The paradigm for the response to hypoxia is erythropoietin gene expression; activation of hypoxia-inducible factor-1 (HIF-1) results in erythropoietin production. Previously, we found that oxygen deprivation induced tissue factor, especially in mononuclear phagocytes, by an early growth response (Egr-1)-dependent pathway without involvement of HIF-1 (Yan, S.-F., Zou, Y.-S., Gao, Y., Zhai, C., Mackman, N., Lee, S., Milbrandt, J., Pinsky, D., Kielis, W., and Stern, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8298–8303). Now, we show that cultured monocytes subjected to hypoxia (pO2 ~ 10 torr) displayed increased Egr-1 expression because of de novo biosynthesis, with a ~10-fold increased rate of transcription. Transfection of monocytes with Egr-1 promoter-luciferase constructs localized elements responsible for hypoxia-enhanced expression to ~424–65, a region including EBS (ets binding site)-SRE (serum response element)-EBS and SRE-EBS-SRE sites. Further studies with each of these regions ligated to the basal thymidine kinase promoter and luciferase demonstrated that EBS sites in the element spanning ~424–375 were critical for hypoxia-enhanceable gene expression. These data suggested that an activated ets factor, such as Elk-1, in complex with serum response factor, was the likely proximal trigger of Egr-1 transcription. Indeed, hypoxia induced activation of Elk-1, and suppression of Elk-1 blocked up-regulation of Egr-1 transcription. The signaling cascade preceding Elk-1 activation in response to oxygen deprivation was traced to activation of protein kinase CβIII, Raf, mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase and mitogen-activated protein kinases. Comparable hypoxia-mediated Egr-1 induction and activation were observed in cultured hepatopler-derived cells deficient in HIF-1β and wild-type hepatoma cells, indicating that the HIF-1 and Egr-1 pathways are initiated independently in response to oxygen deprivation. We propose that activation of Egr-1 in response to hypoxia induces a different facet of the adaptive response than HIF-1, one component of which causes expression of tissue factor, resulting in fibrin deposition.

The cellular response to oxygen deprivation involves a series of metabolic and biosynthetic events associated with adaptation to a hypoxic environment. Enhanced expression of the noninsulin-dependent glucose transporter GLUT1, key glycolytic enzymes, erythropoietin, and vascular endothelial growth factor are well described examples of the host response to hypoxia with obvious advantages for survival in an oxygen-deficient environment (1). Each of these events appears, in large part, to be mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1)1 (1), which is also implicated in angiogenesis, based on the phenotype of HIF-1/α/arylhydrocarbon receptor nuclear translocator (ARNT) and HIF-1α deletionally mutant mice (2–4). Although HIF-1 is critical for restoring cellular homeostasis in hypoxia, biosynthetic events triggered by oxygen deprivation extend beyond HIF-1. For example, hypoxia leads to the induction of c-Fos and appearance of c-Jun/c-Fos AP-1 heterodimers in cultured cells, potentially promoting expression of a range of genes, especially those involved in cell growth (5–8). Oxygen deprivation also activates the transcription factor C/EBPβ (9, 10), which, on binding to nuclear factor-interleukin 6 (NF-IL-6) motifs, results in expression of IL-6, a cytokine associated with acute inflammation. Finally, some investigators have observed activation of NF-kB in hypoxia (11–15), although others have found such activation only during the immediate reoxygenation period associated with oxidant stress (16). These considerations underscore the complexity of the cellular response to environmental challenge by hypoxic and/or oxidative stress.

Venous thrombosis is associated with local hypoxemia and stasis, and has a considerable morbidity and mortality. Although cessation of blood flow perturbs a spectrum of metabolic and hemodynamic factors, we have found that normobaric hypoxia causes de novo expression of tissue factor, especially in mononuclear phagocytes (MPs), resulting in fibrin deposition in pulmonary vasculature (17, 18). Furthermore, we have observed recently that hypoxia-mediated expression of tissue factor is caused by the early growth response gene product Egr-1, based on the parallel absence of enhanced tissue factor expres-

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1 The abbreviations used are: HIF-1, hypoxia-inducible factor-1; ARNT, arylhydrocarbon-receptor nuclear translocator; CA, constitutive active; CMV, cytomegalovirus; DN, dominant-negative; EBS, ets binding site; Elk, early growth response; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated protein kinase; IL-6, interleukin-6; MAP, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase kinase; MP, mononuclear phagocyte; NF, nuclear factor; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; SRE, serum response element; wt, wild-type.
Hypoxia, PKCβII, and Egr-1

sion and fibrin deposition in the pulmonary vasculature of homozygous Egr-1 null mice (19). These observations suggested the possible contribution of Egr-1 to the cell biology of hypoxia, potentially engaging a pathway with quite different outcomes for the adaptive host response than HIF-1. In this study, we analyzed up-regulation of Egr-1 in hypoxic MPs and have found it to occur at the transcriptional level, consequent to activation of protein kinase C-βII (PKCβII), leading to Raf- and MEK-dependent activation of MAP kinases, ERK 1/2, and, ultimately, activation of the ets factor Elk-1. Although the physiologic and pathophysiologic consequences of Egr-1 activation in hypoxia are not yet clear, induction of the trigger of the procoagulant pathway, tissue factor, suggests the basis for pathologic events underlying thrombotic pathology associated with oxygen deprivation by a pathway distinct from HIF-1.

EXPERIMENTAL PROCEDURES

Cell Culture and Induction of Hypoxia—A line of rat mononuclear phagocytes (NR5838; alveolar macrophages) was obtained from ATCC (Manassas, VA), and cells were grown in Ham’s F-12K medium containing 15% heat-inactivated fetal calf serum. Human blood monocytes were harvested from peripheral blood by density gradient centrifugation and plated for 2 days in RPMI 1640 medium containing 10% fetal calf serum (11). The human monocyte line U937 (ATCC) was also grown in RPMI 1640 with fetal calf serum (10%). Mouse macrophages (ATCC; P388D1) were grown in high glucose RPMI 1640 containing 10% fetal calf serum. Wild-type hematoma cells (Hepta1c1c7) or hematoma cells deficient in the ARNT/HIF-1 β function (c4) were generously provided by Dr. Oliver Hankinson (UCLA) and were grown in a minimal essential medium supplemented with 10% fetal calf serum (20). Cells were subjected to hypoxia using an environmental chamber as described previously (17); pO2 in the medium was ∼12–14 torr. Cells subjected to hypoxia were placed in medium preequilibrated with the hypoxic gas mixture just prior to placement in the environmental chamber. Thus, culture were subjected immediately to the oxygen-deprived environment at the time of medium change/placement in the chamber.

Experiments employing mice subjected to hypoxia (final oxygen concentration of 5.5–6.5%) employed C57BL6/J mice (12–15 weeks old; Jackson Laboratories, Bar Harbor ME) and utilized a specially built environmental chamber (17).

Analysis of Egr-1 Expression in Hypoxic Murine Lung—Following hypoxia, mice were killed, and tissues were processed immediately. For Northern analysis, tissue was cut into small pieces, immersed in Trizol (Life Technologies, Inc.), homogenized, and total RNA was extracted and subjected to electrophoresis (0.8% agarose). RNA was transferred to Duralon-UV membranes (Stratagene), and membranes were then hybridized with 32P-labeled cDNA probe for mouse Egr-1 (21). Blots were also hybridized with 32P-labeled β-actin as an internal control for RNA loading. The same procedure was employed to assess Sp1 transcripts in hypoxic murine lung, except 32P-labeled cDNA probe for Sp1 was employed (22).

For Western blotting, nuclear extracts were prepared (see below) and subjected to SDS-PAGE (7.5%). Proteins in the gel were transferred electrophoretically to nitrocellulose membranes, and immunoblotting was performed with rabbit anti-Egr-1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Sp1 IgG (Santa Cruz Biotechnology) according to theblotto procedure (23). Sites of primary antibody binding were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham International, Buckinghamshire, U. K.). The final detection of immunoreactive bands was performed using the enhanced chemiluminescent Western blotting system (Amersham).

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 (19). Sections were first stained with primary antibodies, anti-Egr-1 IgG (Santa Cruz Biotechnology), rat F4/80 monoclonal antibody (Caltag Laboratories, South San Francisco, CA), or rabbit anti-smooth muscle α-actin IgG (Sigma). Then, they were incubated with secondary antibody, an affinity-puriﬁed peroxidase-conjugated IgG, either goat anti-rabbit IgG or goat anti-rat IgG (Sigma).

The electrophoretic mobility gel shift assay (EMSA) was performed on nuclear extracts prepared immediately from hypoxic lung using the method of Dignam et al. (24). Double-stranded oligonucleotide probes for Egr (Santa Cruz Biotechnology) were 5'-end labeled with [32P]ATP by using T4 polynucleotide kinase and standard procedures. Binding reactions were performed as described (9), and samples (5 µg of protein in each lane) were loaded directly onto nondenaturing polyacrylamide/bisacrylamide (6%) gels. Gels were prerun for 20 min before samples were loaded, and electrophoresis was performed at room temperature for 6 h at 300 V. For competition studies, a 100-fold molar excess of unlabeled probes for NF-IL-6 (9), HIF-1 (1), Egr-1 (Santa Cruz Biotechnology), or AP-1 (Santa Cruz Biotechnology) was added. Analysis of Egr-1 Expression in Cultured MPs— MPs were subjected for hypoxia for the indicated times, and total RNA was extracted using Trizol and subjected to Northern analysis. The same protocol as for Egr-1 analysis in mouse lung was employed (see above). Where indicated, Northern analysis was also performed to detect tissue factor transcripts. In this case, 32P-labeled tissue factor cDNA was employed to probe membranes with immobilized RNA. Nuclear run-on analysis, to assess the rate of Egr-1 transcription, was performed on cultured MPs deprived of serum for 24 h and then subjected to normoxia or hypoxia for 30 min. Nuclei were isolated, and in vitro transcription was performed as described (9). EMSA using the consensus 32P-labeled Egr probe on nuclear extracts from hypoxic cultured MPs employed the same methods as for lung tissue above.

Transfection, with Superfect Reagent (Qiagen, Chatsworth, CA), used a series of dele- tional/truncated Egr-1 promoter-reporter (luciferase) constructs: A (the designation given in Fig. 4A) includes a 1.2-kilobase fragment of the murine Egr-1 5′-flanking sequence Egr-1/II/Luc (25–28); B includes sequences from 811 through 1000; C includes sequences from 511 through 1000; D includes sequences from 811 through 1000; E includes sequences from 845 through 1000; G includes sequences from 866 through 1000. The construct labeled V in Fig. 4A denotes the promoterless vector pXP2 (23). The constructs pYSF62b&65 were comprised of 424/–375 and −111/−65, respectively, from the murine Egr-1 promoter, linked to the basal thymidine kinase promoter (−80/−18) and the pGL3 basic vector (Promega). For pYSF63b&64, site-directed mutagenesis was employed to mutate GG to TT located at positions −414/−413 (pYSF63) and −393/−392 (pYSF64), respectively, which flank a CARG box (SRE1) (25–27). Cotransfection with pCMV-β-galactosidase was used as an internal control for efficiency of transfection. Luciferase activity was normalized based on β-galactosidase activity in the same well; this is termed relative luciferase activity and is used in each of the transfection studies.

Analysis of the Role of PKCβII in Hypoxia-induced Expression of Egr-1 and Tissue Factor in Cultured MPs—Prior to experiments, the medium bathing MPs was aspirated, and fresh medium containing 1% fetal calf serum was added for 24 h. Cells were then placed in serum-free medium C inhibitor of Raf (pYSF62b&65) and tissue factor (des- sites (19), and pGL3 basic vector (Promega); for (-111/−14[Luc] constructs (29); CA-PKCβII (rabbit, pcDNA136) (32). The procedure for transfection utilized Superfect Reagent, and the protocol was according to the manufacturer’s instructions. For transfection studies, macrophages were transferred to medium with 1% fetal calf serum for 24 h. Then, cotransfection was performed using either Egr-1 promoter-luciferase (1.5 µg; pYSF62b&65) or tissue factor promoter-luciferase (2 µg; pTF/−111/+14[Luc] constructs, one of the PKC-related constructs (1 µg), and pCMV-β-galactosidase (1 µg). Immediately after transfection, cells were maintained in medium with 1% fetal calf serum for 24 h and were then placed in serum-free medium for 20 h. Cultures were next subjected to hypoxia for 4 h (for Egr-1 studies) or 5 h (for tissue factor studies), and luciferase activity was determined using a luminometer.

Subcellular association of PKCβII was studied by immunoblotting membrane fractions (35%) of hypoxic U937 cells and lungs from mice subjected to oxygen deprivation. Extracts were subjected to non-reduced SDS-PAGE (7.5%) followed by Western blotting with rabbit anti-PKCβII IgG (as primary antibody) and horseradish peroxidase-conjugated goat anti-rabbit IgG (as secondary antibody; Amersham). Similar experiments were performed with extracts of lung. Autophosphorylation of PKC isoforms (PKCα, PKCβII, and PKCε) was studied
by labeling cultures with $^{32}$P, as described (34, 35) followed by immunoprecipitation with isoform-specific antibodies (Santa Cruz Biotechnology), SDS-PAGE, and autoradiography. In brief, cultures were maintained in normoxia, washed with HEPES-buffered phosphate-free high glucose RPMI 1640, and labeled with 100 $\mu$Ci of $^{32}$P for 3 h at 37°C under normoxic conditions. Then, cells were subjected to hypoxia for 5, 10, or 15 min and processed as described (34, 35). Protein concentrations were determined, and supernatants with the same amount of protein were immunoprecipitated with antibodies to PKC$\beta$I, PKC$\beta$II, or PKC$\epsilon$ (Santa Cruz Biotechnology).

Delineation of the Pathway Leading to Expression of Egr-1 in Cultured MPs Subjected to Hypoxia—Cotransfection of MPs used the same procedure as above. To analyze the role of raf, cells were transfected with a dominant-negative (DN)-Raf (pCGN $raf$ N4; generously provided by Dr. Channing Der, University of North Carolina, Chapel Hill) (36). The MEK inhibitor PD98059 (New England Biolabs) was employed at a concentration of 10 $\mu$M and was added 1 h before placement of cells in hypoxia. Other constructs included DN-ERK 1 (pCMV-ERK 1[K→R]), DN-ERK 2 (pCMV-ERK 2[K→R]), and DN-Elk-1 (pCMV-Elk-383A) (all generously provided by Dr. Peter Shaw, Queen’s Medical Center, Nottingham, UK) (38). The Elk-1 chimeric reporter system was employed to detect activation of Elk-1 (39); the GAL4-Elk-1 expression vector and the 5xGAL4-E1b-luciferase reporter were generously provided by Dr. Richard Maurer (Oregon Health Sciences University, Portland). In-gel kinase assays were used to monitor activation of ERK 1/2 (40). In brief, U937 cells in serum-free medium were exposed to hypoxia for 10 min, and cells were washed three times in ice-cold phosphate-buffered saline and suspended in ice-cold extraction buffer. Cell extracts were resolved on SDS-PAGE (10%) containing 0.5 mg/ml myelin basic protein (Sigma).

Data Analysis—Significant differences between experimental groups were detected using analysis of variance for unpaired variables, with post hoc comparisons performed using Tukey’s procedure. Data that are

FIG. 1. Egr-1 expression in hypoxic murine lung. Panel A, mice were subjected to hypoxia for the indicated times followed by sacrifice and extraction of total RNA from the lung. Northern analysis was performed with $^{32}$P-labeled probes for Egr-1, Sp1, and $\beta$-actin. In each case, 20 $\mu$g/lane of total RNA was loaded on the gel. Panels B and C, immunoblotting was performed on nuclear extracts from the lungs of mice subjected to hypoxia for 30 min using antibodies to Egr-1 (panel B) or Sp1 (panel C). In each case, 10 $\mu$g/lane of total protein was loaded on the gel. N and H designate samples from normoxic and hypoxic lung, respectively. Panels D–I, analysis of hypoxic lung for Egr-1. Mice were exposed to hypoxia (panel D) or normoxia (panel E) for 2 h, and lungs were prepared for immunohistologic analysis using antibody to Egr-1. Adjacent sections from hypoxic lung were stained with antibody to Egr-1 (panel F) and F4/80 (panel G) or with antibody to Egr-1 (panel H) and smooth muscle $\alpha$-actin (panel I). The marker bar indicates 25 $\mu$m (panels D, E, H, and I) and 8.3 $\mu$m (panels F and G).
RESULTS

Expression of Egr-1 in Lungs of Mice Subjected to Hypoxia

Mice exposed to hypoxia displayed time-dependent induction of Egr-1 mRNA evident by 15 min, with maximal expression by 30 min (Fig. 1A). Western analysis of nuclear extracts from hypoxic lung showed an increase in Egr-1 antigen, with a major immunoreactive band corresponding to a molecular mass of ~82 kDa (Fig. 1B). In contrast to the observed induction of Egr-1, there was no increase in Sp1 in hypoxic lung, either at the mRNA (Fig. 1A) or antigen level (Fig. 1C; two closely spaced bands corresponding to molecular masses of ~95 and ~105 kDa of similar intensity in normoxic and hypoxic lung were observed) (28). Immunohistochemistry revealed that Egr-1 expression was not confined to a single cell type but that elevated amounts of Egr-1 antigen were present in a range of cells in hypoxic lung (Fig. 1D) compared with normoxic controls (Fig. 1E). Analysis of adjacent sections using markers for mononuclear phagocytes/macrophages (F4/80) (Fig. 1G) and smooth muscle (smooth muscle α-actin IgG) (Fig. 1F) demonstrated colocalization with increased Egr-1 (Fig. 1, F and H). Closely paralleling enhanced levels of Egr-1, a time-dependent appearance of Egr DNA binding activity was observed in nuclear extracts from hypoxic lung (Fig. 2, lanes 2–6). Specificity of the DNA binding activity was shown by disappearance of the gel shift band with excess unlabeled Egr probe (Fig. 2, compare lanes 7 and 8), whereas oligonucleotides corresponding to sequences for AP-1, HIF-1, NF-IL-6, and Sp1 were without effect (Fig. 2, lanes 9–12). Furthermore, in a previous study (19), we have found that the protein responsible for Egr DNA binding activity in hypoxic lung was Egr-1, as shown by complete disappearance of the band in the presence of anti-Egr-1 IgG but not nonimmune IgG. These data indicate that Egr-1 expression and activation of Egr-1 DNA binding activity occur in the lung within minutes of oxygen deprivation.

Mechanisms of Hypoxia-induced Egr-1 Expression

The mechanisms were studied using a MP cell line as a model system. Cultured MPs subjected to oxygen deprivation displayed time-dependent induction of Egr-1 mRNA (Fig. 3A) as well as increased Egr-1 antigen (Fig. 3C) and enhanced Egr DNA binding activity in nuclear extracts (Fig. 3D). Maximal Egr DNA binding activity by EMSA occurred within 30 min of exposure to hypoxia, similar to what was observed in murine lung. The rate of Egr-1 transcription was increased about 10-fold in hypoxic MP cultures compared with normoxic controls (Fig. 3B).

Transient transfection of MPs with series of deletional/truncation Egr-1 promoter-reporter (luciferase) constructs focused our attention on the SRE motifs (Fig. 4A). As an internal control for efficiency of transfection, cotransfection studies were performed with pCMV-β-galactosidase. Results are shown as fold-increase in luciferase activity normalized for
β-galactosidase activity (termed relative luciferase activity). The longest construct (line A) displayed ~5–7-fold increased luciferase activity in hypoxia (this range was observed over four experiments; results of a representative experiment are shown in the figure). Truncation of AP-1 and Sp1 sites in the distal portion of the promoter had virtually no effect on inducibility in hypoxia (Fig. 4A, line C). Consistent with their lack of involvement, a construct spanning the latter elements (Fig. 4A, line B) linked to the most proximal portion of the Egr-1 promoter did not drive luciferase expression in hypoxia. The key portion of the promoter with respect to the effect of hypoxia included the region spanning three SRE elements (Fig. 4A, line D). In contrast, neither of the proximal SRE elements (Fig. 4A, lines E and F) was able to confer enhanced expression of luciferase in response to oxygen deprivation. In view of previous data implicating SRE DNA binding motifs in hypoxic stress-associated up-regulation of GLUT1 and studies of c-Fos expression (5, 41), we further analyzed two types of SRE sites in the Egr-1 promoter, EBS-SRE-EBS (~424/−375 base pairs), from the distal portion of the promoter, and SRE-EBS-SRE (~−114/−65), from the proximal portion of the promoter. Transfection studies were performed with constructs made by ligating EBS-SRE-EBS (~424/−375) or SRE-EBS-SRE (~−114/−65) to the basal thymidine kinase promoter and luciferase, resulting in the vectors pYSF62 and pYSF65, respectively (Fig. 4B). Only transfection with pYSF62 resulted in a hypoxia-mediated increase in luciferase activity; this is consistent with the results in Fig. 4A, line D. Furthermore, in EBS-SRE-EBS (~424/−375), mutational inactivation of the proximal EBS (pYSF64) largely blocked hypoxia-induced expression, whereas mutation of the distal EBS (pYSF63) completely prevented hypoxia-inducibility of luciferase expression (Fig. 4B). These data indicate the essential contribution of the EBS element in the Egr-1 promoter for activation of transcription at the SRE in response to hypoxia. Stimulated by these results, we performed studies to assess a possible role for the ets factor Elk-1 and a kinase cascade leading to its activation, in the up-regulation of Egr-1 consequent to oxygen deprivation (see below).

**Definition of the Hypoxia-induced Cascade Resulting in Activation of Egr-1 Transcription**

Recent studies have demonstrated that reductive stress results in phosphorylation of ERK 1/2 leading to activation of the ets factor Elk-1 (5, 42). The latter, in concert with serum response factor, participates in initiation of Egr-1 gene transcription.
Elk-1 activation in hypoxic MPs. Panel A, Elk-1 activation monitored using the GALA-Elk-1-luciferase expression system. MPs were cotransfected with 100 ng of pcDNA3a-GALA-Elk-1-COOH and 1 μg of 5xGAL4-E1b-luciferase reporter gene as well as 1 μg of pCMV-β-galactosidase and were then subjected to hypoxia (H) or normoxia (N) as described (for details of protocol, see "Experimental Procedures"). Luciferase activity was measured and is reported on the y axis as relative luciferase activity. Where indicated, cultures were also cotransfected with constructs encoding DN-PKCβII (M217, see Fig. 7, below; 1 μg), 1 μg of DN-Raf (pCGN-raf N4), 1 μg of DN-ERK 1 (pCMV-ERK 1(K>R)), 1 μg of DN-ERK 2 (pCMV-ERK 2(K>R)), or 1 μg of pcDNA3 alone. Panel B, effect of Elk-1 on transcription of Egr-1. MPs were cotransfected with 1.5 μg of pYSF62, 1 μg of pCMV-β-galactosidase, and either 1 μg of DN-Erk1-tp(pCMV-Elk-1-833A) or 1 μg of pcDNA3, as indicated. Cultures were maintained in normoxia or hypoxia as described (for details of protocol, see "Experimental Procedures"). Panel C, effect of CA-PKC constructs on Elk-1 activation. MPs were cotransfected with 100 ng of pcDNA3a-GALA-Elk-1-COOH and 1 μg of 5xGAL4-E1b-luciferase in the presence of plasmids encoding CA-PKCβII, CA-PKCα, CA-PKCε, or pcDNA3 alone (1 μg each case). Cultures were harvested after incubation under normoxic conditions. Experiments displayed in the figure were repeated a minimum of three times, and representative results are shown.

**Fig. 6. Effect of PKC inhibitors on hypoxia-induced increased transcripts for Egr-1 (panel A) and tissue factor in MPs (panel B).** MPs were preincubated with 100 μM calphostin C, 10 μM GF109203X, or 20 μM H-7 for 1 h under normoxic conditions. Cultures were then maintained in normoxia or subjected to hypoxia (H) for 30 min (Egr-1) or 4 h (tissue factor), total RNA was harvested, and Northern blotting was performed with 32P-labeled Egr-1 (panel A), tissue factor (panel B), or β-actin probe (panels A and B).

**Role of PKCβII—**Previous studies have shown that activation of PKC stimulates expression of Egr-1 and tissue factor, especially in epithelial cells (28). This led us to speculate that PKC might be the proximal trigger in MPs leading to a kinase cascade, including Raf, MEK, and MAP kinases, which ultimately acts on Elk-1. To test this hypothesis, experiments were performed to examine whether PKC might contribute to hypoxia-induced up-regulation of Egr-1 and tissue factor. MPs were exposed to 100 nM calphostin C, 10 μM GF109203X, or 20 μM H-7 and then subjected to hypoxia. Total RNA was harvested after 30 min, and Northern blotting was performed using radiolabeled mouse Egr-1 cDNA. Hypoxia increased levels of Egr-1 mRNA in hypoxic MPs (Fig. 6A, lane 2), as described above (Fig. 3). Cultures pretreated with PKC inhibitors demonstrated suppression of Egr-1 mRNA in the hypoxic environment (lanes 4, 6, 8); levels were similar to that in normoxic controls (lane 1). Similar results were obtained with respect to the effect of PKC inhibitors on the level of tissue factor transcripts. MPs subjected to hypoxia displayed increased levels of tissue factor transcripts compared with controls maintained in normoxia (Fig. 6B, compare lanes 9 and 10), as reported previously (19). In contrast, this increase in tissue factor mRNA was blocked in cultures pretreated with PKC inhibitors (lanes 11–13). Although these PKC inhibitors are not subtype-specific, they do, at the concentrations employed, show relative selectivity for PKC versus cAMP-dependent and cGMP-dependent protein kinases. Thus, it seemed likely that the hypoxia-associated increase in Egr-1 and tissue factor transcripts in MPs involved PKC.

PKCβII is present in MPs and has been shown, in other cell types, to participate in cellular responses to environmental perturbations, such as increased levels of glucose (44, 45). Quiescent monocyte-like cells (U937) subjected to hypoxia dem-
FIG. 7. PKCβII in hypoxic MPs and lung. Panel A, MPs were maintained in normoxia in medium alone (lane 1; N), subjected to hypoxia for 10 min (lane 2; H), or maintained in normoxia in medium supplemented with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) for 10 min (lane 3). Membranous fraction was prepared and subjected to SDS-PAGE (5 μg of total protein in each lane) and immunoblotting with antibody to PKCβII, PKCβI, and Egr-1.

Panel B, Fold Increase in PKCβII expression in MPs subjected to normoxia (N) or hypoxia (H) for different time periods (5, 10, and 15 min).

Panel C, Relative Luciferase Activity in MPs transfected with pDNA3, pY6F2+, and various constructs expressing PKCβII, PKCβI, or PKCε.

Panel D, Relative Luciferase Activity in MPs transfected with pDNA3, pY6F2+, and various constructs expressing PKCβII, PKCβI, or PKCε.

Panel E, Relative Luciferase Activity in MPs transfected with pDNA3, pY6F2+, and various constructs expressing PKCβII, PKCβI, or PKCε.
onstrated an increase in PKCβII antigen in the membranous fraction compared with normoxic controls (Fig. 7A, lanes 1 and 2). Consistent with these observations in cell culture, extracts from murine lung also demonstrated increased PKCβII antigen in membranous fractions within ~5–10 min of hypoxia compared with normoxic controls (Fig. 7B, lanes 1–3). These data suggested that PKCβII might become activated in response to hypoxia, leading us to test this concept directly by assessing autophosphorylation of PKCβII, the latter closely correlated with PKC activation (35). In addition to PKCβII, our attention was focused on PKCβl and PKCe. Both are expressed by MPs (44), although PKCβl bears strong similarity to PKCβII (both are conventional PKCs and represent alternatively spliced products of the same gene; PKCe represents a structurally distinct class of PKCs, the novel PKCs). Cultured MPs subjected to oxygen deprivation were labeled with 32P and immunoprecipitated with antibodies for PKCβl, PKCβII, or PKCe (Fig. 7C). Strong labeling of PKCβII was observed following hypoxia (~4-fold increased intensity of the immunoprecipitated band by 10 min), whereas there was virtually no change in the intensity of the bands for PKCβl and PKCe.

These data led us to examine the effect of blocking PKCβII by overexpressing a dominant-negative construct. For these studies, we monitored induction of Egr-1 transcription utilizing transient transfection of MPs with the Egr-1 promoter-luciferase construct pYSF62 (Fig. 7D, left panel). Cotransfection of MPs with both pYSF62 and the DN-PKCβII expression vector (M217) blocked the hypoxia-associated increase in luciferase activity observed in cultures transfected with the Egr-1 promoter-luciferase construct alone and subjected to hypoxia (Fig. 7D). In contrast, overexpression of constructs for DN-PKCa or DN-PKCe in the same system had no effect on luciferase expression after transfection of pYSF62 (Fig. 7D). Consistent with the specificity of the inhibition caused by DN-PKCβII, transfection experiments with wtPKCβII (either rabbit (r) or murine (m) in Fig. 7D) in place of the DN-PKCβII construct showed no inhibitory effect. As a further test for specificity of the observed results with DN-PKCβII, “rescue” experiments were performed by cotransferring MPs with DN-PKCβII + wtPKCβII or DN-PKCβII + wtPKCe, and determining the effect on expression of pYSF62 under hypoxic conditions (Fig. 7D). Using these reagents, it has been previously shown that overexpression of wtPKCβII, but not PKCβl, will reverse the phenotype induced by transfection with DN-PKCβII in a skeletal muscle cell culture system (32). In our studies, wtPKCβII, but not wtPKCe, restored expression of pYSF62 under hypoxic conditions in the presence of DN-PKCβII (Fig. 7D). Additional support for a central role of PKCβII in the expression of Egr-1 by MPs was shown by increased expression of the luciferase reporter after cotransfection of constitutively active PKCβII along with pYSF62, even when cells were maintained in normoxia (Fig. 7D, right panel). As expected, cotransfection of cultures with constructs encoding CA-PKCa or CA-PKCe also induced expression of pYSF62 under normoxic conditions, consistent with the well known ability of diverse PKC isoforms to activate downstream effector mechanisms, such as MAP kinases and Egr-1. These data are consistent with an important contribution for activation of PKCβII in hypoxic MPs as a trigger for increased transcription of Egr-1. Specificity appears to occur at the level of preferential PKCβII activation in hypoxic MPs versus apparent lack of comparable PKCβl or PKCe activation.

Inhibition of PKCβII also suppressed activation of tissue factor transcription in hypoxic MPs. Expression of tissue factor was monitored using the tissue factor promoter-luciferase construct spanning the hypoxia responsive Egr-1 elements (pTF[−111/+14]Luc; Fig. 7E) (39). Although hypoxia caused increased luciferase activity/expression with the tissue factor promoter-luciferase construct alone (pTF[−111/+14]Luc), co-transfection with the DN-PKCβII suppressed luciferase activity (Fig. 7E). In contrast, neither mock-transfected controls nor transfection with constructs encoding DN-PKCo or DN-PKCe, in place of DN-PKCβII, demonstrated an inhibitory effect (Fig. 7E). Furthermore, transfection of MPs with wtPKCβII in place of the DN-PKCβII construct did not cause a suppression of luciferase activity due to pTF[−111/+14]Luc in hypoxia. The importance of PKCβII in regulating tissue factor expression was emphasized by the results of studies in which CA-PKCaβII was overexpressed; luciferase activity with pTF[−111/+14]Luc increased even when cells were maintained in normoxia (Fig. 7E, right panel). Consistent with the ability of multiple PKC isoforms to activate downstream targets, increased expression of pTF[−111/+14]Luc was also observed after cotransfection with constructs encoding CA-PKCa or CA-PKCe. As indicated above, these data suggest that specificity for involvement of PKCβII in the cascade leading to Egr-1 and tissue factor transcription consequent to oxygen deprivation occurs at the level of preferential hypoxia-mediated activation of this PKC isoform.

Role of Raf, MEK, and MAP Kinases—Studies were performed to link activation of PKCβII to activation of MAP kinases and Elk-1 in MPs subjected to oxygen deprivation. Activation of ERK 1/2 occurred within 10 min of subjecting MPs to hypoxia, based on an in-gel kinase assay (Fig. 8A). Densitometry demonstrated an ~5–7-fold increase in the intensity of the immunoreactive ERK 1/2 bands after oxygen deprivation (comparing band intensity in normoxia (N) and hypoxia (H) 5 min). The central role of ERK 1/2 in activation of Egr-1 transcription was shown using constructs encoding DN-ERK 1 and DN-ERK 2. Cotransfection of MPs with pYSF62 and DN-ERK 1 or DN-ERK 2 suppressed hypoxia-induced expression of the luciferase reporter (Fig. 8C), consistent with the possibility that either
DN form blocked interaction of ERKs with their downstream targets. These results suggested that MEK 1/2 might also be involved in the pathway leading to hypoxia-mediated Egr-1 transcription. Addition of the MEK inhibitor PD98059 to hypoxic MPs completely suppressed Egr-1 transcripts (Fig. 8B).

Consistent with these results, hypoxia-mediated ERK 1/2 activation, assessed by in-gel kinase assay, was suppressed in the presence of PD98059 (Fig. 8A). At the concentration employed (10 μM), the MEK inhibitor is relatively selective for MEK 1 (IC₅₀ ~ 1–3 μM), although it may also have a lesser effect on MEK 2 (IC₅₀ ~ 50 μM) (37).

Next, we sought to define a possible role for Raf in events leading to Elk-1 and Egr-1 activation. Cotransfection of MPs with DN-Raf and pYSF62 prevented increased transcription of Egr-1, based on evaluating luciferase activity after exposure of cells to hypoxia (Fig. 8D).

It was important to relate activation of Elk-1 in hypoxic MPs to each of the steps examined above. The GAL4 system was used to assess Elk-1 activation (46), along with transfection of one of the following constructs: either DN-PKCβII, DN-Raf, DN-ERK 1, DN-ERK 2, or pcDNA3 alone. After transfection, MPs were transferred to the hypoxia chamber, and expression of luciferase activity was monitored subsequently. In each case, the dominant-negative construct blocked expression of luciferase activity, whereas pcDNA3 alone was without effect (Fig. 5A). The GAL4-Elk-1-luciferase expression system was tested further by transfecting cultured MPs with CA-PKCβII; under these conditions, increased luciferase activity was observed in normoxia (Fig. 5C).

As expected, overexpression of CA-PKCα or CA-PKCε in normoxic MPs also resulted in increased Elk-1-

**Fig. 8. Involvement of MAP kinases, MEK, and Raf in hypoxia-mediated induction of Egr-1. Panel A, MAP kinases (ERK 1 and ERK 2). MPs were placed in hypoxia (H) for 10 min, cells were lysed, and lysates were subjected to electrophoresis for the in-gel kinase assay using myelin basic protein as the substrate. Autoradiograms show two bands of ~42 and ~44 kDa, corresponding to ERK 1 and ERK 2, respectively. Where indicated, cells were maintained in normoxia only (N), or cultures were pretreated for 1 h with 10 μM PD98059 in normoxia and were then exposed to hypoxia for 10 min. In the lane labeled PMA, 50 ng/ml phorbol 12-myristate 13-acetate was added for 10 min under normoxic conditions. Panel B, MPs were preincubated with 10 μM PD98059 under normoxia for 1 h and were then placed in hypoxia for 30 min. Total RNA was extracted for Northern analysis with ³²P-labeled Egr-1 or β-actin probe. Panel C, effect of ERK activation on Egr-1 transcription. MPs were cotransfected with 1.5 μg of pYSF62, 1 μg of pCMV-β-galactosidase, and either DN-ERK 1 (pCMV-ERK 1[K→R]), DN-ERK 2 (pCMV-ERK 2[K→R]), or pcDNA3 alone (1 μg in each case). Cultures were placed in hypoxia, and relative luciferase activity was determined as described under “Experimental Procedures.” Panel D, MPs were cotransfected with DN-Raf (pCGN raf N4) or pcDNA3 alone (1 μg in each case) along with 1.5 μg of pYSF62 and 1 μg of pCMV-β-galactosidase, placed in hypoxia, and relative luciferase activity was then determined as described under “Experimental Procedures.”
pose that this sequence of events, initiated virtually at the onset of hypoxic stress, sets the stage for vascular dysfunction by providing a nidus for generation of procoagulants and fibrin.

The most far-reaching implication of these results concerns the potential of activated PKCβII and Egr-1 in monocytes to recruit multiple downstream cellular targets thereby engaging a range of cellular effector mechanisms. Thus, induction tissue factor, presumably, represents just one of many possible cellular responses modulated by this pathway. Nonetheless, in view of the importance of tissue factor for initiating coagulation, its expression in the intravascular space is of significance and serves as an appropriate starting point for analyzing roles of PKCβII and Egr-1 in ischemic stress. There are intriguing parallels between our results concerning Egr-1 expression and activation using hypoxia as the stimulus and observations of others concerning the role of Egr-1 in the cellular response to urea and shear stress (23, 47).

Hypoxia-mediated activation of transcription via Elk-1 in MPs, presumably in complex with serum response factor (43), is analogous to previous observations in HeLa cells concerning expression of c-fos (5, 42). This pathway of hypoxia-associated induction of transcription at SREs may also explain, in part, SRE-dependent induction of the noninsulin-dependent glucose transporter (GLUT1) associated with oxygen deprivation and inhibitors of aerobic respiration (41). In the latter case, hypoxic induction of GLUT1 resulted from two interactive mechanisms: direct effects of oxygen deprivation, due to an HIF-1 DNA binding motif, and a response to inhibition of mitochondrial respiration, mediated by sequences within 100 nucleotides 5’ of the HIF-1 site, which contained an SRE. These considerations suggest that the PKCβII/Egr-1 pathway under study in our work is most likely a hypoxic stress-associated pathway rather than a direct response to a change in ambient oxygen levels, the latter an apparent property of HIF-1α (1). However, the capacity of cellular mechanisms activated by PKCβII/Egr-1 to modulate cellular behavior emphasizes their relevance to the host response to ischemic challenge.

In the setting of ischemia, much attention has been focused on the contribution of PKC isoforms to preconditioning of the myocardium and acute myocardial injury, although their role remains controversial (48, 49). Changes in PKC activity and isoform-specific increases in PKC in the membrane fraction have also been noted during ischemic neurodegeneration in the cerebellum and hippocampus in a canine model (50, 51). Fewer experiments have addressed the role of PKCβII in ischemia, although a recent study has employed the PKCβ inhibitor LY333531 in a porcine model of ischemia-induced preretinal neovascularization (52). Administration of the PKCβ inhibitor suppressed neovascularization, consistent with a contribution of PKCβ to cellular events underlying angiogenesis. In this context, tissue factor expression (shown to be induced by hypoxia in our studies) (19) has been associated with angiogenesis, especially in tumor neovasculature (53–55), and it is possible that hypoxia-regulated PKCβ activation provides a link between these two events. However, most attention with regard to participation of PKCβII in vascular pathology has focused on cultured endothelial cells exposed to high levels of ambient glucose and implications of the observed PKCβII activation for vascular complications in diabetes (45). Our study in cultured MPs adds a new facet to the biology of PKCβII in the setting of oxygen deprivation: activation of PKCβII causes subsequent expression of Egr-1 and, downstream, induction of tissue factor.

Egr-1 is an ubiquitous transcription factor previously ascribed roles in a range of physiologic and pathophysiologic processes (26). With the generation of Egr-1 knockout mice, the
biologic contexts of Egr-1 function have been defined (56). Egr-1 null mice developed normally, and the only defect noted early on was infertility in females caused by failure to produce luteinizing hormone-releasing hormone (56). Subsequently, we demonstrated that expression of tissue factor and fibrin deposition in response to hypoxia were severely blunted in Egr-1 null mice, indicating a role for this transcription factor in ischemic stress (19). Previous studies have shown Egr-1 activation in other ischemic situations, such as renal and cardiac ischemia/reperfusion (57, 58), and it is tempting to speculate that induction of tissue factor in monocytes subjected to local hypoxemia might magnify the ischemic response. Because increased expression and activation of Egr-1 in response to oxygen deprivation occurred independently of HIF-1, as shown by our experiments in hepatoma cells deficient in HIF-1b function, Egr-1-mediated effects in ischemia may drive other facets of the host response. An important level of HIF activation is known to participate in the induction of tissue factor, especially in epithelial-like cells and vascular smooth muscle (28). Induction of tissue factor has also been shown previously to involve PKC, based on studies with phorbol esters and general PKC inhibitors (59). This is the first study to report, to our knowledge, linking hypoxia to activation of PKCβII, induction of Egr-1, and expression of tissue factor in MPS. Although activation of PKCβII and Egr-1 is clearly not specific for hypoxia-associated cell stress, by recruiting multiple cellular effector mechanisms, this pathway may have considerable impact on the outcome of ischemic events. Further studies will be required to determine the contribution and consequences of the PKCβII/Egr-1 pathway in hypoxia and ischemia.

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