Pulmonary expression of early growth response-1: biphasic time course and effect of oxygen concentration

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Yan, Shi-Fang, Jiesheng Lu, Linna Xu, Yu Shan Zou, Joern Tongers, Walter Kisiel, Nigel Mackman, David J. Pinsky, and David M. Stern. Pulmonary expression of early growth response-1: biphasic time course and effect of oxygen concentration. J Appl Physiol 88: 2303–2309, 2000.—Hypoxia induces complex adaptive responses. In this report, induction of early growth response-1 (Egr-1) transcripts in lungs of mice subjected to hypoxia is shown to be dose and time dependent. Within 30 min of hypoxia, Egr-1 transcripts were ~20-fold elevated in 6% oxygen, ~5.2-fold increased by 10% oxygen, and returned to the normoxic baseline by 12% oxygen. Time course studies up to 48 h showed a biphasic profile with an initial steep rise in Egr-1 transcripts after 0.5 h of hypoxia and a second elevation beginning after 20–24 h. Hypoxic induction of Egr-1 was paralleled by enhanced expression of the downstream target gene tissue factor. Egr-1 and tissue factor antigen were visualized in bronchial and alveolar macrophages. Egr-1 has the capacity to modulate expression of genes involved in the remodeling of the extracellular matrix and properties of smooth muscle, thus possibly contributing to the pulmonary response to chronic hypoxia.

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The possible contribution of Egr-1 to the pathobiology of acute hypoxemic vascular injury was demonstrated by analyzing expression of the procoagulant regulator tissue factor. In vitro experiments showed that tissue factor induction occurred at the transcriptional level and was mediated by Egr-1 interacting with its cognate DNA binding sites in the serum response region of the promoter (7). In vivo, hypoxia caused expression of tissue factor in the hypoxic lung, and this resulted in vascular fibrin accumulation. A cause-effect relationship between hypoxia-mediated Egr-1 expression, induction of tissue factor, and fibrin deposition in pulmonary blood vessels was shown in homozygous Egr-1 null mice. In contrast to wild-type controls, Egr-1 null animals subjected to hypoxia did not display tissue factor expression or vascular fibrin deposits. Egr-1 induction in hypoxia was shown to be independent of hypoxia-inducible factor (HIF) 1 (38), the best characterized adaptive mechanism triggered by oxygen depletion (30).

These studies suggested a possibly distinct contribution of Egr-1 to the biology of the cellular response to hypoxia. However, Egr-1 induction had been studied only under conditions of severe oxygen depletion (6% oxygen) and only for brief periods (up to 6 h). The experiments in this study were designed to examine expression of Egr-1 in lungs of mice exposed to a range of oxygen concentrations (6–20%) and time points (30 min to 48 h) to better assess possible physiological and/or pathophysiological roles for this transcription factor in the hypoxemic lung.

**EXPERIMENTAL PROCEDURES**

Induction of hypoxia. Mice were subjected to hypoxia in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Columbia University. Female C57BL/6 mice (3–4 mo of age) were obtained from Jackson Laboratories (Bar Harbor, ME). Normobaric hypoxia (n = 5 mice per experimental group) was induced for the indicated times by the regulated addition of nitrogen to a chamber equipped with circulating fans, carbon dioxide and ammonia elimination systems, and an on-line oxygen sensor (Horiba, Kyoto, Japan) (18). The environment within the chamber (including temperature and humidity) was regulated by a custom-built interface (K + K Interface), which used a computer-driven environmental control program. This system resulted in the indicated oxygen concentrations ± 0.2%. Mice placed in the chamber in their usual cages were allowed free access to food and water, and the system parameters were adjusted to the indicated final oxygen concentration. Animals exposed to hypoxia were not in distress (although, at more severe levels of oxygen deprivation, they were tachypneic), and there was no mortality even at the lowest oxygen tension (6%) and the longest incubation period (48 h). At the indicated times, animals were killed, and tissues were studied as described below.

Northern analysis. Tissue was cut into small pieces, immersed in TRIzol (GIBCO BRL, Grand Island, NY), and homogenized; total RNA was extracted and electrophoresed on 0.8% agarose gels. RNA was transferred to Duralon-UV membranes (Stratagene), and the latter were then hybridized with 32P-labeled cDNA probes for mouse Egr-1 (39), Sp1 (39), tissue factor (39), or HIF-1α (31). Hybridization of blots with 32P-labeled β-actin was used as an internal control for RNA loading.

**RESULTS**

Dependence of Egr-1 mRNA expression on oxygen tension. Previous studies demonstrated that exposure of mice (C57BL/6) to severe hypoxia (6%) caused a rapid elevation of Egr-1 transcripts (38). However, the relationship between Egr-1 expression and the degree of hypoxia has not been analyzed. To address this issue, mice were placed in environments with different concentrations of oxygen (6, 6.5, 7, 8, 9, 10, 12, and 15% and ambient), and expression of Egr-1 transcripts in the lung was assessed after 30 min (Fig. 1A). Egr-1 mRNA was strongly elevated at 6, 6.5, and 7% oxygen and began to fall off at 8 and 9%, approaching baseline (normoxic) levels by 12%. Densitometric analysis (Fig. 1B) of several gels similar to those in Fig. 1A showed a 20-fold increase in Egr-1 mRNA at 6% oxygen, which declined to ~10-fold at 8% oxygen and which was only slightly above the normoxic baseline (~2-fold) at 10% oxygen. When the same blot was hybridized with a 32P-labeled probe for Sp1, a transcription factor that also interacts with a GC-rich DNA binding motif (often overlapping with Egr-1 DNA binding sites) (1, 2, 10, 15), no increase in Sp1 mRNA was detected in hypoxic animals (Fig. 1C and D). Similarly, levels of β-actin mRNA were unchanged in samples harvested from normoxic and hypoxic animals (Fig. 1, E and F). Experiments performed to detect HIF-1α transcripts in hypoxic lung under the same conditions demonstrated no increase at any of the oxygen tensions tested (6–15% oxygen; not shown).

Dependence of Egr-1 mRNA expression on the duration of hypoxia. Although the 30-min time point appeared to provide a sensitive index of acute induction of Egr-1 transcripts in hypoxic lung (Fig. 1A), it was essential to undertake a time course study. For this...
were unchanged (Fig. 3, C). Although Sp1 and B-III oxygen (Fig. 3). Although Sp1 and at the 48-h time point, comparing the levels of transcripts displayed dose-dependent modulation; for example, at 6 and 10% oxygen, they were approximately ninefold and approximately threefold above normoxia, respectively (Fig. 3, A and B). Thus the increase in Egr-1 mRNA in hypoxic lung during the second phase of Egr-1 induction was also dose dependent. To assess sites of Egr-1 expression after 48 h of hypoxia, immunohistological analysis was performed (Fig. 4A). Although Egr-1 antigen was virtually undetectable in normoxic lung (Fig. 4A, normoxia), in the presence of severe hypoxia (6% oxygen), Egr-1 was evident in vascular smooth muscle (Fig. 4A-I), bronchial smooth muscle (Fig. 4A-II), alveolar macrophages (Fig. 4A-III; note arrows), and bronchial epithelium (Fig. 4A-II) at the 48-h time point. Immunoreactivity in bronchial epithelium and smooth muscle and vascular smooth muscle was not uniform, as evidenced by considerable cell-to-cell variation in staining intensity. With more mild hypoxia (Fig. 4A, 10% oxygen), Egr-1 antigen was still detectable in the same locations, but the intensity of staining was reduced compared with 6% oxygen.

Expression of tissue factor in hypoxic lung. To assess the possible functional significance of Egr-1 expression in hypoxic lung, its activation of a downstream target gene was studied. Previous work has shown that expression of tissue factor is regulated by Egr-1 in the lung consequent to acute hypoxia (up to 6 h) (39). Thus tissue factor was selected as a readout for Egr-1 functional activity at the different oxygen concentrations after 48 h of hypoxia. Consistent with the concept that hypoxia-induced expression of Egr-1 had effects that lasted beyond the acute period, increased levels of tissue factor were observed. Expression of tissue factor transcripts in hypoxic lung was dependent on the oxygen tension; mRNA levels were highest at 6% (6-fold above the normoxic baseline) and appeared to progressively decline with higher oxygen concentration, although at 10% oxygen they were ~2.2-fold above normoxia (Fig. 3, E and F). The effect of oxygen concentration on expression of tissue factor transcripts appeared, in general, to parallel that for Egr-1 (Fig. 3, A and B). Tissue factor antigen (Fig. 4B) was also observed in hypoxic lung compared with virtually no identifiable tissue factor in normoxic samples. Figure 4B shows that there was increased expression of tissue factor in hypoxic vascular smooth muscle, with greater staining intensity at 6% oxygen compared with at 10% oxygen (although, of course, this is not a quantitative technique). Enhanced expression of tissue factor in alveolar macrophages and bronchial smooth muscle from hypoxic lung was also observed (not shown). These data indicate that the distributions of Egr-1 and tissue factor antigen in hypoxic lung overlap.
DISCUSSION

The results of our studies indicate that Egr-1 induction in hypoxic murine lung occurs over a range of oxygen concentrations, not only in the presence of the most severe hypoxia (≤6% oxygen). Furthermore, the temporal pattern of Egr-1 expression is biphasic, with an initial peak after 0.5 h of hypoxia and a second phase beginning after ~20–24 h. With more moderate hypoxia (an oxygen concentration of 9–10%), the initial peak of Egr-1 transcripts at 0.5 h was less striking, but there was still a second phase of Egr-1 induction after 24 h.

Immunohistological analysis of hypoxic lung indicated that Egr-1 was especially evident in smooth muscle of the walls of bronchi and vasculature as well as in alveolar macrophages, cell types critical for tissue remodeling in response to chronic hypoxia. Furthermore, the list of genes subject to regulation by Egr-1 is provocative. Our initial studies have focused on tissue factor. Although this procoagulant regulator has a central role in pulmonary vascular fibrin formation in the setting of acute hypoxia (39), the significance of procoagulant events in the pulmonary response to chronic hypoxia is unclear (10). However, these studies need to be extended to consider the possibility that other genes, previously shown to be regulated by Egr-1 in vitro, might be involved. For example, pulmonary hypertension is associated with increased production of extracellular matrix components, including tenascin-C (6, 12, 27). A previous study has shown the presence of functionally active Egr-1 sites in the murine tenascin promoter (5). Matrix metalloproteinases have also been shown to contribute to the vascular hypertrophy that accompanies pulmonary hypertension (12, 26, 27). Mem-
brane type 1 metalloproteinase (MT1-MMP or MMP14), a potentially important contributor to remodeling of extracellular matrix, has been shown to be regulated in culture by Egr-1 (9). In this context, endothelial and serum factors, including apolipoprotein A-I, tether elastin to smooth muscle cells, inducing serine elastase activity (33). Apolipoprotein A-I has also been shown to be under control of Egr-1; this is partly based on

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**Fig. 3.** Expression of Egr-1 transcripts in the lung after 48 h of hypoxia: dependence on oxygen concentration. Mice were subjected to an environment with the indicated oxygen tension for 48 h, lungs were harvested, and total RNA was prepared. Northern blotting was performed using 32P-labeled cDNA probes for Egr-1 (A), Sp1 (C), tissue factor (TF: E), and β-actin (G) (in each case, 20 µg of RNA were added to a lane of the gel). B, D, F, and H: gels from 3–5 experiments similar to those in A, C, E, and G, respectively, were analyzed as described in the text to determine relative intensity of the bands (y-axis shows increase in the band (fold) compared with normoxia). P < 0.001 or **P < 0.05.

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**Fig. 4.** Immunostaining of hypoxic lung for Egr-1 (A) and tissue factor (B) antigens. Mice were exposed to normoxia or hypoxia (6% and 10% oxygen, as indicated) for 48 h, and lung was harvested and subjected to immunostaining to detect Egr-1 and tissue factor antigens. A-I and A-II: Egr-1 antigen in smooth muscle of vasculature and bronchi, respectively. A-III: Egr-1 in alveolar macrophages (arrows point to macrophages). Bars = 5 µm in A-I, 2 µm in A-II, and 1 µm in A-III. In B, immunostaining for tissue factor antigen in vascular smooth muscle is seen under the same conditions (normoxia, 6% oxygen, and 10% oxygen). Bars = 5 µm. Micrographs shown are representative of 3 different experiments.
comparative studies in Egr-1(-/-) mice and littermate-matched Egr-1(+/-) controls (44). Egr-1-mediated regulation of transforming growth factor-β (16), as well as insulin-like growth factor II (3) and the insulin-like growth factor receptor 1 (35, 36), may also be relevant to changes in expression of extracellular matrix components and smooth muscle cell properties in hypoxic lung. In vivo studies will be required to determine whether products of these genes are expressed in response to hypoxia and subject to control by Egr-1. Furthermore, evaluation of the expression of other genes, such as those related to vasoreactivity, will be important for comparison of Egr-1 null and wild-type mice.

Increased expression of HIF-1α transcripts was not demonstrated by Northern blotting at any of the time points or oxygen concentrations (6–10%) in our studies, based on analysis of the same samples (not shown) described above for Egr-1. This is consistent with increased expression of HIF-1α in ferret lungs ventilated with 0–1.3% oxygen but not at higher oxygen concentrations (42). However, it is clear that HIF-1α participates in the pulmonary vascular response to chronic hypoxia elicited in the presence of 10% oxygen, as shown by the blunted response in heterozygous null chronic hypoxia elicited in the presence of 10% oxygen, but not at higher oxygen concentrations (42). Furthermore, evaluation of the expression of other genes, such as those related to vasoreactivity, will be important for comparison of Egr-1 null and wild-type mice.

In view of these observations, our inability to detect HIF-1α transcripts in hypoxic lung might be because an important component of HIF-1α regulation occurs posttranslationally (30, 31). However, it is well known that there is also significant control of HIF-1α during hypoxia at the transcriptional level (30, 31). This suggests the likely possibility that our assays lacked sufficient sensitivity to detect small changes at low levels of gene expression. In terms of HIF-1α transcripts in hypoxic lung, increased levels of mRNA have been observed in a previous study in rats placed in an atmosphere with 10% oxygen for 2 wk (25), consistent with elevated expression of this component of the HIF-1 heterodimer at a considerably later time point. Our present results indicate that expression of Egr-1 can be detected at this same oxygen concentration (10%) in total RNA extracted from mouse lungs.

Together, these data indicate that increased pulmonary expression of Egr-1 in mice subject to hypoxia, with oxygen concentrations ranging from 6 to 10%, occurs in a graded fashion and with a biphasic time course. Egr-1 transcripts are detectable at each of these oxygen concentrations, although they are undetectable at 15–20% oxygen. Furthermore, expression of an Egr-1 downstream target gene, tissue factor, was also observed, suggesting that Egr-1 was transcriptionally active in the hypoxic lung. These findings suggest the relevance of examining the possible impact of Egr-1 on the vascular response to chronic hypoxia.


