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Phenylbutyrate Decreases Type I Collagen Production in Human Lung Fibroblasts

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Abstract Fibrotic lung diseases are characterized by excess extracellular matrix production, in particular type I collagen. Phenylbutyrate (PB) is a non-toxic pharmacological compound that functions as a weak histone deacetylase inhibitor. In hepatic stellate cells, the synthesis of type I collagen expression is decreased by inhibiting histone acetylation. Our studies examined the regulation of type I collagen by PB in human lung fibroblasts. We found that PB decreases basal and transforming growth factor-β-stimulated α1(I) collagen mRNA and protein levels. Northern blot analyses demonstrated that PB decreases steady-state α1(I) collagen mRNA levels by 78% without significantly changing the stability of the mRNA transcript. PB stimulates cAMP production and increases the acetylation of histone H4, but does not affect the activity of two transforming growth factor-β (TGF-β)-responsive luciferase reporter constructs. These data suggest that PB regulates type I collagen expression in human lung fibroblasts by mechanisms that include cAMP production and histone acetylation. PB may have therapeutic use in fibrotic lung diseases. J. Cell. Biochem. 91: 740–748, 2004.

Key words: type I collagen; phenylbutyrate; cAMP; histone acetylation

Phenylbutyrate (PB) (4-phenylbutyric acid sodium salt) is a non-toxic pharmacological compound that is approved for clinical use in urea cycle disorders such as ornithine transcarbamylase deficiency [Maestri et al., 1996]. In addition, PB is currently under evaluation for the treatment of cystic fibrosis, cancer, sickle cell disease, and thalassemia [Dover et al., 1994; Carducci et al., 2001; Resar et al., 2002; Zeitlin et al., 2002]. PB is a weak histone deacetylase (HDAC) inhibitor, however, the precise mechanisms by which PB exerts its effects on these diseases are incompletely understood [Goh et al., 2001]. In hepatic stellate cells, a major source of extracellular matrix proteins in chronic liver diseases, the potent HDAC inhibitor trichostatin A (TSA) inhibits the synthesis of type I collagen and α-smooth muscle actin [Niki et al., 1999]. In these studies, it appears that TSA interferes with the differentiation of stellate cells into myofibroblasts by inhibiting the acetylation of histone H4.

Fibrosis is characterized by the excess production and deposition of collagen within tissues. In particular, dysregulated type I collagen expression leads to unresolved tissue fibrosis and results in organ dysfunction. Idiopathic pulmonary fibrosis is a progressive illness of unknown etiology that has no effective treatment at this time [Gross and Hunninghake, 2001]. In our studies, the regulation of α1(I) collagen mRNA expression by PB in human lung fibroblasts was examined. Our findings suggest that PB decreases type I collagen expression by several mechanisms including stimulating cAMP production and inhibiting HDAC activity.

MATERIALS AND METHODS

Tissue Culture

Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research) were grown in

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Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U penicillin/ml, 10 μg streptomycin/ml, 0.1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. After confluence was achieved, the serum content of the medium was reduced to 0.4% FBS for 48 h to render the cells quiescent. Cell numbers were determined using an electronic particle counter (Beckman Coulter, Miami, FL).

Northern Blot Analysis

Total cellular RNA was isolated using RNAwiz (Ambion, Austin, TX). RNA was quantified by absorbance at 260 nm and purity was determined by absorbance at 280 and 310 nm. RNA (10 μg) was electrophoresed through a 1% agarose/6% formaldehyde gel and transferred to a nylon membrane. The membrane was exposed to X-ray film for autoradiography at several time points to ensure that the bands could be quantified by densitometry within the linear range. The α1(I) collagen cDNA probe was derived from a rat α1(I) collagen cDNA and specifically binds human α1(I) collagen mRNA [Genovese et al., 1984]. The human connective tissue growth factor (CTGF) cDNA probe was generated by polymerase chain reaction using primers previously reported [Ricupero et al., 2000]. RNA loading was assessed by ethidium bromide staining of ribosomal bands and by cohybridization with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blot Analysis

The cell layer was dissolved in RIPA buffer containing a protease inhibitor cocktail (Roche, Applied Science, Indianapolis, IN) at 4°C and centrifuged 14,000*g* for 20 min. The protein yield was determined by assay (Bio-Rad Laboratories, Hercules, CA) and SDS–PAGE was performed using 4–12% gradient Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were transferred to a nitrocellulose membrane and blocked for 1 h at room temperature in 5% evaporated milk or bovine serum albumin in phosphate-buffered saline containing 0.1% Tween. The membrane was then incubated overnight at 4°C in a primary antibody solution: (1) 1:1,000 rabbit anti-α1(I) collagen antibody (LF-39) (generously provided by Larry W. Fisher, National Institutes of Health, Bethesda, MD) [Fisher et al., 1989]; (2) 1:1,000 rabbit anti-acetyl-histone H4 antibody (Upstate, Waltham, MA); or (3) 1:3,000 mouse anti-α-tubulin antibody (Sigma-Aldrich, St. Louis, MO). Finally, the membrane was incubated for 1 h at room temperature in a secondary antibody solution: (1) goat anti-rabbit antibody conjugated to horseradish peroxidase (Promega, Madison, WI) or (2) goat anti-mouse antibody conjugated to horseradish peroxidase (Promega). Proteins were detected using a chemiluminescence kit (PerkinElmer Life and Analytical Sciences Boston, MA) and the membrane was exposed to X-ray film for autoradiography.

Luciferase Assay

Two transforming growth factor-β (TGF-β)-inducible luciferase reporter constructs were employed: (1) 3TP-lux [Lagna et al., 1996] and (2) $(CAGA)_{12}$-lux [Dennler et al., 1998]. Plasmids were transiently transfected into fibroblasts using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) and luciferase activity was measured by an assay according to the manufacturer's instructions (Promega).

cAMP Assay

cAMP was quantified as previously described [Johnson et al., 1994; Choung et al., 1998]. Briefly, the cells were labeled with ^{3}H-adenine for 24 h, washed, and then incubated for 15 min in medium containing IBMX with or without the indicated reagents. Reactions were stopped and cell lysates were applied to Dowex AG50-X8 columns (Dow Chemical, Midland, MI) containing analytical grade resin (Bio-Rad). The columns were washed and cAMP was eluted from the Dowex columns onto Alumina columns (Sigma). cAMP was then eluted from the Alumina columns and the radioactivity of the cAMP fraction was quantified by liquid scintillation spectrometry.

Reagents

PB was obtained from Triple Crown America. Porcine platelet-derived TGF-β1 was obtained from R & D Systems (Minneapolis, MN). Actinomycin D, cycloheximide (CHX), and sodium butyrate were obtained from Sigma. Trichostatin A was obtained from Upstate. Apicidin was obtained from Calbiochem (San Diego, CA).

Statistical Analysis

A *t*-test was used for means of unequal size. Probability values less than 0.05 were considered significant.

RESULTS

The effect of PB on α1(I) collagen protein expression was examined in cultured IMR-90 human embryonic lung fibroblasts incubated in serum free medium in the presence or absence of TGF-β for 24 h. Western blot analysis of cell-associated protein using an antibody to the carboxyl terminal region of α1(I) collagen demonstrated that PB decreased basal and TGF-β-stimulated levels of α1(I) collagen protein levels (Fig. 1A). PB also decreased α1(I) collagen mRNA levels in identically treated fibroblasts as assessed by Northern blot analysis (Fig. 1B). Densitometry analyses of Northern blots from three separate experiments showed that PB 5 mM decreased basal α1(I) collagen mRNA expression, normalized to GAPDH mRNA expression, by 78% ($P < 0.0001$, $n = 3$) at 24 h suggesting that regulation occurs primarily at the mRNA level.

Dose–response and time-course studies were performed to further characterize the effect of PB on α1(I) collagen mRNA expression. A dose–response relation between PB and α1(I) collagen mRNA levels was determined in fibroblasts treated with PB for 24 h (Fig. 2A). PB at a low

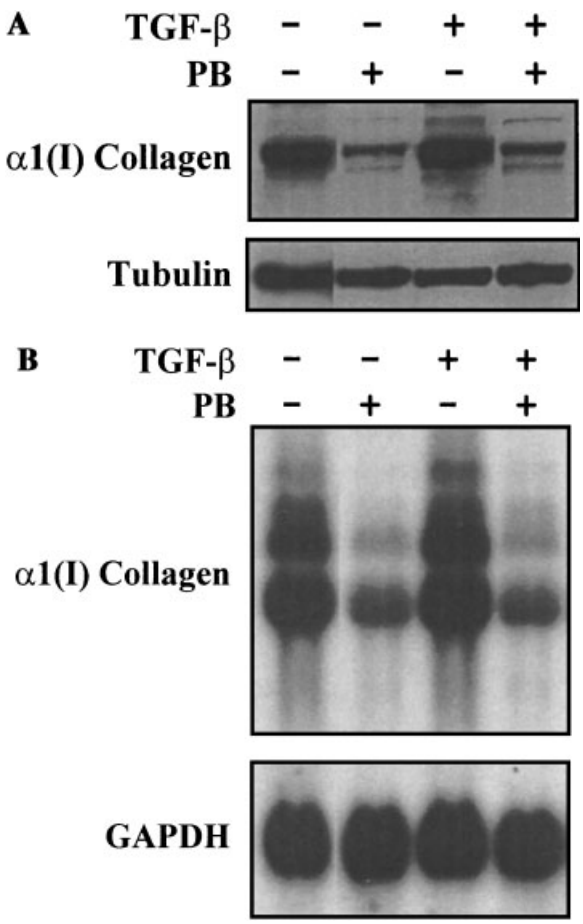

Fig. 1. The effect of phenylbutyrate (PB) on α1(I) collagen protein and mRNA expression. Quiescent lung fibroblast cultures were left untreated or treated with PB 5 mM with or without transforming growth factor -β (TGF-β) 1 ng/ml as indicated for 24 h. **A**: Western blot analyses of cell-associated protein were performed using antibodies to α1(I) collagen and tubulin (representative of two separate experiments). **B**: Northern blot analyses were performed using probes for α1(I) collagen and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (representative of three separate experiments).

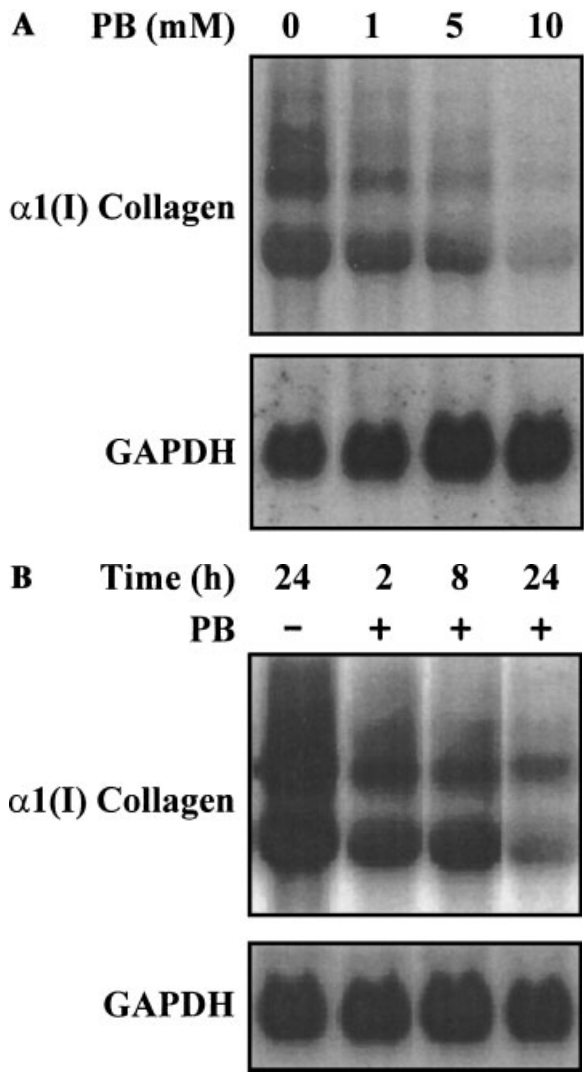

Fig. 2. Dose–response and time-course analyses of the effect of PB on α1(I) collagen mRNA expression. **A**: Quiescent lung fibroblast cultures were left untreated or treated with PB at varying concentrations as indicated for 24 h. Northern blot analyses were performed using probes for α1(I) collagen and GAPDH (representative of three separate experiments). **B**: Quiescent lung fibroblast cultures were left untreated or treated with PB 5 mM for varying times as indicated. Northern blot analyses were performed using probes for α1(I) collagen and GAPDH (representative of two separate experiments).

dose (1 mM) slightly decreased α1(I) collagen mRNA levels whereas PB at higher doses (5 and 10 mM) markedly decreased α1(I) collagen mRNA levels. These results were consistent with previous reports indicating that PB exerts its biologic activity at concentrations in the millimolar range [Comte et al., 2002]. A time-course study revealed that PB (5 mM) decreased α1(I) collagen mRNA expression at 2 h with a maximal decrease at 24 h (Fig. 2B).

The stability of the α1(I) collagen mRNA transcript in fibroblasts treated with PB was then assessed using the transcriptional inhibitor actinomycin D (Fig. 3). Northern blot analyses of two separate experiments revealed that the rate of decay of α1(I) collagen mRNA in fibroblasts treated with PB and actinomycin D was only slightly greater than that observed in fibroblasts treated with actinomycin D alone suggesting that PB did not markedly change the stability of the α1(I) collagen mRNA transcript. Similar to previous reports, the half-life of α1(I) collagen mRNA in fibroblasts was approximately 16 h [Krupsky et al., 1997; Stefanovic et al., 2000].

The potential toxicity of PB was examined by initially incubating the fibroblasts in medium containing PB 10 mM for 24 h followed by incubation in medium without PB for an additional 24 h. Under these conditions, α1(I) collagen mRNA levels returned to those seen in untreated fibroblasts indicating PB was not toxic (data not shown). In addition, examination by light microscopy did not reveal any apparent morphological changes between untreated and treated fibroblasts.

The expression of other matrix molecules by fibroblasts treated with PB was also examined. Northern blot analysis demonstrated that basal and TGF-β-induced CTGF mRNA expression were decreased in fibroblasts treated with PB for 6 h (Fig. 4A). PB had no effect on basal fibronectin mRNA levels at 24 h, but it decreased TGF-β-stimulated fibronectin mRNA levels at this time point (Fig. 4B).

Previous studies have shown that prostaglandin E_2 (PGE_2) decreases the rate of α1(I) collagen mRNA transcription by stimulating cAMP production and that this mechanism is dependent on de novo protein synthesis [Fine et al., 1992]. Using ion exchange chromatography to measure cAMP production, a significant increase in cAMP production was found in fibroblasts treated with PB ($P < 0.00005$) (Fig. 5A). The effect of PB on α1(I) collagen mRNA levels appeared partially CHX dependent (Fig. 5B). In experiments using indomethacin, a non-selective cyclooxygenase inhibitor, to inhibit PGE_2 synthesis, the effect of PB on α1(I) collagen mRNA levels was not altered (data not shown).

The effect of PB and other HDAC inhibitors on α1(I) collagen mRNA expression was determined by Northern blot analysis (Fig. 6). PB decreased α1(I) collagen mRNA levels to a greater extent than the more potent HDAC inhibitors sodium butyrate, and apicidin. Of note, escalating doses of TSA appeared to have effects comparable to PB. At high doses, sodium butyrate (20 mM) also decreased α1(I) collagen mRNA levels (data not shown).

The effect of PB and TSA on histone acetylation and α1(I) collagen mRNA expression was further examined (Fig. 7). Western blot analysis using an antibody to acetylated histone H4 demonstrated that TSA was a much more

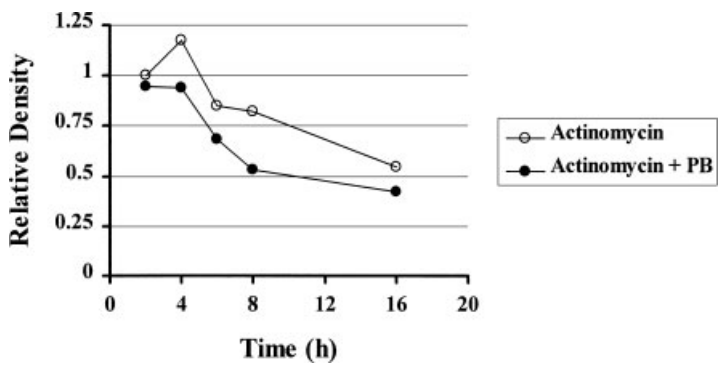

Fig. 3. The effect of PB on the stability of the α1(I) collagen mRNA transcript. Quiescent lung fibroblast cultures were treated with actinomycin D with (closed circles) and without (open circles) PB 5 mM for varying times as indicated. Northern blot analysis was performed using a probe for α1(I) collagen and the bands were quantified by densitometry (representative of two separate experiments).

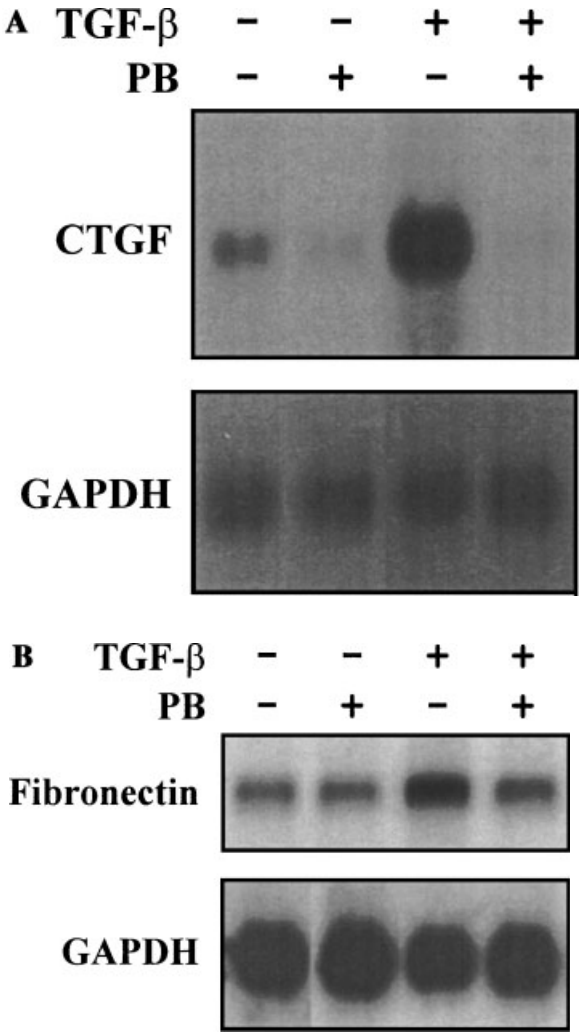

Fig. 4. The effect of PB on CTGF and fibronectin mRNA expression. **A**: Quiescent lung fibroblast cultures were left untreated or treated with PB 10 mM with or without TGF-β 1 ng/ml as indicated for 6 h. Northern blot analyses were performed using probes for CTGF and GAPDH (representative of two separate experiments). **B**: Quiescent lung fibroblast cultures were left untreated or treated with PB 10 mM with or without TGF-β 1 ng/ml as indicated for 24 h. Northern blot analyses were performed using probes for fibronectin and GAPDH (representative of two separate experiments).

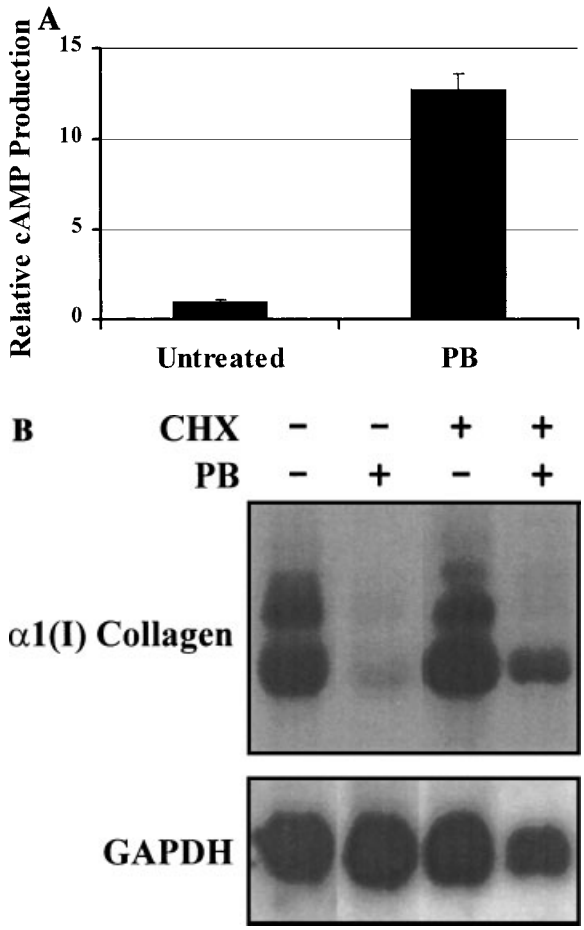

Fig. 5. **A**: The effect of PB on cAMP production. Lung fibroblast cultures were left untreated or treated with PB 10 mM as indicated for 15 m and cAMP was measured as described in ''Materials and Methods'' (mean + SD, representative of two separate experiments). **B**: The effect of PB and cycloheximide (CHX) on α1(I) collagen mRNA expression. Quiescent lung fibroblast cultures were left untreated or treated with PB 10 mM with or without CHX 5 μM as indicated for 24 h. Northern blot analyses were performed using probes for α1(I) collagen and GAPDH (representative of three separate experiments).

potent HDAC inhibitor than PB. In fibroblasts treated with TSA, increased levels of acetylated histone H4 were detected at 1 h and persisted at least 24 h (Fig. 7A). Furthermore, PB and low dose TSA had similar effects on histone H4 acetylation (Fig. 7B), but had marked differences in their effect on α1(I) collagen mRNA levels (Fig. 7C). The dose of PB that induced small increases in acetylated histone H4 resulted in large decreases in α1(I) collagen mRNA expression, whereas high levels of histone H4 acetylation were necessary to decrease α1(I) collagen mRNA levels in fibroblasts treated with TSA suggesting that PB may be acting in several different ways.

The mechanism by which PB attenuated TGF-β-stimulated α1(I) collagen and fibronectin mRNA levels was examined (Fig. 8). Fibroblasts were transiently transfected with the TGF-β-responsive luciferase constructs 3TP-lux (Fig. 8A) or $(CAGA)_{12}$-lux (Fig. 8B) and were then left untreated or treated with PB with or without TGF-β for 4 h. The results of three separate experiments using each reporter construct indicated that PB did not significantly alter basal (3TP-lux, $P = 0.52$; $(CAGA)_{12}$-lux $P = 0.22$) or TGF-β-stimulated (3TP-lux, $P = 0.44$;

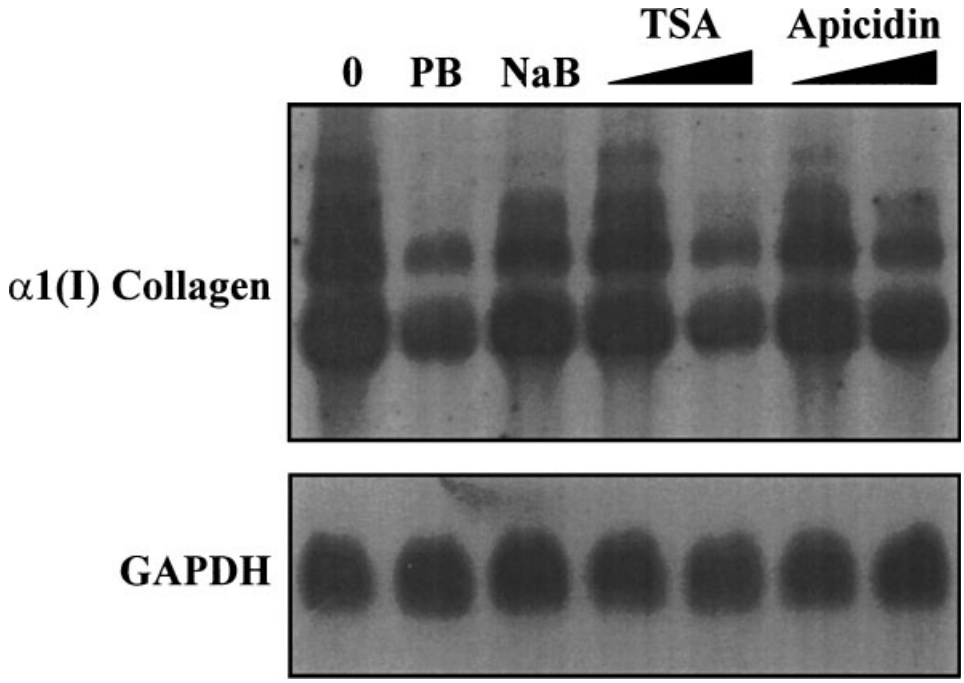

Fig. 6. The effect of HDAC inhibitors on α1(I) collagen mRNA expression. Quiescent lung fibroblast cultures were left untreated or treated with PB 5 mM, sodium butyrate 5 mM, TSA 1 and 10 μM, or apicidin 0.1 and 1 μM as indicated for 24 h. Northern blot analyses were performed using probes for α1(I) collagen and GAPDH (representative of two separate experiments).

$(CAGA)_{12}$-lux, $P = 0.25$) luciferase activity in either construct.

DISCUSSION

Our results demonstrate that PB decreases type I collagen expression in human lung fibroblasts primarily by decreasing the transcription of the *α1(I)* collagen gene and to a lesser extent the stability of the resulting mRNA transcript. Several mechanisms likely account for these effects, including activation of the adenylate cyclase-cAMP-protein kinase A pathway and inhibition of HDAC activity. PB and PGE_2 are both potent stimulators of cAMP production and their effect on basal α1(I) collagen mRNA levels is partially dependent on de novo protein synthesis. PB may act in a similar fashion to PGE_2, a well-described antifibrogenic effector substance. PGE_2 inhibits basal and TGF-β-stimulated type I collagen expression in human lung fibroblasts mainly by inhibiting transcription of the *α1*(I) collagen gene [Fine et al., 1989]. In addition, PGE_2 inhibits collagen synthesis in a variety of other fibroblastic cell types [O'Keefe et al., 1992; Raisz et al., 1993; Brilla et al., 1995]. In these model systems, PGE_2 appears to limit fibrosis by stimulating the production of cAMP. Our experiments employing indomethacin to inhibit cyclooxygenase activity indicated that PGE_2 is not a mediator of PB.

The mechanisms by which PB and PGE_2 decrease basal α1(I) collagen expression may have similarities, however, they are distinguished by the ability of PB to inhibit HDAC activity. Transcriptional regulation may be controlled by the acetylation state of histones. For example, the histone acetyltransferases p300 and CBP regulate the activity of the cyclin *E* gene promoter [Bandyopadhyay et al., 2002]. Our results demonstrated that the potent HDAC inhibitor TSA decreases α1(I) collagen mRNA expression indicating that histone acetylation state may regulate *α1(I)* collagen gene transcription. However, high levels of acetylated histone H4 observed following treatment with TSA are not associated with correspondingly low levels of α1(I) collagen mRNA. Moreover, further decreases in α1(I) collagen mRNA levels are seen following the addition of the weak HDAC inhibitor PB to TSA-treated fibroblasts (data not shown). Our results suggest that histone acetylation is only partially responsible for the effect of PB on α1(I) collagen mRNA expression.

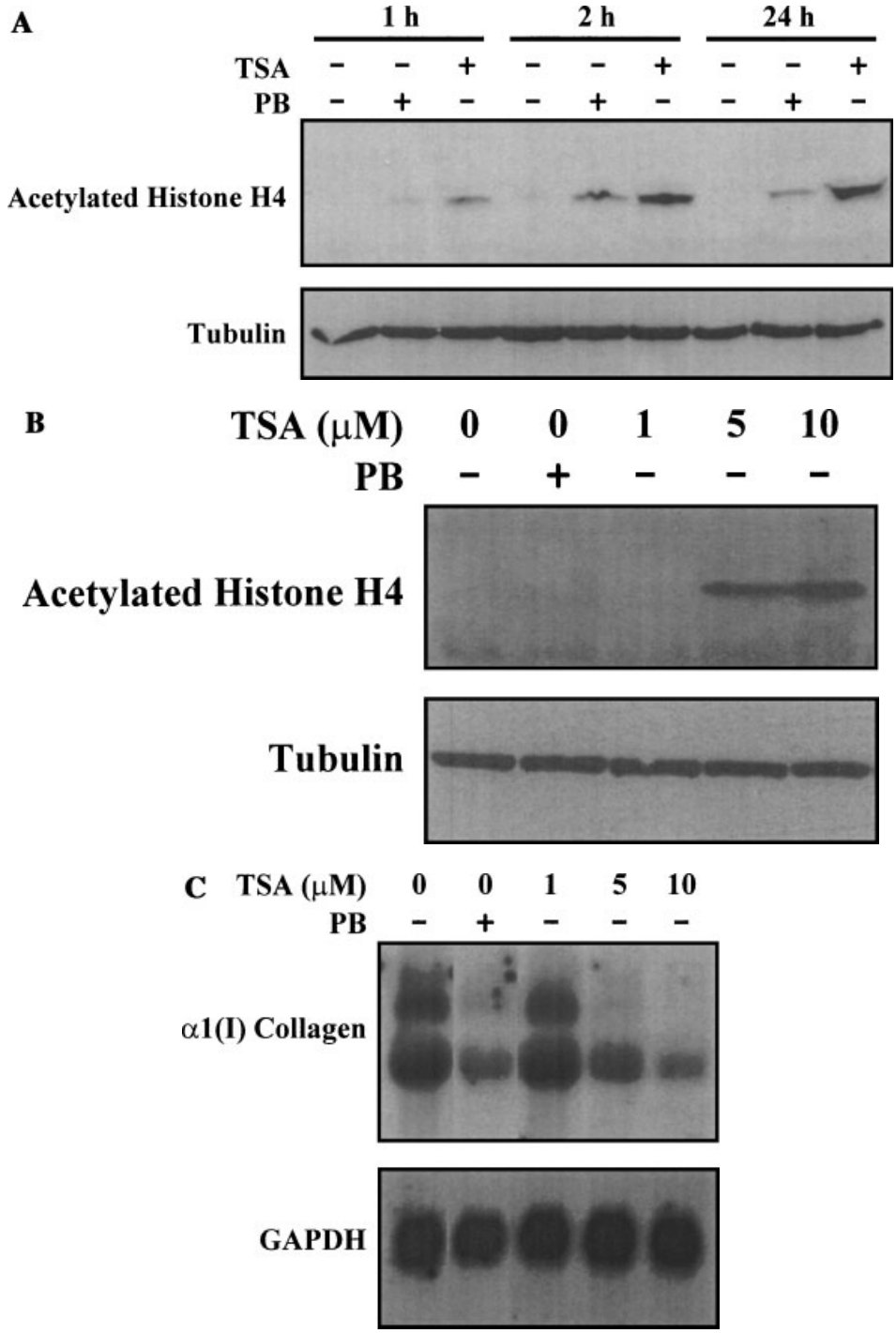

Fig. 7. The effect of TSA on histone H4 acetylation and α1(I) collagen mRNA expression. **A**: Quiescent lung fibroblast cultures were left untreated or treated with PB 5 mM or TSA 10 μM for varying times as indicated. Western blot analyses of cell-associated protein were performed using antibodies to acetyl-histone H4 and tubulin (representative of two separate experiments). **B**: Quiescent lung fibroblast cultures were left untreated or treated with PB 5 mM or TSA at varying concentrations as indicated for 24 h. Western blot analyses of cell-associated protein were performed using antibodies to acetyl-histone H4 and tubulin (representative of two separate experiments). **C**: Quiescent lung fibroblast cultures were left untreated or treated with PB 5 mM or TSA at varying concentrations as indicated for 24 h. Northern blot analyses were performed using probes for α1(I) collagen and GAPDH (representative of two separate experiments).

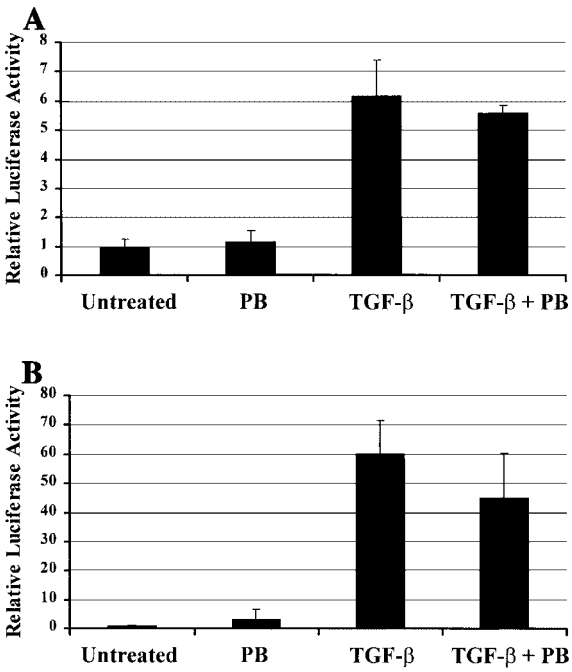

Fig. 8. The effect of PB on TGF-β signaling. Lung fibroblast cultures were transiently transfected with the TGF-β-responsive luciferase constructs **A**: 3TP-lux and **B** $(CAGA)_{12}$-lux and were then left untreated or treated with PB 5 mM with or without TGF-β 1 ng/ml as indicated for 4 h. Luciferase activity was measured as described in "Experimental Procedures" (mean + SD, representative of three separate experiments).

TGF-β signal transduction from the cell surface to the nucleus is mediated in large part by the Smad family of intracellular proteins [Massague, 1998]. Following TGF-β stimulation, Smad2 and Smad3 are phosphorylated and form complexes with Smad4. These complexes translocate to the nucleus and regulate gene transcription by binding DNA directly or in association with other transcription factors. Transcriptional activation of the target gene may be further regulated by cofactors that bind these Smad complexes. Although, our results indicated that PB does not interfere with Smad-mediated signaling to two TGF-β-responsive reporter constructs, PB may alter the association of Smads with co-factors necessary for specific TGF-β-induced *α1(I)* collagen gene transcription. Smads bind the consensus sequence CAGAC with low affinity, but the association of Smads with DNA-binding co-factors increases their affinity and their regulatory specificity [Chen et al., 1997; Wong et al., 1999]. PB may be directly or indirectly antagonizing the association of Smads with specific cofactors necessary for TGF-β-stimulated transcriptional activation of the *α1(I)* collagen gene. The transcriptional coactivators p300 and CBP may interact with Smad proteins [Pouponnot et al., 1998]. Of note, PGE_2 does not affect the activity the 3TP-lux reporter construct following TGF-β stimulation [Ricupero et al., 1999].

Although Smads are integral to TGF-β signal transduction, other signaling proteins such as JNK, ERK1/2, and Ras are activated by TGF-β in several systems [Yan et al., 1994; Hocevar et al., 1999; Sowa et al., 2002]. In a human fibrosarcoma-derived cell line, TGF-β-induced expression of fibronectin is Smad-independent and requires JNK activation [Hocevar et al., 1999]. Consistent with this report, our results demonstrated that PB decreases TGF-β-stimulated fibronectin mRNA levels and does not affect TGF-β signaling to 3TP-lux or $(CAGA)_{12}$-lux, suggesting that PB interferes with Smad-independent TGF-β signaling. In other studies employing cell lines with a TGF-β type I receptor defective in Smad activation but with retained kinase activity, fibronectin protein production appears dependent on Smads [Itoh et al., 2003]. In these investigations, activation of JNK is independent of Smads. However, our studies revealed that PB does not affect JNK or ERK1/2 activation (data not shown). Our results do not exclude a role for Smad-dependent TGF-β signaling in mediating the effects of PB on type I collagen, however, they suggest that Smad-independent pathways may exert some effect on TGF-β-induced Smad signaling.

In summary, PB significantly decreases type I collagen expression in cultured human lung fibroblasts by mechanisms that include stimulation of cAMP production and alteration of histone acetylation state. PB is a non-toxic pharmaceutical compound that is already approved by the Food and Drug Administration to treat ornithine transcarbamylase deficiency. The effect of PB on type I collagen and its favorable safety profile indicate that it may have therapeutic benefit for fibrotic lung diseases, including idiopathic pulmonary fibrosis (IPF) and subepithelial fibrosis associated with asthma.

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