Placenta Growth Factor-induced Early Growth Response 1 (Egr-1) Regulates Hypoxia-inducible Factor-1α (HIF-1α) in Endothelial Cells*\(^{1,2}\)

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Leukotrienes, the lipid inflammatory products derived from arachidonic acid, are involved in the pathogenesis of respiratory and cardiovascular diseases and reactive airway disease in sickle cell disease. Placenta growth factor (PIGF), elaborated from erythroid cells, increased the mRNA expression of 5-lipoxygenase and 5-lipoxygenase-activating protein (FLAP) in human pulmonary microvascular endothelial cells. PIGF-induced both promoter activity and mRNA expression of hypoxia-inducible factor-1α (HIF-1α), which was abrogated by early growth response-1 (EGR-1) small interfering RNA. PIGF showed a temporal reciprocal relationship in the mRNA levels of EGR-1 and NAB2, the latter a repressor of Egr-1. Moreover, Nab2, but not mutant Nab2, significantly reduced promoter activity and mRNA expression of HIF-1α and also reduced expression of the HIF-1α target gene FLAP. Furthermore, overexpression of Egr-1 led to increased promoter activities for both HIF-1α and FLAP in the absence of PIGF. Additionally, the Egr-1-mediated induction of HIF-1α and FLAP promoters was reduced to basal levels by EGR-1 small interfering RNA. The binding of Egr-1 to HIF-1α promoter was corroborated by electrophoretic mobility shift assay and chromatin immunoprecipitation assay, which showed increased Egr-1 binding to the HIF-1α promoter in response to PIGF stimulation. These studies provide a novel mechanism for PIGF-mediated regulation of HIF-1α via Egr-1, which results in increased FLAP expression. This study provides a new therapeutic target, namely Egr-1, for attenuation of elevated leukotriene levels in patients with sickle cell disease and other inflammatory diseases.

Leukotrienes (LT)3 are physiological lipid mediators that have roles in innate immune responses and pathological roles in inflammatory diseases, such as asthma, airway hyper-reactivity, allergic rhinitis, acute lung injury, and atherosclerosis (1–5). LT are synthesized from substrate arachidonic acid (AA) by 5-lipoxygenase (5-LO) in concert with 5-lipoxygenase-activating protein (FLAP). FLAP itself does not have enzymatic activity; however, it enhances the binding of AA to 5-LO and thus is essential for LT biosynthesis (6). 5-LO oxidizes AA to 5-hydroperoxyicosatetraenoic acid, which is converted into leukotriene A4 (LTA4). LTB4 and cysteinyl leukotrienes-LTC4, LTD4, and LTE4 are formed from LTA4. Although leukocytes generate large amounts of LT from AA, nonleukocyte cells, such as endothelial cells, do not have sufficient 5-LO and FLAP to mediate synthesis of LT (5). However, endothelial cells can take up leukocyte-derived LTA4 and convert it into bioactive LT by transcellular biosynthesis (7, 8). LTB4 is a potent chemoattractant for leukocytes and mediates inflammation, although cysteinyl-LT are potent bronchoconstrictors that are involved in edema, inflammation, and secretion of mucus in asthma (9). In sickle cell disease (SCD), steady state levels of plasma and urinary LTB4 are elevated, which can undergo a further increase during vaso-occlusive crises and acute chest syndrome (10).

Increased urinary LTE4 levels are associated with a higher incidence of pain, both in adults and children with SCD (11). However, the molecular mechanisms of induction of LT in SCD are not yet completely understood. Previously, we showed that plasma levels of placenta growth factor (PIGF) are higher in SCD subjects compared with healthy individuals and correlate with increased incidence of vaso-occlusive events (12). In our recent studies, we show that PIGF increases mRNA expression of both hypoxia-inducible factor-1 (HIF-1α) and FLAP in monocytic cells (13).

HIF-1α is the key transcription factor involved in the expression of genes induced under low oxygen conditions (14, 15). Under normoxia, HIF-1α protein is subjected to tightly regulated prolyl hydroxylase (PHDs 1–3)-mediated degradation. However, the degradation of HIF-1α is prevented under hypoxic conditions, resulting in its accumulation and translocation to the nucleus where it forms active heterodimers with mobility shift assay; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; FLAP, 5-lipoxygenase-activating protein; AA, arachidonic acid; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; PAEC, pulmonary aortic endothelial cells; RPA, RNase protection assay; PI3K, phosphoinositide 3-kinase.

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* The on-line version of this article (available at http://www.jbc.org) contains supplemental “Experimental Procedures,” Table S1, and Figs. S1 and S2.

1 The abbreviations used are: LT, leukotriene; 5-LO, 5-lipoxygenase; Egr-1, early growth response 1; FLAP, 5-lipoxygenase-activating protein; HIF-1α, hypoxia-inducible factor-1α; HPVMVE, human pulmonary microvascular endothelial cells; HRE, hypoxia-response element; PIGF, placenta growth factor; SCD, sickle cell disease; t-HBEC, transformed brain endothelial cells; qRT-PCR, quantitative real-time PCR; WT, wild type; EMSA, electrophoretic

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Egr-1 Regulates PlGF-induced HIF-1α

HIF-1β leading to transcription of hypoxia-related genes (16). Recently, we showed that up-regulation of HIF-1α mRNA levels in response to PlGF was independent of hypoxia both in endothelial cells and monocytes (13, 17). However, the molecular mechanisms of PlGF-mediated elevation in HIF-1α expression are not yet studied. Moreover, growth factors (18), lipopolysaccharide (19), and TNF-α (20) have been shown to increase HIF-1α mRNA expression, independently of hypoxia. Importantly, hypoxia, lipopolysaccharide, and TNF-α have been shown to increase HIF-1α mRNA expression via activation of NF-κB (20–22). Studies by Karin and co-workers (23) show that depletion of IKKβ in macrophages results in the down-regulation of HIF-1α mRNA, showing that NF-κB regulates HIF-1α transcription in vivo. However, only limited information is available with respect to the mechanisms of HIF-1α transcriptional regulation by other transcription factors. The HIF-1α gene promoter is TATA-less and consists of a GC-rich sequence, and its constitutive expression has been shown to be regulated by Sp1-binding sites (24, 25). The early growth response-1 (Egr-1) transcription factor has been shown to regulate gene expression by interacting with GC-rich promoter elements and also by displacing Sp1 from its binding sites in the promoter, upon induction by various stimuli (26, 27). We therefore examined the role of Egr-1 in PlGF-induced HIF-1α and FLAP gene expression.

In this study, we show that PlGF augments 5-LO and FLAP mRNA expression in human pulmonary microvascular endothelial cells (HPMVEC), which required activation of the transcription factors Egr-1 and HIF-1α. Our studies showed that treatment of HPMVEC with PlGF leads to a rapid increase in EGR-1 mRNA expression. We identified the mechanisms of PlGF-regulated HIF-1α transcription through Egr-1 by reporter gene assay, electrophoretic mobility shift assay (EMSA), and analysis (ChIP). Importantly, we showed that overexpression of Nab2, a repressor of Egr-1 activity, attenuated PlGF-mediated HIF-1α promoter activity and its transcription. Moreover, we showed that PlGF-mediated activation of Egr-1 results in the up-regulation of HIF-1α, in a hypoxia-independent manner, which in turn activated the downstream target gene FLAP. The absence of Egr-1-binding elements in the FLAP promoter thus obviates directed activation by Egr-1 and supports a sequential mechanism requiring HIF-1α.

EXPERIMENTAL PROCEDURES

Cells and Reagents—HPMVEC were obtained and cultured as described previously (28). Transformed human brain endothelial cells (t-HBEC) were obtained from Dr. Stins (29) and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 1 mM glutamine, 1 mM sodium pyruvate, 5 mM Hepes, minimal essential medium vitamins, and nonessential amino acids (one time), 50 μg/ml endothelial cell mitogen, and heparin (20 units/ml). Unless otherwise indicated, both HPMVEC and t-HBEC were kept overnight in their respective medium containing serum, followed by serum-free media for 3 h, prior to treatment with PlGF (250 ng/ml) or any other experimental conditions.

Reagents were obtained as follows: human recombinant PlGF and vascular endothelial growth factor (VEGF-A) were obtained from R & D Systems (Minneapolis, MN); pharmacological inhibitors of cell signaling (supplemental Table S1) were obtained from Tocris Bioscience (Ellisville, MO); [32P]UTP was from (ICN Biomedical Inc. (Irvine, CA); primary antibodies against Egr-1, HIF-1α, FLAP, 5-LO, vascular endothelial growth factor receptor-1 (VEGFR1), β-actin, and secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise specified, all other reagents were purchased from Sigma. HIF-1α siRNA and scrambled (sc) siRNA were synthesized at the Microchemical Core Facility of the University of Southern California Norris Comprehensive Cancer Center. TranSilent siRNA vectors for EGR-1 were from Panomics Inc. (Fremont, CA).

mRNA Analysis—Total RNA was isolated using TRIzol reagent (Invitrogen), and the RNase protection assay (RPA) was carried out using custom-made Riboquant multiprobe templates consisting of HIF-1α, FLAP, 5-LO, and GAPDH (BD Biosciences), as described previously (30, 31). The band intensities were analyzed on an Alphalmager 2000 gel documentation system (Alpha Innotech Corp., San Leandro, CA). Quantitative real time (qRT)-PCR was performed with the iScript one-step RT-PCR kit using SYBR Green (Bio-Rad) as per the supplier’s instructions on an ABI PRISM 7900 instrument (Applied Biosystems, Foster City, CA). Briefly, 40 cycles of amplification were carried out after reverse transcription at 95 °C for 10 s and 60 °C for 30 s, using the primers listed in Table 1. The mRNA levels were normalized to GAPDH mRNA for both RPA and qRT-PCR.

Transient Transfection—HPMVEC (1 × 10⁶) were resuspended in 100 μl of unsupplemented plain RPMI 1640 medium containing appropriate siRNA constructs (50 nM) or luciferase reporter plasmids or several expression plasmids and transfected by nucleofection using the S-05 program in a nucleofector device (Lonza, Basel, Switzerland) (32). The β-galactosidase plasmid (0.5 μg) was cotransfected with reporter constructs (0.5 μg) to monitor transfection efficiency. After nucleofection, the cells were kept in complete medium overnight followed by serum-free medium for 3 h and treated with PlGF for the indicated times. The cell lysates were analyzed for luciferase and β-galactosidase activity using kits (Promega, Madison, WI). Luciferase values were normalized to β-galactosidase values. Data are expressed relative to the activity of the promoter-less pGL3 basic vector.

Western Blot Analysis—The cytosolic and nuclear extracts were prepared from untreated and PlGF-treated cells. Briefly, 2 × 10⁶ cells were resuspended in 400 μl of cell lysis buffer for 20 min, and cytosolic supernatants were collected by centrifugation at 10,000 × g for 30 s. The nuclear extract was obtained by resuspending the pellet in 50 μl of nuclear extraction buffer for 1 h at 4 °C. The cytosolic extracts isolated from t-HBEC were subjected to SDS-PAGE analysis for Egr-1, HIF-1α, and FLAP protein expression. Blots were stripped and reprobed with β-actin antibody to monitor protein loading. The protein bands were visualized using Immobilon Western reagents (Millipore Corp., Billerica, MA). The membrane for 5-LO blot was developed using Lumigen TMA-6 detection reagent (GE Healthcare) and visualized...
Egr-1 Regulates PIGF-induced HIF-1α

RESULTS

PIGF Augments mRNA Expression of 5-LO and FLAP in HPMVEC—Previous studies have shown that cultured human pulmonary arterial endothelial cells (PAEC) express low levels of 5-LO and therefore do not form LT. However, overexpression of 5-LO in human PAEC results in an increase in LT formation (33). Thus, we examined whether expression of 5-LO and FLAP, key regulatory molecules in LT formation, were modulated in HPMVEC upon PIGF stimulation. As shown in Fig. 1A, there was a time-dependent (1–6 h) increase in FLAP mRNA expression, which was optimum (∼2-fold increase) at 6 h. However, 24 h post-induction, FLAP mRNA expression returned to the basal levels. The endogenous mRNA expression of 5-LO was undetectable, which was increased upon PIGF treatment for 6 h (Fig. 1A). PIGF-mediated increase in FLAP mRNA expression was also verified by qRT-PCR (Fig. 1B), which showed results similar to those observed in RPA. In addition, qRT-PCR results showed increased HIF-1α expression in PlGF-treated HPMVEC at the indicated times.

Using a Fujifilm LAS-4000 Luminescent Image Analyzer (Stamford, CT). The same membrane was stripped and reprobed for β-actin to examine protein loading.

EMSA—The single-stranded wild type (WT) and mutant oligonucleotides used as probes are listed in Table 1. The oligonucleotides were biotin-labeled as per the manufacturer’s instructions (Pierce) followed by annealing of the complementary strands in an equimolar amount. The DNA binding reaction consisted of nuclear extract (10 μg), 5% glycerol, 5 mM MgCl₂, 50 ng/μl poly(dI-dC), 0.05% Nonidet P-40, and 1 ng of biontinylated probe. The specificity of DNA-protein interaction was demonstrated using 50-fold excess unlabeled probe. In supershift assays, nuclear extracts were preincubated for 1 h on ice with the indicated antibody (2 μg). The samples were then subjected to native 6% PAGE in 0.5× TBE, transferred to a Hybond-N⁺ nylon membrane (Amersham Biosciences), and developed with streptavidin-horseradish peroxidase/chemiluminescence.

ChIP Assay—HPMVEC (5 × 10⁶ cells) were treated with PIGF in the presence or absence of indicated inhibitors for indicated times. ChIP analysis was performed utilizing either Egr-1 antibody or HIF-1α antibody or control rabbit IgG as described previously (28). Briefly, 5 μl of DNA sample was subjected to PCR amplification utilizing primers for HIF-1α and FLAP promoter region (primers are listed in Table 1). PCR was performed for 30 cycles under the following conditions: 94°C for 60 s, 56°C for 60 s, and 72°C for 45 s. The PCR products were subjected to agarose gel electrophoresis. Densitometric quantification of ChIP products was performed using Spot-Denso software on Alpha Imager 2000 gel documentation system. The values were normalized to input DNA.

Statistical Analysis—Data are presented as means ± S.E. The significance of difference in mean values between multiple groups was analyzed with parametric one-way analysis of variance followed by a Tukey-Kramer test using Instat 2 software program (GraphPad, San Diego). Student’s t test was used to evaluate the significance of difference between two groups of experiments.

**RESULTS**

**Egr-1 Regulates PIGF-induced HIF-1α in HPMVEC.** A, RPA analysis of FLAP, 5-LO, and GAPDH in total RNA isolated from untreated and PIGF (250 ng/ml)-treated HPMVEC at the indicated times. B, qRT-PCR analysis of HIF-1α and FLAP mRNA in PIGF-treated HPMVEC at the indicated times. C, qRT-PCR analysis of Egr-1, NAB2, HIF-1α, FLAP, 5-LO, and VCAM-1 mRNA in VEGF-treated (250 ng/ml) HPMVEC at the indicated times. D, HPMVEC were pretreated for 30 min with pharmacological inhibitors of HIF-1α, ascorbate (25 μM), PI3K, LY294002 (10 μM), NADPH oxidase, and diphenyleneiodonium (DPI) (10 μM) followed by PIF treatment for 6 h. Total RNA was isolated and subjected to RPA for the expression of the indicated genes. E, HPMVEC were transfected with indicated siRNA or scrambled siRNA constructs followed by PIF treatment for 6 h. Total RNA was subjected to RPA analysis of HIF-1α, FLAP, 5-LO, and GAPDH. Data are representative of three independent experiments.
mRNA expression in PIGF-treated HPMVEC (Fig. 1B). To assess the specificity of PIGF action, we treated HPMVEC with VEGF-A, the most potent angiogenic factor of the VEGF family. The mRNA expression of FLAP and HIF-1α was unchanged, whereas expression of 5-LO remained undetected upon VEGF stimulation at the indicated times (Fig. 1C). However, VEGF led to a 4.5-fold increase in VCAM-1 mRNA expression at 2 h, as expected, and showed that the VEGF was biologically active. Next, we carried out estimation of immunoreactive LT in culture supernatants of PIGF-treated HPMVEC, which showed a 4.5-fold increase in VCAM-1 mRNA expression at 2 h, as expected, and showed that the VEGF was biologically active. We then examined the effect of PIGF on the mRNA expression levels of EGR-1 and its repressor NAB2 (35, 36). Treatment of HPMVEC with PIGF showed an increase in EGR-1 mRNA expression within 30 min, which returned to the basal levels by 4 h (Fig. 2A). In contrast, the levels of NAB2 mRNA declined by half after 30 min, which were further reduced at 60 min. Remarkably, NAB2 mRNA levels showed recovery after 60 min of stimulation, as evident from levels observed at 4 and 8 h, respectively (Fig. 2A). These data showed a temporal reciprocal association between the expression levels of EGR-1 and NAB2 mRNA in response to PIGF in HPMVEC. In contrast to the effect of PIGF, VEGF stimulation of HPMVEC did not alter the mRNA expression of EGR-1 and NAB2 compared with untreated cells (Fig. 1C). We then examined whether PIGF increased functional Egr-1 DNA binding activity in the nuclear extracts of HPMVEC. As shown in Fig. 2B, bind-
expression in cytosolic extracts of stimulated t-HBEC (Fig. 2C). The levels of Egr-1 protein peaked during the first 2 h after stimulation followed by a decrease from 4 to 8 h. In contrast, the levels of HIF-1α, FLAP, and 5-LO proteins were relatively low during the first 2 h followed by an increase from 4 to 8 h after stimulation. These results showed that PlGF increased Egr-1 protein expression at an early time period, although the increase in HIF-1α, FLAP, and 5-LO proteins occurred at a later time point.

**Nab2 Represses PlGF-induced Egr-1 Transcriptional Activity**—We performed transcription reporter assays to examine the known repressor effect of Nab2 on Egr-1 transcriptional activity. HPMVEC were transfected with a luciferase reporter construct containing four Egr-1-binding sites (pEgr-1-Luc). Upon PlGF treatment of HPMVEC transiently transfected with pEgr-1-Luc, we observed a 4-fold increase in luciferase activity, whereas HPMVEC transfected with plasmid lacking Egr-1-binding sites (pCtrl-Luc) did not show increased luciferase activity above the basal levels (Fig. 2D). When HPMVEC were cotransfected with Nab2 WT plasmid in addition to pEgr-1-Luc, there was a significant reduction in luciferase reporter expression (Fig. 2D). This result was specific because cotransfection of reporter with the Nab2 mutant (Nab2:ΔNCD2) caused no significant reduction in reporter expression (Fig. 2D). The interaction of a physiological repressor of Egr-1, namely Nab2, corroborates our observation that PlGF-induced responses are mediated through Egr-1.

**PlGF Induced Egr-1 Binding to HIF-1α Promoter**—Because EGR-1 siRNA reduced PlGF-induced HIF-1α mRNA expression in HPMVEC, we analyzed the HIF-1α promoter for the presence of cis-acting Egr-1-binding elements. In silico analysis of the HIF-1α promoter (−863/+5 bp) revealed the presence of high GC content (67%), multiple Egr-1/Spi1, NF-kB, and AP-1-binding sites, and the absence of a canonical TATA box. The presence of a physiological repressor of Egr-1, namely Nab2, corroborates our observation that PlGF-induced responses are mediated through Egr-1.

**PIGF Regulates Egr-1 and HIF-1α Expression in t-HBEC**—Because HIF-1α expression in t-HBEC is critical for wound healing and angiogenesis, we investigated the effect of PlGF on HIF-1α expression. h-HBEC were transfected with a luciferase reporter construct containing four Egr-1-binding sites (pEgr-1-Luc). Upon PlGF treatment of h-HBEC, we observed a 4-fold increase in luciferase activity, whereas HPMVEC transfected with plasmid lacking Egr-1-binding sites (pCtrl-Luc) did not show increased luciferase activity above the basal levels (Fig. 2D). When HPMVEC were cotransfected with Nab2 WT plasmid in addition to pEgr-1-Luc, there was a significant reduction in luciferase reporter expression (Fig. 2D). This result was specific because cotransfection of reporter with the Nab2 mutant (Nab2:ΔNCD2) caused no significant reduction in reporter expression (Fig. 2D). The interaction of a physiological repressor of Egr-1, namely Nab2, corroborates our observation that PlGF-induced responses are mediated through Egr-1.

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**Egr-1 Regulates PI GF-induced HIF-1α**

**Role of Egr-1 and Nab2 in PI GF-induced HIF-1α promoter activity.** HPMVEC transfected with the HIF-1α promoter construct pHHF1A (−863/+5 bp)-Luc along with β-galactosidase plasmid were either cotransfected with indicated siRNA (A) or with expression plasmids (B) prior to 6 h of PI GF stimulation. Each construct was used at 0.5 μg for transfection. Estimation of luciferase and β-galactosidase activities was carried out as described under “Experimental Procedures.” The data represent the means ± S.E. of three independent experiments. ***, p < 0.001; ns, nonsignificant.

**Gene/fragment location**

| Method | Forward sequence | Reverse sequence |
|--------|------------------|------------------|
| qRT-PCR | ctcagaagctgagcctca | aaggcataagcgaagcag |
| qRT-PCR | tcgagcctgacccagacgct | cggcctgacccagacgcc |
| qRT-PCR | gacccctgaccccgacgct | cttgagcctgaccccgacgct |
| qRT-PCR | cgggacccagcccagcttc | acggcataagcgaagcag |
| ChIP | atiggcctgacccggagcagca | cccctggagacagactagagpa |

**Oligonucleotide primers used in this study**

| Gene/fragment location | Method | Forward sequence | Reverse sequence |
|------------------------|--------|------------------|------------------|
| Human HIF-1α | qRT-PCR | ctcagaagctgagcctca | aaggcataagcgaagcag |
| Human FLAP | qRT-PCR | tcgagcctgacccagacgct | cggcctgacccagacgcc |
| Human EGR-1 | qRT-PCR | gacccctgaccccgacgct | cttgagcctgaccccgacgct |
| Human NAB2 | qRT-PCR | cgggacccagcccagcttc | acggcataagcgaagcag |
| ERE (−380/−372 bp) in HIF-1α promoter | EMSA | ctcagaagctgagcctca | aaggcataagcgaagcag |
| ERE mutant in HIF-1α promoter | EMSA | tcgagcctgacccagacgct | cggcctgacccagacgcc |
| HRE (−170/−167 bp) in FLAP promoter | EMSA | gacccctgaccccgacgct | cttgagcctgaccccgacgct |
| HRE mutant in FLAP promoter | EMSA | cgggacccagcccagcttc | acggcataagcgaagcag |
| FLAP promoter (−310/+9 bp) | ChIP | atiggcctgacccggagcagca | cccctggagacagactagagpa |
| HIF-1α promoter (−455/+154 bp) | ChIP | atiggcctgacccggagcagca | cccctggagacagactagagpa |

**Egr-1 Regulates PI GF-induced HIF-1α**

Promoter Activation Requires Egr-1

Further validation of Egr-1 protein binding to the HIF-1α promoter was obtained by ChIP analysis of the native chromatin from PI GF-treated HPMVEC. As shown in Fig. 3C, chromatin samples immunoprecipitated with Egr-1 antibody led to a 3-fold increase in the expected PCR product size of 323 bp, corresponding to the HIF-1α promoter (−455/−132 bp) containing at least two Egr-1-binding sites. The amplification of the ChIP product was significantly reduced by pretreatment of cells with either antibody to VEGFR1 or curcumin, a putative Egr-1 inhibitor (37). The amplification of input DNA was equal in all the samples (Fig. 3C, 2nd panel), and immunoprecipitation with control rabbit IgG did not amplify any product (Fig. 3C, 3rd panel). Next, we examined whether Egr-1 also regulated FLAP mRNA expression by directly binding to its promoter in vivo. As shown in Fig. 3C, bottom panel, immunoprecipitation of chromatin from PI GF-treated HPMVEC with an Egr-1 antibody did not show amplification of a PCR product corresponding to the −310/+9 bp region of the FLAP promoter. Taken together, these results showed that Egr-1 binds specifically to the HIF-1α promoter to augment HIF-1α transcription but not to the FLAP promoter in HPMVEC.

**Nab2 Represses PI GF-induced HIF-1α Transcriptional Activity**

Next, we examined whether PI GF mediated Egr-1 increase was directly associated with transcriptional activation of HIF-1α. Transfection of HPMVEC with a luciferase reporter construct (p9HIF-1α-Luc) containing nine hypoxia-response elements (HRE), showed a 5-fold increase in luciferase activity upon PI GF treatment (Fig. 3D, lane 2) compared with untreated cells (lane 1). PI GF-induced HRE luciferase activity was significantly attenuated in cells overexpressing Nab2 WT protein (Fig. 3D, lane 3). However, expression of Nab2 mutant protein did not significantly reduce HRE luciferase activity (Fig. 3D, lane 4). The overexpression of Nab2 WT and Nab2 mutant proteins had no effect on HRE luciferase activity, in the absence of PI GF treatment (data not shown). These results showed that PI GF mediated induction of HRE activity could be repressed by Nab2, a repressor of Egr-1, supporting the role of Egr-1 in transactivation of HIF-1α.

**PI GF-induced HIF-1α Promoter Activation Requires Egr-1 and Is Repressed by Nab2**

To further verify the role of Egr-1 in the regulation of HIF-1α transcription, HPMVEC were cotransfected with HIF-1α promoter plasmid (pHIF1A (−863/+5)-Luc) and EGR-1 siRNA. As shown in Fig. 4A, PI GF increased HIF-1α promoter activity by 4-fold, which was reduced by EGR-1 siRNA but not with scEGR-1 siRNA. Moreover, cotransfection of pHIF1A (−863/+5)-Luc with Egr-1 expression plasmid in HPMVEC resulted in a 5-fold increase in HIF-1α promoter activity, in the absence of PI GF treatment (Fig. 4B, lane 2) compared with cells transfected with an empty vector (Fig. 4B, lane 1). Additionally, Egr-1-mediated HIF-1α promoter activation was attenuated by cotransfection with Nab2 WT expression plasmid. However, cotransfection with Nab2 mutant lacking Egr-1 binding ability, did not affect HIF-1α reporter activity. These results showed that overex-
expression of Egr-1 protein stimulated \(HIF-1\alpha\) promoter activity, in the absence of PIGF stimulation. Taken together, these results clearly suggest that Egr-1 was directly responsible for inducing \(HIF-1\alpha\) promoter activity and that induction was antagonized by the physiological repressor Nab2.

**Egr-1 Increases FLAP mRNA Expression via HIF-1\(\alpha\)—In silico analysis of the FLAP promoter did not reveal the presence of Egr-1-binding sites, thus we examined whether the Egr-1 mediated induction of FLAP promoter activity was dependent on HIF-1\(\alpha\). As shown in Fig. 5A, lane 2, PIGF increased FLAP promoter (\(-371FLAP-Luc\)) activity by 4-fold. Similarly, overexpression of Egr-1 achieved the same result, leading to a 6-fold increase in FLAP promoter activity (Fig. 5A, lane 3), in the absence of PIGF treatment. Moreover, cotransfection of HPMVEC with Egr-1 expression plasmid along with \(HIF-1\alpha\) siRNA, resulted in significantly reduced FLAP promoter activity (Fig. 6A, lane 4). Cotransfection of Egr-1 expression plasmid with \(sCHIF-1\alpha\) siRNA did not show any inhibition in Egr-1 induced FLAP promoter activity, as expected (Fig. 5A, lane 5). The involvement of HIF-1\(\alpha\) in FLAP promoter activation, as a positive control, was confirmed in HPMVEC by overexpression of HIF-1\(\alpha\), which led to a 5-fold increase in FLAP promoter activity (Fig. 5A, lane 6), independent of PIGF stimulation. These results showed that Egr-1 mediated FLAP promoter activation occurred via HIF-1\(\alpha\).

Next, we examined whether Nab2 affected the mRNA levels of both \(HIF-1\alpha\) and FLAP. As shown in Fig. 5B, PIGF treatment of HPMVEC for 6 h resulted in a 3- and 4-fold increase in the mRNA levels of \(HIF-1\alpha\) and FLAP, respectively. However, overexpression of WT Nab2, but not mutant Nab2 protein, led to significantly reduced levels of both \(HIF-1\alpha\) and FLAP mRNA in response to PIGF treatment of transfected HPMVEC. The overexpression of both WT and mutant \(NAB2\) mRNA was confirmed by qRT-PCR, which showed an increase of both WT and mutant \(NAB2\) mRNAs by 2.5-fold, compared with untransfected cells (Fig. 5B). In addition, transfection of Nab2 WT but not Nab2 mutant reduced PIGF-mediated immunoreactive LTB4 release (Supplemental Fig. S1C). These results showed that Nab2 reduced PIGF-induced mRNA expression of both \(HIF-1\alpha\) and FLAP corroborating the upstream role of Egr-1.

**PIGF-mediated FLAP Expression**

![FIGURE 5. Egr-1 regulates FLAP mRNA expression through HIF-1\(\alpha\). A, HPMVEC transfected with \(-371FLAP-Luc\) promoter construct along with \(\beta\)-galactosidase plasmid were either cotransfected with the indicated expression plasmid or with siRNA construct prior to PIGF exposure for 6 h. Each construct was used at 0.5 \(\mu\)g for transfection. Luciferase and \(\beta\)-galactosidase activities were estimated as described under "Experimental Procedures." ***, \(p < 0.001\); ns, nonsignificant. B, qRT-PCR analysis of EGR-1, NAB2, HIF-1\(\alpha\), and FLAP in untreated and PIGF-treated HPMVEC. Where indicated, HPMVEC were transfected with either \(NAB2\) WT or \(NAB2\) mutant construct. C, HPMVEC were cotransfected with either WT (\(-371FLAP-Luc\)) or mutant FLAP promoter constructs (HRE-M1 or HRE-M2 or HRE-M1 + 2 or NF-\(\kappa\)B) and \(\beta\)-galactosidase plasmid, followed by PIGF treatment for 6 h. The luciferase activity was normalized with that of the promoter-less pGL3 basic vector. The data represent means \(\pm\) S.E. of three independent experiments. ***, \(p < 0.001\); **, \(p < 0.01\); ns, nonsignificant. D, HPMVEC were pretreated with either ascorbate or LY294002 or diphenyleneiodonium (DPI) prior to PIGF stimulation for 4 h. The chromatin samples were subjected to immunoprecipitation with either HIF-1\(\alpha\) antibody (1 \(\mu\)g, upper panel) or control rabbit IgG (1 \(\mu\)g, lower panel). Purified DNA was PCR-amplified with the primers (listed in Table 1) corresponding to the region containing both HRE sites (\(-310/+9\) bp) in the FLAP promoter. The input DNA panel represents the amplification of samples before immunoprecipitation. Data are representative of three independent experiments.

| FLAP | WT | - | WT | WT | WT | WT |
|------|----|---|----|----|----|----|
| PIGF | -  | - | +  | +  | +  | +  |
| Nab2WT | - | - | +  | +  | +  | +  |
| Nab2Mut | - | - | -  | -  | -  | -  |
| Egr-1 | - | - | +  | +  | +  | +  |
| Nab2WT + PIGF | - | - | +  | +  | +  | +  |
| Nab2Mut + PIGF | - | - | -  | -  | -  | -  |

In HPMVEC Involves Activation of HIF-1\(\alpha\) but Not of NF-\(\kappa\)B—Because transcriptional activation can be cell- and tissue-sensitive, we examined whether PIGF-mediated FLAP expression in HPMVEC involved the same set of transcription factors as was observed in THP-1 monocytic cells and peripheral blood monocytes (13). We used WT \(-371FLAP-Luc\) and different mutant constructs as described previously (13). There was a 4-fold increase in WT FLAP promoter activity, in response to PIGF treatment of HPMVEC, whereas cells transfected with promoter mutations in HRE site-1 (HIF-1\(\alpha\)-M1) or HRE site-2 (HIF-1\(\alpha\)-M2) showed reduced activity by 50% (Fig. 5C). Moreover, mutation of both HRE sites (HIF-1\(\alpha\)-M1 + 2) in the FLAP promoter showed maximum inhibition (80%) in PIGF-induced FLAP promoter activity. In contrast, an NF-\(\kappa\)B mutant construct did not affect PIGF-induced FLAP promoter activity (Fig. 5C). These results demonstrated that both HRE sites, but not the promoter proximal NF-\(\kappa\)B site, were essential for PIGF-induced FLAP promoter activity in HPMVEC, as was observed previously in monocytes (13).
Egr-1 Regulates PIGF-induced HIF-1α

The role of HRE sites in PIGF-induced FLAP expression was further substantiated by demonstrating increased HIF-1α-DNA binding activity in vitro by EMSA (supplemental Fig. S2) and in vivo by ChIP (Fig. 5D). HPMVEC treated with PIGF showed a 4-fold increase in expected PCR product of 319-bp size corresponding to the FLAP promoter region, whereas only IgG did not amplify any products (Fig. 5D, upper panel). These results indicate that PIGF increases FLAP mRNA expression by promoting HIF-1α binding to FLAP promoter in HPMVEC. Thus, PIGF mediated FLAP transcription in hematopoietic cells, and endothelial cells utilize the same set of transcription factors.

DISCUSSION

In this study, we showed that PIGF, a member of VEGF family, increased mRNA expression of 5-LO and FLAP in human pulmonary microvascular endothelial cells. Most importantly, we identified the molecular mechanisms of PIGF-mediated HIF-1α induction in a hypoxia-independent manner. The effect was specific for PIGF, as VEGF-A, a potent angiogenic factor, did not augment the expression of HIF-1α, 5-LO, and FLAP mRNA. Our results show that PIGF increased levels of the Egr-1 transcription factor, which in turn regulated the transcription of HIF-1α. To the best of our knowledge, this work is the first report that defines the relationship between Egr-1 and HIF-1α.

Our studies identified a role for the transcription factor Egr-1 in regulation of PIGF-induced HIF-1α mRNA expression and its downstream target gene FLAP. Our results showed that EGR-1 siRNA was effective in reducing PIGF-mediated 5-LO and FLAP mRNA expression. The role of Egr-1 in 5-LO transcription is consistent with the presence of several GC-boxes in the human 5-LO gene promoter that are proximal to the transcription start site and are recognized by transcription factors Sp1 and Egr-1 (34, 38). However, PIGF-induced FLAP mRNA expression was attenuated by both EGR-1 siRNA and HIF-1α siRNA, indicating direct or indirect roles of these transcription factors. Previous studies showed that TNF-α- and lipopolysaccharide-induced FLAP promoter activities in THP-1 cells require the first 134 bp of the promoter (−134/+12 bp), which contains binding sites for NF-κB and CCAAT/enhancer-binding protein (39, 40). However, we showed that PIGF-induced FLAP promoter activity in THP-1 cells required HRE, but not the NF-κB site, in the −371/+12 region of the FLAP promoter (13). In silico analysis of the human FLAP promoter did not reveal the presence of bona fide Egr-1/Sp1 sites. Thus, we hypothesized that Egr-1 may have acted through HRE to up-regulate the expression of an HIF-1α-regulated target gene, i.e. FLAP. This study showed that PIGF-induced promoter activity and mRNA expression of FLAP were attenuated by HIF-1α siRNA, which was consistent with our previous findings in THP-1 cells, indicating that both monocytes and endothelial cells utilize the same signaling pathways for FLAP expression. Thus, we examined the role of Egr-1 and its repressor Nab2 (36) in PIGF-induced transcription of HIF-1α.

Egr-1, a zinc finger transcription factor, preferentially binds the GC-rich sequence 5′-TGCCT(G/A)GGCGGT-3′. Egr-1 belongs to a group of early response genes, as stimulation by extracellular stimuli such as growth factors and cytokines rapidly induces EGR-1 gene expression (41–45). Moreover, Egr-1 plays important roles in development, growth control, and differentiation (45). Egr-1-mediated gene transcription is tightly regulated by the repressor proteins NAB1 and Nab2 (35, 47). The function of Nab2 as Egr-1 regulator is more important because its expression is shown to be induced by the same signals that lead to EGR-1 expression, whereas NAB1 is constitutively expressed in most cells (36). Our studies showed that PIGF induced EGR-1 in the early phase of induction (first 30 min), whereas the NAB2 levels were down-regulated in HPMVEC. This was followed by a reduction in EGR-1 mRNA and a concomitant increase in NAB2 mRNA at a later phase of PIGF induction (1–8 h). This relationship suggested a reciprocal mode of regulation in PIGF-mediated EGR-1 and NAB2 expression in HPMVEC. These findings are in concordance with a previous study, which showed a temporal association in the expression of EGR-1 and NAB2, in response to VEGF in endothelial cells (48). However, in this study, stimulation of HPMVEC with VEGF did not alter mRNA expression of both EGR-1 and NAB2. The possible explanation for the differences seen in the studies is perhaps the result of differences in endothelial cell type, dose, and/or duration of VEGF stimulation of HPMVEC. Furthermore, our results showed that WT Nab2 aborted PIGF-driven Egr-1 transcriptional activity, whereas a dominant negative mutant Nab2 expression plasmid (Nab2: ΔNCD2) had no effect on PIGF-induced Egr-1 transcriptional activation. There are at least two separable repression domains.
Egr-1 Regulates PI GF-induced HIF-1α

in Nab2 (NCD1 and NCD2), and the NCD2 region in Nab2 is essential for repression transcription of Egr-1 (49).

Egr-1 coregulates expression of a number of genes containing similar GC-rich sequences, by displacing Sp1 binding from these promoters (26, 27, 34). An examination of the 5′-UTR region of the HIF-1α gene shows several GC-rich sequences, known to be constitutively regulated by Sp1-binding sites (25).

Our results showed that Egr-1 has an essential role for PI GF-induced HIF-1α transcription through direct interaction with the HIF-1α promoter in HPMVEC. We identified several putative Egr-1-binding sites in the promoter of HIF-1α, and we demonstrated that PI GF promotes the functional binding of Egr-1 to at least one of its binding sites (∼74/−68 bp) present in the HIF-1α promoter, as demonstrated by EMSA. Consistent with this observation, ChIP analysis showed increased amplification of the HIF-1α promoter region (∼455/+154 bp) containing Egr-1-binding sites in the chromatin of PI GF-treated cells when immunoprecipitated with Egr-1 antibody. Furthermore, PI GF-mediated HIF-1α promoter activation was inhibited by EGR-1 siRNA. In addition, Egr-1 overexpression stimulated HIF-1α promoter activity, which was completely abolished by coexpression of WT Nab2 protein but not by Nab2 mutant protein. Thus, our results are in line with previous findings where Nab2 inhibited VEGF-induced tissue factor promoter activity (48). Recent studies have implicated cross-talk between NF-κB and HIF-1α genes, where NF-κB regulates HIF-1α promoter activity and mRNA expression in response to H2O2, short duration hypoxia, and TNF-α (20, 21, 50). Thus, the possibility of PI GF-mediated activation of HIF-1α through indirect stimulation of NF-κB cannot be ruled out.

Endothelial cells are generally thought to be incapable of carrying out the conversion of AA to LTA4, because these cells do not express 5-LO enzyme (51). However, they express LTA4 hydrolase and LTC4 synthetase enzymes, which can generate LTB3 and LTC4, respectively, from exogenous LTA4. In addition, other studies have identified the transcellular mode of cysteinyl-LT biosynthesis (8, 52) by uptake of LTA4, which is secreted from neighboring activated peripheral blood leukocytes after their adhesion to vascular endothelium (53). Consequently, a direct role of endothelial cells as an independent source of LT has not been widely accepted. However, low levels of expression of 5-LO have been detected in PAEC (33). In this study, 5-LO mRNA expression was undetectable in HPMVEC at resting stage by both RPA and qRT-PCR, which was induced upon PI GF treatment. Our findings of PI GF-induced 5-LO expression in HPMVEC are consistent with previous studies of increased 5-LO expression in PAEC of patients with primary pulmonary hypertension (54) and in rats exposed to chronic hypoxia (46). Our results showed that PI GF up-regulates FLAP mRNA expression by activation of PI3K, NADPH oxidase, and HIF-1α in HPMVEC as reported previously for PI GF-induced FLAP expression in THP-1 monocytes (13). Previously, we showed that PI GF increases ET-1 mRNA expression in HPMVEC (17). Consistent with our earlier findings (17), we observed an increase in HIF-1α mRNA in HPMVEC in response to PI GF. In this study we show that PI GF-induced 5-LO and FLAP expression occur in both HPMVEC and t-HBEC.

Finally, the functional significance of Egr-1-induced HIF-1α was established by showing the transactivation of its target genes such as FLAP. In silico analysis showed that the FLAP promoter lacks Egr-1-binding sites. However, overexpression of Egr-1 in HPMVEC resulted in increased FLAP promoter activity, which was completely abrogated upon silencing of HIF-1α. Thus, we concluded that Egr-1 regulates FLAP mRNA expression by first activating HIF-1α gene expression. In addition, the Egr-1 repressor Nab2 significantly reduced the expression of both downstream genes, HIF-1α and FLAP, in response to PI GF stimulation of HPMVEC. The direct role of HIF-1α in PI GF-induced FLAP expression was confirmed by different approaches, including silencing with HIF-1α siRNA, site-directed mutagenesis of the FLAP promoter, EMSA (supplemental Fig. S2), and ChIP analysis. The results obtained in this study were consistent with our previous report of PI GF-induced FLAP expression in monocytes (13).

In conclusion, we show that PI GF-induced HIF-1α expression was mediated by Egr-1 and that the Egr-1/HIF1α pathway carried out PI GF-mediated induction of FLAP mRNA expression in endothelial cells (as illustrated in Fig. 6). Experiments defining the molecular signaling pathway(s) responsible for PI GF-mediated Egr-1 induction are currently in progress and will further enhance our understanding of the role of PI GF in pathophysiological complications of SCD.

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