Endothelin-1–Induced Macrophage Inflammatory Protein-1 β Expression in Monocytic Cells Involves Hypoxia-Inducible Factor-1α and AP-1 and Is Negatively Regulated by microRNA-195

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Endothelin-1–Induced Macrophase Inflammatory Protein-1β Expression in Monocytic Cells Involves Hypoxia-Inducible Factor-1α and AP-1 and Is Negatively Regulated by microRNA-195

Caryn Gonsalves and Vijay K. Kalra

Patients with sickle cell disease (SCD) exhibit a chronic inflammatory state manifested by leukocytosis and increased circulating levels of proinflammatory cytokokines. Our studies show that placenta growth factor levels are high in SCD, and placental growth factor induces the release of the vasoconstrictor endothelin-1 (ET-1) from pulmonary microvascular endothelial cells. In this study, we observed that ET-1 increased the expression of the chemokines MIP-1β and CCL4. ET-1–induced MIP-1β mRNA expression in THP-1 cells and human peripheral blood monocytes occurred via the activation of PI3K, NADPH oxidase, p38 MAPK, and JNK-1 but not JNK-2. ET-1–induced MIP-1β expression involved hypoxia-inducible factor-1α (HIF-1α), independent of hypoxia, as demonstrated by silencing with HIF-1α small interfering RNA, EMSA, and chromatin immunoprecipitation analysis. ET-1–induced MIP-1β promoter luciferase activity was attenuated when any of the five hypoxia-response elements, AP-1, or NF-κB binding motifs in the proximal MIP-1β promoter (−1053/+43 bp) were mutated. Furthermore, ET-1 significantly downregulated the expression of a key microRNA, microRNA-195a, which showed a complementary binding site in the 3′ untranslated region of MIP-1β mRNA. Moreover, ET-1–induced MIP-1β mRNA expression in either THP-1 cells or peripheral blood monocytes was reduced upon expression of microRNA-195a. Conversely, transfection of monocytes with anti–microRNA-195a oligonucleotide augmented several-fold ET-1–induced MIP-1β expression. Taken together, these studies showed that ET-1–mediated MIP-1β gene expression is regulated via hypoxia-response elements, AP-1, and NF-κB cis-binding elements in its promoter and negatively regulated by microRNA-195, which targets the 3′ untranslated region of MIP-1β. These studies provide what we believe are new avenues, based on targets of HIF-1α and microRNAs, for ameliorating inflammation in SCD. The Journal of Immunology, 2010, 185: 6253–6264.

Sickle cell disease (SCD) is the result of a point mutation in the β-globin gene leading to the formation of sickle hemoglobin. The latter upon deoxygenation leads to the polymerization of hemoglobin, culminating in the sickle shape of RBCs. The clinical manifestations of SCD include chronic hemolytic anemia, frequent infections, and recurring episodes of painful crises (1–4). Another salient feature of SCD is a chronic inflammatory state characterized by leukocytosis, monocytes, and elevated circulating levels of proinflammatory cytokokines, which occurs in the absence of acute infection or an acute vasocclusive event (5, 6). Studies have shown higher levels of IL-1β, TNF-α (7, 8), and IL-8 (9) in the plasma of SCD patients.

Monocytes, neutrophils, and endothelial cells are in an activated state in SCD patients at steady state (5, 10, 11); however, relatively little is understood about the causes of basal inflammation in SCD.

Our recent studies (12) show that plasma levels of placental growth factor (PlGF), a member of the vascular endothelial growth factor family, are higher in SCD patients when compared with those of healthy controls. Furthermore, PlGF significantly increases the expression of proinflammatory cytokokines (IL-1β, MIP-1β, MCP-1, and IL-8) from monocytes in healthy subjects (12, 13). PlGF also shows an increase in the expression of endothelin-1 (ET-1) in human pulmonary endothelial cells (14) via activation of hypoxia-inducible factor-1α (HIF-1α), independently of hypoxia.

Because levels of ET-1 are elevated in SCD patients with acute chest syndrome (15), we examined whether ET-1 contributed to the inflammatory state seen in SCD.

In this report, we show that monocytic cells were activated by ET-1, resulting in the increased expression of MIP-1β or CCL4, a member of the CC chemokine family (16). Human MIP-1β is an acidic protein of 8000 Da that is upregulated in T cells, monocytes, and lymphocytes and is involved in the migration of subsets of leukocytes (17). Both LPS (18, 19) and IL-1β (20, 21) induce the expression of MIP-1β. Studies have shown that MIP-1β blocks the entry of HIV-1 into CD4+ T cells and CCR5+ T cells. Elevated plasma levels of MIP-1β have been shown to antagonize HIV-1 infection (22, 23). It also has been shown that the AP-1/cAMP response element (CRE)-like motif in the MIP-1β promoter is involved in the LPS-mediated expression of MIP-1β (18). Furthermore, MIP-1β transcription in T cells is regulated by transcription
factors of the CREB modulator family (24). In this study, we show that ET-1-mediated cellular signaling led to the upregulation of MIP-1β in monocyctic cells, which involved PI3K, NADPH oxidase, p38 MAPK, JNK-1, and the transcription factor (HIF-1α). microRNA-195a was found to negatively regulate ET-1–induced MIP-1β expression. microRNA-195a was one of several microRNA candidates that were identified based on the presence of potential binding sites in the 3′ untranslated region of human MIP-1β mRNA. To the best of our knowledge, this is the first report showing that ET-1 induction of MIP-1β was dependent on HIF-1α, in a hypoxia-independent manner. Additionally, we also report that microRNA-195a negatively regulated MIP-1β expression through a posttranscriptional mechanism.

Materials and Methods

Peripheral blood monocytes, THP-1 monocyctic cells, and reagents

THP-1, a promonocytic cell line, from American Type Culture Collection (Manassas, VA), was cultured in RPMI 1640 medium containing 10% heat-inactivated FBS (13). Unless otherwise indicated, THP-1 cells were kept overnight in serum-free RPMI 1640 prior to treatment with ET-1. Peripheral blood monocytes (PBMs) were isolated, using Percoll gradients from blood collected in EDTA from normal healthy volunteers. All of the donors consented to donation as per an informed consent protocol approved by the institutional review board at University of Southern California-Los Angeles County Hospital (13). PBMs were resuspended in RPMI 1640 complete medium and treated with ET-1 for the indicated time periods.

Double HRE1M and HRE4M-Luc

The mutant constructs of the human MIP-1β promoter were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) utilizing the wild-type –1065/+43 bp MIP-1β-Luc construct as a template. The mutations in five HIF-1α binding sites [hypoxia response element (HRE) sites] of the MIP-1β promoter designated as HRE1M (bases –192 to –189), HRE2M (–320 to –317), HRE3M (–440 to –437), HRE4M (–476 to –473), and HRE5M (–706 to –703) and mutations in the NF-κB and AP-1 binding sites designated as NF-κBM (bases –87 to –77) and AP-1M (bases –109 to –91 bp) were carried out using the primers shown in Table I. Double mutations in HRE1 and HRE4 sites in the –1065/+43 bp MIP-1β-Luc construct were performed utilizing the HRE1M construct as a template. Triple mutations at HRE1, HRE4, and AP-1 sites were performed utilizing the double HRE1M and HRE4M-Luc construct as a template. The mutations were confirmed by DNA sequencing (Microchemical Core Facility, Norris Comprehensive Cancer Facilities, University of Southern California).

Isolation and quantification of mRNA

Total RNA was isolated from monocyctic cells using TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA). mRNA expression was quantified by quantitative real-time PCR (qRT-PCR) utilizing specific primers listed in Table I. qRT-PCR of mRNA templates (100 ng) was performed using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) and the ABI Prism 7900HT Sequence Detection System, version 2.3 (Applied Biosystems, 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 PCR for 40 cycles entailing 95˚C for 10 s, followed by annealing at 60˚C for 30 s and detection. Values are expressed as relative expression of mRNA normalized to mRNA for the housekeeping gene GAPDH. Relative quantification (RQ) values for mRNA expression were calculated as 2−ΔΔCT by the comparative threshold cycle (Ct) method (26), where ΔΔCT = (Ct target gene of treated sample – Ct GAPDH of treated sample) – (Ct target gene of control sample – Ct GAPDH of control sample).

Isolation and quantification of microRNAs

A total of 5 × 10^6 THP-1 cells were kept in serum-free media for 8 h and treated with ET-1 for the indicated time periods. microRNAs were isolated and purified using the mirVana Kit (Applied Biosystems/Ambion) according to the manufacturer’s protocol. microRNA levels were detected using microRNA-specific primers (Applied Biosystems/Ambion) by qRT-PCR. Ct values obtained were normalized to Ct values for 5S rRNA, which is expressed constitutively. RQ values for microRNA expression were calculated as 2−ΔΔCT by the comparative Ct method (26), where ΔΔCT = (Ct target microRNA of samples – Ct 5S of treated sample) – (Ct target gene of control sample – Ct 5S of control sample).

Transient transfection

THP-1 cells (1 × 10^6) or PBMs (1 × 10^6) were transfected with the indicated siRNA constructs (50 nM), expression plasmids for microRNA-195a (1 μg), anti–microRNA-195a oligonucleotides, and Luciferase reporter plasmids by nucleofection utilizing Nucleofector Kit V (Amaxa Biosystems, Cologne, Germany), as described previously (27). Briefly, the reporter construct 1065/+43 bp full-length MIP-1β promoter plasmid (0.5 μg) was cotransfected to control for transfection efficiency. Twenty-four hours posttransfection, the media was removed, and cells were kept in serum-free medium for 3 h followed by treatment with ET-1 for the indicated time periods. The cells were harvested, lysed, and quantified for both luciferase activity on a luminometer (Lumat LB 950; Berthold, Bad Wildbad, Germany) and β-galactosidase activity by absorbance at 520 nm, using assay kits (Promega, Madison, WI). Luciferase values were normalized to β-galactosidase values. Data are expressed relative to the activity of the promoterless pGL3 basic vector. Where indicated, microRNA was extracted from cells transfected with siRNA, overexpression plasmids for microRNA-195a, and anti–microRNA-195a oligonucleotides and quantified by qRT-PCR.

Preparation of nuclear extracts and Western blot analysis

Nuclear extracts were prepared from THP-1 cells as described previously (14). Briefly, 5 × 10^6 cells were washed with cold PBS, lysed, and centrifuged in a microfuge at 10,000 × g for 30 s. The cytosolic supernatant was collected, and the nuclear pellet was resuspended in 50 μl nuclear extraction buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 420 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF, and 1 μl of protease inhibitor mixture) followed by incubation on ice for 60 min with intermittent vortexing. The nuclear extract was obtained by centrifuging at 10,000 × g for 10 min at 4˚C. Nuclear extracts were subjected to Western blotting using HIF-1α Ab (1:250), HIF-1α Ab (1:250), NF-κB, and β-actin Abs. Cytosolic extracts were subjected to Western blotting and probed with Abs to MIP-1β (1:250). The protein bands were detected with Immobilon Western Reagents (Millipore, Billerica, MA). Blots were stripped and reprobed using a β-actin Ab (1:2500) to confirm equal protein loading.

EMSA

Double-stranded complementary oligonucleotides corresponding to the MIP-1β promoter containing a proximal HRE site at position –192 to –189 and MIP-1β with a mutation in the same HRE site (Table I) were biotin-labeled using a Lightshift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) and used for the EMSA (27). Briefly, the DNA binding reaction consisted of 5 μg nuclear protein extract from THP-1 cells, 5% glycerol, 5 mM MgCl2, 50 ng/μl poly (deoxyinosinic:deoxyycytidylic), 0.05% Nonidet P-40, and 0.5 ng biotinylated MIP-1β oligonucleotide, which was incubated at room temperature for 20 min. The samples were

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subjected to PAGE, transferred to a Hybond-N+ nylon membrane (Ameri-
sham Biosciences, Piscataway, NJ) followed by detection of the DNA–protein
complex bands with streptavidin-HRP/chemiluminescence reagent (27).

Chromatin immunoprecipitation assay
A total of $10^6$ THP-1 cells were kept overnight in serum-free RPMI
1640, followed by treatment with ET-1 for the indicated time period in
the presence or absence of inhibitors. Chromatin immunoprecipitation
(ChIP) analysis was performed utilizing HIF-1α Ab as described pre-
viously (27). 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, 48˚C for 60 s, and
72˚C for 120 s, using primers listed in Table I. The PCR products were
subjected to 2% agarose gel electrophoresis followed by densitometric
analysis of the amplified product. The values were normalized to input
DNA.

Quantification of MIP-1β by ELISA
A total of $10^6$ THP-1 cells were treated with ET-1, in serum-free
medium, in the presence or absence of the indicated pharmacological
inhibitors for 8 h. The supernatants of treated cells were assayed for MIP-
1β protein release using an ELISA kit (R&D Systems, Minneapolis, MN).
Cells were lysed, and protein concentrations were determined using the
Bradford method. ELISA values were normalized to the cell protein con-
centrations.

Chemoattract assay
Chemoattract of THP-1 cells was assayed in 96-well plates (Neuroprobe,
Gaithersburg, MD) using Transwell inserts of 5-μm pore size as described
previously (28). The effect of neutralizing Ab against MIP-1β was de-
termined by adding Ab to the chemoattractant in the lower compartment of
the chemoattract chamber. Where indicated, THP-1 cells were preincubated
with CC55 Ab for 30 min at room temperature prior to treatment with ET-1.

Statistical analysis
Control and ET-1–treated samples were compared using the Student t test.
One-way ANOVA followed by the Turkey-Kramer test was used for
multiple comparisons using the Instat-2 software program (GraphPad, San
Diego, CA). Data are presented as mean ± SD. Values of $p < 0.05$ were
considered statistically significant.

FIGURE 1. ET-1 augments MIP-1β mRNA expression in THP-1 cells via PI3K, p38 MAPK, NADPH oxidase, and NF-κB. A, MIP-1β mRNA ex-
pression in THP-1 exposed to ET-1 for the indicated time points. THP-1 cells were treated with acetic acid as a negative control. B, ET-1 induced MIP-1β mRNA
expression in THP-1 cells pretreated with the pharmacological inhibitors, LY294002 (PI3K inhibitor), PD98059 (MAPK inhibitor), SB203580 (p38 MAPK),
and SP600125 (JNK inhibitor). C, MIP-1β mRNA expression in THP-1 cells, pretreated with inhibitors sulfasalazine (NF-κB inhibitor), DPI
(NADPH oxidase inhibitor), ascorbate, R59949 (HIF-1α inhibitor), BQ610 (ET2 receptor inhibitor), and BQ788 (ET4 receptor inhibitor) and subjected to
ET-1 for 30 min. Total RNA was isolated for all of the experiments and subjected to qRT-PCR. qRT-PCR data represent fold changes in MIP-1β mRNA
expression, following treatment with ET-1, compared with no treatment. MIP-1β mRNA expression was normalized to GAPDH mRNA levels. D, Levels of
MIP-1β in cell culture media from THP-1 cells, pretreated with LY294002 (PI3K inhibitor), PD98059 (MAPK inhibitor), SB203580 (p38 MAPK),
SP600125 (JNK inhibitor), sulfasalazine (NF-κB inhibitor), DPI (NADPH oxidase inhibitor), ascorbate (HIF-1α inhibitor), R59949 (HIF-1α inhibitor),
BQ610 (ET2 receptor inhibitor), and BQ788 (ET4 receptor inhibitor), followed by treatment with ET-1. Data are expressed as mean ± SD of three in-
dependent experiments. **p < 0.001; ***p < 0.01; *p < 0.05; NS, $p > 0.05$. 

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Results
ET-1–mediated MIP-1β mRNA induction involves PI3K, MAPK, p38 MAPK, and JNK

Because levels of ET-1 are higher in patients with SCD (15), we examined whether circulatory ET-1 activated monocytes caused the expression of proinflammatory cytokines. As shown in Fig. 1A, ET-1 (250 μM) in a time-dependent manner (15–120 min) showed increased mRNA expression of MIP-1β in THP-1 cells, and optimal expression occurred between 30 and 60 min after ET-1 treatment. Pretreatment of THP-1 cells with pharmacological inhibitors of PI3K (LY294002), MAPK (PD98059), p38 MAPK (SB203580), and JNK (SP600125) inhibited ET-1–mediated MIP-1β mRNA expression by 89 ± 5%, 104 ± 7%, 102 ± 8%, and 118 ± 1%, respectively, when compared with that of untreated cells (Fig. 1B). These data showed that ET-1–dependent cellular signaling for MIP-1β mRNA expression involved PI3K, MAPK, p38 MAPK, and JNK.

ET-1–induced MIP-1β mRNA expression also involves the ET-BR receptor, NADPH oxidase, and HIF-1α

Next, we examined whether ET-1–mediated signaling involved its cognate receptors, endothelin receptor-A (ET-AR) or endothelin receptor-B (ET-BR) expressed on monocytic cells. As shown in Fig. 1C, BQ788 (ET-BR receptor antagonist) but not BQ610 (ET-AR receptor antagonist) completely reduced ET-1–mediated MIP-1β mRNA expression (118 ± 6%) in THP-1 cells. Furthermore, ET-1–induced MIP-1β mRNA expression was reduced by sulfasalazine (113 ± 7%), an inhibitor of NF-κB; DPI (96 ± 3%), an inhibitor of NADPH oxidase; and R59949 (110 ± 2%), an inhibitor of HIF-1α (Fig. 1C). As shown in Fig. 1D, ET-1–mediated release of MIP-1β from THP-1 cells was reduced in cells pretreated with LY294002 (134 ± 3%), PD98059 (126 ± 4%), SB293580 (80 ± 2%), SP600125 (107 ± 1%), sulfasalazine (97 ± 2%), DPI (100 ± 3%), ascorbate (109 ± 6%), R59949 (128 ± 3%), and BQ788 (125 ± 2%). Taken together, these results showed that...
ET-1–mediated mRNA expression of MIP-1β involved activation of ET-BR receptor, NADPH oxidase, and HIF-1α. Because pharmacological inhibitors may have nonspecific inhibitory effects, we used a gene silencing approach for key protein kinases and NADPH oxidase associated with ET-1–mediated MIP-1β expression. As shown in Fig. 2A, THP-1 cells transfected with siRNAs for p38 MAPK and p47^phox showed complete inhibition in ET-1–mediated MIP-1β mRNA expression, whereas control scRNA had no effect. Western blots showed specific knockdown of the target protein with the appropriate siRNA but not with control scRNA (Fig. 2). Additionally, siRNA for p65, a subunit of NF-κB, also exhibited complete inhibition of ET-1–mediated MIP-1β mRNA expression, whereas transfection with a control scRNA for p65 had no effect on MIP-1β expression (Fig. 2B). As shown by Western blots, siRNA for p65 specifically knocked down p65; however, a similar effect was not observed with scRNA. However, transfection with siRNAs for JNK-1 and JNK-2 had differential effects on MIP-1β expression. As shown in Fig. 2C, ET-1–mediated MIP-1β expression was inhibited completely by JNK-1 siRNA, whereas JNK-2 siRNA showed no such inhibition. The knockdown of JNK-1 protein was observed with siRNA for JNK-1, whereas control scRNA had no effect as demonstrated by Western blot analysis (Fig. 2C, right panel). Taken together, these data showed that the effects of pharmacological inhibitors were corroborated by siRNAs and supported the putative roles of p38 MAPK, NF-κB, NADPH oxidase, and JNK-1 in ET-1–mediated induction of MIP-1β expression.

**FIGURE 3.** ET-1–mediated MIP-1β mRNA expression involves HIF-1α. A. MIP-1β mRNA expression in THP-1 cells transiently transfected with siRNA for HIF-1α, HIF-2α siRNA, and HIF scRNA, prior to stimulation with ET-1 for 30 min. B. MIP-1β mRNA expression in THP-1 cells, transfected with PHD-2 siRNA and scRNA for PHD-2. C. MIP-1β mRNA expression in human monocytes, transfected with siRNA for HIF-1α and HIF scRNA and treated with ET-1 for 30 min. Total RNA was isolated for all of the experiments and subjected to qRT-PCR. qRT-PCR data are expressed as fold changes in MIP-1β mRNA expression, when treated with ET-1, compared with that of no treatment. mRNA levels for MIP-1β were normalized to GAPDH mRNA levels. Western blots for HIF-1α and PHD-2 were performed to demonstrate the specificity of the respective siRNA. Cells were treated with ET-1 for 30 min. Western blots were performed as described in Materials and Methods. Blots were probed with β-actin to demonstrate equal loading. D. ET-1–mediated MIP-1β release promotes chemotaxis of THP-1 cells. THP-1 cells were exposed to conditioned media elaborated from PBMs treated with ET-1 for 24 h. Monocytes were pretreated with inhibitors for PI3K (LY294002), HIF-1α (R59949), and NADPH oxidase (DPI). THP-1 cells were incubated with Ab to CCR5 prior to exposure to conditioned media from ET-1–treated monocytes. Where indicated, Ab to MIP-1β was added to the conditioned media. Data are expressed as mean ± SD of three independent experiments. ***p < 0.001; **p < 0.01; *p < 0.05; NS, p > 0.05.
3C, treatment of PBMs with ET-1 elicited a ~5-fold increase in MIP-1β mRNA levels, which was completely reduced to the basal level in PBMs transfected with HIF-1α siRNA (Fig. 3C, lane 4). However, PBMs transfected with HIF-1α scRNA did not show inhibition in ET-1–mediated MIP-1β mRNA expression (Fig. 3C, lane 3) compared with that of PBMs treated with ET-1 (Fig. 3C, lane 2). As shown in Fig. 3C, bottom panel, knockdown with HIF-1α siRNA attenuated >80% of HIF-1α protein in ET-1–treated PBMs, whereas scRNA had no effect. Additionally, we determined whether MIP-1β released upon treatment of PBMs with ET-1 was functional in mediating the chemotaxis of monocytes. rMIP-1β (20 ng/ml) caused a ~4-fold increase in the chemotaxis of THP-1 monocytes (Fig. 3D, lane 2). The conditioned media elaborated from ET-1–treated PBMs, which contained MIP-1β at a concentration of 20 ng/ml, also increased by ~4-fold the chemotaxis of THP-1 monocytes (Fig. 3D, lane 3). Furthermore, the effect was specific for MIP-1β, because the addition of Ab to MIP-1β reduced chemotaxis by 77 ± 3% (Fig. 3D, lane 4). The conditioned media elaborated from PBMs that were pretreated with inhibitors (LY294002, R59949, and DPI) followed by ET-1 treatment also showed reduced (~50%) chemotaxis of THP-1 cells (Fig. 3D, lanes 5–7). Additionally, blockage of MIP-1β receptor CCR5 on THP-1 cells, by utilizing an anti-CCR5 Ab, also reduced chemotaxis by 95 ± 5% (Fig. 3D, lane 8). Taken together, these results showed that MIP-1β released from ET-1–treated PBMs was biologically functional in mediating chemotaxis of THP-1 cells via the CCR5 receptor.

**FIGURE 4.** ET-1 augments MIP-1β-Luc promoter via HIF-1α, NF-κB, and AP-1. A, Schematics of the MIP-1β (~1065/+43 bp) promoter region, containing binding sites for five HRE sites, NF-κB, and AP-1. Mutations in binding sites for HIF-1α, NF-κB, and AP-1 in the MIP-1β luciferase constructs are indicated, where specific nucleotide mutations are denoted by asterisks. B, Deletion analysis of MIP-1β promoter. THP-1 cells were cotransfected with the indicated deletion constructs and β-galactosidase plasmid, followed by treatment with ET-1 for 4 h. The luciferase activity was normalized to the promoterless pGL3 vector. C, ET-1–induced MIP-1β (~1065/+43 bp) promoter activity required five HRE sites. The MIP-1β HRE1M (~1065/+43 bp) contains a mutation in the HRE site at position −192 to −189 bp. The MIP-1β HRE2M and MIP-1β HRE3M (~1065/+43 bp) contain mutations in the HRE sites at positions −320 to −317 and −440 to −437 bp, respectively. The MIP-1β HRE4M and HRE5M (~1065/+43 bp) contain mutations in the HRE site at position −476 to −473 and −706 to −703 bp. The MIP-1β HRE1M and HRE4M (~1065/+43 bp) contain mutations in the HRE site at position −192 to −189 and −476 to −473 bp. D, ET-1–induced MIP-1β (~1065/+43 bp) promoter activity required the AP-1 binding sites. The MIP-1β NF-κBM (~1065) has a mutation in the NF-κB binding site at position −87 to −77 bp. The MIP-1β AP-1M (~1065) has a mutation in the AP-1 binding site at position −109 to −91. The MIP-1β AP-1M (~1065) and HRE1M and HRE4M have mutations in the AP-1 site, the HRE1 site at position −192 to −189 bp, and in HRE4 site at position −476 to −473 bp. E, MIP-1β-Luc expression in THP-1 cells transiently transfected with siRNA for c-Fos and scRNA prior to stimulation with ET-1 for 4 h. 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 ~1065/+43 bp MIP-1β-luc construct and to that of the transfection efficiency of β-galactosidase. Data are expressed as mean ± SD of three independent experiments. ***p < 0.01; **p < 0.05; NS, p > 0.05.

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**Schematics of human MIP-1β promoter (Accession no: S56704)**

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**A**

- Schematics of human MIP-1β promoter (Accession no: S56704).

**B**

- Relative fold luciferase activity (lanes 1-6).

**C**

- Relative fold luciferase activity (lanes 1-8).

**D**

- Relative fold luciferase activity (lanes 1-5).

**E**

- Relative fold luciferase activity (lanes 1-5).
"ET-1–mediated MIP-1β expression requires HREs, NF-κB, and AP-1 binding motifs in its promoter"

Previous studies (24) have shown that the promoter region of MIP-1β has overlapping consensus sites for AP-1 and CRE-like binding motifs at positions −70 to −65 bp relative to the transcriptional start site. In silico analysis of the −1065/+43 bp MIP-1β promoter (Gene Accession No. S56704; www.ncbi.nlm.nih.gov/nuccore/236042) revealed the presence of several HREs, characterized by RCGTG and an NF-κB binding motif, as depicted in the schematic of the promoter (Fig. 4A). THP-1 cells transfected with a reporter luciferase plasmid, under control of the −1065/+43 bp segment of the MIP-1β promoter, showed a ∼20-fold increase in luciferase activity in response to treatment with ET-1 (Fig. 4B, lane 2). Analysis of truncated MIP-1β promoter constructs showed that the −485/+43 bp segment was ∼60% effective (Fig. 4B, lane 3) compared with the full-length promoter (−1065/+43 bp) (Fig. 4B, lane 2), whereas truncated promoter constructs (−264/+43 bp, −192/+43 bp, and −74/+43 bp) showed no increase in luciferase activity in response to ET-1 (Fig. 4B, lanes 4–6) compared with that of the promoterless reporter plasmid (pGL3) (Fig. 4B, lane 1). Thus, we used the MIP-1β −1065/+43 bp promoter for further studies. As shown in Fig. 4C, mutation of individual HREs, HRE1 to HRE5, designated as HRE1M (−192/−189 bp), HRE2M (−320/−317 bp), HRE3M (−440/−437 bp), HRE4M (−476/−473), and HRE5M (−706/−703 bp) within the −1065/+43 bp MIP-1β promoter led to ∼50% reduction in ET-1–induced promoter activity. Additionally, double mutants with mutations in HRE1 and HRE4 sites (HRE1M + HRE4M) of the MIP-1β luciferase promoter construct resulted in a further reduction (94 ± 3%) in ET-1–induced promoter activity (Fig. 4C, lane 8) compared with that of a single mutation of either HRE1 (Fig. 4C, lane 3) or HRE4 site (Fig. 4C, lane 6). Taken together, these results showed that each individual HRE site in the MIP-1β proximal promoter was essential for ET-1–mediated MIP-1β promoter activity, and double mutations in HRE sites of the MIP-1β promoter led to a further reduction in ET-1–induced promoter activity.

Next, we examined the role of transcription factors NF-κB and AP-1 in ET-1–mediated induction of MIP-1β. The mutation of the NF-κB binding motif (−87 to −77 bp) in the −1065/+43 MIP-1β promoter resulted in a 19 ± 4% inhibition in reporter activity (Fig. 4D, lane 3). However, mutation of the AP-1 site (−109 to −91 bp) resulted in 70 ± 4% reduction in ET-1–mediated reporter activity (Fig. 4D, lane 4). Combined mutations in the AP-1 binding site as well as HRE1 and HRE4 inhibited MIP-1β promoter activity by 103 ± 1% (Fig. 4D, lane 5) compared with ET-1–induced promoter activity (Fig. 4D, lane 2). Because AP-1 complex appeared to play an important role in ET-1–mediated MIP-1β activity, we used knockdown of c-Fos, a component of AP-1, by siRNA. siRNA for c-Fos inhibited MIP-1β promoter activity by 102 ± 3% (Fig. 4E, lane 4) versus control (Fig. 4E, lane 2). We did not observe significant inhibition of promoter activity with a control scRNA. These results showed that the AP-1 motif along with the HREs were essential components of the transcriptional complex for ET-1–mediated MIP-1β transcription. Because mutation of the NF-κB site led to a modest reduction in MIP-1β luciferase activity, it may not play a major role in ET-1–mediated induction of MIP-1β.

**FIGURE 5.** ET-1 augments the binding of HIF-1α protein to MIP-1β HREs in THP-1 cells. A, ET-1–induced HIF-1α protein expression is attenuated by pharmacological inhibitors LY294002 (PI3K), R59949 (HIF-1α), and BQ788 (ETα receptor inhibitor) in THP-1 cells. Cells were treated with ET-1 for 30 min. Data are representative of three independent experiments. B, EMSA of nuclear extracts from THP-1 cells utilizing oligonucleotide probes for the proximal HRE1 site (−192 to −189) in the MIP-1β promoter. Where indicated, oligonucleotide probes with mutations in the HRE1 (−192 to −189) site were used. As indicated, a 50-fold excess of cold probe was added to the nuclear extracts. Data are representative of three independent experiments. Nuclear extracts were obtained from THP-1 cells treated with ET-1 for 4 h. Repositioned gel lanes are indicated by vertical lines. C, ET-1 augments binding of HIF-1α to HRE1 (−192 to −189) in the MIP-1β promoter as determined by ChIP analysis. THP-1 cells were pretreated with pharmacological inhibitors for 30 min prior to treatment with ET-1 for 30 min. HIF-1α Ab (top panel) or control rabbit IgG (middle panel) was used for immunoprecipitation of soluble chromatin. Primers used to amplify the PCR products flanking the HRE1 in the MIP-1β promoter are indicated in Table I. The bottom panel represents the amplification of input DNA before immunoprecipitation. Data are representative of two independent experiments.
ET-1 stabilizes HIF-1α protein under normoxia

It is known that hypoxia causes stabilization of HIF-1α protein, whereas normoxia leads to its degradation (30, 31). We observed that ET-1 treatment of THP-1 cells resulted in increased HIF-1α protein levels (Fig. 5A, lane 2), which occurred under normoxia. The ET-1–mediated increase in HIF-1α protein was reduced below the basal level by PI3K inhibitor (LY294002) (Fig. 5A, lane 3). Moreover, an inhibitor of HIF-1α (R59949) and ET-BR receptor antagonist (BQ788) reduced (>50%) ET-1–mediated HIF-1α protein expression (Fig. 5A, lanes 4–5). These results showed that ET-1 augmented HIF-1α protein expression, independent of hypoxia, and occurred via upstream activation of ET-BR and PI3K.

ET-1 stimulated HIF-1α DNA binding activity in vitro (EMSA) and in vivo (ChIP) to the MIP-1β promoter

As shown in Fig. 5B, nuclear extracts from ET-1–treated THP-1 cells showed increased binding to wild-type oligonucleotide probe spanning the HRE site at position −189 to −192 bp (Table I) of the MIP-1β promoter in an EMSA. Both LY294002 and R59949 reduced by ~60% binding of HIF-1α to the wild-type oligonucleotide probe (Fig. 5B, lanes 3–4). Furthermore, a 50-fold excess of unlabeled probe competed out HIF-1α binding (Fig. 5B, lane 6). The mutant HRE oligonucleotide probe (RCGTG to RCATA) (HRE mutant) (Table I) exhibited negligible HIF-1α DNA binding (Fig. 5B, lane 5), when compared with the wild-type probe (Fig. 5B, lane 2). These results also were substantiated by ChIP analysis, which showed increased HIF-1α binding to the MIP-1β promoter in the native chromatin. ET-1–treated THP-1 cells showed ~6-fold increase in expected PCR product size of 142 bp, corresponding to the MIP-1β promoter region containing the HRE site at positions −198 to −189 bp, utilizing primers listed in Table I. These results corroborated the MIP-1β reporter assay results as described in Fig. 4B. Pretreatment of THP-1 cells with LY294002, BQ788, and R59949 reduced by ~95% the levels of expected PCR products (Fig. 5C, top panel). As shown in the middle panel of Fig. 5C, the amplification of input DNA before immunoprecipitation with HIF-1α Ab was equal in all of the samples. Furthermore, immunoprecipitation of chromatin samples with control rabbit IgG did not display any amplification of the expected PCR products (Fig. 5C, bottom panel). Taken together, these results showed that ET-1 augments HIF-1α binding to the MIP-1β promoter in vivo and upregulates its transcription.

Identification of microRNAs inhibiting ET-1–mediated MIP-1β mRNA expression

Because ET-1 induced the mRNA expression of MIP-1β, we examined microRNAs that may be involved in posttranscriptional regulation of MIP-1β mRNA expression. Previous studies (32, 33) have shown several microRNAs that are regulated by hypoxia and HIF-1α in cancer cells. Analysis of the Sanger microRNA database revealed that microRNA-20, microRNA-194, microRNA-195a, and microRNA-223 have binding sites in the 3′ untranslated region (UTR) of human MIP-1β mRNA, and the binding of hsa-microRNA-195a and hsa-microRNA-223 is depicted in schematic of Fig. 6A. Thus, we analyzed the expression of these potential microRNA regulators in response to ET-1 in THP-1 cells. As shown in Fig. 6B, there was a 60–80% reduction of microRNA-20, microRNA-194, and microRNA-195a mRNA levels in ET-1–treated THP-1, when compared with those of untreated THP-1 cells. By contrast, microRNA-223 did not change significantly in ET-1–treated THP-1 cells (Fig. 6B, lane 5) compared with that of the control (Fig. 6B, lane 1). Of these microRNAs, the binding sites for microRNA-195a in the 3′UTR of MIP-1β showed the best
complementarity among various species, and thus it was studied in detail. We therefore examined the role of microRNA-195a in the ET-1–mediated expression of MIP-1β.

As shown in Fig. 6C, transfection of anti–microRNA-195a oligonucleotide into THP-1 cells, followed by ET-1 treatment, increased MIP-1β mRNA levels by 22-fold (Fig. 6C, lane 4) compared with that of ET-1–treated THP-1 cells alone (~7-fold) (Fig. 6C, lane 3). To determine, if the observed effect was specific for microRNA-195a, THP-1 cells were transfected with a microRNA-195a expression plasmid. As shown in Fig. 6C, overexpression of microRNA-195a reduced ET-1 induced levels of MIP-1β from 7-fold (Fig. 6C, lane 2) to the basal level (Fig. 6C, lane 3). Next, we determined whether the same effect could be seen in primary human PBMs. As shown in Fig. 6D, transfection of PBMs with microRNA-195a expression plasmid reduced ET-1–mediated MIP-1β mRNA expression to the basal level (Fig. 6D, lane 3) compared with ET-1–treated PBMs which showed an increase of ~3-fold in MIP-1β expression (Fig. 6D, lane 2). We also determined if these changes in mRNA levels of MIP-1β corresponded to changes in MIP-1β protein levels. THP-1 cells treated with ET-1, showed increased levels of MIP-1β protein (Fig. 6D, lane 3) compared with that of untreated cells (Fig. 6D, lane 1). Cells transfected with anti–microRNA-195a followed by treatment with ET-1 showed increased levels of MIP-1β protein, when compared with that of cells treated with ET-1 alone (Fig. 6D, lane 3 versus lane 2). However, overexpression of microRNA-195a attenuated MIP-1β protein expression to the basal levels (Fig. 6D, lane 4 versus lane 2) Taken together, these results showed that microRNA-195a reduced, whereas anti-microRNA-195a oligonucleotides augmented, ET-1–mediated MIP-1β expression in both THP-1 cells and human PBMs (Fig. 7).

**Discussion**

ET-1 levels are elevated in patients with SCD at steady state and further increase during vaso-occlusive crises and in SCD patients afflicted with acute chest syndrome (34). ET-1, a potent vasconstrictor, likely contributes to pulmonary complications, such as pulmonary hypertension, acute chest syndrome, and inflammation, which are risk factors for early mortality in SCD (5, 6, 35, 36). We have shown previously (14) that an erythroid cell-derived angiogenic growth factor, PGF, induces ET-1 expression in human pulmonary microvascular endothelial cells. ET-1 also upregulates the expression of its cognate receptor, ET-BR, in monocytes, thus amplifying the expression of the chemokines MCP-1 and IL-8.

**FIGURE 6.** microRNA-195a regulates posttranscriptional expression of FLAP. A, Putative binding sites for microRNA-195a and microRNA-223 in the MIP-1β 3' UTR. B, Affect of ET-1 on the expression levels of microRNA-20, microRNA-194, microRNA-195a, and microRNA-223 in THP-1 cells. Cells were treated with ET-1 for 30 min. microRNA was isolated as described in Materials and Methods. C, Effect of anti-microRNA inhibitors for microRNA-195a and overexpression of microRNA-195a on ET-1–induced MIP-1β mRNA expression in THP-1 cells. Cells were treated with ET-1 for 30 min. D, MIP-1β protein levels in THP-1 cells transfected with anti-microRNA inhibitors and microRNA-195a overexpression plasmids. Cells were treated with ET-1 for 4 h. Data are representative of three independent experiments. E, Effect of anti-microRNA inhibitors and overexpression of microRNA-195a in human monocytes treated with ET-1 for 30 min. Total mRNA was isolated and subjected to qRT-PCR. qRT-PCR data represent fold increases in FLAP mRNA levels, upon treatment with hypoxia, compared with that of no treatment and is normalized to GAPDH mRNA levels. Data are presented as mean ± SD and are representative of three independent experiments.
In this study, we observed the role of the transcription factor HIF-1α in the transcription of the MIP-1β gene. The ET-1–mediated upregulation of MIP-1β occurred via HIF-1α, independent of hypoxia. This was demonstrated by a number of approaches. Silencing with HIF-1α siRNA inhibited ET-1–mediated MIP-1β expression. Furthermore, transfection with PHD-2 siRNA, which increases HIF-1α protein levels (42), led to increased MIP-1β expression in the absence of ET-1 treatment. Transfection with HIF-2α siRNA did not affect ET-1–induced MIP-1β mRNA expression, showing that HIF-2α was not involved. It is pertinent to mention that HIF-1α is expressed ubiquitously, whereas HIF-2α is expressed mostly in vascular endothelial cells during development, and all of the transformed cell lines show HIF-2α expression (43). Our studies showed an increase in HIF-1α protein in response to ET-1, independent of hypoxia. As illustrated in the schematics of Fig. 7, the increase in HIF-1α protein occurred via activation of both the PI3K and the NADPH oxidase pathways, because pharmacological inhibitors of PI3K and NADPH oxidase attenuated ET-1–mediated HIF-1α protein expression. However, the mechanism(s) by which ET-1 stabilizes HIF-1α protein in a hypoxia-independent manner is not known. The stabilization of HIF-1α protein may occur by reactive oxygen species pathways, as has been observed previously (27, 44). It may involve the inactivation of the PHD-2 enzyme by reactive oxygen species, as demonstrated previously for PI3K-mediated HIF-1α activation (27). TGF-β1–induced HIF-1α stabilization also has been shown to involve selective inhibition of the PHD-2 enzyme (45). The activation of the PI3K pathway (Fig. 7) by ET-1 may affect HIF-1α expression via phosphorylation as noted previously (46, 47).

The role of HIF-1α in the expression of MIP-1β was supported further by analysis of the MIP-1β promoter. Our studies showed that ET-1 induced full-length (−1065/+43) MIP-1β promoter luciferase activity. The truncated MIP-1β promoter (−485/+43) showed ~80% activity, whereas further deletion (−264/+43) did not exhibit ET-1–mediated promoter activity compared with that of the full-length promoter (−1065/+43). An in silico analysis of the MIP-1β full-length promoter (Gene Accession No. SS6704; www.ncbi.nlm.nih.gov/nuccore/236042) revealed the presence of five HREs, designated as HREs 1–5, as illustrated in the schematics of Fig. 4A. In addition, there were motifs for NF-κB (−87 to −77) and AP-1/Cres-lik (−109 to −91) in the MIP-1β promoter. Mutation of each of HRE singly (HRE1 to HRE5, designated as HRE1M, HRE2M, HRE3M, HRE4M, and HRE5M) in the full-length MIP-1β promoter led to a 50% reduction in promoter activity, indicating that each HRE site was essential for ET-1–induced MIP-1β promoter activity. Moreover, mutation of the putative AP-1 site in the MIP-1β promoter reduced ~80% of the ET-1–mediated promoter activity. Mutation of two HRE sites (HRE1 and HRE4) also resulted in ~90% reduction in ET-1–induced MIP-1β promoter activity, suggesting that a combination of two or more HRE sites can be sufficient for transcription of MIP-1β. Moreover, mutations of two HRE sites (HRE1 and HRE4) and one AP-1 site completely abrogated promoter activity, showing that both AP-1 and HRE binding sites are essential for ET-1–mediated MIP-1β transcription. However, mutation of the NF-κB motif only modestly (~20%) reduced ET-1 promoter activity. These results thus showed that all five HREs, AP-1, and NF-κB cis-elements in the MIP-1β promoter were essential for optimum ET-1–mediated MIP-1β promoter activity. Using EMSAs, we observed increased binding of HIF-1α protein to the HREs of the MIP-1β promoter in nucleic extracts from ET-1–treated THP-1 cells. Additionally, HIF-1α protein was observed to bind to the HREs of MIP-1β in native chromatin as demonstrated by ChIP analysis utilizing Ab to HIF-1α. Furthermore, our studies showed that ET-1–mediated expression of
MIP-1β involved ET-BR receptor. It is pertinent to mention that both MIP-1α and MIP-1β are derived from separate genes; however, they are highly homologous (~60% identity) and yet differentially affect mucosal and systemic adaptive immunity (48). We, however, did not investigate the effect that ET-1 may have on the expression of MIP-1α.

Recent studies show that gene expression can be regulated posttranscriptionally by microRNAs (49, 50). Accordingly, we identified the microRNAs that likely regulate MIP-1β gene expression in response to ET-1. In silico analysis of the 3′ UTR of the MIP-1β mRNA showed the complementary binding sites for microRNA-20, microRNA-194, and microRNA-195 as indicated in the schematics of Fig. 6A. The expression of these microRNAs was reduced in ET-1-treated THP-1 cells, implying that upregulation of MIP-1β expression required downregulation of these microRNAs. We selected microRNA-195a among several microRNAs that showed complementary target sites in 3′-UTRs of MIP-1β mRNA, and also the microRNA-195a binding site was conserved among species, suggesting a possible role for microRNA-195a in the regulation of MIP-1β mRNA and protein expression. Our studies showed that overexpression of microRNA-195a completely inhibited ET-1–induced MIP-1β expression, whereas anti–microRNA-195a augmented ET-1–mediated MIP-1β expression, in both PBMs and THP-1 cells, showing that microRNA-195a is a likely negative regulator of ET-1–mediated MIP-1β transcription and translation. MicroRNAs have the potential of silencing gene expression by several different mechanisms other than translational repression of target mRNA (51). Recently, microRNAs have been identified that target sites in the 3′-UTRs of selected genes involved in the innate immune response (52), expression of TLRs (53), and erythropoiesis (54). The functional role of characterized microRNA-195a in vitro warrants validation in vivo. Recent studies (55) have shown that microRNA-195 plays an important role in cardiac stress. Specifically, transgenic mice that were made hypertrophied showed increased expression of microRNA-195 and microRNA–199a-5p (55). Additionally, overexpression of microRNA-195 induced hypertrophic growth in vitro, whereas microRNA–199a-5p overexpression led to a pronounced morphological response with a phenotype of eccentric cardiac hypertrophy (55), indicating that regulated expression of these microRNAs in vivo causes cardiac remodeling. Our previous studies have shown the importance of microRNA–199a-5p in the regulation of HIF-1α (56). However, a search for potential microRNA binding sites on the 3′ UTR of HIF-1α mRNA, using the miRANDA (http://www.microrna.org) software, did not reveal a binding site for microRNA-195. Additionally, in silico analysis did not show any potential binding sites for microRNA–199a-5p on the 3′ UTR of MIP-1β. Thus, we suggest that microRNA-195 can directly modulate MIP-1β expression, whereas microRNA-199 that affects HIF-1α levels may indirectly affect expression of MIP-1β via the HIF-1α pathway. We suggest that these microRNAs individually or together can modulate chemokine (CCL4) expression and thus chemotaxis of leukocytes by their effect on their specific targets. Studies have shown that microRNA-192 levels are high in tissues from type 1 and type 2 diabetic mice and also in diabetic kidney glomeruli (57, 58). The locked nucleic acid-modified oligoacetolides, as microRNA-inhibitors have been delivered successfully in mice (59), and locked nucleic acid-modified anti–microRNA-192 have been shown to specifically reduce microRNA-192 levels in mouse kidney and reduce diabetic nephropathy (58). Our studies demonstrate for the first time, to our knowledge, that posttranscriptional repression by microRNA-195a provides another avenue by which ET-1–mediated MIP-1β expression can be modulated. Our recent studies (60) show mice that overexpress Pigf induce ET-1 and concomitantly result in pulmonary hypertension. Additionally, these findings were corroborated in 123 patients with SCD (60) in whom plasma Pigf levels were associated with increased tricuspid regurgitant velocity, the latter a measure of peak pulmonary artery pressure. Because HIF-1α plays an important role in innate immune function (61) and ET-1 expression (14), therapeutic strategies utilizing inhibitors of HIF-1α and also inhibitors of microRNAs provide new avenues to ameliorate inflammation and pulmonary hypertension.

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Disclosures
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