**[1], **Randall J. Ruch**[2], and **Sheldon W. May**[3]

[1]Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Mercer University, Atlanta, Georgia 30341

[2]Department of Biochemistry and Cancer Biology, University of Toledo, Toledo, OH, 43614

[3]School of Chemistry and Biochemistry and the Petit Institute for Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, Georgia 30332

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 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.

Keywords

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

[*]Correspondence to: Diane Matesic, Ph.D., Department of Pharmaceutical Sciences, Mercer University, 3001 Mercer University Drive, Atlanta, GA 30341, Ph: 678-547-6241, FAX: 678-547-6423, matesic_df@mercer.edu.

types [Raingeaud et al., 1995; Wang et al., 1997; Lewis et al., 1998; Schaeffer et al., 1999; Pearson et al., 2001; Engelberg et al., 2004]. p38 MAPK transmits signals from the cell membrane to the nucleus in response to oxidative or osmotic stress, cytokines, and radiation [Zarubin et al., 2005; Dhillon et al., 2007]. Activation of p38 MAPK via phosphorylation activates the transcription factors ATF-2 and HSP-27 [Raingeaud et al., 1995; Cobb et al, 1995; Rouse et al., 1994], 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 [Dhillon et al., 2007; Timofeev et al., 2005; Bradham and McClay, 2006]. 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 5-fold 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, 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, 25x 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 were 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 4 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 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 hours. 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 minutes between pulses). Samples were centrifuged at 14,000 × g for 30 minutes 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 minutes 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 60V until the samples had passed through the stacking gel, then at 120–150V. Proteins were transferred on to PVDF membranes at 20V 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 second 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 20V or 1 h at 180 V. Membranes were stained with Ponceau Red, then incubated in block buffer for 1–2 hours. 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 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 35mm 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 ml 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 3 times with PBS and once with Ca^{2+}/Mg^{2+} PBS. Cells were fixed with 1.5 ml of 4% paraformaldehyde for 30 minutes, 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 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 (S.D). 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. A probability of $P < .05$ 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 hr increased phosphorylation of p38 MAPK on key activation sites, Thr180/Tyr182 (Figure 1A). Lanes 4 and 5 (separately treated, replicate samples) of Figure 1A show 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 (Figure 1A lower panel). Densitometric scans of blots (Figure 1 B) revealed a 3-fold 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 (Figure 1 C, top panel, lanes 4 and 5 compared to vehicle-treated control lanes 2 and 3), and had no effect on total p38 MAPK levels (Figure 1C, bottom panel, lanes 2–5). Densitometric scans revealed a ~2.5 fold increase in phospho-p38 MAPK in PBA–treated H2009 cells (Figure 1D). This increased p38 MAPK phosphorylation by PBA was sustained for as long as 14

days post treatment (Figure 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 hours (Figure 1 G and H) compared to WB-*ras*1 cells treated over the same time (Figure 1 A and 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 hr which was not significant (Figure 1 I, $p>0.05$), while at 24 h treatment, a ~1.7 fold increase in p38 MAPK phosphorylation was observed (Figure 1 J, $p<0.05$). 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 (Figure 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 0.1mg/ml PBA for 48 h increased the phosphorylation of HSP-27 and ATF-2 at key activation sites by approximately 2–6 fold in identical samples (Figure 3A and B, compare lanes 4 and 5 with vehicle control lanes 2 and 3 in the top and 3rd panels and quantification in B,C,E and F). Treatment of H2009 cells with PBA (0.1mg/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 10-fold lower concentration (10 μg/ml) than the parent compound. (Figure 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 (Figure 4A, lanes 6 and 7 with lanes 4 and 5, and quantification in Figure 4B). Similarly, 20 μg/ml PBA-Me stimulated p38 MAPK and HSP-27 phosphorylation in WB-*ras*1 cells by more than two-fold (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 (Figure 5A, top panel, lanes 2 ,3, 6 and 7) were detected and the stimulation of p38 MAPK phosphorylation by PBA was not substantially affected (Figure 5A, top panel, lanes 8 and 9 compared to lanes 6 and 7, quantification on 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 (Figure 5A, 4th panel from the top, lanes 8 and 9 compared to lanes 4 and 5 and quantification in Figure 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_o (non-phosphorylated) and P_1 phosphoforms in WB-*ras*1 cells (Figure 6A,B), as we previously reported [Sunman et al., 2004]. SB203580 prevented the increase in connexin 43-P_2 and had no effect on the basal levels of this phosphoform (Figure 6A,B). PBA also increased cell-cell communication approximately 3.5 fold in these cells, but this increase was negated by SB203580 (Figure 6C). SB203580 had no effect on the basal level of cell-cell communication in these cells. Treatment of H2009 cells with PBA (0.1 mg/ml or 0.2 mg/ml for 48 h or 5 d) did not increase cell cell-communication or connexin 43 P_2 content. However, a 3-fold increase in the amount of P_o 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 d 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 (Figure 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 (Figure 8A, B). PBA (0.1 mg/ml PBA for 48 h) similarly decreased JNK phosphorylation in H2009 human lung tumor cells (Figure 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, and PKC (Figure 9). Densitometric evaluations revealed no differences between controls and PBA-treated cells ($p > 0.1$; data not shown).

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 [Raingeaud et al., 1995; Cobb et al, 1995; Rouse et al., 1994]. However, in our experiments, increased activation of p38 MAPK and downstream effectors was only observed with treatment durations of greater then 4 h (Figure

1 I). 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, 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 (Figure 1 E and F) and effects on cell growth that occurred over 2–14 d (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 (Figure 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 3-fold 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 6-fold and 8-fold 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 (Figure 1 E and F) and its effects on cell growth were observed as long as 14 d 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 2-fold 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 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 (Figure 6). The P_2 phosphoform has been associated with high level 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 d 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 [Losa et al., 2003; Olson and Hallahan, 2004; Iyoda et al., 2003]. 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].

Acknowledgments

Grant sponsor: National Institutes of Health; Grant numbers: 1RO1CA096973 and 1R15CA135415

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

REFERENCES

Atlanta, GA: 2011. American Cancer Society: Cancer Facts and Figures.

Antonyak MA, Kenyon LC, Godwin AK, James DC, Emlet DR, Okamoto I, Tnani M, Holgado-Madruga M, Moscatello DK, Wong AJ. Elevated JNK activation contributes to the pathogenesis of human brain cancers. Oncogene. 2002; 21:5038–5046. [PubMed: 12140754]

Aranvindakshan J, Cyr DG. Nonylphenol alters connexin 43 levels and connexin 43 phosphorylation via an inhibition of the p38-Mitogen-Activated Protein Kinase pathway. Biol Repro. 2005; 72:1232–1240.

Bradham C, McClay DR. p38 MAPK in development and cancer. Cell Cycle. 2006; 5:824–828. [PubMed: 16627995]

Bauer JD, Sunman JA, Foster MS, Thompson JR, Ogonowski AA, Cutler SJ, May SW, Pollock SH. Anti-inflammatory effects of 4-phenyl-3-buteonic acid and 5-(acetylamino)-4-oxo-6-phenyl-2-hexenoic acid methyl ester, potential inhibitors of neuropeptide bioactivation. J Pharmacol Exper Therap. 2007; 320:1171–1177. [PubMed: 17138865]

Brognard J, Dennis PA. Variable apoptotic response of NSCLC cells to inhibition of the MEK/ERK pathway by small molecules or dominant negative mutants. Cell Death Differ. 2002; 9:893–904. [PubMed: 12181740]

Bulavin DV, Phillips C, Nannenga B, Timofeev O, Donehower LA, Anderson CW, Appella E, Fornace AJ. Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16 (Ink4q)-p19 (Arf) pathway. Nat Genet. 2004; 36:343–350. [PubMed: 14991053]

Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ. Mechanism of p38 MAP kinase activation in vivo. Genes Dev. 2003; 17:1969–1978. [PubMed: 12893778]

Cao J, Semenova MM, Solovyan VT, Han J, Coffey ET, Courtney MJ. Distinct requirements for p38alpha and c-Jun N-terminal kinase stress-activated protein kinases in different forms of apoptotic neuronal death. J Biol Chem. 2004; 279:35903–35913. [PubMed: 15192112]

Cho BC, Im CK, Park MS, Kim SK, Chang J, Park JP, Choi HJ, Kim YJ, Shin SJ, Sohn JH, Kim H, Kim JH. Phase II study of erlotinib in advanced non-small cell lung cancer after failure of gefitinib. J Clin Oncol. 2007; 25:2528–2533. [PubMed: 17577030]

Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Biol Chem. 1995; 270:14843–14846. [PubMed: 7797459]

Cohen P. The search for physiological substrates of MAP and SAP kinases in mammalian cells. Trends Cell Biol. 1997; 7:353–361. [PubMed: 17708980]

Comis RL. The current situation: erlotinib (Tarceva) and gefitinib (Iressa) in non-small cell lung cancer. Oncologist. 2005; 10:467–470. [PubMed: 16079313]

De Feijter AW, Ray JS, Weghorst CM, Klaunig JE, Goodman JI, Chang CC, Ruch RJ, Trosko JE. Infection of rat liver epithelial cells with v-Ha-ras: correlation between oncogene expression, gap junctional communication, and tumorigenicity. Mol Carcinog. 1990; 3:54–67. [PubMed: 2346586]

Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signaling pathways in cancer. Oncogene. 2007; 26:3279–3290. [PubMed: 17496922]

Engelberg D. Stress-activated protein kinases—tumor suppressors or tumor initiators? Semin Cancer Biol. 2004; 14:271–282. [PubMed: 15219620]

Enslen H, Raingeaud J, Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem. 1998; 273:1741–1748. [PubMed: 9430721]

Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J. MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science. 2002; 295:1291–1294. [PubMed: 11847341]

Hui L, Bakiri L, Mairhorfer A, Schweifer N, Haslinger C, Komnenovic V, Scheuch H, Beug H, Wagner EF. P38α suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. Nature Genetics. 2007; 39:741–749. [PubMed: 17468757]

Iwai N, Martinez A, Miller MJ, Vos M, Mulshine JL, Treston AM. Autocrine growth loops dependent on peptidyl alpha-amidating enzyme as targets for novel tumor cell growth inhibitors. Lung Cancer. 1999; 23:209–222. [PubMed: 10413197]

Iyoda K, Sasaki Y, Horimoto M, Toyama T, Yakushijin T, Sakakibara M, Takehara T, Fujimoto J, Hori M, Wands JR, Hayashi N. Involvement of the p38 mitogen-activated protein kinase cascade in hepatocellular carcinoma. Cancer. 2003; 97:3017–3026. [PubMed: 12784337]

Jou YS, Matesic DF, Dupont E, Lu SC, Rupp HL, Madhukar BV, Oh SY, Trosko JE, Chang CC. Restoration of gap-junctional intercellular communication in a communication-deficient rat liver cell mutant by transfection with connexin 43 cDNA. Mol Carcinog. 1993; 8:234–244. [PubMed: 8280372]

Katopodis AG, May SW. Novel substrates and inhibitors of peptidylglycine α-amidating monooxygenase. Biochemistry. 1990; 29:4541–4548. [PubMed: 2372538]

Katopodis AG, Ping D, May SW. A novel enzyme from bovine neurointermediate pituitary catalyzes dealkylation of alpha-hydroxyglycine derivatives, thereby functioning sequentially with peptidylglycine alpha-amidating monooxygenase in peptide amidation. Biochemistry. 1990; 29:6115–6120. [PubMed: 2207061]

Kumar S, Boehm J, Lee JC. P38 MAP kinases: key signaling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov. 2003; 2:717–726. [PubMed: 12951578]

Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev. 2001; 81:807–817. [PubMed: 11274345]

Lee KW, Jung JW, Kang KS, Lee HJ. p38 is a key signaling molecule for H-ras-induced inhibition of gap junction intercellular communication in rat liver epithelial cells. Ann NY Acad Sci. 2004; 1030:258–263. [PubMed: 15659805]

Levitzki A. Protein kinase inhibitors as a therapeutic modality. Acc Chem Res. 2003; 36:462–469. [PubMed: 12809533]

Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Adv Cancer Res. 1998; 74:49–139. [PubMed: 9561267]

Liberto MC, Matera G, Lamberti AG, Barreca GS, Foca D, Quirino A, Soria MR, Foca A. Bartonella quintana-induced apoptosis inhibition of human endothelial cells is associated with p38 and SAPK/JNK modulation and with stimulation of mitosis. Diagn Micr Inf Dis. 2004; 50:159–166.

Losa JH, Parada Cobo CP, Viniegra JG, Sánchez-Arevalo Lobo VJ, Ramón y Cajal S, Sánchez-Prieto R. Role of the p38 MAPK pathway in cisplatin-based therapy. Oncogene. 2003; 22:3998–4006. [PubMed: 12821934]

Lowe LC, Senaratne SG, Colston KW. Induction of apoptosis in breast cancer cells by apomine is mediated by caspase and p38 mitogen activated protein kinase activation. Biochem Biophys Res Commun. 2005; 329:772–779. [PubMed: 15737653]

Matesic DF, Rupp HL, Bonney WJ, Ruch RJ, Trosko JE. Changes in gap-junction permeability, phosphorylation, and number mediated by phorbol ester and non-phorbol-ester tumor promoters in rat liver epithelial cells. Mol Carcinog. 1994; 10:226–236. [PubMed: 8068183]

Mitsudomi T, Steinberg SM, Nau MM, Carbone D, D'Amico D, Bodner S, Oie HK, Linnoila RI, Mulshine JL, Minna JD. p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical futures. Oncogene. 1992; 7:171–180. [PubMed: 1311061]

Musil LS, Goodenough DA. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. J Cell Biol. 1991; 115:1357–1374. [PubMed: 1659577]

Ogawa T, Hayashi T, Kyoizumi S, Kusunoki Y, Nakachi K, MacPhee DG, Trosko JE, Kataoka K, Yorioka N. Anisomycin downregulates gap-junctional intercellular communication via the p38 MAP-kinase pathway. J Cell. Sci. 2004; 117:2087–2096. [PubMed: 15054109]

Olson JM, Hallahan AR. P38 MAP kinase: a convergence point in cancer therapy. Trends Mol Med. 2004; 10:125–129. [PubMed: 15102355]

Pearson G, Robinson F, Gibson TB, Xu B-e, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001; 22:153–183. [PubMed: 11294822]

Prise KM, O'Sullivan JM. Radiation-induced bystander signalling in cancer therapy. Nat Rev Cancer. 2009; 9:351–360. [PubMed: 19377507]

Pruitt K, Pruitt WM, Bilter GK, Westwick JK, Der CJ. Raf-independent deregulation of p38 and JNK mitogen-activated protein kinases are critical for Ras transformation. J Biol Chem. 2002; 277:31808–31817. [PubMed: 12082106]

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. Pro-inflammatory cytokines and environmental stress cause p38 Mitogen-Activated Protein Kinase activation by dual phosphorylation on Tyrosine and Threonine. J Biol Chem. 1995; 270:7420–7426. [PubMed: 7535770]

Rennefahrt U, Illert B, Greiner A, Rapp UR, Troppmair J. Tumor induction by activated JNK occurs through deregulation of cellular growth. Cancer Lett. 2004; 215:113–124. [PubMed: 15374640]

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell. 1994; 78:1027–1037. [PubMed: 7923353]

Ruch RJ, Cesen-Cumming K, Malkinson AM. Role of gap junctions in lung neoplasia. Exp Lung Res. 1998; 24:523–539. [PubMed: 9659581]

Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol. 1999; 19:2435–2444. [PubMed: 10082509]

Sunman JA, Foster MS, Folse SL, May SW, Matesic DF. Reversal of the transformed phenotype and inhibition of peptidylglycine alpha-monooxygenase in Ras-transformed cells by 4-phenyl-3-butenoic acid. Mol Carcinog. 2004; 41:231–246. [PubMed: 15468302]

Timofeev O, Lee TY, Bulavin DV. A subtle change in p38 MAPK activity is sufficient to suppress in vivo tumorigenesis. Cell Cycle. 2005; 4:118–120. [PubMed: 15611662]

Trosko JE, Ruch RJ. Cell-cell communication in carcinogenesis. Front Biosci. 1998; 3:208–236.

Tsao MS, Smith JD, Nelson KG, Grisham JW. A diploid epithelial cell line from normal adult rat liver with phenotypic properties of ‘oval’ cells. Exp Cell Res. 1984; 154:38–52. [PubMed: 6468534]

Wang XS, Diener K, Manthey CL, Wang S-W, Rosenzweig B, Bray J, Delaney J, Cole CN, Chan-Hui P-Y, Mantlo N, Lichenstein HS, Zukowski M, Yao Z. Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. J Biol Chem. 1997; 272:23668–23674. [PubMed: 9295308]

Prise KM, O'Sullivan JM. Radiation-induced bystander signalling in cancer therapy. Nat Rev Cancer. 2009; 9:351–360. [PubMed: 19377507]

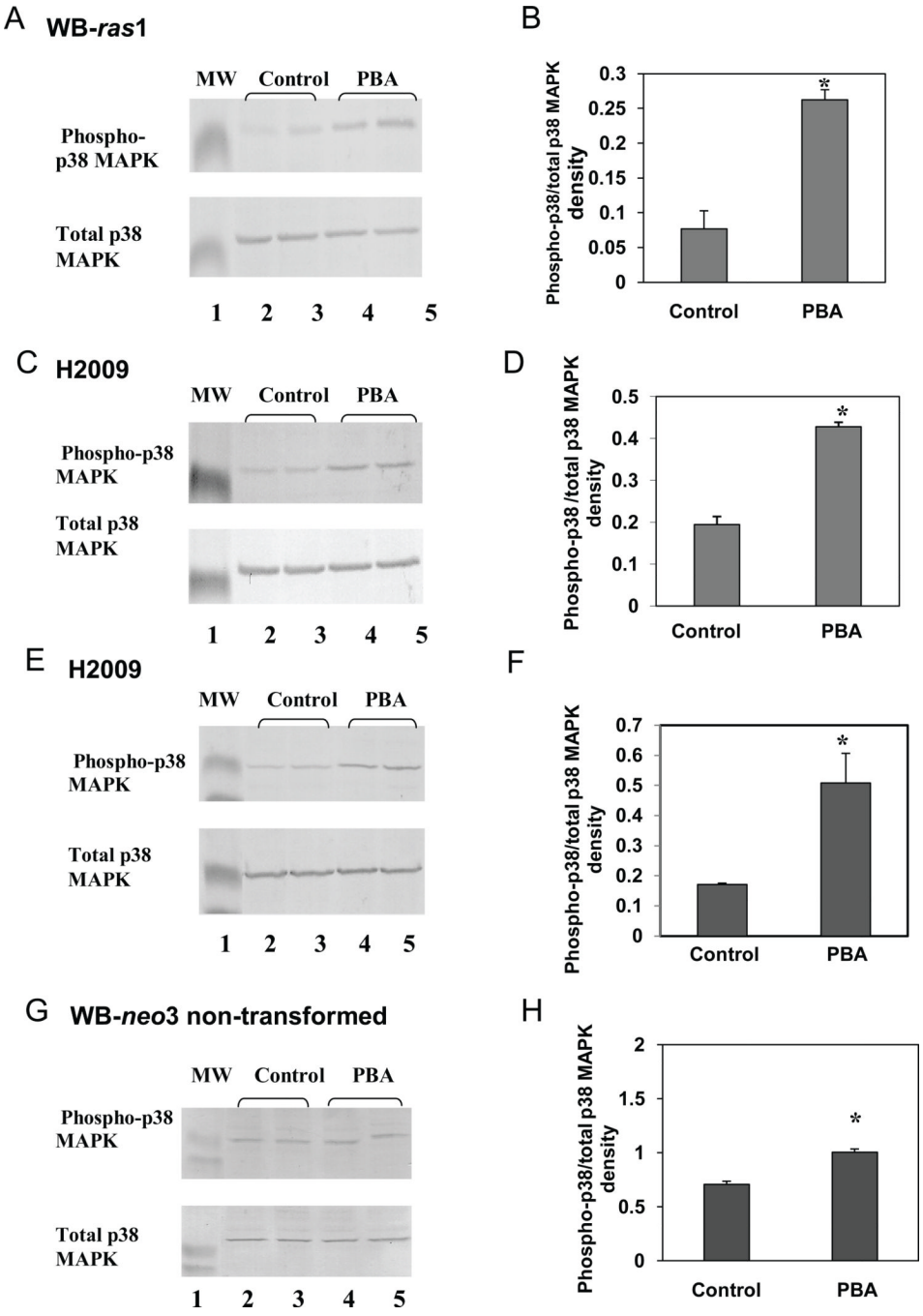
Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nature Rev Cancer. 2009; 9:537–549. [PubMed: 19629069]

Wong MK, Lo AI, Lam B, Lam WK, Ip MS, Ho JC. Erlotinib as salvage treatment after failure to first-line gefitinib in non-small cell lung cancer. Cancer Chemother Pharmacol. 2010; 65:1023–1028. [PubMed: 19680652]

Yamasaki H, Naus CC. Role of connexin genes in growth control. Carcinogenesis. 1996; 17:1199–1213. [PubMed: 8681433]

Yang SH, Galanis A, Sharrocks AD. Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. Mol Cell Biol. 1999; 19:4028–4038. [PubMed: 10330143]

Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. Cell Res. 2005; 15:11–18. [PubMed: 15686620]



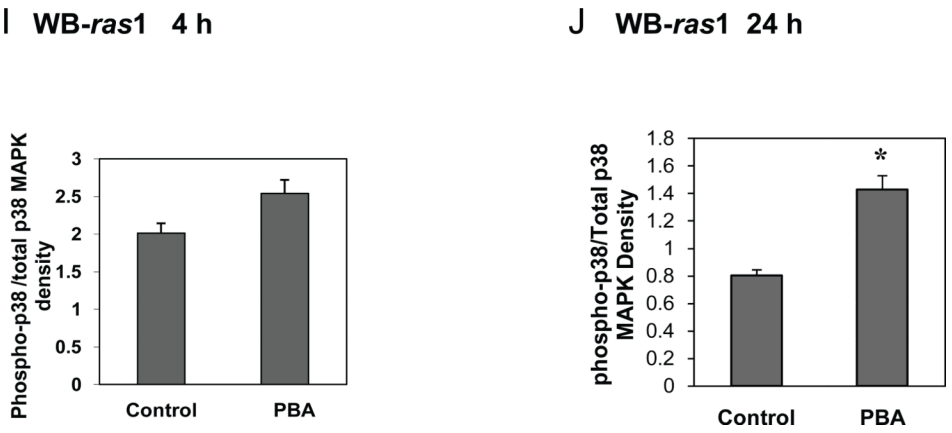


Figure 1.

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 d (E) and extracted for total protein for Western blot analysis as described in 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 densiometric quantification of bands in scanned blots (panels B, D, F and H) are presented as the mean ± S.D., and are representative of six (WB-*ras*1), three (H2009, 48 h), one (H2009 14d), and two (WB-*neo*3) independent experiments (astericks 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.

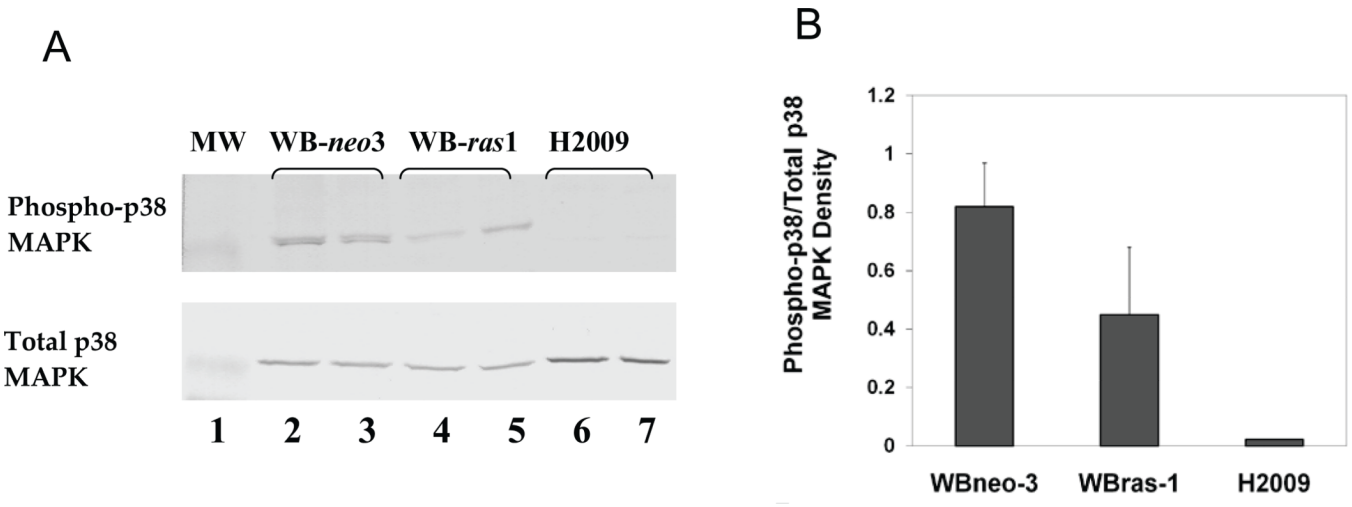


Figure 2.

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 ± S.D. (MW= 41 kD pre-stained molecular weight marker). Phospho-p38 MAPK antibodies used are specific for Thr180/Tyr182.

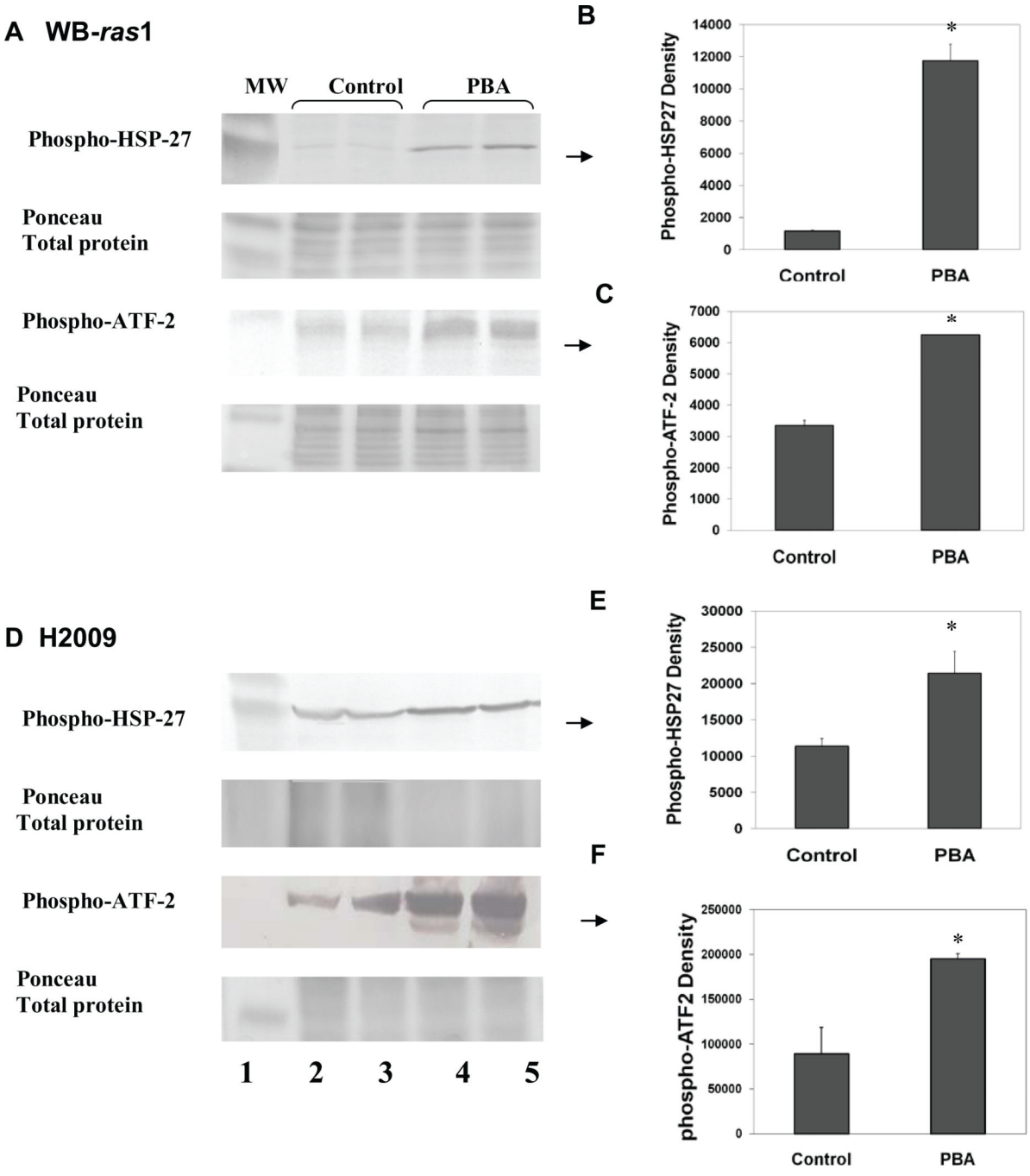


Figure 3.

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 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 ± S.D., ($p<0.05$ 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.

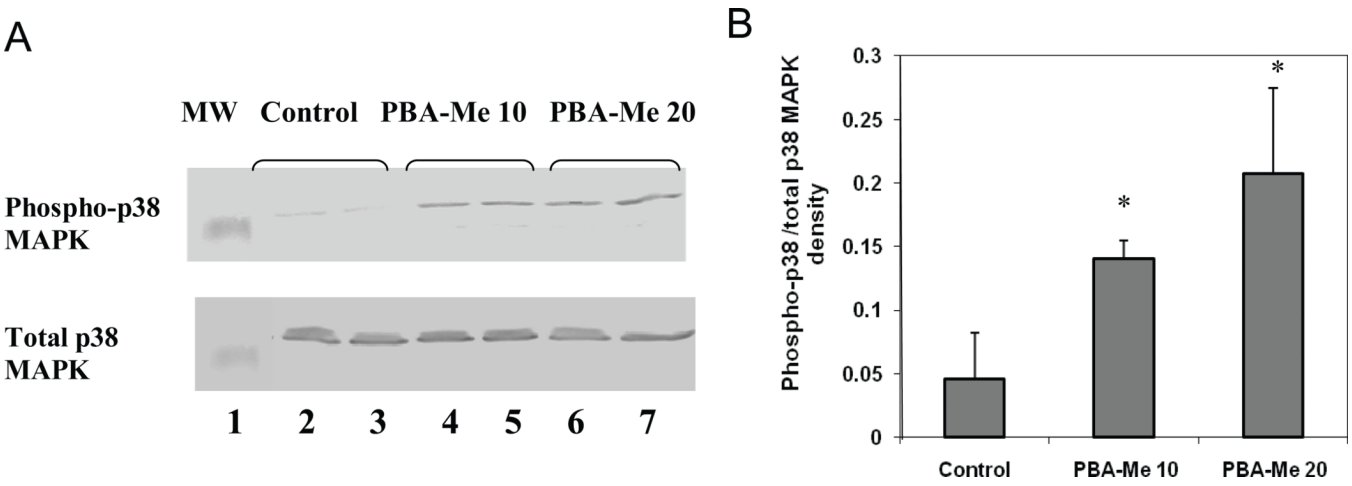


Figure 4.

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 ± S.D. ($p<0.05$ compared to control).

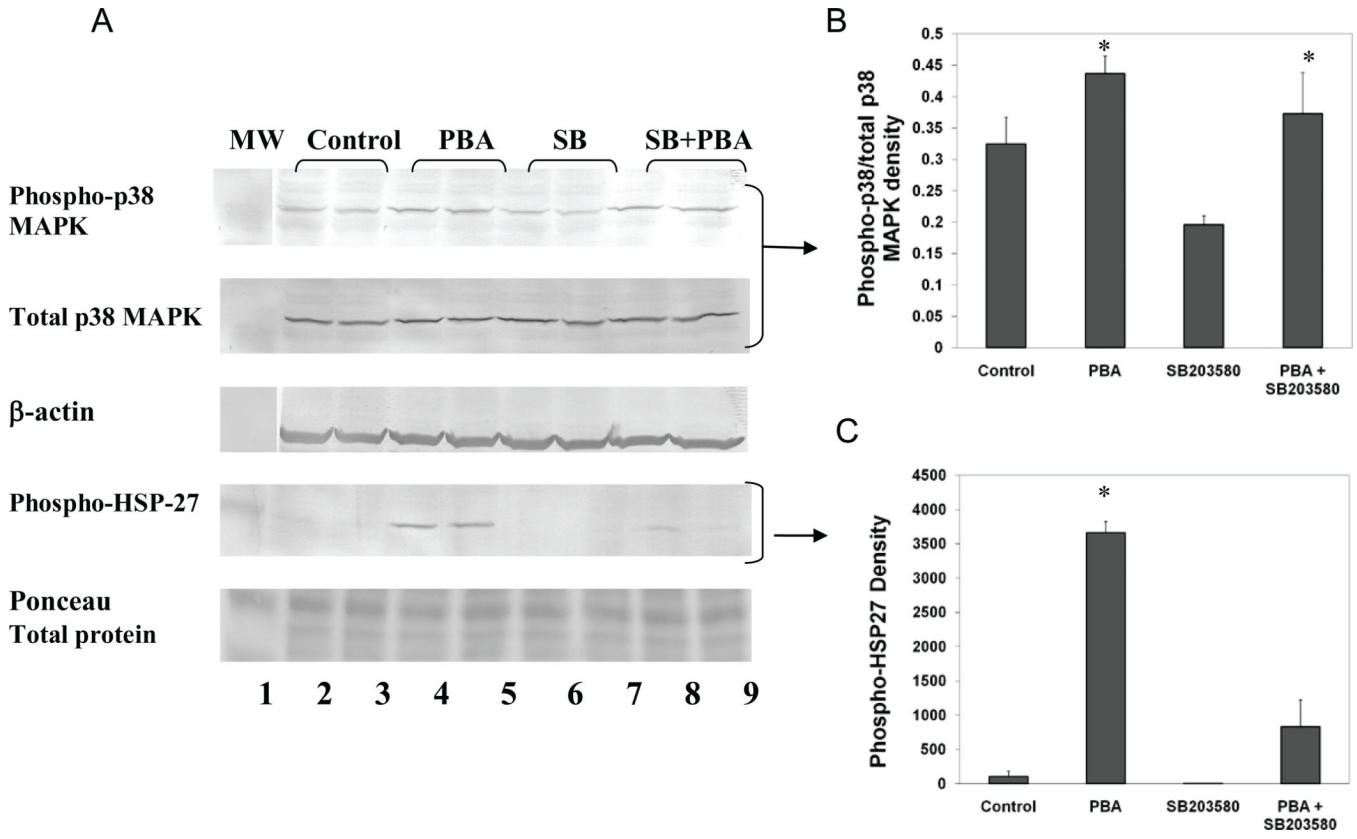


Figure 5.

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 hrs, then extracted for Western blot analyses. as described in Methods. Two duplicate vehicle-control lanes (lanes 2 & 3), 0.1 mg/ml PBA-treated lanes (lanes 4 & 5), 2 μM SB203580 (lanes 6 & 7), or 0.1 mg/ml PBA + SB203580 (lanes 8 & 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.

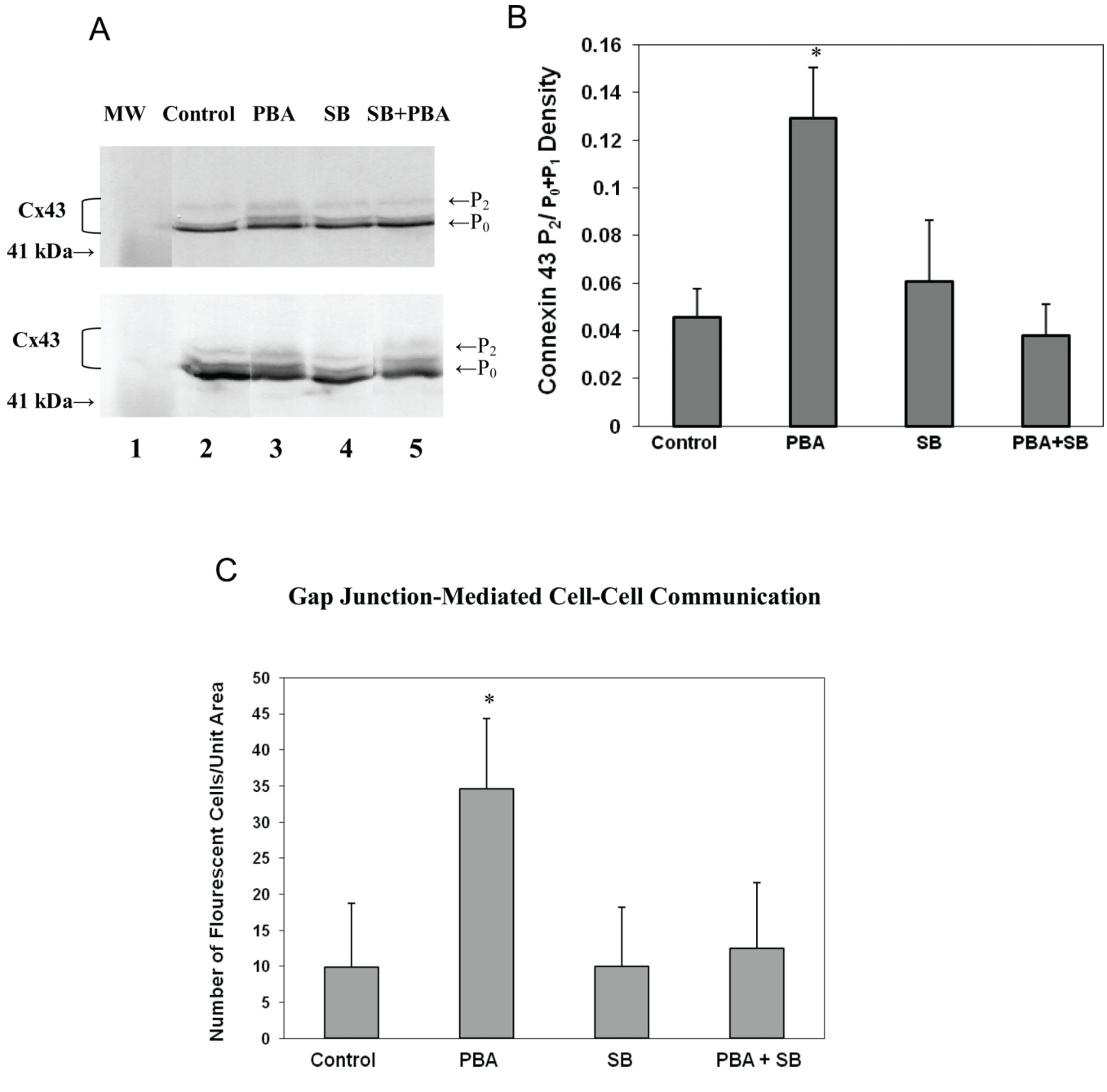


Figure 6.

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 ± S.D. IN panel C, cell-cell communication in similarly treated cultures was determined by dye-transfer (mean ± S.D., n= 11–13 replicate cultures. Asterisks indicate statistical significance ($p<0.05$).

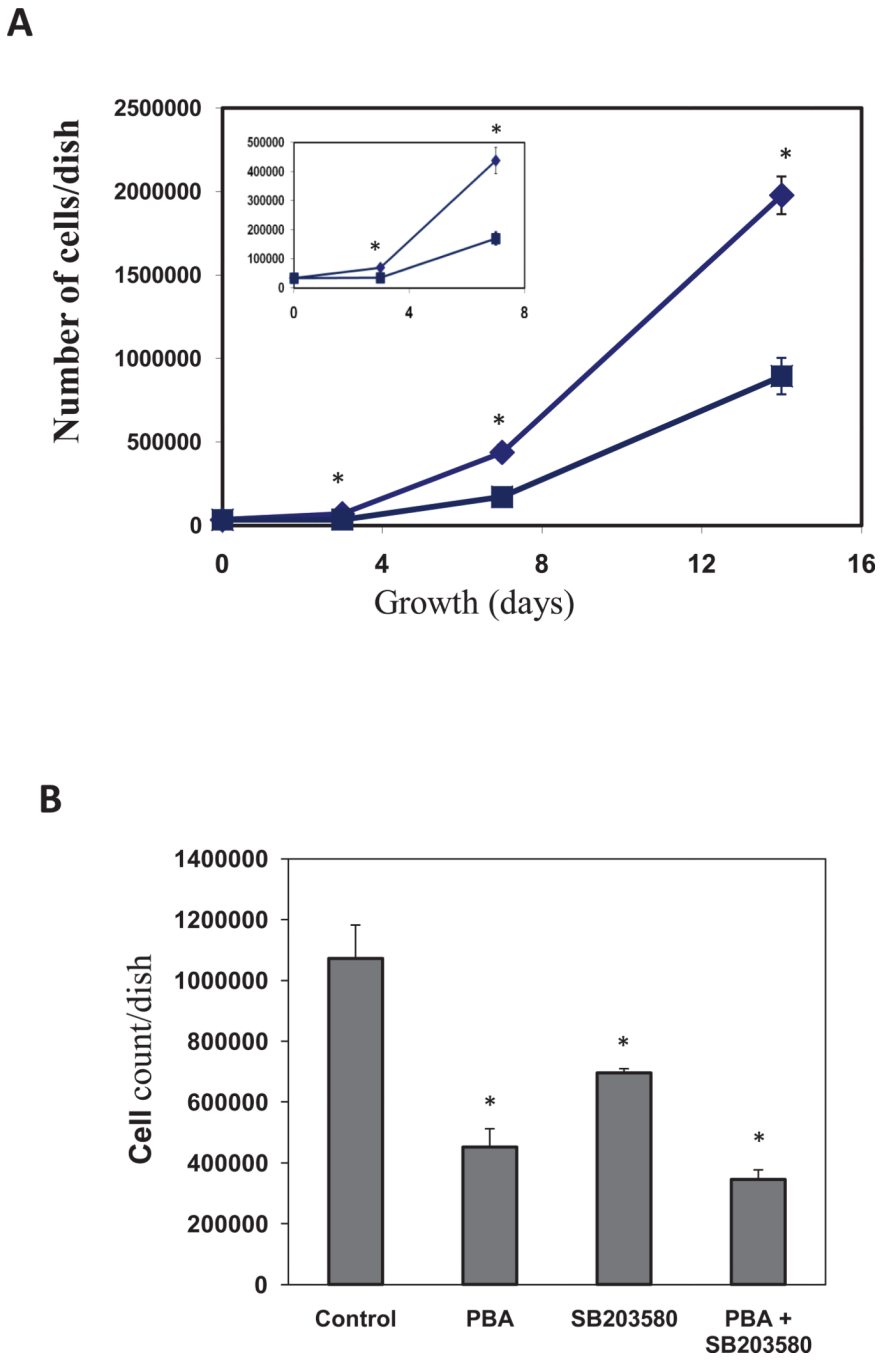


Figure 7.

Effect of PBA on H2009 anchorage-dependent cell growth. **(A)** Cells were grown on 35mm^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 ± S.D. (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 ± S. D.; asterisks indicate $p < 0.05$ versus control).

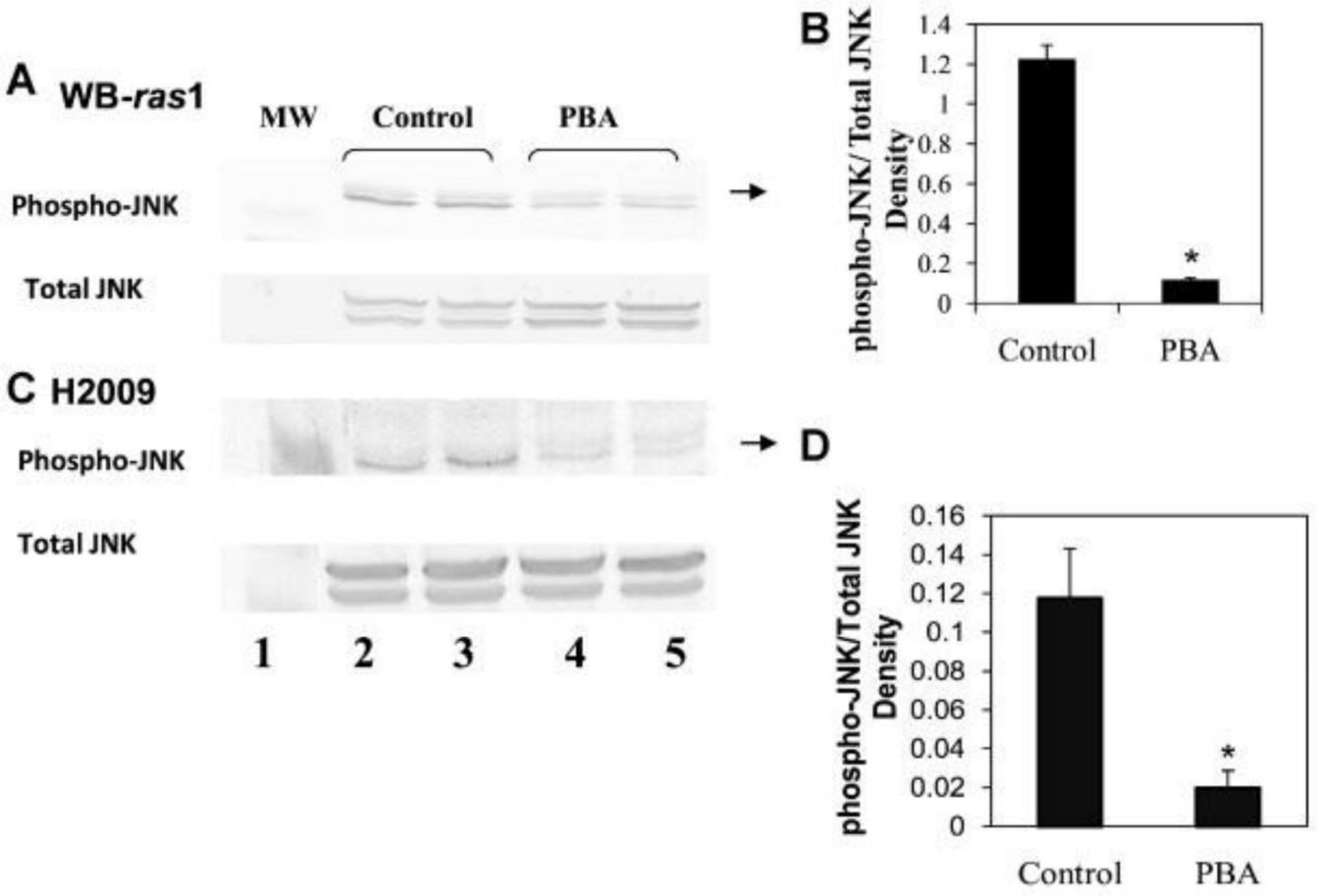


Figure 8.

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 ± S.D.; asterisks indicate $p<0.05$ versus control. Phospho-JNK antibodies used are specific for Thr183/Tyr185.

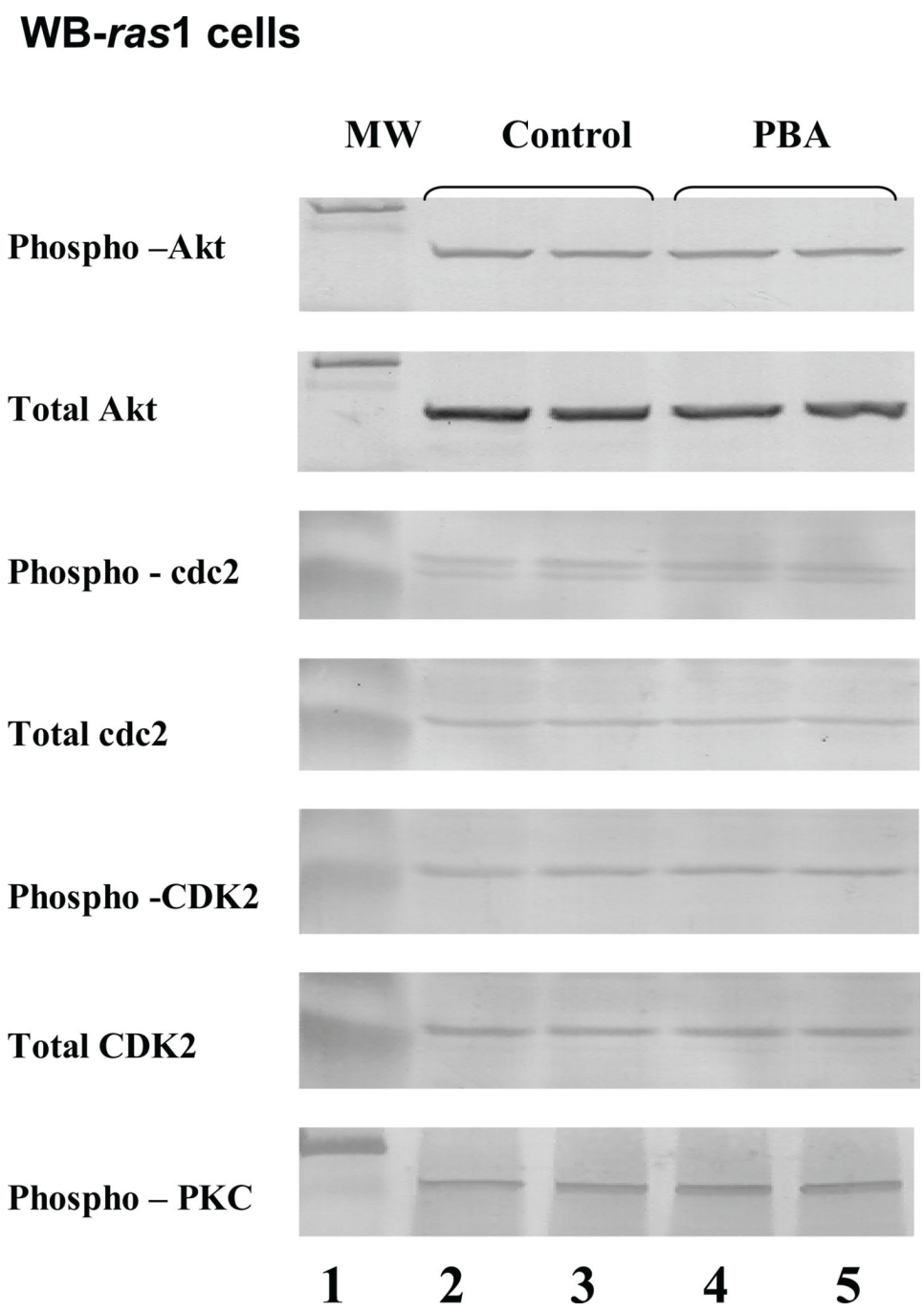


Figure 9.
Effects of PBA on the content of activated Akt, cdc2, CDK2 and PKC in WB-*ras*1 cells. The cells were treated with 0.1 mg/ml PBA for 48 hrs (pAkt/Akt) or 24 hrs and total proteins were extracted for Western blot analysis.