Available online at www.sciencedirect.com
ScienceDirect
Microvascular Research 73 (2007) 163–172
Microvascular Research
www.elsevier.com/locate/ymvre

Hypoxia inducible factor-1 modulates hemin-induced IL-8 secretion in microvascular endothelium

Ramesh Natarajan, Bernard J. Fisher, Alpha A. Fowler III *

Division of Pulmonary Disease and Critical Care Medicine, Department of Internal Medicine, Virginia Commonwealth University Medical Center, Box 980050, Richmond, VA 23298, USA

Received 18 October 2006; revised 5 January 2007; accepted 27 January 2007
Available online 8 February 2007

Abstract

Ischemia/Reperfusion injury and hemolysis are characterized by erythrocyte lysis and release of free heme into the microcirculation. Following substantial erythrocyte lysis, heme overwhelms circulatory heme-binding protein networks rapidly forming hemin, the oxidized form of iron protoporphyrin IX. Hemin's role in modulating inflammatory responses in microvascular endothelium (MVEC) remains ill-defined. We studied the impact of hemin exposure on human MVEC interleukin-8 (IL-8) expression. Hemin significantly up-regulated MVEC IL-8 secretion and was associated with cellular iron loading. Hemin-induced IL-8 up-regulation was significantly attenuated by increasing environmental serum concentrations. As well, hemin-induced IL-8 secretion was significantly reduced in a concentration-dependent fashion following pyrrolidine dithiocarbamate exposure, suggesting that induction occurred via an oxidant-sensitive mechanism. Interestingly, transfection studies revealed that oxidant-driven transcription factors NF-κB and AP-1 played no role in hemin-induced IL-8 transcription. In studies employing actinomycin D, hemin was found to dramatically lengthen IL-8 mRNA half-life. Of major importance in the current report was the finding that hypoxia inducible factor-1 (HIF-1), a powerful transcription factor mediating tissue responses to hypoxia, potently regulated hemin-induced IL-8 secretion in human MVEC. Activation of HIF-1 via the prolyl hydroxylase inhibitor dimethyloxalylglycine attenuated hemin-induced IL-8 secretion. These studies were confirmed via DNA-directed siRNA silencing of HIF-1α. In conclusion, hemin induces a serum protein-sensitive pro-inflammatory phenotype in MVEC via an oxidant-sensitive mechanism that is powerfully regulated by HIF-1.

Keywords: Hypoxia inducible factor-1; Hemin; Heme; Interleukin-8; Human microvascular endothelium; HMEC-1; Erythrocyte lysis; Pyrrolidine dithiocarbamate; Reactive oxygen species; Iron

Introduction

Hypoxia inducible factor-1 (HIF-1) is a potent α, β transcription factor that mediates tissue responses to hypoxia. Once activated the heterodimer binds to the DNA consensus sequence 5′-RCGTG-3′ driving transcription of many genes involved in oxygen homeostasis, including inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and heme oxygenase-1 (HO-1) (Wang and Semenza, 1993; Wenger, 2002). The activity of HIF-1 importantly depends upon the expression and activity of the α subunit which is regulated via post-translational hydroxylation of proline residues mediated by one of three prolyl hydroxylase isoforms (PHD 1, 2, and 3) (Jiang et al., 1996; Hirsila et al., 2003). Proline hydroxylation targets HIF-1α for proteosome degradation following binding by the von Hippel Lindau tumor suppressor protein E3 ubiquitin ligase complex (Huang et al., 1998). Cellular hypoxia produced by exposure to diminished environmental oxygen tensions or by "chemical hypoxia" mediated through prolyl-hydroxylase inhibition and cobalt chloride stabilizes HIF-1α, leading to αβ heterodimerization and activation. Beyond regulation of genes involved in oxygen homeostasis, emerging information suggests that HIF-1 activation regulates genes that mediate inflammatory responses which occur following cytokine release (e.g., tumor

Abbreviations: FAC, ammonium iron (III) citrate; DMOG, dimethyloxalylglycine; PDTC, pyrrolidine dithiocarbamate; Sn(IV)PPIX, tin protoporphyrin; Zn(II)PPIX, zinc protoporphyrin.

* Corresponding author. Fax: +1 804 828 3559.
E-mail address: afowler@vcu.edu (A.A. Fowler).

0026-2862/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.mvr.2007.01.002

necrosis factor) and ischemia/reperfusion (I/R) injury. Recently, we showed that activating HIF-1 by systemic administration of the prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG) significantly attenuated post-ischemic infarct size, serum IL-8 surges, and myocardial polymorphonuclear neutrophil (PMN) infiltration in a rabbit model of cardiac ischemia/reperfusion injury (Ockaili et al., 2005). Attenuated cardiac I/R injury in this study was associated with robust expression of cardiac HO-1.

Heme, an omnipresent iron III–protoporphyrin IX complex present in erythroid and non-erythroid cells, appears in plasma most frequently following erythrocyte lysis (Uzel and Conrad, 1998). At tissue sites where micro-hemorrhage or significant stasis or cessation of blood flow occurs, heme released from erythrocytes in the presence of chloride ions is rapidly converted to hemin, the oxidized form of iron protoporphyrin IX (Tsiftsoglou et al., 2006). Under ordinary circumstances, heme-binding plasma proteins, such as hemopexin, haptoglobin, and albumin remove hemin produced intravascularly for disposal in hepatocytes (Müller-Eberhard and Nikkila, 1989). Heme-binding proteins prevent nonspecific cellular hemin uptake, thus, attenuating hemin-catalyzed oxidative reactions. However, following substantial erythrocyte lysis (e.g., sickle cell crisis, immune mediated hemolysis) large concentrations of heme are released into the circulation over short time periods potentially overcoming the heme-binding protein network. Hemin formed in this way permeates endothelial cell membranes or cell membranes of adjacent cell populations releasing free intracellular iron. Bioavailable intracellular iron drives transcription of oxidant-dependent genes (e.g., chemokines). Balla et al. and Glei et al. showed that the release of free iron enables hemin to play a significant role in catalyzing oxidant-mediated (e.g., H_2O_2) reactions that result in lipid peroxidation and cell membrane damage, inducing cell injury or death (Balla et al., 1991, 1993; Glei et al., 2006). Other work underscores the crucial role hemin accumulation plays in circulating cell populations such as PMN. Graca-Souza et al. showed that hemin promotes significant increases in reactive oxygen species generation and IL-8 production in human PMN, suggesting that hemin plays a key role in initiation and amplification of inflammatory responses through its effects on PMN as well (Graca-Souza et al., 2002).

Ischemia/Reperfusion injury is characterized by erythrocyte lysis and release of free heme at microvascular injury sites that arise from such local factors as tissue acidosis and reactive oxygen species generation (Bhakuni et al., 2005; Bracci et al., 2002; Tavazzi et al., 2000). The extent to which hemin accumulation modulates inflammatory responses in microvascular endothelium (MVEC) remains unclear. A further area of uncertainty is the role that HIF-1 might play in modulating pro-inflammatory conditions induced by the interactions between hemin and MVEC. In the current study, we examined the impact of hemin exposure on the expression of the pro-inflammatory chemokine IL-8 in human MVEC. Our work shows that hemin significantly up regulates MVEC IL-8 expression through an oxidant-sensitive mechanism that prolongs IL-8 mRNA half life. Importantly, this work shows that activation of HIF-1 in MVEC dramatically attenuated hemin-induced IL-8 secretion.

Methods

Reagents and chemicals

Iron protoporphyrin (hemin) as well as zinc, and tin protoporphyrins were obtained from Frontier Scientific, Inc. (Logan, UT). Stock solutions of protoporphyrins (10 mM) were prepared in 1.4 N NH_4OH and stored at −20 °C. Dimethyloxalylglycine (DMOG) was purchased from Cayman Chemical Company (Ann Arbor, MI). Recombinant human TNFα was purchased from Collaborative Biomedical Products (Bedford, MA). The human IL-8 ELISA kit was obtained from R&D Systems (Minneapolis, MN). Thermoscript RT-PCR system, MCDB-131 culture medium, and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA). Sterile tissue culture plasticware was obtained from Corning Inc. (Corning, NY). The Brilliant SYBR Green QPCR Master Mix was obtained from Stratagene (La Jolla, CA). The IL-8 promoter, pGL3-BF^2-Luc, contains the proximal 421 bp inclusive of NF-κB and AP-1 binding sites as previously reported (Fowler et al., 1999). The dual luciferase assay system and pHRL-null vector were purchased from Promega Corp. (Madison, WI). RNA isolation kits and Effectene™ transfection reagents were obtained from Qiagen Inc. (Valencia, CA). Rabbit anti-HIF-1α antibody (sc-10790) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were obtained from Sigma-Aldrich Company (St. Louis, MO).

Endothelial cell culture

The human microvascular endothelial cell line (HMEC-1) was obtained from the Centers for Disease Control and Prevention (Atlanta, GA) (Ades et al., 1992). Cells were cultured in medium MCDB-131 supplemented with 10% FBS, hydrocortisone (1 μg/ml), and epidermal growth factor (10 ng/ml) under a 5% CO_2 atmosphere at 37 °C as previously reported (Natarajan et al., 2002). Cells were cultured in 24-well plates for transfections, cytokine, and iron assays. For total RNA preparation and nuclear extracts HMEC-1 was cultured in 35 mm (9.6 cm^2) dishes. Growth media were replaced with reduced serum buffered media (MCDB-131, 2% FBS, 10 mM HEPES) prior to experimentation.

Quantification of HMEC-1 cytotoxicity

Cell viability was determined by quantifying reduction of a fluorogenic indicator, Alamar blue (Alamar Biosciences, Sacramento, CA), according to the manufacturer's instructions. Alamar Blue was added to a final concentration of 10%. Viable cells metabolically reduce this non-toxic redox indicator. The reduction of the dye results in the Alamar Blue conversion of resazurin to resarufin, which is measured at 530 nm excitation and 590 nm emission using a microplate fluorometer. The reduction of Alamar Blue compared to untreated cells is utilized as an index of viability and expressed as percentage of control medium exposed cells (Ghosh et al., 2003).

Quantification of intracellular iron

Intracellular iron was quantified as described previously (Riemer et al., 2004). HMEC-1 in 24-well plates were incubated with hemin (10–100 μM) for 1 h. Cells were washed ×2 with ice-cold PBS containing 1 mM desferroxamine (DFX), and lysed with 50 mM NaOH. Cell lysates were exposed to an acid permanganate solution (iron-releasing reagent) and incubated for 2 h at 60 °C. Lysates were cooled to 22 °C then incubated for 30 min with the iron detection reagent ferrozine. Absorbance was measured at 550 nm and compared to the absorbance of equally treated iron (III) chloride ($FeCl_3$) standard solutions (1–300 μM). Intracellular iron concentrations determined were normalized to micrograms of adherent cell protein.

IL-8 protein quantification

Output of IL-8 protein was quantified in conditioned medium from HMEC-1 cultures using a sandwich ELISA prepared with human IL-8 DuoSet antibody pairs (R&D Systems) according to manufacturer's instructions. Absorbance at 450 nm was recorded and IL-8 concentrations determined from a four-parameter