Hypoxia-induced Proliferative Response of Vascular Adventitial Fibroblasts Is Dependent on G Protein-mediated Activation of Mitogen-activated Protein Kinases*

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Hypoxia has been shown to act as a proliferative stimulus for adventitial fibroblasts of the pulmonary artery. The signaling pathways involved in this growth response, however, remain unclear. We tested the hypothesis that hypoxia-induced proliferation of fibroblasts would be dependent on distinct (compared with serum) activation and utilization patterns of mitogen-activated protein (MAP) kinases initiated by Gαi/o proteins. We found that hypoxia stimulated increases in DNA synthesis and growth of quiescent fibroblasts in the absence of exogenous mitogens and also markedly augmented serum-stimulated growth responses. Hypoxia caused a transient activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), the time course and pattern of which was somewhat similar to that induced by serum but which was of lesser magnitude. On the other hand, hypoxia-induced activation of p38 MAP kinase was biphasic, whereas serum-stimulated activation of p38 MAP kinase was transient, and the magnitude of activation was greater for hypoxia compared with that of serum stimulation. ERK1/2, JNK1, and p38 MAP kinase, but not JNK2 were necessary for hypoxia-induced proliferation because PD98059, SB202190, and JNK1 antisense oligonucleotides nearly ablated the growth response. JNK2 appeared to act as a negative modulator of hypoxia-induced growth because JNK2 antisense oligonucleotides led to an increase in DNA synthesis. In serum-stimulated cells, antisense JNK1 oligonucleotides and PD98059 had inhibitory effects on proliferation, whereas SB202190 led to an increase in DNA synthesis. Pertussis toxin, which blocks Gαi/o-mediated signaling, markedly attenuated hypoxia-induced DNA synthesis and activation of ERK and JNK but not p38 MAP kinase. We conclude that hypoxia itself can act as a growth promoting stimulus for subsets of bovine neonatal adventitial fibroblasts largely through Gαi/o-mediated activation of a complex network of MAP kinases whose specific contributions to hypoxia-induced proliferation differ from traditional serum-induced growth signals.

Change in the architecture of the blood vessel wall occurs in response to a wide range of physiologic stimuli and various injurious insults. In response to chronic hypoxic exposure, the pulmonary arteries (PA)1 undergo concentric thickening with medial and adventitial changes predominating (1, 2). When the hypoxic exposure occurs in early neonatal life, the adventitial changes are particularly striking and include early and dramatic increases in fibroblast proliferation (1, 3, 4). Much of the work done to date in defining this process has focused on the growth factors that are produced under hypoxic conditions and their potential effects on cell proliferation. Little work, however, has been done on the growth promoting signaling pathways that might be induced by hypoxia itself and thus might affect vascular wall cell proliferation directly and/or markedly influence the response to locally produced growth factors. A better knowledge of this process is important for an understanding of the remodeling process in many vascular diseases because hypoxemic or ischemic conditions are thought to contribute to this process.

Mitogen-activated protein (MAP) kinase family members including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase have been proposed to be important signaling components linking extracellular stimuli to cellular responses including cellular growth, differentiation, and metabolic regulation (5–8). Mitogen-activated protein kinases can be activated by various external stimuli such as growth factors, hormones acting via G protein-coupled receptors, and physical stresses (6, 9). Hypoxia has also shown to stimulate ERK, JNK, and p38 MAP kinase in different cell types (10–15). Recently, Jin et al. (16) demonstrated the activation of all three MAP kinases in whole pulmonary arterial wall of rat in response to hypoxia, although this study did not evaluate the activation patterns in specific cell types in the vessel wall. Activation of p38 MAP kinase has been demonstrated to be necessary for the proliferation of adult bovine PA adventitial fibroblasts and renal mesangial cells in response to hypoxia (12, 14). By contrast, the mitogenic response to hypoxia of cultured human osteoblastic periodontal

1 The abbreviations used are: PA, pulmonary artery; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum.
ligament cells is mediated by the selective phosphorylation and activation of ERK1/2 (15). These observations support the hypothesis that the pathways used to achieve cell proliferation in response to a specific stimulus may be cell type-specific. There is insufficient information on the MAP kinase pathways activated by hypoxia in cells at a developmental stage of heightened susceptibility to hypoxia-induced change, i.e. the neonate.

Moreover, little is known of the upstream signals that lead to MAP kinase activation and ultimately cell proliferation in response to hypoxia. One possible candidate is a family of heterotrimeric guanine nucleotide binding proteins (G proteins). It has been shown that G proteins can be activated by a number of environmental stimuli and play critical roles in cell proliferation (17, 18). Previous work in our laboratory demonstrated that specific subpopulations of smooth muscle cells utilized pertussis toxin-sensitive G proteins (Go(13)) for growth to a far greater extent than other smooth muscle cells populations and that these cell populations proliferated in response to hypoxia, whereas the others did not (19). Further, activation of G proteins has been speculated to be a critical early event in hypoxia-induced cell responses in different cell types (20–23). However, the role of G proteins in hypoxia-induced activation of MAP kinases and proliferative responses of vascular adventitial fibroblasts is unknown.

We therefore hypothesized that hypoxia-induced proliferation of neonatal PA adventitial fibroblasts is transduced through G protein-mediated activation of MAP kinases in a manner that is different from serum-induced MAP kinase activation and cell proliferation. To assess hypoxia-induced signaling, we utilized a primary cell system generated from animals where hypoxia-induced fibroblast proliferation has been documented in vivo (1). Cultured cells from these animals were amenable to serum deprivation for 5 days, which allowed assessment of hypoxia-induced signaling in the absence of exogenous stimuli that could influence the MAP kinase signaling pathways. Further, an approach in which the effects of serum and hypoxia were simultaneously evaluated in the same cells and was utilized to determine whether there was a unique aspect of MAP kinase signaling in hypoxia-induced proliferation.

MATERIALS AND METHODS

EGF’s minimum essential medium (MEM), trypsin-EDTA 10× suspension, penicillin, streptomycin, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and β-mercaptoethanol were purchased from Sigma. Fetal bovine serum (FBS) was obtained from Gemini Bio-Products, Inc. (Calabasas, CA). Molecular weight markers and reagents for protein determination were from Bio-Rad. Antibodies for total p38, JNK1, and ERK1/ERK2 and horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat immunoglobulin G were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride membrane and rabbit anti-goat immunoglobulin G were from Santa Cruz and ERK1/ERK2 and horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat immunoglobulin G were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride membrane was obtained from Amersham Pharmacia Biotech. Renaissance chemiluminescence reagent plus was from PerkinElmer Life Sciences. SB202190 was obtained from CalBiochem (San Diego, CA). Lipofectin was obtained from Amersham Pharmacia Biotech. Renaissance Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride membrane was obtained from Amersham Pharmacia Biotech. Renaissance chemiluminescence reagent plus was from PerkinElmer Life Sciences.

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To test whether hypoxia affects the serum-stimulated growth response of fibroblasts, cells were seeded sparsely (250 cells/cm²) in 10% FBS/MEM and allowed to attach overnight according to the previously described method (25). From day 1, fibroblasts were exposed to normoxia and hypoxia up to 11 days. The hypoxia chambers were repurged with the gas mixtures every 24 h. Results were expressed as cell counts × 10⁶/well.

Phosphorylation of MAP Kinases in Response to Hypoxia—Cells (500–10⁵) were plated in 100-mm Petri dishes in medium containing 10% FBS and growth arrested for 5 days with 0.1% FBS/MEM. At the end of 5 days, medium was removed to leave 2 ml, so that the cell monolayer was just covered, and then cells were exposed to hypoxia (3% O₂) for the indicated times (0, 10, 30, and 60 min and 24 h). Cells were lysed with the homogenization buffer (20 mM Tris, pH 7.5, containing 0.25 M sucrose, 3 mM EDTA, 3 mM EGTA, 50 mM mercaptoethanol, 50 µg/ml leupeptin, 50 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100) (25), scraped off the dish, freeze-thawed, and centrifuged at 6,000 rpm for 10 min at 4 °C. Supernatant was used as whole cell lysates. Protein concentrations were determined by using a Bio-Rad protein assay. 5–30 µg of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% milk in TBS-Tween for 1 h at room temperature and then incubated with anti-phospho (active) MAP kinase antibodies in TBS-Tween with 5% milk (1:500 to 1:1000) overnight at 4 °C. Antibodies against activated MAP kinases were developed using their phosphorylation pattern at threonine/tyrosine residues. Anti-ERK1/2 is dually phosphorylated at Thr202/Tyr204, anti-JNK is dually phosphorylated at Thr183/Tyr185, and anti-p38 is dually phosphorylated at Thr180/Tyr182. After three washes with TBS-Tween, membranes were incubated with anti-rabbit antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The bands were identified by using chemiluminescence reagents, and the emitted light was recorded on film.

To compare the phosphorylation of MAP kinases in response to hypoxia and serum deprivation, the growth arrested adventitial fibroblasts were stimulated with 10% FBS/MEM for similar lengths of time as hypoxia. The cell lysates were analyzed for the phosphorylated and nonphosphorylated forms of MAP kinases by Western blot analysis. The protein loading for each sample was evaluated by stripping the membrane with buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) and reprobed with anti-β-actin antibody.

Film images of Western blots obtained from ECL detection were analyzed on a scanner and visualized using a Vista Scan software package. Densitometric quantitation of the bands was performed using NIH Image 1.58 program. The bands were scanned, and the area under the curve of each band was determined. This value represents the band intensity in arbitrary units. The value at 0 h time was considered as 100%, and fold increases in response to hypoxic exposure and serum stimulation were calculated with respect to the 0 h.

Effects of JNK1 and JNK2 Antisense Oligonucleotides on Hypoxia and Serum-induced Proliferative Responses of Fibroblasts—All oligonucleotides used in this study were phosphorylamine oligonucleotides prepared according to a previously described method (26). The transient transfection of fibroblasts with scrambled and antisense constructs was performed using Lipofectin. JNK1 and JNK2 antisense oligonucleotides were mixed with Lipofectin (10 µM) and added to the cell monolayer. After 5–6 h, the medium was replaced with MEM containing 0.1% FBS. For determination of JNK protein levels, cell extracts were prepared with homogenizing buffer. An equal amount of protein was resolved on SDS-10% polyacrylamide gels and visualized by Western analysis with specific anti-JNK1 antibodies.
For the proliferation assay, growth-arrested fibroblasts were transiently transfected with 200 nM of antisense and scrambled oligonucleotides. As described above, after the 5 h of lipofection, the medium was replaced with MEM supplemented with 0.1% FBS. The next day, [\textsuperscript{3}H]thymidine was added to the cells, and cultures were either exposed to normoxia and hypoxia, or stimulated with 10% FBS for 24 h. Cells were processed for the measurement of DNA synthesis according to a previously described method (24).

**Effects of PD98059 and SB202190 on Hypoxia-induced and Serum-stimulated Growth Responses of PA Adventitial Fibroblasts—**To detect the role of ERKs and p38 MAP kinases in hypoxia-induced and serum-stimulated growth, fibroblasts were seeded at a density of $10 \times 10^4$ cells/cm$^2$ in 10% FBS/MEM, grown for 1 day, and then growth-arrested for 5 days with 0.1% FBS/MEM. PD98059 (10 \muM), specific blocker of ERK (27), and SB202190 (100 nM), specific antagonist of p38 MAP kinase (28), were added to the medium and incubated at 37°C for 1 h. The cells were exposed to normoxia and hypoxia in the presence of [\textsuperscript{3}H]thymidine for 24 h and processed for the measurement of DNA synthesis (24).

**Effects of Pertussis Toxin on Proliferation and MAP Kinase Activation of Fibroblasts in Response to Hypoxia—**To evaluate the role of G protein in proliferative responses of fibroblasts, quiescent cells were incubated with 100 ng/ml of pertussis toxin at 37°C for 1 h and then either exposed to normoxia and hypoxia or stimulated with 10% FBS for 24 h in the presence of [\textsuperscript{3}H]thymidine. DNA synthesis was measured according to a previously described method (24).

To examine the function of G protein in hypoxia-induced activation of MAP kinases, growth-arrested fibroblasts were incubated with 100 ng/ml pertussis toxin overnight at 37°C and then exposed to hypoxia. Cells were harvested with homogenizing buffer for the preparation of whole cellular lysates. 5–30 \mug of protein were electrophoresed on 10% polyacrylamide gels followed by transfer to polyvinylidene difluoride membrane and immunoblotting with anti-ERK, anti-JNK, and anti-p38 MAP kinase antibodies detecting the phosphorylated forms. Immuno-reactive bands were visualized with ECL Western blotting detection reagents.

**Data Analysis—**All data are expressed as arithmetic means ± S.E.; n equals the number of replicate wells/test condition in representative experiments. One-way and two-way analyses of variance followed by the Student-Newman-Keuls multiple comparisons within and between groups of data points were utilized. Data were considered significantly different if $p < 0.05$.

**RESULTS**

**Hypoxia in the Absence of Exogenous Mitogens Induces Growth of Adventitial Fibroblasts and Also Augments Serum-induced Proliferation—**To evaluate the possibility that hypoxia itself acts as a growth-promoting stimulus for fibroblasts isolated from the PA adventitia of neonatal calves, we examined the effect of low oxygen concentrations on DNA synthesis in fibroblasts in the absence of any co-mitogens. Quiescent fibroblasts were exposed to gas mixtures with varying amounts of oxygen (21%, 10%, and 3–1%) for 24 h in the presence of [\textsuperscript{3}H]thymidine, and DNA synthesis was measured. We found that oxygen concentrations of 3–1% initiated a 2–4-fold increase in thymidine incorporation in fibroblasts compared with 21% O$_2$ (Fig. 1A). To determine whether hypoxia-induced increases in DNA synthesis resulted in a net increase in cell number, growth-arrested cells were exposed to hypoxia (3% O$_2$ because this is where maximal DNA synthesis was observed) for 72 h, and cell counts were performed. An increase in the number of fibroblasts after chronic exposure to hypoxia was found (Fig. 1B).

To determine whether the hypoxia-induced proliferative response was unique to adventitial fibroblasts from the pulmonary circulation, we compared the effects of hypoxia on DNA synthesis in aggregate fibroblast populations derived from both the PA and the thoracic aorta of eight newborn calves. PA adventitial fibroblasts from all eight calves consistently demonstrated an increase in replication upon hypoxic exposure (Fig. 1C). In contrast, in six of eight animals, no increase in proliferation was observed in quiescent adventitial fibroblasts isolated from the aorta in response to hypoxia. However, in two aortic fibroblast populations, hypoxia induced DNA synthesis to the same levels as observed in PA adventitial fibroblasts (Fig. 1C).

To evaluate whether hypoxia could effect the serum-induced proliferative response of fibroblasts, growth curves in the presence and absence of hypoxia were evaluated. We found that the growth of fibroblasts in serum was significantly enhanced under hypoxic conditions (Fig. 1D). These data suggest that hypoxia-induced and serum-stimulated growth of fibroblasts might be driven by distinct and additive/synergistic signaling pathways in neonatal bovine PA adventitial fibroblasts.

**Hypoxia Induces Activation of MAP Kinase Members in a Pattern Distinct from That Induced by Serum—**In an effort to evaluate the possibility that distinct signaling pathways are involved in hypoxia-induced growth responses of fibroblasts, we examined the effects of hypoxia on the activation of MAP kinase family members in quiescent, serum-starved fibroblasts and compared them to those induced by serum. Hypoxia induced a rapid and somewhat sustained activation of both ERK1 and ERK2 (Fig. 2A). The pattern of activation was similar for ERK1 and ERK2, although the magnitude of change was greater for ERK1. Further, the time course of activation for hypoxia was similar to that for serum, although the magnitude of the activation was not as great as serum (Fig. 2B). At 24 h, hypoxia-exposed fibroblasts had slightly higher levels of phospho-ERK1 and phospho-ERK2 than serum-stimulated cells (Fig. 2). No modification in the protein level of total ERK kinases was observed either in response to hypoxic exposure or serum stimulation for up to 24 h (Fig. 2A), demonstrating that the increase in ERK phosphorylation in response to both stimuli was not due to an increase in total ERK protein.

JNK was transiently activated in fibroblasts following hypoxic exposure (at 10 min) (Fig. 3A). p46 JNK appeared to undergo a greater increase than p54 JNK. In serum-stimulated cells, JNK was also activated at 10 min, but the activation was maintained up to 30 min (Fig. 3A). Densitometric quantitation of the blots demonstrated that the increase in phosphorylation of p46 JNK in hypoxic fibroblasts was ~8-fold greater than that of normoxic controls (Fig. 3B). The increase in p46 JNK phosphorylation in response to serum reached its maximum after 10 min (15-fold) (Fig. 3B). The increases in phosphorylation of p54 JNK in response to serum stimulation and hypoxic exposure were 7- and 5-fold, respectively (Fig. 3C). Western blots using pan JNK antibody demonstrated that JNK protein levels in adventitial fibroblasts did not change during either hypoxia or serum exposure up to 24 h (Fig. 3A).

Using the same experimental paradigm, we also found that hypoxia stimulated extensive phosphorylation of p38 MAP kinase (Fig. 4A). The hypoxia-induced activation of p38 MAP kinase was biphasic. Activation was already observed at 10 min, sustained up to 30 min, and then decreased to basal levels. However, a second increase in phosphorylation of p38 MAP kinase was observed in hypoxia-stimulated fibroblasts at 24 h. Serum stimulated a rapid phosphorylation of p38 MAP kinase that was also maintained up to 30 min (Fig. 4A). The maximum increases in phosphorylation induced by hypoxia and 10% serum were 26- and 17-fold, respectively (Fig. 4B). There was no effect of hypoxic exposure on total p38 protein level for up to 24 h (Fig. 4A). The p38 protein levels were increased at 60 min and 24 h of serum stimulation in fibroblasts; however, at these time points there was no detectable phosphorylation of the protein (Fig. 4B). Collectively these data demonstrate that both hypoxia and serum activate ERK, JNK, and p38 MAP kinases, but there are differences in the kinetics of activation of these kinases between the two stimuli with the most striking differences being in p38 MAP kinase activation.

**MAP Kinase Activation Is Necessary for Hypoxia-induced Proliferative Responses of PA Adventitial Fibroblasts—**The hypoxia-induced increase in DNA synthesis in serum-starved fi-
ERK, JNK, and p38 MAP Kinases in Fibroblast Hypoxic Growth

Fig. 1. Hypoxia induces proliferation of neonatal bovine adventitial fibroblasts in vitro. A, hypoxic stimulation of DNA synthesis is greatest at 3–1% O₂. Cells were plated at a density of 10 × 10^3/cm² in 24-well plates and made quiescent by serum deprivation (0.1% FBS) for 5 days. Cells were then exposed to 21, 10, and 3–1% O₂ in the presence of [³H]thymidine for 24 h. Values are the means ± S.E. for this and all subsequent figures. n = 4 replicate wells. *, p < 0.05 compared with 21% O₂ results. Similar results were obtained in three independent experiments with cell populations isolated from at least three independent animals. B, chronic hypoxia exposure increases cell density above normoxic levels. Growth-arrested fibroblasts were exposed to normoxia (21% O₂) and hypoxia (3% O₂) for 3 days, trypsinized, and counted under light microscope. n = 4 replicate wells. *, p < 0.05 compared with cell counts under both normoxic (24 and 72 h) and 24-h hypoxic exposure. C, hypoxia consistently induces an increase in proliferation in all fibroblast populations isolated from PA but only in selective fibroblast populations derived from aorta. Adventitial fibroblasts were isolated from both aorta and PA of the same neonatal calf and plated according to the hypoxic conditions. PA and adventitial fibroblasts were plated at the density of 250 cells/cm² in medium containing 10% FBS and allowed to attach overnight. On day 1, cells were exposed to normoxia and hypoxic gas for 30 min/day for up to 11 days. *, p < 0.05 compared with the corresponding normoxic value. Similar results were reproduced with at least two other cell populations.

Because pharmacological inhibitors of JNKs are currently not commercially available, we employed specific antisense oligonucleotides designed to inhibit expression of specific JNK isoforms (JNK1 and JNK2) and to determine whether activation of JNK was functionally related to the hypoxia-induced proliferative response of fibroblasts (27). As shown in Fig. 6A, the antisense oligonucleotides of JNK1 inhibited the increase in [³H]thymidine incorporation by fibroblasts in response to hypoxia. In the presence of scrambled oligonucleotides, adventitial fibroblasts maintained their unique hypoxia-induced proliferative capability (Fig. 6A). We confirmed that antisense oligonucleotides blocked the expression of JNK1 by demonstrating that JNK1 protein levels were dramatically reduced in cells treated with the antisense oligonucleotides (Fig. 6A). JNK1 antisense oligonucleotides did not affect the rate of apoptosis under normoxic or hypoxic conditions (data not shown). The role of JNK1 activation in serum-stimulated growth responses of fibroblasts was also evaluated using the antisense oligonucleotides. Serum-induced growth was also attenuated by JNK1 antisense oligonucleotides (Fig. 6B). As opposed to the inhibition of DNA synthesis observed with JNK1 antisense oligonucleotides, JNK2 antisense oligonucleotides appeared to enhance the hypoxia-induced proliferative responses of fibroblasts (Fig. 6C). In these experiments, the slight increase in apoptosis observed under hypoxic conditions was blocked by JNK2 antisense oligonucleotides (data not shown).

To evaluate the role of p38 MAP kinase in hypoxia-induced and serum-stimulated proliferative responses of adventitial fibroblasts, we used SB202190, a selective inhibitor of p38 MAP kinase (28). Pretreatment of cells with SB202190 significantly reduced the hypoxia-induced increase in DNA synthesis (Fig. 7A). To confirm that the inhibitory effect of SB202190 was due to the inhibition of DNA synthesis but not due to an increase in apoptosis, the apoptotic rate of hypoxic fibroblasts were also examined in the presence and absence of SB202190. The slight increase in apoptosis induced by hypoxia was also blocked by SB202190, thus eliminating the possibility that the inhibition of DNA synthesis was simply due to an increased rate of apoptosis (data not shown). In contrast to the inhibition of hypoxia-induced
growth by SB202190, the serum-stimulated proliferative response of fibroblasts was potentiated by a similar concentration of the inhibitor (100 nM) (Fig. 7B), suggesting that activation of p38 MAP kinase has distinct functional roles in hypoxia-induced and serum-stimulated growth of fibroblasts.

We also evaluated hypoxia-induced activation patterns of ERK, JNK, and p38 MAP kinase in aortic adventitial fibroblasts that lack the ability to proliferate in response to hypoxia (see Fig. 1C, nonresponsive cells). No increase in ERK and p38 MAP kinase activation was observed in these cells in response to hypoxia. However, hypoxia did induce a slight increase in JNK activation (1.5 fold) that was of lesser magnitude than observed in cells induced to proliferate by hypoxia (data not shown).

*Pertussis Toxin-sensitive G Proteins Are Essential Upstream Signaling Components of Proliferation and Activation of MAP Kinases in Response to Hypoxia*—To evaluate the role of Go alpha protein in hypoxia-induced proliferative responses, PA adventitial fibroblasts were treated with pertussis toxin, which inhibits Gi/Go activation by ADP-ribosylation. The hypoxia-induced increase in DNA synthesis was markedly attenuated by pertussis toxin (Fig. 8A). Proliferation in response to serum stimulation was also attenuated by pertussis toxin (Fig. 8B), demonstrating an important role for Go alpha activation in both hypoxia-induced and serum-stimulated growth responses of neonatal adventitial fibroblasts.

To determine whether the inhibitory effects of pertussis toxin on hypoxia-induced proliferation were mediated through inhibition of MAP kinases, quiescent fibroblasts were pre-

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**Fig. 2.** Hypoxia induces an increase in phosphorylation of ERK1 and ERK2. A, Western blot analysis of ERK1 and ERK2 phosphorylation in response to hypoxia and 10% serum. Representative immunoblots for phospho-ERKs and total ERKs. Growth-arrested PA adventitial fibroblasts were stimulated with either hypoxia (3% O2) or 10% PBS containing medium for 0, 10, 30, and 60 min and 24 h. Total protein was extracted and blotted on polyvinylidene difluoride membrane. Blots were probed with an anti-phospho p42/p44 antibody. Western blots were performed on the same extracts using anti-ERK1 and -ERK2 antibodies to examine the level of total amount of ERKs. Similar results were obtained from three separate experiments. For each experiment, cells from different animals were used. B, quantitative comparison of hypoxia and serum-induced ERK1 phosphorylation in PA adventitial fibroblasts. The bands on the radiographic film were scanned, and the area under the curve was measured for individual band using NIH Image analysis program. The value for 0 min of stimulation was considered as 100%. C, quantitative comparison of hypoxia and serum-induced ERK2 phosphorylation in PA adventitial fibroblasts.
treated with pertussis toxin and exposed to hypoxia, and MAP kinase activation was then evaluated using antibodies against activated forms of the kinases. The hypoxia-induced activation of ERK and JNK was almost completely blocked by pertussis toxin (Fig. 8C). In contrast, pretreatment of fibroblasts with 100 ng/ml pertussis toxin for 24 h had no effect on the hypoxia-induced phosphorylation of p38 MAP kinase (Fig. 8C). Therefore, hypoxia induces ERK and JNK through the activation of Gaq proteins, whereas hypoxia-induced activation of p38 MAP kinase occurs through other G proteins or other as yet undefined pathways.

DISCUSSION

The present study demonstrates that hypoxia can stimulate quiescent neonatal adventitial fibroblasts to proliferate in the absence of any exogenous mitogens and that this proliferative response is dependent, in large part, on Gaq-mediated activation of ERK and JNK pathways and Gaq-independent activation of p38 MAP kinase. Equally as important is the observation that not all adventitial fibroblast populations possess the capability of proliferating in response to hypoxia and that in the unresponsive cells hypoxia does not stimulate ERK or p38 activation. We showed that the hypoxia-induced proliferative response appears to be mediated through different mechanisms than those activated when the cells are stimulated to proliferate with serum. This was demonstrated by experiments showing that: 1) activation patterns of ERK, JNK, and especially p38 MAP kinases were different in response to hypoxia versus serum; 2) blocking Gaq ERK, and JNK1 nearly ablated the proliferative response induced by hypoxia yet had only moderate effects on serum-induced proliferation; 3) blocking p38 MAP kinase with SB202190 attenuated hypoxia-induced proliferation yet led to an increase in serum-induced growth; and 4) hypoxia led to synergistic augmentation of serum-induced growth.

Previous studies have demonstrated variable patterns of ac-
tivation of ERK, JNK, and p38 MAP kinase in response to hypoxia depending on the cell type studied and the conditions under which the experiments were done. Seko et al. (10, 11) demonstrated that hypoxia and hypoxia/reoxygenation activate ERK as well as JNK and p38 MAP kinase in cultured rat cardiac myocytes. However, the physiological end point of hypoxia-induced activation of MAP kinases in these cells was not examined. It has been reported that hypoxia activates JNK and p38 MAP kinase and stimulates proliferation in adult bovine PA adventitial fibroblasts (12, 29). Interestingly, in these cells hypoxia has little effect on ERK. In PC12 cells, hypoxia has been shown to activate ERK and p38 MAP kinase but not JNK (13). These observations suggest a complex integration of signaling pathways in the regulation of cellular responses upon hypoxic exposure that is cell type-specific and perhaps even developmentally regulated. Our experimental design, i.e. the withdrawal of serum for 5 days before hypoxic exposure, was designed to allow assessment of MAP kinase signaling in the absence of residual co-mitogenic stimuli. MAP kinase activity was induced by hypoxia in cells that were also stimulated to proliferate. In other cells, also from the adventitia of neonatal animals, hypoxia did not induce proliferation, and MAP kinase activity was not stimulated. Thus, the possibility exists that subsets of adventitial fibroblasts exist that exhibit different sensitivities to MAP kinase activation by hypoxia.

We have demonstrated a rapid and dramatic activation of ERK in growth-arrested neonatal bovine fibroblasts that were stimulated to proliferate with hypoxia, consistent with reports that these kinases are critical for proliferation in a variety of cell types (30). A relatively higher activation level of ERK1 compared with ERK2 was observed in response to hypoxia, although under basal conditions, the cells express a greater abundance of phosphorylated ERK2 than ERK1. A high ratio of activated ERK1/ERK2 has been shown to be associated with proliferation in muscle cells (31), supporting our hypothesis that activation of ERK might be necessary for hypoxia-induced proliferation in fibroblasts. The pattern of ERK activation was similar to that observed under serum stimulation, although less in magnitude. In our studies, inhibition of ERK activation during hypoxic exposure blocked almost completely the hypoxia-induced proliferative responses of fibroblasts. In contrast, serum-stimulated growth of fibroblasts was only partially blocked by the inhibition of ERK activation. These observations are consistent with the idea that ERKs play a more critical role in hypoxia-induced proliferative response than in serum-induced growth.

In our studies, activation of JNK occurred at 10 min of hypoxic challenge. This is unlike observations in adult fibroblasts where activation of JNK in response to hypoxia was not observed until after 3–6 h of hypoxic exposure (12). However, the time course of JNK activation in response to hypoxia in our study is comparable with that of the well known growth stimulus, serum. Three different JNK genes, JNK1, -2, and -3, and at least 10 different splice variants with molecular weights between 46 and 54 exist. Our data suggest that hypoxia, like serum, activates p46 JNK to a greater degree than p54 JNK. Many growth promoting factors, including serum, activate JNK (32). However, most studies have suggested that this stress-activated pathway is involved in growth inhibition rather than mitogenesis (33). Our antisense oligonucleotide data suggest that JNK1 activation in response to both hypoxia and serum stimulation exerts positive effects on cell proliferation, consistent with other reports demonstrating a role for JNK1 in proliferation (27, 34, 35). In contrast, the JNK2 antisense oligonucleotide results suggest that JNK2 might exert negative effects on hypoxia-induced cell growth. Differential functional roles of JNK isoforms have also been reported in cultured small cell lung cancer (36). Thus, it is possible that hypoxia may actually simultaneously activate positive and negative growth regulatory pathways as a safeguard against excessive growth and that JNK1/2 are critical components of this response.

We found a significant increase in p38 MAP kinase phosphorylation in response to hypoxia. The biphasic activation pattern of p38 MAP kinase was different than that observed for ERK and JNK and interestingly different from the activation pattern induced by serum. The majority of existing reports have suggested that p38 is involved in growth inhibition (37, 38). However, a few reports have suggested positive roles for this kinase in promoting cell hypertrophy (39), ischemic preconditioning (40), and hemopoietic (41) and T cell proliferation or differentiation (42, 43). Our results with the widely used pyr-
idinyl imidazole inhibitor of p38, SB202190, demonstrating attenuation of hypoxia-induced proliferative responses of neonatal PA fibroblasts are consistent with previous reports of the role of p38 MAP kinase in the hypoxia-induced proliferative response of adult PA fibroblasts and mesangial cells (14, 29). The fact that the same dose of SB202190 that attenuated hypoxia-induced proliferation augmented serum-induced proliferation also suggests potentially unique roles for p38 isozymes in hypoxic cell responses. The p38 family of protein kinases consists of several isoforms, including p38α, p38β, p38δ, p38γ, and p38ε (44). The inhibitor, SB202190, is nearly equipotent against p38α and p38β but does not inhibit p38γ or p38δ (45). Therefore, the inhibitory effects of SB202190 on the hypoxia-induced growth response of fibroblasts suggest that activation of selective p38 isoforms might play an important role in hypoxic growth responses of fibroblasts. Selective activation of p38α and p38γ isoforms in response to hypoxia has
also been demonstrated in PC12 cells (46). Future studies will be aimed at delineating the specific activation patterns of p38 MAP kinase isoforms and their role in cellular responses upon hypoxic exposure in neonatal adventitial fibroblasts.

Little is known of the upstream events necessary for MAP kinase activation in response to hypoxia. In glioblastoma cell lines, hypoxia induces activation of phosphatidylinositol 3-kinase/Akt pathway in a PTEN-regulated manner (47). However, studies in carotid body and PC12 cells have implicated hypoxia-induced activation of G proteins as an early event in the modulation of ion channel activity and cell depolarization (20–23). Gαi proteins have been shown to be involved in mediating mitogenic responses and to be activated by environmental stresses such as shear stress, mechanical stretch, and reactive oxygen species (16, 17, 48). Previous experiments have also established that certain subtypes of vascular wall cells utilize Gαq proteins to a far greater degree than others for proliferative responses (18). Our results with pertussis toxin, which inhibits signaling through Gαqi, strongly supports the hypothesis that activation of Gαqi proteins is necessary for hypoxia-induced proliferation. Our data also demonstrate that hypoxic activation of ERK and JNK is mediated through Gαqi, but the activation of p38 MAP kinase occurs independent of this protein. It is possible that hypoxia might also activate pertussis toxin-insensitive G proteins, e.g. Gαq11 or Gα12/13, and hypoxia-induced activation of p38 MAP kinase might be mediated through these G proteins. Heterogeneous involvement of G proteins in hypoxia-induced activation of MAP kinases is consistent with the observation that activation of ERK but not p38 MAP kinase appears to be dependent at least in part on Gα proteins during contraction of collagen matrices by fibroblasts under isometric tension (49). Stimulation of the sphingosine 1-phosphate receptor has also recently been shown to be coupled to activation of ERK and p38 by pertussis toxin-sensitive and -insensitive mechanisms, respectively (50). Pertussis toxin-sensitive G proteins have also been implicated in activation of ERK in cardiac and endothelial cells subjected to changes in mechanical forces (51, 52). Thus, our results indicate that Gαqi proteins are a necessary upstream component of hypoxia-induced proliferation in neonatal fibroblasts. Further, our observations that hypoxia in the absence of exogenous ligands stimulates MAP kinase activation and cell proliferation raises the possibility that G protein activation and subsequent cellular events occur in a ligand-independent manner. This is consistent with recent observations of Nishida et al. (48), who found that the Gβγ-responsive ERK activation induced by H2O2 is independent of ligands binding to Gi-coupled receptors. Future work will be directed at evaluating the pathways through which hypoxia activates G proteins.

In summary, we have shown that hypoxia stimulates proliferation of certain adventitial fibroblast populations isolated from neonatal calves in the absence of co-mitogens. Hypoxia-induced proliferation is inhibited by pertussis toxin as is the activation of ERK and JNK, implying an upstream role of Gαqi proteins in hypoxic growth regulation. Importantly, the MAP kinase signaling pathways induced by hypoxia, especially p38 MAP kinase, appear distinct from that induced by serum, allowing potentially additive or synergistic interactions with growth factors under pathologic conditions. Further, our results suggest the existence of different subpopulations of fibroblasts, only some of which are capable of transducing a hypoxic stimulus into a proliferative response.

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