Fibrin deposition is a salient feature of hypoxic vasculature and results from induction of tissue factor. Such tissue factor expression in an oxygen deficient environment is driven by the transcription factor Early Growth Response (Egr)-1. Using homozygous null mice for the protein kinase C β-isoform gene (PKCβ null), PKCβ is shown to be upstream of Egr-1 in this oxygen deprivation-mediated pathway for triggering procoagulant events. Whereas wild-type mice exposed to hypoxia (6%) displayed a robust increase in tissue factor transcripts and antigen, and vascular fibrin deposition, PKCβ null animals showed a markedly blunted response. Consistent with a central role for Egr-1 in hypoxia-induced expression of tissue factor, PKCβ null mice subjected to oxygen deprivation displayed at most a minor elevation in Egr-1 transcripts, antigen, and intensity of the gel shift band by electrophoretic mobility shift assay, compared with normoxic animals. These data firmly establish PKCβ as a trigger for events leading to induction of Egr-1 and tissue factor under hypoxic conditions, and provide insight into a biologic cascade whereby oxygen deprivation recruits targets of PKCβ and Egr-1, thereby amplifying the cellular response.

Oxygen deprivation is a frequently encountered physiologic stress accompanying disorders of the lung and cardiovascular system, as well as consequent to high altitude exposure. The cellular response to hypoxia is complex and involves a range of mechanisms, some occurring within minutes of oxygen deprivation, whereas others reset multistep biosynthetic and physiologic programs (1–9). For example, hypoxia-induced translocation of P-selectin to the endothelial cell surface, thereby promoting adherence of leukocytes at sites of ischemic stress, occurs within minutes and is due to a transient rise in cytosolic calcium (4). The best characterized mechanism triggering biosynthetic adaptation to oxygen deprivation involves the transcriptional regulator hypoxia-inducible factor-1 (HIF-1) (10). Activation of HIF-1 at low ambient oxygen tension results in expression of an array of genes, which, in a highly integrated manner, redirects metabolic and other cellular mechanisms enhancing cell survival in the hypoxic environment (2, 3, 7, 10). HIF-1 increases expression of the noninsulin-dependent glucose transporter (GLUT1) (11) and multiple glycolytic enzymes (12), thereby promoting glucose uptake and glycolysis, as the efficacy of aerobic respiration is diminished due to limited oxygen availability. In a highly complementary manner, increased levels of erythropoietin and vascular endothelial growth factor expand oxygen carrying capacity of the blood and, over time, enhance ingrowth of vessels to sites of hypoxia, respectively (2, 3, 13). The positive impact of these changes for cellular and organ homeostasis is evident from the lethal phenotype of homozygous null mice for either vascular endothelial growth factor or HIF-1α (14–17).

Another facet of the biosynthetic response to hypoxia concerns expression of oxygen-regulated proteins (ORPs), which occurs within the first 48 h of hypoxia (8). The ORPs comprise a group of polypeptides whose members overlap with glucose-regulated proteins (GRP), such as GRP78 and GRP94 (18), well known for their role as chaperones in the endoplasmic reticulum (ER). Studies of ORP150, a recently described member of this group cloned from hypoxic astrocytes (19), also places it in the ER; enhanced expression of ORP150 in response to severe oxygen deprivation promotes cell survival (20). These data indicate that ER stress, likely due to accumulation of incompletely folded/misfolded proteins in the ER, as energy depletion and changes in protein biosynthesis impair normal protein processing, is an important feature of the intracellular milieu in hypoxia.

Our recent studies have highlighted a quite distinct pathway triggered by oxygen deprivation. Hypoxia causes transcriptional activation of tissue factor, the key procoagulant cofactor that initiates the coagulation mechanism resulting, ultimately, in vascular fibrin formation (6, 21, 22). Increased tissue factor in hypoxic vasculature is especially evident in mononuclear phagocytes (MPs) (6, 21). Further analysis of this pathway showed that induction of tissue factor expression resulted from up-regulation of the transcription factor Early Growth Response (Egr)-1 (22). For example, homozygous Egr-1 null mice subjected to hypoxia did not display either enhanced tissue factor expression or vascular fibrin deposition. As fibrin accumulation in vessels subject to hypoxiaemia could have far-reaching...

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
This paper is available on line at http://www.jbc.org
ing pathophysiological consequences (23, 24) and increased levels of Egr-1 could recruit multiple other target genes, we have performed further studies to elucidate mechanisms underlying transcriptional activation of Egr-1 in response to oxygen deprivation. Our first studies using cultured cell lines suggested that events leading to Egr-1 expression could be traced back to protein kinase C isoform βII (PKCβII) (22). Because of the complex array of PKC isoforms and their overlapping properties, as well as the likelihood that results in established cell lines subject to transient transfection with multiple expression constructs would not faithfully model all aspects of the in vivo milieu, we have turned to experiments in genetically manipulated mice. In this regard, mice deficient in the PKCβ gene (since PKCβII and PKCβIII isoforms are encoded by the same gene, these mice are deficient in both) have been produced by homologous recombination (termed PKCβ null mice) (25). The phenotype of these animals includes immunologic abnormalities, impaired humoral immune responses and reduced cellular responses to B cells, similar to those observed in X-linked immunodeficiency, although survival, reproductive status, and other general properties of these mice appear to be intact. Using PKCβ null animals, we have established a central role for PKCβ in the pathway triggered by hypoxia leading to activation of Egr-1 and tissue factor expression, and resulting in fibrin deposition in lung vasculature. These studies delineate a new facet of the response to oxygen deprivation, distinct from HIF-1, which is likely to initiate a complex pattern of biosynthetic changes in hypoxic/hypoxemic environments.

**EXPERIMENTAL METHODS**

**Induction of Hypoxia in Vivo and in Vitro—**Experiments employing mice subjected to hypoxia were performed according to protocols approved by the Institutional Animal Care and Use Committee at Columbia University, in accordance with the Association for the Accreditation of Laboratory Animal Care guidelines. Homozygous PKCβ null mice were prepared as described (25). Both PKCβ null and wild-type controls (12–15 weeks old) were in a similar mixed background (129xC57BL/6). However, since the 129 strain actually comprises a collection of genetically heterogeneous substrains, it is more than likely that the control mice (which were most likely derived from a different strain with a different embryonic stem cell than the PKCβ null mice) are not genetically identical to the knockout mice. Mice were subjected to normobaric hypoxia (n = 5 per experimental condition unless indicated otherwise) for the indicated times by the regulated addition of nitrogen to a hypoxic lung by the method of Dignam et al. (33). Double-stranded oligonucleotide probes for Egr (Santa Cruz Biotechnology) and GLUT1 (27) were also hybridized with 32P-labeled β-actin as an internal control for RNA loading.

For Western blotting of Egr-1, nuclear extracts were prepared (see below), and subjected to SDS-PAGE (7.5%; nonreduced). Proteins in the gel were transferred electrophoretically to nitrocellulose membranes, and immunoblotting was performed with rabbit anti-Egr-1 IgG (Santa Cruz Biotechnology) according to the Blotto procedure (28). Sites of primary antibody binding were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham International, Buckinghamshire, United Kingdom). Final detection of immunoreactive bands was performed using the enhanced chemiluminescent Western blotting system (Amersham). A similar strategy was utilized to detect ERK1/2, except that an extract of total cell protein was employed, and anti-MAPK IgG (active with ERK1/2 regardless of phosphorylation status; Zymed Laboratories Inc.) or anti-active MAPK IgG (1:5000 dilution; the latter selective for phosphorylated ERK1/2; Promega) was used as the primary antibody (29, 30).

For immunoblotting of tissue samples for fibrin, lung was harvested from animals treated with heparin (10 units/g, resulting in an activated partial thromboplastin time >300 s) prior to sacrifice (21). Tissue was placed in buffer (0.05 mM Tris, 0.15 M NaCl, 500 units/ml heparin; final pH 7.6) on ice and homogenized. Plasmid digestion was performed by a modification of the method of Francis (31) by adding human plasmid (0.32 units/ml, Sigma) and incubating the sample for 6 h at 37 °C. This was followed by addition of more plasmid (0.32 units/ml) and additional incubation for 2 h, followed by centrifugation (2,300 × g for 15 min), and aspiration of the supernatant. Samples of plasmid-treated hypoxic and normoxic lung homogenates (200 μg of total protein) were boiled in reducing SDS-sample buffer for 5 min and subjected to SDS-PAGE (10%; reduced). Samples were electrophoretically transferred to nitrocellulose, and blots were reacted with rabbit anti-fibrin antibody made to γ-γ chain cross-links (21, 32), followed by affinity-purified peroxidase-conjugated anti-rabbit IgG (Sigma). Final detection of the bands was as above.

For immunocytochemical studies, lung tissue was harvested, cut into small pieces, washed with phosphate-buffered saline (pH 7.0) to remove blood, fixed in formalin, and embedded in paraffin (6, 22). Sections were first stained with primary antibodies, rabbit anti-Egr-1 IgG (8 μg/ml; Santa Cruz), rat anti-F4/80 monoclonal antibody (10 μg/ml; Caltag Laboratories, South San Francisco, CA), rabbit anti-tissue factor IgG (63 μg/ml) (21), rabbit anti-phosphorylated ERK1/2 (1:500 dilution; New England Biolabs, Beverly, MA), or rabbit anti-fibrin antibody (4 μg/ml). Then, they were incubated with secondary antibody, an affinity-purified peroxidase-conjugated anti-rabbit or anti-rat IgG (Sigma).

The electrophoretic mobility gel shift assay was performed on nuclear extracts prepared immediately after harvest of the hypoxic lung by the method of Dignam et al. (33). Double-stranded oligonucleotide probes for Egr (Santa Cruz Biotechnology) were 5′ end-labeled with [32P]ATP (3,000 Ci/mmol)
using T4 polynucleotide kinase and standard procedures. Binding reactions were performed as described (34), and samples (5 μg of protein in each lane) were loaded directly onto nondenaturing polyacrylamide/bisacrylamide (6%) gels prepared in 0.5x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Gels were pre-run for 20 min before samples were loaded, and electrophoresis was performed at room temperature for 1.5–2 h at 200 V. For competition studies, an 100-fold molar excess of unlabeled probes for either Egr (Santa Cruz Biotechnology) or Sp1 (Promega) was added.

**RESULTS**

**Expression of Egr-1 and Activation of ERK1/2 in Hypoxic Lung: Effect of PKCβ Deletion**—In a previous study, hypoxia was shown to induce Egr-1 expression and Egr-1-dependent transcription of tissue factor (22). In order to assess whether PKCβ might participate in upstream events leading to Egr-1 up-regulation, PKCβ null mice were subjected to oxygen deprivation and Egr-1 expression was compared with that observed in age- and strain-matched controls. Wild-type mice demonstrated strong up-regulation of transcripts for Egr-1 mRNA in the lung within 30 min of exposure to the environment with 6% oxygen (Fig. 1A, lanes 1 and 2; ~16-fold increase in intensity of the band in samples from hypoxic lung compared with normoxic controls). In contrast, PKCβ null mice subjected to hypoxia for the same time showed only a weak increase in Egr-1 transcripts (Fig. 1A, lanes 3 and 4; ~1.6-fold increase in hypoxia compared with normoxia). Immunoblotting of nuclear extracts from hypoxic lung confirmed that increased amounts of Egr-1 antigen were present in samples from wild-type mice after 30 min, compared with normoxic controls (Fig. 1B, lanes 1 and 2). In fact, the rapidity of the rise in Egr-1 antigen...
suggests that even post-translational mechanisms related to protein stabilization might also be involved. Analogous to the lower levels of Egr-1 mRNA in PKCβ null mice, the knockouts showed an immunoreactive band of low intensity after exposure to hypoxia (Fig. 1B, lanes 3 and 4), versus the robust response in wild-type mice. In hypoxic lung from wild-type mice, Egr-1 antigen was especially evident in smooth muscle cells and in mononuclear phagocytes (Fig. 1D; panel C shows Egr-1 immunostaining in the normoxic control). Staining of adjacent sections with F4/80 demonstrated colocalization of this murine monocyte marker (Fig. 1F) in cells expressing Egr-1 (Fig. 1E). The PKCβ null mice showed only low levels of Egr-1 antigen in lungs of normoxic and hypoxic mice (Fig. 1, G and H). Gel shift analysis of nuclear extracts from lungs of wild-type mice using 32P-labeled consensus oligonucleotide probe for Egr demonstrated a gel shift band whose intensity increased strongly in response to hypoxia (Fig. 1I, lanes 3 and 4). Nuclear binding activity was sequence-specific, based on competition with excess unlabeled Egr probe, but not unlabeled oligonucleotide probe for Sp1 (data not shown). When PKCβ null mice were exposed to hypoxia, the intensity of the gel shift band from nuclear extracts of lung increased minimally compared with normoxia (Fig. 1I, lanes 4 and 5). These data demonstrate that deletion of the PKCβ gene strongly inhibits signaling events in the lung leading to induction of Egr-1. The small residual increase in Egr-1 in PKCβ null mice was not reproducible (often being similar to normoxic controls) and, thus, was difficult to characterize further.

In a cultured monocyte-like cell line, we had observed previously that hypoxia caused translocation of PKCβII to the membranous fraction and autophosphorylation, and closely correlated with events leading to Egr-1 expression by a pathway that involved activation of MAP kinases ERK1/2 (22). This led us to examine whether activation of ERK1/2 occurred in hypoxic lung, and if this would be altered in PKCβ null mice. Wild-type mice were exposed to hypoxia for 10 min, and lung homogenates were prepared for immunoblotting with antibody specific for phosphorylated ERK1/2 (29, 30). A strong increase in intensity of immunoreactive material with mass ~42/44 kDa, corresponding to the migration of phospho-ERK1/2, was observed in hypoxic, versus normoxic, samples (Fig. 2A, lanes 2 and 1, respectively). Similar experiments performed with lung homogenates from PKCβ null mice showed only low levels of phosphorylated ERK1/2 antigen in normoxic and hypoxic mice (Fig. 2A, lanes 3 and 4). The latter result was obtained after 10 min of hypoxia (a time point when strong ERK1/2 activation was observed in wild-type mice); experiments performed at shorter and longer incubation times using PKCβ null mice did not show increased phospho-ERK1/2, indicating the apparent absence of ERK1/2 activation, rather than just an altered time course (data not shown). Panel B displays the presence of similar levels of total ERK1/2 antigen in lung harvested from wild-type and PKCβ null mice under normoxic and hypoxic conditions. Immunostaining was performed on lung tissue from normoxic and hypoxic mice with antibody to phosphorylated ERK1/2. Compared with normoxic controls (Fig. 2C), immunoreactive material was found lungs of wild-type hypoxic mice (Fig. 2D) in a distribution corresponding to vascular smooth muscle cells (main panel in D) and mononuclear phagocytes (inset to D). Studies on lung tissue from PKCβ null animals demonstrated little immunoreactivity in lungs from either normoxic and hypoxic animals (Fig. 2, E and F). Thus, deletion of PKCβ prevents an hypoxia-mediated signaling pathway in which ERK1/2 activation/phosphorylation is tied to up-regulation of Egr-1.

Expression of Tissue Factor and Induction of Fibrin Formation in Hypoxic Lung: Effect of PKCβ Deletion—Wild-type mice exposed to hypoxia display activation of Egr-1, which, in turn,
triggers increased transcription of the tissue factor gene (6). Thus, wild-type mice subjected to oxygen deprivation showed elevated levels of steady-state tissue factor mRNA (Fig. 3A, lane 2; ~20-fold increase comparing lanes 2 and 1), which was followed by enhanced expression of tissue factor antigen in the lung (Fig. 3C), versus normoxic controls (Fig. 3B). Further studies showed the distribution of tissue factor antigen in lungs from oxygen-deprived animals to overlap that observed for hypoxia-induced Egr-1 expression and activated ERK1/2, being found principally in smooth muscle cells and MPs (data not shown). When the same protocol was followed with PKCβ null mice, animals subjected to hypoxia showed only a slight increase in tissue factor transcripts on Northern blots (Fig. 3A, lane 4; ~2.2-fold increase comparing lanes 3 and 4) and virtually no elevation of immunoreactive tissue factor in the lung (Fig. 3E), compared with PKCβ null mice maintained in normoxia (Figs. 3, A, lane 3, and D). As with levels of Egr-1 in hypoxic PKCβ null mice, the residual increase in tissue factor was variable and at extremely low levels, making it difficult to analyze in detail. The key role of tissue factor in fibrin deposition in hypoxic lung was displayed by immunoblotting plasmin-digests of lung extracts using an antibody specific for a neo-epitope in fibrin (21). Lung from wild-type mice subjected to hypoxia displayed a strong band immunoreactive with anti-fibrin antibody, which was only weakly stained in normoxic controls (Fig. 4A, lanes 4/5). Consistent with these results, immunostaining for fibrin in hypoxic lung tissue from wild-type mice displayed deposits of fibrin immunoreactive material (Fig. 4C), versus their absence in normoxic lung (Fig. 4B). In parallel with low levels of tissue factor in hypoxic PKCβ null mice, there was no evidence of fibrin epitopes in lung tissue harvested from these animals after exposure to oxygen deprivation by immunoblotting or immunostaining (Fig. 4, panels A (lane 5) and E; note that panels A (lane 4) and D display results in normoxic PKCβ null mice). Our data are consistent with a cause-effect relationship for induction of Egr-1 and tissue factor in hypoxic lung and the appearance of fibrin deposits, and indicate that PKCβ isoforms are upstream of these events.

Specificity of Suppressed Gene Expression in PKCβ Null Mice: Hypoxia and LPS—Although PKCβ null mice demonstrated inhibition of Egr-1 and tissue factor gene activation following exposure to hypoxia, this did not reflect a general suppression of gene expression. For example, systemic infusion of lipopolysaccharide into PKCβ null mice resulted in a strong increase in Egr-1 and tissue factor transcripts compared to that observed in wild-type mice (Fig. 5, A and B, respectively, compare lanes 1 and 2). Also, other pathways regulating the cellular response to hypoxia, such as HIF-1, remained intact. Up-regulation of the noninsulin-dependent glucose transporter (GLUT-1) is a well-known feature of the protective response to oxygen deprivation, which depends, in large part, on HIF-1 (11). Hypoxic PKCβ null mice displayed strong induction of GLUT-1 transcripts in the lung (Fig. 5C, lane 4; lane 3 shows normoxic PKCβ null control), which was comparable to that observed in wild-type an-
by Southern blotting with a 32P-labeled tissue factor probe (Fig. 5C, lane 2; lane 1 shows the normoxic wild-type control). As in our studies with alveolar macrophages (NR8383) and assessed the effect of PKCβ deletion. Wild-type (+/+ ) and PKCβ null (−/−) mice were treated as in A (above) except that the incubation time in hypoxia was 4 h, and Northern blotting was performed with 32P-labeled cDNA probes for GLUT1 and β-actin.

Macrophage Expression of Tissue Factor in Response to Oxygen Deprivation: Effect of PKCβ Deletion—One critical site in hypoxic lung for activation of gene expression in the hypoxiainduced Egr-1-tissue factor pathway is the MP (22). It was important to perform additional experiments to be certain that the observed inhibitory effect in PKCβ null mice reflected events at the level of MPs, rather than complex intercellular compensatory mechanisms possibly operative within the PKCβ null phenotype (as has been observed with other knockouts). For example, mice in whom the Interleukin 6 gene had been deleted displayed high levels of tumor necrosis factor-α, the latter contributing to the response of these mice to a range of stimuli (38, 39). Resident peritoneal macrophages (also termed MPs) were obtained by lavage from wild-type and PKCβ null mice. Following exposure of MPs from wild-type mice to hypoxia (pO2 ~14 torr) for 4 h, induction of tissue factor transcripts was observed by RT-PCR (Fig. 6A, lane 3; lane 2 shows normoxic control). The identity of this amplicon was confirmed by Southern blotting with a 32P-labeled tissue factor probe (Fig. 6B, lane 3). In contrast, similar experiments with resident peritoneal macrophages from PKCβ null mice displayed virtually no increase in tissue factor transcripts (Fig. 6, A and B, lane 7; lane 6 shows the normoxic control). As in our studies using the intact animal, induction of tissue factor in response to other stimuli, such as phorbol ester and lipopolysaccharide, remained intact in the PKCβ null mice; both of these stimuli caused an increase in transcripts in MPs from wild-type (Fig. 6, A and B, lanes 4 and 5) and PKCβ null mice (Fig. 6, A and B, lanes 8 and 9).

Hypoxia-induced Expression of Egr-1 in Alveolar Macrophages: Effect of PKCβ Inhibition—The coagulant properties of alveolar macrophages are likely to be relevant to the procoagulant environment of hypoxic lung. In a previous study with monocyte-like U937 cells, hypoxia was shown to induce mem-

brane translocation and autophosphorylation of PKCβII, compared with lack of such changes in PKC isoforms α and ε (22). Furthermore, transient transfection studies demonstrated that expression of dominant-negative PKCβII selectively suppressed hypoxia-mediated activation of Egr-1 and tissue factor transcription (22). In order to determine the applicability of these results to macrophages in the lung, we turned to a line of cultured rat alveolar macrophages (NR8383) that were treated as in A (above) except that the incubation time in hypoxia was 4 h, and Northern blotting was performed with 32P-labeled cDNA for GLUT1 and β-actin.

FIG. 5. LPS-mediated induction of Egr-1 (A) and tissue factor (B) and hypoxia-mediated induction of GLUT1 (C); effect of PKCβ deletion. Wild-type (+/+ ) and PKCβ null (−/−) mice were infused with LPS (20 μg) and after either 30 min (A) or 6 h (B) total lung RNA was isolated and subjected to Northern analysis using 32P-labeled cDNA for Egr-1 (A) or TF (B). In other experiments (C), mice were exposed to hypoxia (H; 6% oxygen) or normoxia (N) for 4 h, sacrificed, and total RNA was isolated from the lung, and subjected to Northern analysis (15 μg/lane) with 32P-labeled cDNA probes for GLUT1 and β-actin.

FIG. 6. Expression of tissue factor by peritoneal macrophages exposed to LPS, phorbol 12-myristate 13-acetate, or hypoxia: effect of PKCβ. Resident peritoneal macrophages were isolated from age- and strain-matched wild-type control (+/+ , lanes 2–5) and PKCβ null mice (−/−, lanes 6–9). Cells were exposed to normoxia (N) or hypoxia (H) for 4 h (lanes 2 and 3 or lanes 6 and 7). In other experiments, cultures were maintained in normoxia and exposed to phorbol myristate acetate (50 ng/ml; lanes 4 and 8 labeled P) for 1 h or to LPS (1 μg/ml; lanes 5 and 9 labeled L) for 6 h. Following each of these treatments, RNA was isolated, and RT-PCR was performed with primers for murine tissue factor (TF) or β-actin. Amplicons were visualized by ethidium bromide staining, TF (A) or β-actin (C), or were transferred to membranes and hybridized with a 32P-labeled cDNA for murine tissue factor (B). Lane 1 shows the migration of molecular weight markers (100-base pair DNA ladder; Promega).

FIG. 7. Hypoxia-mediated expression of Egr-1 and GLUT1; effect of PKCβ inhibition by LY379196. A, cultured rat alveolar macrophages (NR8383) were preincubated with LY379196 (0.2 μM) for 60 min, and were then incubated under normoxic (N) or hypoxic (pO2 ~12–14 torr; H) conditions for 30 min. Then, total RNA was isolated and Northern analysis was performed (10 μg of RNA/lane) using 32P-labeled cDNA for Egr-1 or β-actin. Note that controls in which LY379196 was incubated with normoxic NR8383 cells showed no differences in Egr-1 transcripts. B, cultured alveolar macrophages were treated as in A (above) except that the incubation time in hypoxia was 4 h, and Northern blotting was performed with 32P-labeled cDNA for GLUT1 and β-actin.
ent increase in Egr-1 transcripts in the hypoxic cells (Fig. 7A, lane 3). The apparent specificity of the inhibitor for the hypoxia-induced pathway under study was consistent with the observation that when the alveolar macrophage cell line was exposed to hypoxia, induction of GLUT-1 mRNA in hypoxic macrophages was maintained in the presence of LY379196 (Fig. 7B).

**DISCUSSION**

Isomers of PKC have been linked to many physiologic and pathophysiologic processes (40–44). Recent studies have particularly focused attention on the β-isomers because of their potential role in cardiovascular dysfunction, especially as it relates to endothelial cells and cardiac myocytes (45–53). Most studies analyzing PKCβ isomers have relied on inhibitors that show apparent specificity for β1/βII versus other members of the PKC family (44, 45, 54–59). Alternative experimental approaches have employed overexpression of wild-type PKCβII or inducible expression of a constitutively active variant in cardiac myocytes (46, 47). Another means to dissect the contribution of PKCβ is use of PKCβ null mice. The advantage of this approach is that selective ablation of the PKCβ gene would be not be predicted to interfere with other PKC family members or to impact on downstream targets of PKCβ. Of course, the possibility that compensatory mechanisms are recruited in the absence of PKCβ must be kept in mind, as in any experiment with knockout mice. Previously described properties of PKCβ null mice made them ideal for our studies, as their growth, reproductive capacity, and survival are intact (25). Furthermore, although their phenotype remains to be fully defined, based on their characteristics reported to date (involving mainly immunologic defects which would not be predicted to affect phenomina in our study a priori), it appears that multiple physiologic responses, such as induction of GLUT1 consequent to hypoxia and expression of tissue factor following LPS, remain intact.

Studies reported herein using PKCβ null mice delineate a central role for PKCβ in hypoxia-mediated induction of Egr-1 transcription, which leads to increased expression of tissue factor. Our previous studies with cultured monocytic-like cell lines demonstrated that oxygen deprivation triggered PKCβII activation within minutes, and was followed by a pathway including Raf, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, and ERK1/2 (22). Subsequent ERK1/2-induced activation of elk-1, resulting in formation of a complex with serum response factor, promotes transcription of Egr-1, and leads directly to tissue factor transcription. The current results in PKCβ null mice confirm the broad outlines of this pathway in vivo, and indicate a cause-effect relationship between PKCβ activation in hypoxic lung and subsequent events leading to tissue factor induction: 1) enhanced expression of Egr-1 and tissue factor observed in response to oxygen deprivation in wild-type mice was not observed to any appreciable extent in PKCβ null animals; 2) hypoxia-mediated activation of ERK1/2, key contributors to the pathway linking PKCβ activation to up-regulation of Egr-1 transcription, was also not seen in PKCβ null mice; and 3) levels of tissue factor antigen remained low and pulmonary vascular fibrin deposition was not observed in hypoxic PKCβ null mice. These data provide strong support for a pathway in which activation of PKCβ triggers events leading to accumulation of fibrin in pulmonary vasculature. This response occurs rapidly and could potentially have long term effects on vascular properties by several mechanisms; deposited fibrin could block blood flow and trigger vascular remodeling directly, thrombin formed within the vessel could activate thrombin receptors on cells within the vessel wall (60), and other target genes of Egr-1 and PKCβ could be expressed.

The biologic significance of the PKCβ-Egr-1-tissue factor pathway induced by hypoxia will require considerably more study to delineate. However, it is clear that these hypoxia-mediated events can be differentiated from HIF-1-induced responses. Whereas hypoxia-induced up-regulation of Egr-1 and tissue factor was strongly suppressed in PKCβ null mice, oxygen deprivation led to comparable induction of GLUT-1 (which is largely due to HIF-1) (11) in PKCβ null and wild-type mice. This is consistent with our previous observation that a cultured hepatoma line unable to form active HIF-1, due to deficient ARNT/HIF-1β function, demonstrated comparable induction of Egr-1 transcripts, versus that in wild-type controls, when exposed to hypoxia (22). Furthermore, expression of tissue factor in response to other stimuli, such as phorbol ester and LPS, also remained intact in PKCβ null mice. In this context, the adaptive advantage of a pathway causing induction of local vascular fibrin formation remains uncertain; though it could allow sequestration of hypoxic areas from nonischemic tissues, the possibility that subsequent obstructive thrombus formation might prevent blood flow leading to necrosis also exists. Future studies examining the range of responses triggered by PKCβ and Egr-1 activation in hypoxic tissues will be required to appreciate the impact of these events on the vascular adaptation to hypoxemia. Another important issue concerns the specificity of this pathway for different cells and in different tissues. Our studies in hypoxic lung have shown strong up-regulation of Egr-1 and tissue factor at the antigen level in smooth muscle cells and MPs. However, in view of the presence of Egr-1 in virtually every cell, and the similar potential of a wide range of cells to express tissue factor, it will be essential to know why certain cells are especially susceptible to hypoxia-induced responses in this pathway. Nonetheless, the contribution of the current work is to firmly establish that PKCβ has an integral role in an hypoxia-induced pathway leading to activation of MAP kinases, and transcription of Egr-1 and tissue factor. These observations form a strong foundation for future studies to further analyze in detail mechanisms underlying this pathway in vitro and physiologic consequences for such effector mechanisms in vivo.

**REFERENCES**

1. Guillemin, K., and Kranzow, M. (1997) Cell 89, 9–12
2. Semenza, G. (1996) Trends Cardiovasc. Med. 6, 151–157
3. Wood, S. M., Gleadle, J., Pugh, C., Hankinson, O., and Ratcliffe, P. (1996) J. Biol. Chem. 271, 15117–15123
4. Pinsky, D. J., Nakai, Y., Liao, H., Oz, M. C., Wagner, D. D., Tanya, N. M., Johnson, R. C., Heath, M., Lawson, C. A., and Stern, D. M. (1996) J. Clin. Invest. 97, 493–500
5. Muller, J., Kraus, B., Kaltschmidt, C., Baeuerle, P., and Rupe, R. (1997) J. Biol. Chem. 272, 23435–23439
6. Yan, S.-F., Zou, Y.-S., Gao, Y., Zhao, C., Mack, N., Lee, S., Milbrandt, J., Pinsky, D., Kiel W., and Stern, D. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 8298–8303
7. Bunn, H., and Poyton, R. (1996) Respir. Physiol. 76, 839–885
8. Heacock, C., and Sutherland, R. (1996) Int. J. Radiat. Oncol. Biol. Phys. 35, 1287–1290
9. Michiels, C., Arnold, T., Knott, I., Dieu, M., and Remacle, J. (1993) Am. J. Physiol. 264, C866–C874
10. Zhu, H., and Bunn, H. F. (1999) Respir. Physiol. 115, 239–247
11. Iyer, N., Kotche, L., Agani, F., Laughner, E., Wenger, R., Gassmann, M., Gearhart, J., Lawler, A., Yu, A., and Semenza, G. (1998) Genes Dev. 12, 149–162
12. Semenza, G., Roth, P., Fang, H.-M., and Wang, G. (1994) J. Biol. Chem. 269, 23757–23763
13. Shweiki, D., Tux, D., Abassi, D., and Keshet, E. (1992) Nature 359, 843–845
14. Carmeliet, P., Ferreira, V., Breier, G., Pollelley, S., Kiechans, L., Gerstenrain, M., Fahrig, M., Vandenhooca, A., Harpal, K., Eberhardt, C., Decler, C., Pawling, J., Kohn, K., and Moos, D. (1996) Nature 380, 435–439
15. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K., Powell, R., and Braxton, L., Hillan, K., and Moore, M. (1996) Nature 380, 439–442
16. Iyer, N., Kotche, L., Agani, F., Laughner, E., and Semenza, G. (1999) Dev. Biol. 210, 344–347
17. Carmeliet, P., Dur, Y., Herbert, J., Fukumura, D., Brusselens, K., Dewerchin, M., Neeman, M., Beno, F., Abramovitch, R., Maxwell, P., Koch, C., Ratcliffe, P., Moos, L., Jain, R., Collen, D., and Kesther, E. (1998) J. Biol. Chem. 273, 19411–19416
