Insulin-like Growth Factor-I Signaling Mechanisms, Type I Collagen and Alpha Smooth Muscle Actin in Human Fetal Lung Fibroblasts

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ABSTRACT: Bronchial wall remodeling is a major morbidity component in oxidant injury in bronchopulmonary dysplasia (BPD) and asthma. Hypothesis: IGF-1 enhances alpha smooth muscle expression and collagen synthesis in developing lung fibroblasts leading to fibrosis through nuclear NF-$_k$B -dependent transcription. We studied NF-$_k$B dependent transcription by transfecting HFLF with a NF-$_k$B responsive promoter driving the luciferase gene and treating with IGF-1 (100 ng/mL) and measuring luciferase activity. We exposed cells to the PI-3 kinase inhibitor or the Erk1/2 inhibitor one hr before stimulating with IGF-1. We also used IGF-1 receptor antibody to inhibit the action of IGF-1 and studied its effect on alpha-sma and type I collagen. IGF-1 treatment significantly increased luciferase activity. This was attenuated by PI-3 kinase and MAP-Kinase inhibitors. Western blot analysis showed PI-3 kinase mediates IGF-1 activation of NF-$_k$B independent of I$_K$B phosphorylation. We found an up-regulation of phospho NF-kB in the nuclear extract compared with total NFKB showing that IGF-1 regulates NF-$_k$B transcriptional activity downstream of NF-$_k$B nuclear translocation. IGF-1-induced increase in alpha-sma expression and type-I collagen was significantly inhibited by pretreatment with LY294002 and IGF-1 receptor antibody. IGF-1 cell signaling leading to collagen synthesis in fetal lung fibroblasts is mediated by PI3 Kinase acting through NF-$_k$B in HFLF. (*Pediatr Res* **60: 389–394, 2006**)

In asthma, chronic inflammation leads to pathologic changes in the airways with marked remodeling (1). An increased number of myofibroblasts beneath the bronchial epithelial basement membrane has been described in asthma. Bronchial biopsies of asthmatic patients show thickening of the subepithelial layer due to the deposition of fibrillar collagen (2). The production of mediators by epithelial cells in close proximity to myofibroblasts during epithelial repair after repeated damage is one possible mechanism for airway remodeling (3). IGF-1 and IGF binding proteins (IGFBPs) are active in the pulmonary cellular environment to modulate cell proliferation and function (4). IGF-1 and its receptor, IGF-1R, are also known to modulate repair processes after lung injury (5). Others and we have observed that IGF-1R expression is more intense in areas of increased cell proliferation in developing lung (6,7). These studies prompted us to investigate the hy-

pothesis that IGF-1 mediated signaling in developing lung cells may induce some of the phenotypic changes that are observed in asthma. We examined the signaling pathway activated by IGF-1 in fetal lung fibroblasts leading to an increase in myofibroblast proliferation and collagen synthesis, and whether this involves nuclear NF-kappaB (NF-$_k$B)-dependent transcription.

MATERIALS AND METHODS

Reagents were obtained as follows: Recombinant IGF-1 and IGF-1 receptor antibody were purchased from R&D Systems (Minneapolis, MN). Goat anti-rabbit IgG was purchased from Upstate Biotechnology (Charlottesville, VA). Penicillin G, acrylamide, DMSO, SDS, pepstatin and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). L-glutamine, Hanks balanced salt solution (HBSS), Dulbecco's modified PBS (D-PBS), Dulbecco's minimal essential medium (DMEM) and FCS were obtained from GIBCO (Grand Island, NY). The PI-3 kinase inhibitor LY 294002, ERK-1/2 inhibitor PD 98059, and antibodies against phospho-AKT, AKT, phospho-ERK and ERK were purchased from Cell Signaling (New England Biolabs, Ipswich, MA).

Cell culture. The cell line chosen in this study is a human fetal lung fibroblast cell line (HFLF), which was derived from fetal lung fibroblasts. We have used this cell line successfully to model pulmonary cell-cell interactions in co-cultures with pulmonary epithelial cells in a three-dimensional collagen gel (8). HFLF from American Type Culture Collection (Rockville, MD) were cultured in DMEM containing 10% FCS, penicillin G (100U/mL), streptomycin (100 μg/mL), amphotericin (2 μg/mL) and L-glutamine (500 μM) at 37°C in 5% CO_2/ 95% air. Cells were plated at a density of $2X10^5$ per well in six well plates. Sub-confluent cultures were serum-starved for 24 h followed by treatment with DMEM containing IGF-1 with and without the inhibitors. The concentration of IGF-1 used (100 ng/mL) was determined in experiments described below and this dose has also been used by others in fibroblasts and other cells (9,10). To see if IGF-1 receptor (IGF-1R) has any effect on IGF-1 stimulated responses we used 5 μg/mL of IGF-1R antibody (A12) kindly provided by Imclone systems, NY.

Cell proliferation assays. Assays were performed in quadruplicate using a calorimetric method for determining the number of viable cells in proliferation. MTS, a tetrazolium compound was bio-reduced into a colored formazon product by living cells in proliferation. Serum-starved cells plated in 96 well plates were pretreated with PI-3 kinase inhibitor, LY 294002 (50 μM/mL), MAP kinase inhibitor PD 98059 (5 μM/mL), or IGF-1 receptor antibody (5 μg /mL) before treatment with IGF-1 (100 ng/mL). The concentration of IGF-1 was determined by prior dose response experiments. At the completion of the experiment, cells were incubated with MTS (ONE Solution Reagent, Promega) for two hours and the absorbance was recorded at 490 nm in a 96 well plate reader.

PI-3 kinase and MAP-kinase activities. Cells were plated in six well plates at a density of 2×10^5 cells per well. 95% confluent cells were serum-starved with 0.5% BSA for four hours, exposed to either the PI-3 kinase inhibitor (LY 294002) or the Erk1/2 inhibitor (PD 98059) at 50 μM concentration. After one-hour incubation with the inhibitor, IGF-1 (100 ng/mL) was added and cultures continued for 12 h. The proteins were separated from cell lysates by PAGE and the

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Abbreviations: α-sma, alpha smooth muscle actin; **BPD,** bronchopulmonary dysplasia; **ERK,** extracellular signal regulated kinase; **IGFBP-3,** IGF binding protein-3; **MAP,** mitogen activated protein kinase; **PI-3 kinase,** phosphatidylinositol 3-kinase

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activation of PI-3 kinase and MAP kinase evaluated by Western blot analysis. The duration of exposure to IGF-1 was determined by preliminary time series experiments. The doses used for LY294002 and PD98059 have been found to be physiologic and used by others in fibroblast cell cultures to block PI-3 kinase and MAP Kinase pathways (11) (12).

Western blot analysis. A 50-μg protein sample was separated by PAGE on a 10% acrylamide-SDS gel then transferred to a nitrocellulose membrane. The membranes were probed using antibodies to phospho-AKT, AKT, phospho- I_KB, I_KB and phospho-ERK and ERK (Cell Signaling) at 1:1000 dilution. The membranes were incubated in the presence of primary antibody at 4°C overnight. Following a sequence of washes in TBST, the membranes were incubated with goat anti-rabbit IgG (1:30,000 in TBST) for one hour at room temperature. Antigens were identified using chemiluminescence (Amersham, Life Science, Bio-Rad) and exposure to X-ray film. Comparisons of relative changes were made by densitometry scanning. Actin was used as an internal standard. The membranes were also probed with Type-I anti-collagen antibody, and with mouse monoclonal anti-human α-sma antibody (R&D Systems).

Transient transfection and luciferase assay. Cells were transiently transfected with a DNA plasmid construct expressing the luciferase gene under the control of NF-$_k$B DNA binding site. pTK81- NF-$_k$B -Luc containing two copies of the annealed oligonucleotide sequence 5′- CGCGGGGACTTTCCC gtac GGGGACTTTCCCG-CGgtac-3′ in the antisense orientation 5′ to the luciferase gene in the *Kpn*I site in the vector pTK81-Luc was kindly provided by William L Lowe Jr, M D (Northwestern University Medical School). Since renilla based reporters are frequently used as transfection controls for luciferase transcriptional reporter assays, we used this system as an internal standard. For transfection assays, the fibroblasts were plated onto 12-well plates at a density of 1×10^5 cells per well. Plasmid DNA was transfected into cells using the cationic lipid Lipofectin (Life Technologies, Inc, Gaithersburg, M.D.), according to the manufacturer's instruction. Cells incubated overnight in Opti-mem (Promega, Madison, WI) with Lipofectin and plasmid DNA.

Luciferase assay. Transfected cells were transferred to serum-free DMEM + 0.25% BSA for four hours. The inhibitors to PI-3 kinase and MAP kinase were added at 50μM concentration one hour before treating with IGF-1 (100 ng/mL) overnight. The cells were harvested, and luciferase activity measured by luminometer (Monolight, BD Biosciences) as described (13), and normalized to renilla luciferase. All assays were performed in triplicate.

Western blot analysis of P65 subunit of phospho NF-$_k$B analysis in nuclear extracts. Cells were serum-starved overnight before treatment with inhibitors and the growth factor as described above. Nuclear extracts were prepared using the Celytic™ NuClear Extraction kit (Sigma Chemical Co.) and stored at −70º until further analysis. The nuclear extract was separated by PAGE, and the membrane was probed with antibody directed against phospho NF-$_k$B and total NF-$_k$B p65 subunit of NF-$_k$B (1:1000 dilution) (Cell Signaling). The Phospho NF-$_k$B p65 (Ser536) antibody detects NF-$_k$B p65 only when phosphorylated at serine 536.

Immunohistochemistry of alpha smooth muscle actin expression analysis. Fixed, permeabilized cells were blocked with PBS containing 0.1% Triton X-100, 1% BSA, and 10% normal donkey serum at room temperature for 45 min. Cells were incubated with a mouse anti-α-sma MAb (1:1000) overnight at 4°C followed by Rhodamine Red-coupled anti-mouse IgG at RT in the dark for one hour. Images were acquired on a Zeiss Axiovert microscope equipped with a Sutter DG-4 Xenon fluorescence excitation source, Hamamatsu cooled-CCD digital camera, and a PC running Metamorph 5.0 (Universal Imaging Systems) at the GRASP Center/Tufts Center for Neuroscience Research Imaging Facility. Color was added in Metamorph after image acquisition. α-sma expressing cells from four independent experiments were scored from a total of 20 cells in each of three different fields and the percentage of α-sma-expressing cells was determined using Scion imaging software to quantitate fluorescence.

Data analysis. Densitometry was used for quantitative analysis for Western blots. Data are represented as mean ± SEM. Statistical analysis to determine the effect of IGF-1 with and without the inhibitors was performed using one-way analysis of variance with Bonferroni correction for multiple comparisons. Differences between control and treated cells were considered statistically significant if p values were <0.05.

RESULTS

Effect of IGF-1 on the PI-3 kinase-signaling pathway in human fetal lung fibroblasts. Since we have shown in our earlier studies (14,15) that IGF-1 plays an important role in oxidant injury in developing lung, we chose to investigate the signaling pathway of IGF-1 in human fetal lung fibroblasts. Western blots were used to evaluate the phosphorylated form of AKT and total AKT, known also as protein kinase$_B$ (Fig. 1*A*).
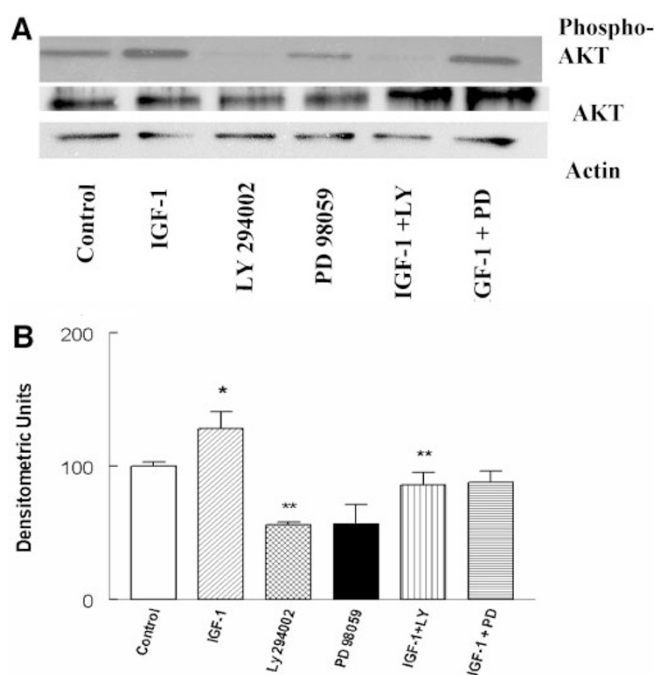

Figure 1. Effect of IGF-1 on PI 3 kinase pathway in human fetal lung fibroblasts. Western blots were probed for phospho Akt and re-probed with AKT antibody. (*A*) Representative Western blot for phospho AKT and AKT. (*B*) The histogram shows results of quantitative densitometry of protein levels of the ratio of Phospho AKT to AKT in the cells. Bars represent the mean ± SEM of $n = 3$ experiments. *$p < 0.05$ compared with control cells; **$p < 0.05$ compared with cells treated with IGF-1 (100 ng/mL).

IGF-1 treatment significantly increased Phospho AKT, suggesting that IGF-1 signaled through the PI-3 kinase pathway. This was confirmed by the ability of LY 294002 to block AKT phosphorylation with IGF-1 treatment (Fig. 1*B*). The ability of IGF-1 to increase AKT phosphorylation was not changed in the presence of the MAP-kinase inhibitor PD 98059 (Fig. 1*A*) showing that there is no cross talk between these two pathways.

Effect of IGF-1 on ERK phosphorylation in HFLF. Next we examined the effects of IGF-1 on ERK1/2 members of the MAP-Kinase signaling pathway. There was no significant increase in ERK 1/2 phosphorylation with IGF-1 (100 ng/mL), nor did either PD 98059 or LY 294002 alter ERK 1/2 phosphorylation in the presence of IGF-1 (Fig. 2*A,B*).

Effect of IGF-1 on NF-$_k$B transcriptional activity in HFLF. Having shown that IGF-1 activates the PI-3 kinase pathway we investigated the ability of IGF-1 to increase NF-$_k$B transcriptional activity. We transfected HFLF using pTK81- NF-$_k$B-Luc, a fusion gene as described in Methods. IGF-1- increased luciferase activity was significantly inhibited by PI-3 kinase and by MAP-kinase inhibitors at 50 μM (Fig. 3) suggesting that these pathways contribute to IGF-1-induced activation of NF-$_k$B.

Since we found an increase in NF-$_k$B activity in IGF-1 treated cells we further investigated the mechanism of IGF-1-induced NF-$_k$B activation. An important event in the activation of NF-$_k$B is its release from the inhibitory protein I_KB followed by translocation into the nucleus. To evaluate the release for nuclear translocation of NF-$_k$B, we studied I_KB phosphorylation and degradation. Surprisingly we did not find any change in I_KB phosphorylation or degradation with IGF-1
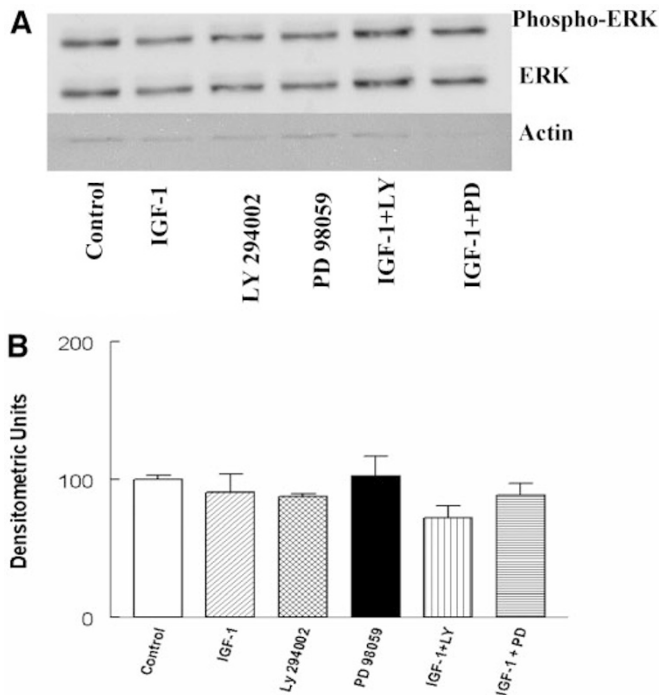
Figure 2. Effect of IGF-1 on ERK phosphorylation in human fetal lung fibroblasts. Western blots were probed with the antibody for phospho-ERK-1/2 and re-probed with antibody for total ERK1/2. (*A*) Representative Western blot for phospho ERK and ERK. (*B*) The histogram shows results of quantitative densitometry of protein levels of Phospho ERK and ERK in the cells. Bars represent the mean $\pm$ SEM of $n = 3$ experiments. 1: Control; 2: IGF-1; 3: LY294002; 4: PD 98059; 5: IGF-1+Ly; 6: IGF-1+PD.
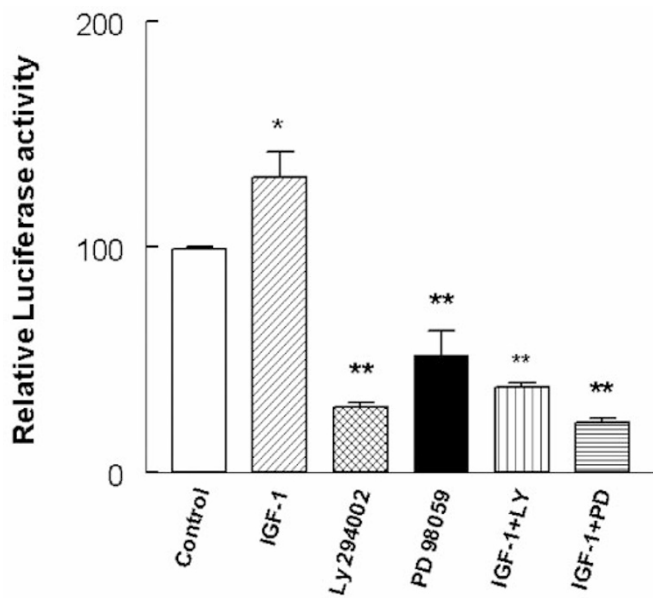

Figure 3. Effect of IGF-1 on $NF_K B$ activity in human fetal lung fibroblasts. The histogram shows quantitative representations of Luciferase activity. The values represent relative luciferase activity compared with control. Bars represent the mean $\pm$ SEM of $n = 3$ experiments. *$p < 0.05$ compared with control cells; **$p < 0.05$ compared with cells treated with IGF-1.

(Fig. 4). A similar stimulation of NF-$_k$B-derived gene transcription without increased Ik -B phosphorylation has been previously reported in bovine pulmonary artery endothelial cells (16).

To investigate the nuclear translocation of NF-$_k$B, nuclear extracts were prepared from fetal lung fibroblasts stimulated

with IGF-1 (100 ng/mL) overnight after pretreatment for 1h with PI-3 kinase or MAP kinase inhibitors as described above. The nuclear extracts were separated by PAGE, transferred to membranes, and the membranes probed with antibody directed against the p65 subunit of phospho NF-$_k$B and total NF-$_k$B. Phospho-NF-$_k$B p65 (Ser536) antibody detects NF-$_k$B p65 only when phosphorylated at serine 536. The antibody does not cross-react with the p50 subunit or other related proteins (17). NF-$_k$B p65 antibody detects endogenous levels of total NF-$_k$B. We found that IGF-1-induced increase in nuclear phospho NF-$_k$B was inhibited both by the PI-3 kinase and the MAP kinase inhibitors at 50 μM concentrations, and by IGF-1 receptor antibody at 5 μg/mL (Fig. 5a and b). These data suggest that IGF-1 regulates NF-$_k$B nuclear transcriptional activity independent of changes in $I_K B$ phosphorylation.

IGF-1 stimulation of collagen synthesis in HFLF. Based on our previous studies showing that hyperoxia increased collagen synthesis in neonatal rat lung explant cultures, and also increased IGF-1 in epithelial cells (7,18), we investigated the effect of IGF-1 on Type I collagen in fetal lung fibroblasts with and without PI-3 kinase and MAP-kinase inhibitors. Figure 6 shows a representative Western blot for Type I collagen and β-Actin and the summary of densitometry analysis. There was a significant increase in collagen in cells treated with IGF-1 compared with controls, which was significantly inhibited in the presence of LY 294002. These data
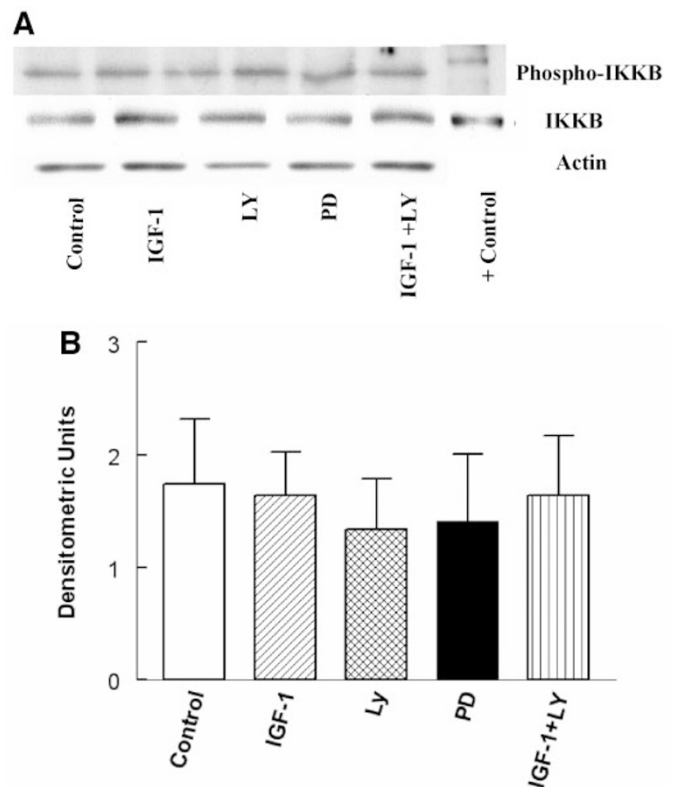

Figure 4. Effect of IGF-1 on $I_K B$ phosphorylation in human fetal lung fibroblasts. Cell lysates were prepared and Western blots were probed for phospho-$I_K B$ and re-probed with $I_K B$ antibody. (*A*) Representative Western blot for phospho $I_K B$ and $I_K B$. Jurkat whole cell lysate (Santa Cruz Biotechnology) was used as positive control for $I_K B$ (Fig. 4*A,B*). (*B*) Densitometry of protein levels of phospho $I_K B$/Actin. Bars represent the mean $\pm$ SEM of $n = 3$ experiments.
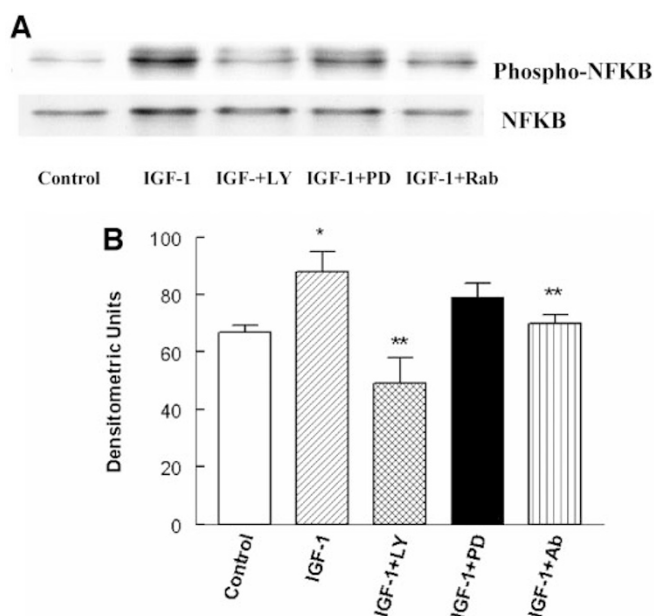
Figure 5. NF-$_k$B (P65 subunit) analysis in nuclear extracts from HFLF. The nuclear extract was separated by PAGE and the membranes were probed with antibody directed against the p65 subunit of NF-$_k$B and phospho NF-$_k$B (Ser536). (*A*) Representative blot of phospho NF-$_k$B and NF-$_k$B p65 (*B*) The histogram shows quantitative representations of protein levels of Ser536 Phospho *NF-$_k$B (P65 subunit)* in the nuclear extracts of the cells (Bars represent mean ± SEM of $n = 3$ experiments). *$p < 0.05$ compared with control cells; **$p < 0.05$ compared with cells treated with IGF-1 100 ng/mL. Rab, receptor antibody.
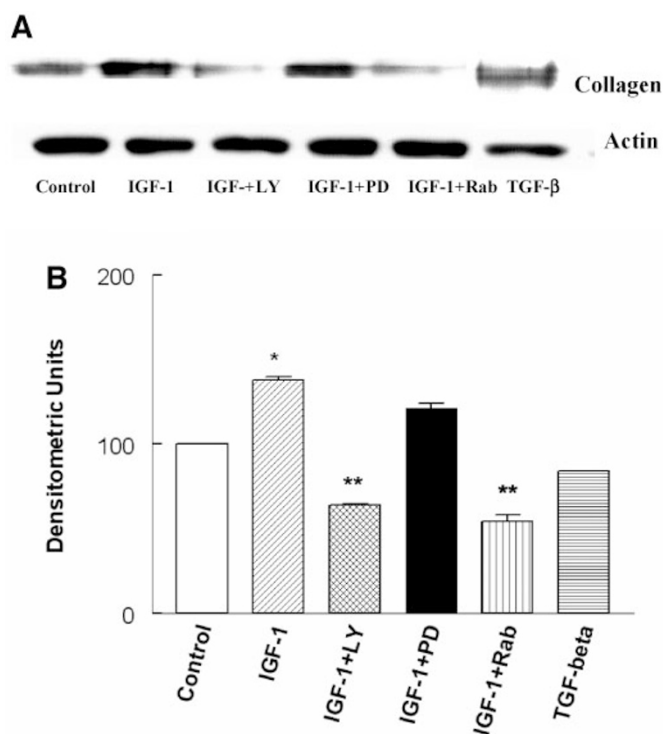

Figure 6. Effect of PI3 kinase inhibitors on IGF-1 stimulated collagen synthesis in HFLF. Western blots were probed for Type I collagen and re-probed for actin antibody. (*A*) Representative Western blot for Type I collagen and Actin. (*B*) The histogram shows quantitative densitometry of protein levels of Type I collagen/actin ratio in the cells. Bars represent the mean ± SEM of $n = 3$ experiments. *$p < 0.05$ compared with untreated cells; **$p < 0.05$ compared with cells treated with IGF-1. Rab, receptor antibody.

show that PI-3 kinase plays an important role in IGF-1 stimulated type I collagen production in fetal lung fibroblasts.

PI-3 kinase inhibition and IGF-1 receptor antibody block IGF-1 stimulated increases in α-sma and cell proliferation. Previous studies have documented a stimulatory effect of IGF-1 on myofibroblast proliferation at sites of inflammation (19,20), although the signal transduction pathways for increased α-sma in fetal lung fibroblasts have not been identified. We found that IGF-1 stimulated a 2-fold increase in α-sma protein. There was a robust inhibition of IGF-1-stimulated increase in α-sma by Ly 294002, and by IGF-1 receptor antibody (Fig. 7*A,B*)). We also observed a moderate inhibition of IGF-1-stimulated increase in α-sma by PD98059. Immunohistochemistry confirmed these changes in α-sma (Fig. 8*A,B*). In addition, the IGF-1 - induced increase in cell proliferation was significantly inhibited by IGF-1 receptor antibody and PI-3 kinase and MAP kinase inhibitors (Fig. 9*A*).

DISCUSSION

The main aim of the present study was to evaluate the mechanisms by which IGF-1 alters type-I collagen and α-sma expression in HFLF, since these cells are involved in airway remodeling in neonatal hyperoxic lung injury leading to CLD, airway hyper-reactivity, and increased incidence of subsequent asthma. In our earlier studies we have shown that IGF-1 is up-regulated in type II epithelial cells after hyperoxic exposure of neonatal rat lung explant cultures (8) (7). In addition, profibrotic cytokines are increased in bronchoalveolar fluid of preterm infants who developed chronic lung disease (21). IGF-1 and other profibrotic cytokines lead to airway remodeling with increased thickness of interstitial matrix (22)
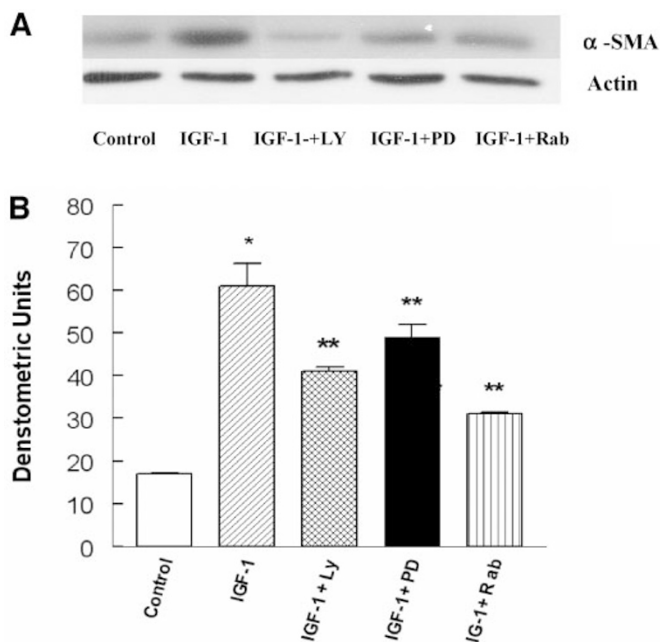

Figure 7. Effect of PI3 kinase inhibitors on IGF-1 stimulated α-sma in HFLF. Western blots were probed for α-*sma* antibody and re-probed for actin (*A*) Representative Western blot for α-*sma* and actin. (*B*) Densitometry of the α-sma/actin protein ratio Bars represent the mean ± SEM of $n = 3$ experiments. *$p < 0.05$ compared with control cells; **$p < 0.05$ compared with cells treated with IGF-1.

(23). The airway hyper-responsiveness is primarily a function of contraction of airway smooth muscles (24).

In this study we sought to identify the point(s) in the IGF-1 signal transduction pathways, which are activated to increase the population of α-sma-positive cells in the airways. We found out that IGF-1 stimulated AKT phosphorylation which was inhibited by pre-treatment with LY294002, strongly suggesting that PI-3 kinase is activated by IGF-1. PI-3 kinase activation due to IGF-1 has been shown in other systems, including bovine pulmonary vascular endothelial cells (25). We also showed that activation of the ERK-1 and ERK-2 members of MAPK family in response to IGF-1 was not significant in these cells, suggesting that PI-3 kinase activation is the major pathway involved in IGF-1 mediated increase in α-sma expressing cells. The doses used for LY294002 and PD98059 used in this study act against physiologic levels of IGF-1 and were used by others in fibroblast cell cultures to block PI-3 kinase and MAP Kinase pathways (26,27).

Having shown the effect of IGF-1 on the PI-3 kinase pathway, we next examined the downstream mechanisms activated by this pathway. One mechanism of IGF-1 mediated effects is NF-$_k$B dependent transcriptional activation. At baseline NF-$_k$B is generally present as a dimer with the inhibitory I$_k$B protein. Upon activation, I$_k$B is phosphorylated allowing NF-$_k$B, to disassociate and translocate to the nucleus to exert its transcriptional regulation properties. Previous studies demonstrated IGF-1 stimulates NF-$_k$B dependent gene transcription in neuronal (13) and endothelial cells (8). In this study we showed that IGF-1 increases NF-$_k$B dependent transcription in human fetal lung fibroblasts using a construct containing a NF-$_k$B responsive promoter driving the luciferase gene. The stimulation of NF-$_K$B dependent transcription was attenuated both by the PI-3 kinase inhibitor LY 294002, and by the MAPK1 inhibitor PD98059 suggesting that both these pathways have a role in IGF-1-induced activation of NF-$_k$B. This is in agreement with the effect of IGF-1 seen in neuronal cells (28).

We further investigated the mechanism of IGF-1-induced NF-$_k$B activity. NF-κB, a heterodimer composed of p50 and p65 subunits, is a potent activator of gene expression from NF-κB DNA biding sites due to the presence of transactivating domains located in the C-terminal region of the p65 protein (29). Regulation of gene expression by NF-$_k$B is controlled through signaling mechanisms that promote nuclear translocation after I$_k$B degradation and through mechanisms that are responsible for up-regulating the transactivation function of NF-$_k$B. Surprisingly, we did not find any increase in either nuclear p65 levels or the phosphorylation or degradation of I$_k$B. These data suggest that IGF-1 regulates NF-$_k$B transcriptional activity downstream of NF-$_k$B nuclear translocation. This is in agreement with a recent study that showed that both PI-3 kinase and AKT were able to stimulate the transcriptional activity of NF-$_k$B by inducing changes in basal activity of nuclear NF-$_k$B rather than influencing nuclear translocation (30). We found an up-regulation of phospho NF-$_k$B in the nuclear extract compared with total NF-$_K$B suggesting that activated PI-3 kinase or AKT stimulates NF-$_k$B -dependent transcription by stimulating the phosphorylation of the nuclear p65 subunit of NF-$_k$B rather than by inducing NF-$_k$B nuclear translocation via I$_k$B degradation.
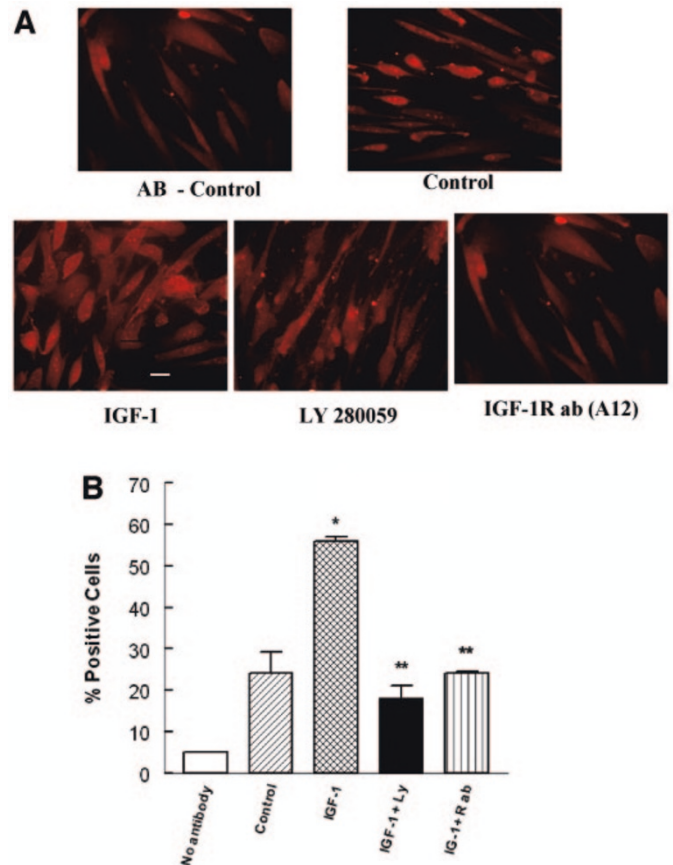

Figure 8. (*A*) Representative immunofluorescence for α-sma in control cells and in cells treated with IGF-1, IGF-1+ PI-3 kinase inhibitor (LY 294002), and IGF-1 + IGF-1R antibody. A negative control (no primary antibody) is also shown (bar, 40 μM). (*B*) Histogram shows the percent of α-sma positive cells in fetal lung fibroblasts stimulated with IGF-1 with and without PI-3 kinase inhibitor or IGF-1R antibody, $n = 3$ experiments. *$p < 0.05$ compared with untreated cells; **$p < 0.05$ compared with cells treated with IGF-1.

Cellular responses to oxidative stress are regulated by the type 1 IGF-1 receptor (31,32). Oxidant injury implicated in the pathophysiology of bronchopulmonary dysplasia acutely up-regulates IGF-1R expression in the lung. This led us to suspect that IGF-1 receptor blockade in lung tissue could prevent the deleterious effects of hyperoxia. It has been shown that IGF-1 receptor antibody fully blocked the IGF-1 mediated inhibition of apoptosis and increase in cell proliferation (33) (34). Our results show that antibody blockade of IGF-1 receptor inhibited IGF-1 stimulation of α-sma expression in fetal lung fibroblasts both by Western blot analysis and by immunohistochemistry, and inhibited stimulation of cell proliferation. The inhibition of IGF-1-induced cell proliferation with these inhibitors has also been shown in other cell lines including human ES cells. Proliferation in these cells is mediated primarily via the PI 3-kinase/Akt signaling pathway, using the IGF-1 receptor as an upstream activator (35).

Increase in α-sma expression in lung injury is often associated with an increase in collagen synthesis. Therefore we studied collagen production in neonatal lung by analyzing the content of type I collagen. As part of the repair process following lung inflammation, fibroblasts proliferate and produce large amounts of collagen. It has been shown that IGF-1
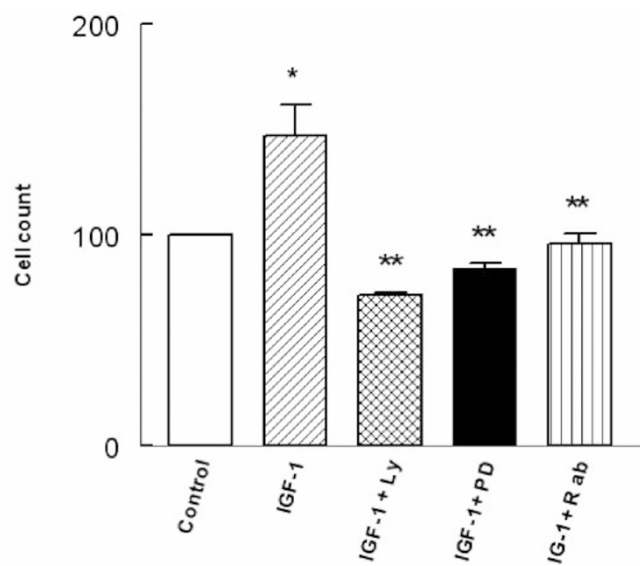
Figure 9. Cell Proliferation. MTS, a tetrazolium compound was bio-reduced into a colored formazon product by living cells in proliferation. The absorbance was measured at 490 nm wavelength using an Elisa plate reader. α-sma expressing cells were scored as described in the Methods section. $*p < 0.05$ compared with control cells; $**p < 0.05$ compared with cells treated with IGF-1.

increases collagen synthesis in several systems including human lung fibroblasts (36,37). In these studies we found that pre-treatment with both a PI-3 kinase inhibitor reduced IGF -1 induced increases in type I collagen.

In conclusion, hyper-reactive airways in infants with bronchopulmonary dysplasia are associated with the development of smooth muscle cell hyperplasia and increased expression of collagen synthesis and α-sma. Our present study demonstrates that these phenotypic changes in fetal lung fibroblasts may be regulated by IGF-1 and the type I IGF-1 receptor, and may be signaled through PI-3 kinase and activation of NF-$_k$B. This provides a basis for developing strategies to interfere with IGF-1 receptor signaling to reduce or prevent the development of hyperoxia induced hyperreactive airway disease.

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