HIGHLIGHTED TOPIC | Pulmonary Circulation and Hypoxia

Egr-1 antisense oligonucleotides inhibit hypoxia-induced proliferation of pulmonary artery adventitial fibroblasts

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Early growth response-1 (Egr-1) transcription factor has been shown to be upregulated by hypoxia in pulmonary artery adventitial fibroblasts. We therefore hypothesized that Egr-1 is a hypoxia-inducible transcription factor that drives fibroblast proliferation under hypoxic conditions. To evaluate the role of Egr-1 in hypoxia-induced proliferation, we employed an Egr-1 antisense strategy. Addition of antisense Egr-1 oligonucleotides, but not sense oligonucleotides, attenuated the hypoxia-induced upregulation of Egr-1 protein and reduced hypoxia-induced DNA synthesis by 50%. Cell proliferation was also significantly inhibited by the addition of antisense Egr-1 oligonucleotides but not the sense oligonucleotides. In addition, hypoxia-induced upregulations of cyclin D and epidermal growth factor receptor were attenuated by Egr-1 antisense oligonucleotides. We conclude that Egr-1 expression is very sensitive to upregulation by hypoxia in pulmonary artery adventitial fibroblasts and that it plays an important role in the autonomous growth phenotype induced by hypoxia in these cells.

Early growth response-1; epidermal growth factor receptor; cyclin D; pulmonary hypertension; vascular remodeling

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spontaneous to hypoxia. Furthermore, our laboratory (5) also demonstrated that hypoxia induced an autonomous growth phenotype in these cells via the activation of a G-protein-coupled receptor and the downstream MAPK ERK-1/2. It remained for us to demonstrate whether the direct inhibition of Egr-1 would attenuate hypoxic proliferation of adventitial fibroblasts. As such, we investigated whether an oligonucleotide antisense strategy would inhibit Egr-1 protein production, inhibit fibroblast proliferation, and inhibit specific downstream regulators of cell division.

MATERIALS AND METHODS

Cell culture. Pulmonary artery adventitial fibroblasts were isolated from tissue explants of neonatal bovine calves exposed to 14 days of hypobaric hypoxia and cultured as previously described (5, 7). All animals were provided care under a protocol approved by the Institutional Animal Care and Use Committee, which operates under the Guiding Principles in the Care and Use of Animals of the American Physiological Society. Our laboratory (7) has previously demonstrated that fibroblasts from chronically hypoxic animals have exaggerated growth responses to hypoxia and may thus contribute to vascular remodeling in a distinct manner. Fibroblasts were used for experiments between passages 2 and 6. Cells were plated at a density of 5 × 10^4 cells/cm^2, grown to 80% confluence, and then growth arrested in DMEM without serum for 3 days before hypoxic exposure. We have found that fibroblasts survive well in the absence of all serum and supplemental growth factors for 3 days. Under these conditions, basal activation of the signaling pathways of interest are at their lowest levels (not absent). This experimental system allows us to study the effects of hypoxia on cells in the absence of comitogens, which stimulate growth-related signaling pathways and thus confound the effects of hypoxia (7).

DNA synthesis. DNA synthesis was determined by [methyl-^-H]thymidine incorporation as described by Das et al. (6). Cells were incubated either with or without Egr-1 sense or antisense oligonucleotides (10 µM) (Integrated DNA Technologies, Coralville, IA) and labeled with 1 µCi of [methyl-^-H]thymidine (NEN Life Science Products, Boston, MA). Cells were placed in a Bellco chamber for 24 h with either 1% O_2 or 21% O_2. At the end of this incubation, fibroblasts were washed and counted in a beta-scintillation counter (Beckman LS 6500).

Cell proliferation. Cell proliferation was determined via a standard proliferation assay as described previously (6). Experimental cell cultures were incubated with or without sense or antisense oligonucleotides and placed in a Bellco chamber with either 1% or 21% O_2 for a period of 48 h. After the incubations, cells were washed and counted.

Egr-1 analysis and oligonucleotide preparation. Specified cell cultures were incubated with either sense or antisense Egr-1 oligonucleotides (10 µM) just before hypoxia or normoxia exposure. Egr-1 sense oligonucleotides were of the following sequence: 5'-AGTG-GCCCGTGACCCCGC-3'. Egr-1 antisense oligonucleotides were arranged as follows: 5'-GCGCGGTTGACGGGACACT-3'. The oligonucleotides were obtained via an NCBI blast search for Mus musculus Egr-1 mRNA. Oligonucleotides were added to the cell cultures without modification or transfection reagent. The final concentration was determined via a dose-response experiment. Cells were placed in Bellco chambers under 1% or 21% O_2 for times specified in the figure legends. At the termination of the experiments, cells were prepared for SDS-PAGE as previously described (11). Samples containing 20 µg of protein underwent electrophoresis, were transferred to a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ) overnight, and then were probed with Egr-1, epidermal growth factor receptor (EGFR), or cyclin D primary antibodies under conditions recommended by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Ponceau staining was used to confirm equal protein loading. Membranes were washed with Tris-buffered saline-Tween 20 and then incubated with peroxidase-conjugated secondary antibody under conditions specified by the manufacturer (Amersham). Immunoreactive bands were detected by chemiluminescence with the use of an enhanced luminal reagent kit (NEN Life Science Products), which was followed by exposure to hyperfilm (Amersham). We then quantitated protein bands using ImageJ densitometry.

Immunohistochemistry. As per our previously described bovine model of chronic hypoxia, the pulmonary artery was harvested from neonatal calves at day 14 of life (27). Tissue specimens were frozen in OCT compound (Sakura Fine Technical, Torrance, CA), sectioned at 5 µm, and placed on glass slides for immunohistochemistry. Slides were stored at −70°C before they were stained. Slides were warmed to room temperature, fixed with 1% paraformaldehyde for 2 min, followed immediately by methanol (10 min at −20°C), and then air dried. The slides were rehydrated with PBS and then blocked with FBS-PBS (1:1) for 30 min. Egr-1 rabbit polyclonal primary antibody (Santa Cruz Biotechnology) was diluted 1:1,000 in 5% PBS in PBS and added to slides overnight in a humidified chamber at 4°C. On day 2, slides were washed with PBS before biotinylated secondary antibody (1:400, Vector Labs, Burlingame, CA) was added to the slides for 45 min at room temperature. Slides were again rinsed with PBS before the addition of streptavidin-conjugated Alexa-594 (1/2,000, Molecular Probes, Eugene, OR) for 45 min at room temperature. Slides were washed three times with PBS, rinsed with distilled H_2O, dried, and embedded into Dapi-containing Vectashield mounting medium (Vector Laboratories). We obtained multichannel images with a Zeiss fluorescent microscope and the AxioVision digital imaging system.

Data analysis. We quantified the density of bands using the NIH Image-J program. Data are expressed as means ± SE. Statistical significance was determined using Prism’s one-way ANOVA followed by the Newman-Keuls test. All experiments were replicated three times (n = 3) unless otherwise specified.

RESULTS

Chronic hypoxia upregulates Egr-1 expression in the pulmonary artery adventitia. We performed immunofluorescent staining on bovine lung tissue sections to investigate whether Egr-1 was upregulated within the pulmonary vasculature in response to hypoxia. Newborn calves were exposed to 14 days of hypoxia (12% oxygen) or normoxia (21% oxygen) before tissue collection. Consistent with our previous findings, we observed marked pulmonary artery adventitial thickening in animals exposed to hypoxia compared with animals maintained under normoxic conditions (Fig. 1). Egr-1 fluorescence was appreciated in endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts in lung tissue from hypoxic and normoxic animals. This observation is consistent with the ubiquitous expression of this transcription factor shown by other authors (11, 25). We observed a significant increase in Egr-1 protein expression within the distal pulmonary artery adventitia of hypoxic animals compared with normoxic controls. This increased fluorescence was particularly noteworthy in the fibroblasts surrounding sites of adventitial neovascularization (arrow).

Hypoxia upregulates Egr-1 protein expression in cultured pulmonary artery fibroblasts. Having demonstrated that Egr-1 protein is upregulated by hypoxia in pulmonary artery adventitial fibroblasts in vivo, we investigated whether hypoxia would induce increased Egr-1 protein expression in cultured pulmonary adventitial fibroblasts. Accordingly, we exposed
cultured adventitial fibroblasts to 1% oxygen, 10% oxygen, or 21% oxygen for 6-h incubations. The respective partial pressures of oxygen in cell culture were 6, 58, and 122 Torr, respectively. Protein expression was determined with Western blot analysis. We chose a 6-h incubation period based on earlier work with adventitial fibroblasts that showed maximal Egr-1 protein expression at between 4 and 6 h (13). As expected, Egr-1 protein expression was significantly increased in both 1% oxygen and 10% oxygen compared with 21% oxygen concentrations (Fig. 2). Thus exposure of cultured pulmonary artery adventitial fibroblasts to hypoxia results in a significant increase in the expression of Egr-1 protein.

**Egr-1 antisense oligonucleotides block Egr-1 protein production in adventitial fibroblasts exposed to hypoxia.** We sought to determine whether Egr-1 antisense oligonucleotides would inhibit hypoxia-induced upregulation of Egr-1 protein. We exposed adventitial fibroblasts to 1% or 21% oxygen for 48 h. Before hypoxic exposure, sense or antisense oligonucleotides (10 μM) were added to the media. As expected, 1% oxygen induced a significant increase in Egr-1 protein expression (Fig. 3). The addition of Egr-1 antisense oligonucleotides significantly inhibited the hypoxia-induced upregulation of Egr-1 protein. In fact, the level of Egr-1 protein expression after the addition of antisense oligonucleotides was similar to that observed in normoxic control cells. Egr-1 sense oligonucleotides did not affect hypoxia-induced upregulation of Egr-1 protein expression. There was no effect of the sense or antisense oligonucleotides on the baseline protein expression under normoxic conditions (data not shown).

**Egr-1 antisense oligonucleotides attenuate hypoxia-induced DNA synthesis and cell proliferation in pulmonary artery adventitial fibroblasts.** We next investigated the effect of Egr-1 antisense oligonucleotides on hypoxia-induced DNA synthesis. We added either Egr-1 sense or Egr-1 antisense oligonucleotides to pulmonary artery adventitial fibroblasts exposed to 1% or 21% oxygen for 24 h as per our previous [3H]thymidine protocol (7). The sense and antisense oligonucleotides (10 μM) had no effect on basal DNA synthesis at 21% oxygen in any experiment (data not shown). We found that 1% oxygen caused a significant increase in DNA synthesis in cultured fibroblasts compared with normoxic controls (Fig. 4). The addition of antisense Egr-1 oligonucleotides, but not sense oligonucleotides, significantly reduced hypoxia-induced DNA synthesis.

We also tested whether the reduction in hypoxia-induced DNA synthesis caused by the addition of antisense oligonucleotides...
tides also reduced the hypoxia-induced increases in cell number. We found that cell number was significantly increased after 48-h incubations in 1% oxygen compared with 21% oxygen (Fig. 5). The addition of Egr-1 antisense oligonucleotides, but not sense oligonucleotides, significantly inhibited the increase in cell replication induced by exposure to 1% oxygen. These experiments suggest that Egr-1 plays a direct role in the autonomous growth phenotype exhibited by adventitial fibroblasts exposed to hypoxia in cell culture.

**Egr-1 antisense oligonucleotides block hypoxia-induced cyclin D and EGFR protein production.** Although it was clear that Egr-1 was upregulated in response to hypoxia and played a key role in the hypoxia-induced proliferation of adventitial fibroblasts, it was not clear how this response was orchestrated. Thus we looked at two key downstream regulators of cell replication: cyclin D and EGFR. The expression of these two proteins is thought to be controlled, at least in part, by Egr-1 (14, 18). Adventitial fibroblasts were incubated in either 1% or 21% oxygen for 48 h as in previous studies. As predicted, significant increases in the expression of these two downstream regulators of cell replication were appreciated in response to...
hypoxia (Figs. 6 and 7). The addition of Egr-1 antisense oligonucleotides caused a significant decrease in the expression of these proteins compared with that seen with hypoxia alone. The addition of sense oligonucleotides did not alter the expression of either protein compared with hypoxia alone. Thus, in our cells under our conditions tested, Egr-1 appears to induce hypoxic fibroblast proliferation, at least in part, via the down-stream proteins EGFR and cyclin D.

**DISCUSSION**

Previous studies in our laboratory (5, 13) have shown that hypoxia induces significant increases in Egr-1 protein and mRNA and DNA binding in cultured pulmonary artery adventitial fibroblasts. However, whether Egr-1 participates directly in the hypoxia-induced proliferative response remained unclear. Furthermore, little was known of the downstream targets of Egr-1 that contribute to the hypoxia-induced proliferative response. We found an increase in Egr-1 expression in the pulmonary artery adventitia of neonatal calves exposed to hypoxia for 2 wk. We confirmed that Egr-1 is significantly increased in cultured pulmonary artery adventitial fibroblasts in response to hypoxia (13). Utilizing an antisense strategy, we found that the direct inhibition of this transcription factor blocked hypoxia-induced adventitial cell proliferation significantly and reproducibly. Furthermore, we demonstrated that Egr-1 contributes to the proliferative phenotype demonstrated by the adventitial fibroblast in response to hypoxia, at least in part, by regulating expression of cyclin D and EGFR.

Several studies have demonstrated that Egr-1 is upregulated by hypoxia in a variety of cell types. Yan et al. (39) showed that exposure of cultured monocytes to hypoxia significantly increased Egr-1 mRNA expression. These investigators also determined that hypoxia-induced Egr-1 protein expression in smooth muscle cells and alveolar macrophages varied depending on the duration and degree of hypoxia (40). Increased expression of Egr-1 by a variety of lung cells has also been described in ischemia-reperfusion models (12, 38). In an in vitro model of adrenergic control during hypoxic stress, Wong et al. (37) found that Egr-1 controlled transcription of the pivotal enzyme, phenylethanolamine N-methyltransferase, responsible for epinephrine synthesis. Our present findings are in complete agreement with these previous studies. Thus it is clear that hypoxia consistently increases Egr-1 protein expression in many cell types, including pulmonary artery adventitial fibroblasts.

Egr-1 has been demonstrated to play an integral part in the proliferative response in numerous cell types. Santiago et al.
fibroblast growth factor gene is transcriptionally autoregulated. Wang et al. (36) found that, in cell culture, the basic response orchestrated by Egr-1 remained incompletely under

variety of stressors. Thus our findings demonstrating that Egr-1 antisense oligonucleotides inhibited neointimal formation after balloon injury. Similar results have also been reported in a murine model (34). In a primate heart transplant model, Wada et al. (35) found increased Egr-1 in smooth muscle cells of rejected hearts even before morphological changes such as intimal thickening were observed. In a mouse heart transplant model, Okada et al. (21) showed a dramatic reduction in coronary allograft vasculopathy and attenuated parenchymal rejection in homozygous Egr-1-null mice compared with wild-type animals. In a murine lung transplant model, these investigators (20) found that Egr-1 antisense oligonucleotides attenuated the pulmonary vascular injury induced by transplant and improved recipient survival by 56%. Thus our findings demonstrating that Egr-1 antisense oligonucleotides can inhibit hypoxia-induced fibroblast proliferation are consistent with the idea that Egr-1 plays a critical role in vascular cells in mediating the proliferative response to a variety of stressors.

The downstream modifiers that control the proliferative response orchestrated by Egr-1 remain incompletely understood. Wang et al. (36) found that that, in cell culture, the basic fibroblast growth factor gene is transcriptionally autoregulated by Egr-1. EGFR initiates the cellular response to epidermal growth factor, transforming growth factor-α, and other ligands (3, 33). Using a human osteosarcoma cell line, Nishi et al. (18) found that the upregulation of EGFR observed in response to hypoxia could be inhibited with the addition of Egr-1 antisense oligonucleotides. Our results in adventitial fibroblasts demonstrate that hypoxia-induced increases in EGFR can be attenuated by Egr-1 antisense oligonucleotides and are in complete agreement with the earlier studies. Together, these results suggest EGFR upregulation may play an important role in hypoxia-induced fibroblast proliferation.

Cyclin D is another well-described chaperone of cell proliferation (14). Recently, Baron et al. (1) showed that they could inhibit the tumor-associated increase in cyclin D and delay the occurrence of prostate tumors by injecting whole animals with Egr-1 antisense oligonucleotides. Our study also supports a role for Egr-1 in regulating cyclin D expression in hypoxic adventitial fibroblasts. Collectively, although our study suggests a role for EGFR and cyclin D in fibroblasts, other factors potentially downstream of Egr-1 need to be further defined. Indeed, in our study, the inhibition of EGFR and cyclin D with antisense Egr-1 was not as profound as what we achieved for Egr-1 itself. This suggests that Egr-1 is not the only transcription factor affecting the induction of these proteins under hypoxic conditions.

Egr-1 is a transcription factor that contributes to the orchestration of cellular responses to numerous insults, including hypoxia (22). To our knowledge, our report is the first to demonstrate that Egr-1 is directly involved in the hypoxia-induced proliferative responses of pulmonary artery adventitial fibroblasts. Moreover, Egr-1 remains a promising target for therapeutic intervention. Although it is clearly upregulated in response to a variety of stresses, it is not an essential transcription factor under normal conditions (27). Egr-1-null mice are phenotypically normal, save for a deficiency in luteinizing hormone (4). Inhibition of Egr-1 in a variety of animal models of heart and lung transplantation, tumor growth, and glomerulonephritis leads to an improved outcome (30, 31, 35, 40). Given the clear role that this master-switch transcription factor has in the pathological adaptations to stress, effective Egr-1 inhibition may significantly attenuate the pulmonary vascular remodeling induced by chronic hypoxic exposure.

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