Hypoxia-Mediated Expression of 5-Lipoxygenase–Activating Protein Involves HIF-1α and NF-κB and MicroRNAs 135a and 199a-5p

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Hypoxia occurs in a number of pathological states, such as pulmonary, hematological, and cardiovascular disorders. In this study, we examined the molecular mechanism by which hypoxia contributes to increased leukotriene formation. Our studies showed hypoxia augmented the expression of 5-lipoxygenase activating protein (FLAP), a key enzyme in leukotriene formation, in both human pulmonary microvascular endothelial cells and a transformed human brain endothelial cell line. Hypoxia-induced FLAP mRNA expression involved activation of NADPH-oxidase, PI-3 kinase, mitogen-activated protein kinase, NF-κB, and hypoxia-inducible factor (HIF)-1α. Hypoxia-induced FLAP promoter activity was attenuated on mutation of hypoxia–response elements (HREs) and NF-κB binding motif in the FLAP promoter. Hypoxia also augmented binding of HIF-1α to HREs in FLAP promoter as demonstrated by EMSA with nuclear extracts. Furthermore, chromatin immunoprecipitation analysis showed HIF-1α bound to disease, and inflammation in individuals who have sickle cell disease.

expression. In addition, NF-κB sites in the FLAP promoter were also required for hypoxia-mediated FLAP expression. As microRNAs (miRNAs) have been generally characterized as negative regulators of gene expression (28–31), we examined the possibility that miRNAs were involved in cytoplastic FLAP mRNA stability. Our studies for the first time, to the best of our knowledge, showed the role of miR-135a and miR-199a-5p in the regulation of hypoxia-mediated FLAP mRNA levels and subsequent protein expression.

Materials and Methods

Cell culture

HPMVECs were grown in EBM-1 media supplemented with EBM-2 bulit kit (Lonza, Cologne, Germany) as previously described (32, 33). SV-40-LT transformed human brain endothelial cells (t-HBEC), a gift from Monique Stins, were cultured in RPMI 1640 supplemented with 1 mM t-glutamine, 1× MEM-vitamins, 1× nonessential amino acids, endothelial cell mitogen (50 μg/mL), heparin (20 μU/mL), penicillin-streptomycin (100 μg/mL), 5 mM HepES, 1 mM sodium pyruvate, and 10% heat inactivated FBS in tissue culture flasks coated with 1% sterilized gelatin solution as described (34). Unless otherwise indicated, cells were kept in serum-free media overnight prior to hypoxia treatment.

Reagents

Diphenyleleniodide chloride (DPI), LY294002, PD98059, SP600125, rapamycin, and SB203580 were obtained from Tocris Bioscience (Ellisville, MO). R59949 (diacyl glycerol kinase inhibitor) and sulfaalazine were purchased from Calbiochem (Gibbstown, NJ). These pharmacological inhibitors were used at the indicated concentrations: DPI (10 μM), LY294002 (15 μM), SP600125 (100 μM), SB203580 (1 μM), PD98059 (10 μM), sulfaalazine (2 μM), R59949 (30 μM), and rapamycin (10 μM) as deemed optimal from literature. Primary Abs for HIF-1α, β-actin, and FLAP, and HRP-conjugated secondary Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). siRNA for HIF-1α, scrambled siRNA for HIF-1α (scRNA), PHD-2 siRNA, and PHD-2 scRNA were synthesized at the Microchemical Core facility of the University of Southern California Comprehensive Cancer Center as previously described (32). siRNA for p47phox, p38 MAP kinase, JNK-2, and control siRNA were purchased from Santa Cruz Biotechnology. PTEN overexpression and PI3K dominant negative plasmids were generous gifts from Dr. Debbie Johnson (Keck School of Medicine, University of Southern California, Los Angeles, CA). The –336FLAP-pGL3 full-length FLAP promoter constructs and the deletion constructs (–956FLAP-pGL3, –371FLAP-pGL3, and –134FLAP-pGL3) were generously provided by Dr. Timothy Bign (25) (Veterans’ Administration Hospital San Diego, La Jolla, CA). The pcDNA3-5 LO vector was kindly provided by Dr. Colin Funk (Queen’s University, Kingston, Ontario, Canada).

mRNA extraction and analysis by qRT-PCR

Endothelial cells (t-HBEC and HPMVEC) were incubated in 1% O2 in a Forma tissue culture incubator, equipped with an oxygen sensor, for various periods. Total mRNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). mRNA expression was determined and quantified using specific primers (Table I) by quantitative real-time PCR (qRT-PCR). The qRT-PCR of mRNA templates (100 ng) was performed using the iScript One-Step RT-PCR kit with SYBR green (Bio-Rad) and the Applied Biosystems ABI Prism 7900HT Version 2.3 Sequence Detection System (Foster City, CA). Amplification was carried out as follows: cDNA synthesis at 50 °C for 10 min, iScript reverse transcriptase inactivation at 95 °C for 5 min, and 30 cycles under the following conditions; 95 °C for 30 s, 58 °C for 60 s, and 72 °C for 30 s and detection. Values are expressed as relative expression of mRNA normalized to the housekeeping GAPDH mRNA. Relative quantification (RQ) values for mRNA expression were calculated as 2 ΔΔCt by the comparative Ct method (35), where ΔΔCt = (Ct target gene of treated sample – Ct GAPDH of treated sample) – (Ct target gene of control sample – Ct GAPDH of control sample).

MicroRNA extraction and purification

Approximately 5 × 106 cells were kept in serum-free media for 8 h and treated with 1% O2 for 8 h. miRNAs were isolated and purified using the mirVANA kit (Applied Biosystems/Ambion, Austin, TX), according to manufacturer’s protocol. miRNA levels were detected using miRNA specific primers (Applied Biosystems/Ambion) by qRT-PCR. Ct values obtained were normalized to Ct values for 5S rRNA. RQ values for miRNA expression were calculated as 2 ΔΔCt by the comparative Ct method (35), where ΔΔCt = (Ct target miRNA of samples – Ct 5S of treated sample) – (Ct target gene of control samples – Ct 5S of control sample).

Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts were prepared as previously described (33). Briefly, 5 × 106 cells were washed with PBS and resuspended in cell lysis buffer and centrifuged for 1 min at 10,000 × g. The supernatant (cytosolic extract) was collected and the pellet resuspended in 100 μl nuclear extraction buffer. Nuclear extractions were obtained by centrifugation at 10,000 × g for 10 min. Protein concentrations were determined using the Bradford method.

Western blot analysis

t-HBEC cells (5 × 106) in serum-free media were treated with 1% O2 for the indicated time periods. Protein extracts were subjected to electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with Ab to HIF-1α (1:250) and with Ab to FLAP (1:250). Membranes were also probed for β-actin (1:2500) levels to determine equal loading. Protein bands were detected using Immunobilin Western reagents (Millipore, Billerica, MA).

EMSA

Double-stranded complementary oligonucleotides corresponding to FLAP promoter containing a proximal HRE site at position −170 to −167, and FLAP with a mutation in the same HRE site (Table I) were biotin labeled using a Lighshift Chemiluminescent EMSA kit (27) (Pierce, Rockford, IL). The DNA binding reaction with 5 μg nuclear protein extract, 5% glycerol, 5 mM MgCl2, 50 μg/ml poly(dI: dC), 0.05% NP-40, and 0.5 ng biotinylated FLAP oligoeucleotide were incubated at room temperature for 20 min. The samples were then subjected to a nondenaturating 6% PAGE in 0.5× TBE, transferred to a Hybond-N+ nylon membrane (Amersham Biosciences, Piscataway, NJ), followed by detection of the DNA-protein complex bands with streptavidin-HRP/chemiluminescence (33). The specificity of the interaction between the protein and DNA was demonstrated using a 50-fold excess of unlabeled probe.

Site-directed mutagenesis of FLAP promoter

Mutations in the HRE binding sites and NF-κB site of FLAP promoter were generated using the Quik-Change site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) using primers shown in Table I. The wild-type −965 FLAP luciferase construct was used as a template. Mutations were confirmed by DNA sequencing.

Transient transfections

Endothelial cells were transfected with various siRNA constructs (50 nM) and luciferase reporter constructs by nucleaseion using the S-005 program (Amaza Biosystems, Cologne, Germany) as previously described (36). Briefly, 105 cells were resuspended in 100 μl RPMI-1640 media with 1 μg FLAP luciferase reporter construct and 0.5 μg β-galactosidase reporter plasmid or siRNA (50 nM). Where indicated, 60 pmol anti-miRNA inhibitor (Applied Biosystems/Ambion) and 1 μg miRNA overexpression constructs (Genscript, Piscataway, NJ) were used for transfections. Transfected cells were kept in complete media overnight, followed by serum-free media for 3 h, and then exposed to hypoxia for the indicated periods. For luciferase assays, cells were harvested and analyzed for luciferase activity (Promega, Madison, WI) using a luminometer (Berthold Technologies; Lumat LB 9501), set for 10 s light collection. β-galactosidase activity was assessed by colormetric assay (Promega). The luciferase values were normalized for β-galactosidase activity and expressed as relative luciferase units. For mRNA analysis, cells were lysed in TRIZol, followed by extraction of mRNA.

Chromatin immunoprecipitation assay

t-HBEC (104 cells) were kept overnight in serum-free RPMI-1640, followed by hypoxia for the indicated period. Chromatin immunoprecipitation (ChiP) analysis was performed using HIF-1α Ab as previously described (27). Briefly, cells were fixed with formaldehyde, lysed, and chromatin was sheared by sonication (6 pulses at 15 sec each, 40% potency). The lysate was centrifuged at 10,000 × g for 10 min at 4°C. The supernatants were precleared for 2 h at 4°C with protein A-Sepharose beads (Sigma-Aldrich, St. Louis, MO). Precleared supernatants were immunoprecipitated with HIF-1α Ab or control normal rabbit IgG Ab at 4°C overnight. The immune complexes, with protein A beads were collected and washed sequentially with low-salt buffer, high-salt buffer, and TE buffer. DNA cross-links were reversed at 65°C overnight, and DNA was extracted by phenol/chloroform/isoamyl alcohol, followed by ethanol precipitation. Immunoprecipitated DNA was air-dried and resuspended in 100 μl nuclease-free water. DNA was subjected to PCR amplification for 30 cycles under the following conditions; 95°C for 30 s, 58°C for 60 s, and 72°C for 120 s, using primers listed in Table I. The PCR products were

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subjected to 2% agarose gel electrophoresis, followed by densitometric analysis of amplified product. The values were normalized to input DNA.

**LT assay**

t-HBEC (1 × 106 cells) were transfected with 5-LO plasmid (1 μg) by nucleofection using the S-005 program (Amaza Biosystems) as previously described (36). Cells were kept in complete media for overnight incubation, followed by replacement of media to serum-free media (2 ml). Cells were again kept overnight and exposed to 1% O2 for 24 h in the presence and absence of indicated pharmacological inhibitors. The cell media was removed for cyst LT assay, whereas cells were lysed in RIPA buffer for protein estimation by Bradford method (37). Cyst LT levels in media was removed for cyst LT assay, whereas cells were lysed in RIPA buffer for protein estimation by Bradford method (37). Cyst LT levels in media were determined using EIA kit (Assay Designs, Ann Arbor, MI) in the presence and absence of indicated pharmacological inhibitors. The cell media was removed for cyst LT assay, whereas cells were lysed in RIPA buffer for protein estimation by Bradford method (37). Cyst LT levels in media was removed for cyst LT assay, whereas cells were lysed in RIPA buffer for protein estimation by Bradford method (37).

**Statistical analysis**

Data are presented as means ± SD. Control and hypoxia-treated samples were compared using a Student *t* test. One-way ANOVA, followed by Tukey-Kramer test was used for multiple comparisons using the Instat-2 software program (GraphPad, San Diego, CA). Values of *p* < 0.05 were considered statistically significant.

**Results**

**Hypoxia augments FLAP expression in human brain endothelial cell line (t-HBEC)**

As shown in Fig. 1A, hypoxia (1% O2) caused a time dependent (2, 4, and 8 h) increase in the expression of FLAP mRNA in transformed human brain endothelial cell line (t-HBEC). At 8 h, there was a significant (∼4-fold) increase in mRNA expression of FLAP. It is pertinent to note that basal levels of 5-LO mRNA were low and not augmented by hypoxia (data not shown).

Hypoxia-induced FLAP mRNA expression involves activation of NADPH-oxidase, PI-3 kinase, MAP kinase, NF-κB, and HIF-1α

Pretreatment of t-HBEC with pharmacological inhibitors of PI-3 kinase (LY294002), MAP kinase (PD98059), and p38 MAP kinase (SB203580), followed by exposure to hypoxia for 8 h resulted in reduction of FLAP mRNA expression by (65 ± 3%, 83 ± 5%, and 66 ± 1%, respectively, compared with noninhibitor treated cells (Fig. 1B). However, an N-terminal Jun Kinase inhibitor (SP600125) had no significant effect (Fig. 1B). Furthermore, hypoxia-induced FLAP mRNA expression was reduced by inhibitors for NF-κB (86 ± 5%), NADPH-oxidase (100 ± 2%), and HIF-1α (104 ± 5%), in comparison with hypoxia-treated cells (Fig. 1C). To determine whether hypoxia-mediated signaling used the same pathways in primary endothelial cell cultures, we used HPMVECs. As shown in Fig. 1D, hypoxia-induced mRNA expression of FLAP by ∼3.5-fold in HPMVECs, which was attenuated by LY294002 and R59949 at basal levels.

As pharmacological inhibitors can act in a nonspecific manner, t-HBEC was transfected with either Dn PI-3 Kinase or a PTEN overexpression plasmid, followed by exposure to hypoxia. As shown in Fig. 2A, both PTEN (lane 3) and Dn PI-3 kinase (lane 4) completely reduced hypoxia-induced FLAP mRNA expression. Similarly, transfection of t-HBEC with siRNA for p47^phox^, a subunit of NADPH-oxidase, completely antagonized hypoxia-induced FLAP mRNA expression (Fig. 2B, lane 4). However, scrambled p47^phox^ siRNA had no effect (Fig. 2B, lane 3). Furthermore, transfection of t-HBEC with siRNA for p38MAP kinase (Fig. 2C, lane 4, but not NFK-B2 siRNA (Fig. 2C, lane 5), also completely attenuated hypoxia induced FLAP mRNA expression. Transfection of t-HBEC with siRNA for p65 (a component of the NF-κB complex) reduced hypoxia-mediated FLAP expression below basal levels (Fig. 2D, lane 4). Taken together, these results indicate that hypoxia-induced FLAP mRNA expression involved PI-3 kinase, p38 MAP kinase, NF-κB, and NADPH-oxidase, but not NFK kinase.

**Hypoxia-induced FLAP expression involves activation of HIF-1α**

Because R59949, a putative HIF-1α pharmacological inhibitor, inhibited hypoxia-mediated FLAP expression, t-HBEC was transfected with siRNA for HIF-1α and HIF-2α, and a corresponding scrambled control HIF siRNA (scRNA) as a control. As shown in Fig. 3A, hypoxia-induced FLAP mRNA expression in t-HBEC was reduced to the basal level by HIF-1α siRNA (lane 4), but not with either HIF-2α siRNA (lane 5) or scrambled control siRNA (lane 3). Furthermore, transfection with prolylhydroxylase-2 (PHD-2) siRNA, which stabilizes HIF-1α protein, resulted in an ∼7-fold increase in FLAP expression, under normoxia (Fig. 3B, lane 4). Transfection with scPHD-2 siRNA under normoxia did not affect FLAP mRNA expression (Fig. 3B, lane 3). Similarly, transfection of HPMVECs with HIF-1α siRNA completely reduced hypoxia-induced FLAP mRNA expression (Fig. 3C, lane 4). However, control scHIF-1α siRNA had no effect (Fig. 3C, lane 3). These results indicated that HIF-1α, but not HIF-2α, was involved in hypoxia-mediated FLAP expression in both t-HBEC and HPMVECs.

**Hypoxia-mediated LT formation in endothelial cells requires expression of 5-LO**

Previous studies (38) have shown that cultured human pulmonary aortic endothelial cells (HPAECs) express low levels of 5-LO and therefore, do not form LTs. However, overexpression of 5-LO in

<table>
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For site-directed mutagenesis (SDM) and the HRE mutant oligonucleotides used for EMSA, the mutated nucleotides are indicated in bold.
HPAEC led to an increase in LT formation in response to A23187 (39). In the current study, we observed that t-HBEC cells also show relatively low levels of 5-LO mRNA, which did not change in response to hypoxia (data not shown). Thus t-HBEC were transfected with a 5-LO expression plasmid. The 5-LO transfected t-HBEC, in response to hypoxia, showed ∼1.9-fold increase in cyst LT formation when compared with normoxic conditions (Fig. 3D). The hypoxia-induced cyst LT formation was attenuated by HPAEC led to an increase in LT formation in response to A23187 (39). In the current study, we observed that t-HBEC cells also show relatively low levels of 5-LO mRNA, which did not change in response to hypoxia (data not shown). Thus t-HBEC were transfected with a 5-LO expression plasmid. The 5-LO transfected t-HBEC, in response to hypoxia, showed ∼1.9-fold increase in cyst LT formation when compared with normoxic conditions (Fig. 3D). The hypoxia-induced cyst LT formation was attenuated by

FIGURE 2. Hypoxia induces FLAP mRNA expression in t-HBECs via PI-3 kinase, p38 MAP kinase, NADPH oxidase, and NF-κB. A, Overexpression of Dn PI-3 kinase and PTEN attenuates FLAP mRNA expression. B, siRNA for p47^phox, a subunit of NADPH oxidase, inhibits hypoxia-induced FLAP mRNA expression. C, Effect of p38 siRNA, JNK-2 siRNA, and control siRNA on hypoxia (8 h) induced FLAP mRNA expression. D, Transfection with siRNA for p65, a subunit of NF-κB, also attenuated hypoxia-induced FLAP mRNA expression. Total RNA was isolated and subjected to qRT-PCR. qRT-PCR data are expressed as fold-changes in FLAP mRNA expression, on treatment with hypoxia compared with without treatment. FLAP mRNA levels were normalized to GAPDH mRNA levels. Data are expressed as means ± SD of three independent experiments. ***p < 0.001; **p < 0.01; *p < 0.05; ns, p > 0.05.
LY 294002 (62 ± 3%), DPI (47 ± 2%), and R59949 (68 ± 5%) (Fig. 3D), the same pharmacological inhibitors that inhibited hypoxia-induced FLAP mRNA expression. The substrate, arachidonic acid, for LT synthesis was likely formed endogenously in response to hypoxia, as hypoxia is known to activate phospholipase A2 in endothelial cells (40, 41).

**Hypoxia-mediated upregulation of the FLAP gene requires proximal HREs and NF-κB motif in its promoter**

Because hypoxia-mediated FLAP expression was attenuated by HIF-1α siRNA, we determined whether this occurred via HREs, which are present in promoter regions of several genes, including FLAP (27) as illustrated in the schematics of Fig. 4A. Hypoxia (1% O2) increased by 3.5-fold (Fig. 4B, lane 2) the activity of the full length FLAP promoter (−3368/+12 bp), when compared with promoterless pGL3 construct (Fig. 4B, lane 1). Analysis of serial deletion constructs of FLAP promoter (Fig. 4B) showed that the −965/+12 bp region of the FLAP promoter (lane 3) showed almost similar activity as the full length FLAP promoter (lane 2), whereas the −593/+12 bp construct (lane 4) showed ~80% reduced hypoxia-mediated promoter activity. Furthermore, deletion constructs corresponding to −371/+12 bp (Fig. 4B, lane 5) and −131/+12 bp (Fig. 4B, lane 6) showed >90% reduced FLAP promoter activity. Thus, we used the −965/+12 bp FLAP promoter construct for further studies. This region of the FLAP promoter, as shown in schematics of Fig. 4A, contains four putative consensus HIF-1α binding (RCGTG) elements (−635 to −632 bp, −517 to −514 bp, −264 to −261 bp, and −170 to −167 bp), relative to transcriptional start site, along with a NF-κB site (located at −43 to −34 bp) and a C/EBP consensus binding site (located at −28 bp and −25 to −12 bp). Bigby and his coworkers (25) have shown that LPS-induced FLAP expression in THP-1 cells requires the binding of both NF-κB and C/EBP to the FLAP promoter. As shown in Fig. 4C (lanes 4–7), mutation of HRE sites (HRE-M1, −170 to −167 bp; HRE-M2, −264 to −261 bp; HRE-M3, −517 to −514 bp; and HRE-M4, −635 to −632 bp) resulted in >90% inhibition of hypoxia-induced FLAP promoter activity in t-HBEC. Moreover, mutation of the NF-κB site (located at −43 to −34 bp) in the −965 FLAP promoter, also resulted in >90% reduction of hypoxia-induced FLAP promoter activity (Fig. 4C, lane 8). We determined whether similar transcriptional elements were also involved in primary endothelial cells (HPMVEC). As shown in Fig. 4D, mutation of HRE sites (lanes 4–7) and NF-κB sites (lane 8) in the FLAP promoter attenuated >80% FLAP promoter activity in response to hypoxia, as was seen in t-HBEC. Taken together, these results indicated the involvement of all four HREs and the NF-κB sites in the proximal promoter of FLAP essential for hypoxia-induced FLAP gene expression.

As shown in Fig. 5A (lanes 4–6, and lanes 8–11), hypoxia-induced FLAP promoter activity (−965 to +12 bp) was reduced by LY 294002 (96 ± 2%), PD98059 (69 ± 3%), SB203580 (77 ± 3%), sulfasalazine (54 ± 2%), R59949 (69 ± 2%), rapamycin (73 ± 2%), and DPI (73 ± 3%) compared with hypoxia alone (lane 3). However, SP600125 did not affect FLAP promoter activity (Fig. 5A, lane 7). As shown in
Fig. 5B, hypoxia-induced FLAP-promoter activity was also attenuated to the basal level by HIF-1α siRNA (lane 5), whereas sHIF-1α siRNA had no affect (lane 4). The effect of these pharmacological inhibitors and HIF-1α siRNA on FLAP promoter activity was similar to that observed on its mRNA expression, supporting the role of PI-3 kinase, p38 MAP kinase, and NF-κB in FLAP mRNA expression (42). As shown in Fig. 6A, hypoxia caused an increase in HIF-1α protein levels (lane 2) in the nuclear extracts of r-HBEC, which was attenuated completely by LY294002, sulfasalazine, and R59949 (lanes 3 and 4). Moreover, 50-fold excess unlabeled probe competed out HIF-1α DNA binding (lane 5). An oligonucleotide with a mutation in the HRE site (−170 to −167 bp, Table I) showed negligible HIF-1α DNA binding (Fig. 6B, lane 2) when compared with the wild-type HRE (Fig. 6B, lane 2) in nuclear extracts from hypoxia exposed cells. These results indicated that HRE sites in the FLAP promoter were specifically bound by HIF-1α. The binding of HIF-1α to HRE sites in the FLAP promoter in vivo, was ascertained by ChIP analysis. Hypoxia-treated cells showed a 5-fold increase, as determined by densitometry, in expected PCR product (∼60% the expected PCR product (Fig. 6C)), as shown in the lower panel, the amplification of input DNA before immunoprecipitation was equal in all the samples. Immunoprecipitation of chromatin samples with control rabbit IgG did not show any amplification (Fig. 6C, middle panel). These data indicated that hypoxia increased HIF-1α binding to HREs in FLAP promoter in vivo, thereby causing upregulation of FLAP expression.
Identification of miRNAs involved in hypoxia-mediated FLAP-1 mRNA expression

Because hypoxia induced the mRNA expression of FLAP gene, we examined the miRNAs that may be involved in stabilization and degradation of FLAP mRNA. Previous studies identified several miRNAs, namely, miR-20, miR-155, miR-106a, miR-135a, miR-17-3p, miR-199a-5p, and miR-203 which are regulated by hypoxia and HIF-1α in endothelial cells. Thus, we analyzed the expression of these putative miRNAs in response to hypoxia in endothelial cells. There was a diminution in the expression of these miRNAs in both r-HBEC (Fig. 7A) and HPMEC (Fig. 7B). Among these set of miRNAs, only miR-135a and miR-199a-5p showed complementary binding sites upon in silico analysis of the 3′ UTR of the FLAP mRNA (Fig. 7C), thus we selected these miRNAs for further studies. We transfected r-HBEC with anti-miR oligonucleotides for miR-135a and miR-199a-5p to determine which of these miRNAs would regulate FLAP mRNA expression. As shown in Fig. 7D, transfection of anti–miR-135a induced FLAP mRNA expression by 8-fold under hypoxic condition, when compared with hypoxia alone (Fig. 7D, lane 3 versus lane 2) in r-HBEC. However, transfection with anti–miR-199a-5p induced FLAP mRNA expression by 4-fold over and above treatment with hypoxia alone (Fig. 7D, lane 4 versus lane 2). Similar results were observed in HPMEC, wherein transfection with anti–miR-135a and anti–miR-199a-5p augmented FLAP mRNA expression by 4-fold (Fig. 7E, lane 3 versus lane 2) and 3-fold (Fig. 7E, lane 4 versus lane 2), respectively, compared with hypoxia alone. These studies indicated that anti–miR-135a and anti–miR-199a-5p could modulate the intracellular levels of endogenous miRNAs to upregulate the expression of FLAP. We performed a converse experiment, namely, how overexpression of miR-135a and miR-199a-5p would affect hypoxia-induced FLAP expression. Transfection with miR-135a expression plasmid in r-HBEC abrogated FLAP mRNA expression below the basal level (Fig. 7F, lane 3), and overexpression of miR-199a-5p reduced FLAP expression to the basal level (Fig. 7F, lane 4). Similar results with miR-135a and miR-199a-5p expression plasmids were observed for FLAP mRNA expression in HPMEC (Fig. 7G, lanes 3 and 4 versus lane 2). Next, we examined whether these miRNAs affected the protein expression of FLAP. As shown in Fig. 7H, hypoxia (1% O2) increased FLAP protein expression by 1.5-fold, which was moderately increased (~20%) by anti–miR-135a and anti–miR-199a-5p. However, overexpression of miR-135a and miR-199a-5p reduced hypoxia-induced FLAP protein expression by 60% and 100%, respectively. Taken together, the data showed that miR-135a and miR-199a-5p reduced hypoxia-induced mediated FLAP mRNA and protein expression, and conversely anti–miR-135a and anti–miR-199a-5p augmented FLAP mRNA and protein expression in both primary HPMVECs and transformed HBECs.

Discussion

In this study, we show that hypoxia (1% O2) increased the expression of FLAP in endothelial cells. The levels of 5-LO were low in r-HBEC and did not significantly change in response to hypoxia. Previous studies have shown that cultured HPAECs express a minimal amount of 5-LO and thus do not synthesize LTs (38). However, endothelial cells can synthesize LTs by transcellular synthesis, wherein arachidonic acid generated by neutrophils or monocytes contributes to the total pool of LTs as a result of adhesion of these WBCs to endothelial cells (45, 46). In the current study, we observed that cyst LT levels were low in r-HBEC under normoxic condition, which did not change in response to hypoxia (1% O2). However, expression of 5-LO in these cells resulted in ~1.9-fold increase in cyst LT levels in response to hypoxia. The substrate, arachidonic acid, required for LT synthesis was likely formed due to hypoxia-mediated activation of phospholipase A2 in endothelial cells as previously shown (40, 41).

Next, we examined the hypoxia-mediated cellular signaling pathway for the expression of FLAP. We observed pharmacological inhibitors of PI-3 kinase (LY294002, MAP kinase (PD 98059), and p38 MAP kinase (SB203580) attenuated hypoxia-mediated FLAP mRNA expression. In addition, NF-κB inhibitor (sulfasalazine), NADPH-oxidase inhibitor (DPI), and putative HIF-1α inhibitor (R59949) also inhibited FLAP mRNA expression. However, an N-terminal Jun Kinase inhibitor (SP600125) had no significant effect. We used the RNA interference (RNAi)-mediated gene knockdown approach to more precisely delineate the signaling pathways involved. Transfection of r-HBEC with siRNAs for p47phox (a subunit of NADPH-oxidase), p38 MAP kinase, p65 (an NF-κB component), and HIF-1α, as well as dominant negative PI-3 kinase transgene attenuated hypoxia-mediated FLAP expression. Our results indicated that hypoxia-induced FLAP

FIGURE 5. Hypoxia-induced FLAP promoter activity involves PI-3 kinase, p38 MAP kinase, NF-κB, NADPH oxidase, and HIF-1α. A, Effect of hypoxia on FLAP promoter (−965 bp+12 bp) luciferase activity in r-HBECs, pretreated with pharmacological inhibitors (LY294002, PD98059, SB203580, SP600125, rapamycin, sulfasalazine, R59949, and DPI) for 30 min, prior to treatment with hypoxia. B, siRNA for HIF-1α attenuates hypoxia-induced FLAP promoter (−965/+12 bp) luciferase activity in r-HBECs. Transfected cells were treated with hypoxia for 8 h. Both luciferase and β-galactosidase activity were measured as described in Materials and Methods. The luciferase activity is expressed as fold-change and has been normalized to that of the untreated −965 FLAP-luc construct and to transfection efficiency of β-galactosidase. Data are expressed as mean ± SD of three independent experiments. ***p < 0.001; **p < 0.01; ns, p > 0.05.
mRNA expression involved activation of NADPH-oxidase, PI-3 kinase, p38 MAP kinase, MAP kinase, NF-κB, and HIF-1α. However, transfection with JNK-2 siRNA or SP60025 (inhibitor of JNK kinase) did not adversely affect hypoxia-mediated FLAP expression, indicating that the JNK pathway was not involved in hypoxia-mediated FLAP induction. Because both HIF-1α and HIF-2α have similar domain architecture and bind to an identical core motif (RCGTG), and display high degree of homology in their oxygen-dependent degradation domains (47), we examined the role of these proteins in hypoxia-mediated FLAP expression. Transfection of t-HBEC with HIF-1α siRNA but not HIF-2α siRNA attenuated hypoxia-induced FLAP expression, indicating that HIF-1α but not HIF-2α was involved in FLAP transcription. HIF-1α and HIF-2α have been previously shown to induce different transcriptional activator genes (47). It is well established that under hypoxic conditions, HIF-1α protein is stabilized and does not undergo degradation as prolylhydroxylases (PHDs 1–3) do not cause hydroxylation of proline residues in HIF-1α, in the absence of oxygen, for subsequent degradation by the ubiquitin-proteasome pathway (42, 48). Cells transfected with PHD-2 hydroxylase-2 siRNA, which stabilized HIF-1α protein, induced FLAP expression under normoxia, supporting the role for HIF-1α protein to FLAP HREs in t-HBECs. In our previous study (27), we showed that PHG, an angiogenic factor, augments FLAP expression via HIF-1α, independently of hypoxia. The PHG-mediated expression of FLAP involves two HRE sites, but not the NF-κB site in the FLAP promoter (27). In the current study, we observed that hypoxia-mediated FLAP expression required four HREs and the NF-κB site in the proximal region of FLAP promoter as demonstrated by mutations of each HRE singly and the NF-κB site. Binding of HIF-1α to HRE sites in the FLAP promoter was further confirmed by EMSA and ChIP, corroborating its activity in FLAP induction. These results indicated that hypoxia mediated signaling leading to FLAP expression involved HIF-1α and NF-κB. LPS-mediated FLAP expression in THP-1 cells was previously shown to require both NF-κB and C/EBP for FLAP promoter activation (25).

Recently, miRNAs have been identified that target sites in the 3′-UTRs of selected mRNAs (29, 30, 49) and specifically affect innate immune responses (50), expression of TLRs (51), and erythropoiesis (52). In addition to several signature miRNAs regulated by hypoxia, which have been characterized and target HIF-1α (31, 43), we have identified miR-199a-5p and miR-135a as potential regulators of hypoxia-induced FLAP mRNA expression. In silico analysis of the FLAP mRNA 3′UTR showed the presence of complementary binding sites for miR-135a and miR-199a-5p. The expression of miR-199a-5p and miR-135a was significantly attenuated by hypoxia, when compared with expression under normoxic conditions. Transfection with anti–miR-199a-5p and anti–miR-135a oligonucleotides induced FLAP expression under hypoxia when compared with normoxia. Moreover, overexpression of miR-135a and miR-199a-5p reduced the hypoxia-induced FLAP mRNA expression to the basal levels. These studies, for the first time, showed a role of miR-135a and miR-199a-5p in regulating hypoxia-mediated FLAP mRNA expression.

In conclusion, our studies show that hypoxia augments the expression of 5-LO activating protein via activation of HIF-1α and NF-κB. The posttranscriptional repression by miR-135a and miR-199a-
5p in human endothelial cells provides another novel mechanism by which expression of FLAP is fine tuned and likely regulated. We suggest that hypoxia-mediated LT formation in conjunction with the previously identified role of PlGF in LT formation (27), may contribute to inflammation and vaso-occlusion in SCD. FLAP inhibitors, such as MK-886, have been demonstrated to attenuate atherogenesis in the experimental model of atherosclerosis (10). Because the crystal structure of FLAP has been elucidated (23), and several FLAP

**FIGURE 7.** miR-135a and miR-199a-5p regulate posttranscriptional expression of FLAP. A. Effect of hypoxia in the expression levels of miRNA-20, miRNA-155, miRNA-106a, miRNA-135a, miRNA-17-3p, miRNA-199a-5p, and miRNA-203 in t-HBECs. B. Effect of hypoxia on the expression of miRNAs-20, -155, -106a, -135a, -17-3p, -199a-5p, and -203 in HPMVECs. Cells were treated with hypoxia (1% O2) for 8 h and miRNA was isolated and subjected to qRT-PCR. qRT-PCR data represents fold-increases in specific miRNA levels, on treatment with hypoxia (1% O2), compared with normoxia and is normalized to 5S miRNA levels. C. Schematics of putative binding sites for miR-135a and miR-199a-5p in the 3’ UTR of FLAP gene. D. Effect of anti-miRNA inhibitors for miR-135a and miRNA-199a-5p on hypoxia-induced FLAP mRNA expression in t-HBECs. E. Anti-miRNA inhibitors for miRNAs-135a and 199a-5p augments FLAP mRNA expression in HPMVECs. F. Overexpression of miRNA-135a and miRNA-199a-5p in t-HBEC cells inhibits FLAP mRNA expression, in response to hypoxia. G. Hypoxia-induced FLAP mRNA expression was attenuated by overexpression of miRNA-135a and miRNA-199a-5p in HPMVECs treated with hypoxia for 8 h. Total mRNA was isolated and subjected to qRT-PCR. qRT-PCR data represents fold-increase in FLAP mRNA levels, on treatment with hypoxia, compared with normoxia, and data are normalized to GAPDH mRNA levels. Data are means ± SD, and are representative of three independent experiments. ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05. H. FLAP protein levels in cytosolic extracts from t-HBECs, transfected with anti-miR135a and anti–miR199a-5p oligonucleotides, and miR-135a and miR-199a-5p expression plasmids, and treated with hypoxia (1% O2) for 12 h. Data were normalized to β-actin as a loading control and expressed as fold-change relative to normoxia. Data are representative of three independent experiments.
inhibitors have shown efficiency in early clinical trials of the treatment of asthma (53), these FLAP inhibitors have potential therapeutic use in attenuating respiratory and cardiovascular diseases. In addition, pharmacological inhibitors, which inhibit HIF-1α and NF-kB mediated activation of FLAP, and hence leukotriene formation have potential as therapeutic modalities to ameliorate hypoxia mediated inflammatory diseases, including lung injury, asthma, vaso-occlusion in SCD, myocardial infarction, and stroke.

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Disclosures

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References


