Vascular Pharmacology 51 (2009) 133–139

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

HIF-1 attenuates Ref-1 expression in endothelial cells: Reversal by siRNA and inhibition of geranylgeranylation

Agnieszka Loboda, Anna Stachurska, Jerzy Dorosz, Marek Zurawski, Joanna Wegrzyn, Magdalena Kozakowska, Alicja Jozkowicz, Jozef Dulak *

Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

ARTICLE INFO

Article history:
Received 5 August 2008
Received in revised form 27 April 2009
Accepted 28 May 2009

Keywords:
Hypoxia
Base excision repair
Ape/Ref-1
Endothelial cells
Statins

ABSTRACT

Redox factor-1 (Ref-1), a multifunctional protein with DNA repairing activities, plays a cytoprotective function by post-translational redox modification of numerous transcription factors, including hypoxia inducible factor-1 (HIF-1).

In the present study, activation of HIF-1 by hypoxia and dimethyloxaloylglycine (DMOG), a hypoxia mimic, diminished Ref-1 mRNA and protein expression in human microvascular endothelial cells (HMEC-1). Similarly, adenoviral delivery of the stabilized form of HIF-1α decreased Ref-1 mRNA and protein levels. Accordingly, HIF-1α siRNA abolished the hypoxia-induced inhibition of Ref-1 expression, indicating the role of HIF-1 in down-regulation of Ref-1. Also, translocation of Ref-1 from nucleus to cytoplasm after HIF-1 activation was noted. Interestingly, we observed the restoration of Ref-1 expression in hypoxia by pharmacologically relevant doses of atorvastatin. This effect was dependent on the inhibition of protein geranylgeranylation, but not farnesylation, as only the inhibitor of the former but not the latter prenylation step restored the Ref-1 expression.

The regulation of Ref-1 by statins may be considered as a novel mechanism of their beneficial effects on endothelium.

1. Introduction

Ref-1 (redox factor-1), which is also called human apurinic/apyrimidinic endonuclease (hAPE-1), Apex or HAP-1, is a bifunctional protein possessing redox and endonuclease properties (Xanthoudakis et al., 1994). The endonuclease activity of Ref-1 is essential in the DNA repair process called base excision repair (BER) (Barzilay and Hickson, 1995) and is involved in the repair of spontaneous and oxidative DNA damage, as well as in the processing of DNA alkylation damage (Fritz et al., 2003). Moreover, Ref-1, together with thioredoxin, mediates redox activation and DNA binding of various transcription factors involved in cell growth, differentiation, and stress response (reviewed in Evans et al., 2000). Among them, cooperation with hypoxia inducible factor-1 (HIF-1) can be important in regulation of hypoxia-dependent gene expression.

Tissue hypoxia (low oxygen concentration in tissues) is a state that characterizes several pathophysiological situations, like ischemia, atherosclerosis or cancer. Hypoxia changes expression of proteins involved in erythropoiesis (Wang and Semenza, 1993), angiogenesis (Risau, 1997), vasomotor tone (Kourembanas et al., 1991) and metabolic pathways (Firth et al., 1995), and its effect can be mediated by reactive oxygen species (ROS) (reviewed in Semenza, 2000).

Hypoxia-dependent regulation of gene expression occurs mostly at the transcriptional level due to HIF-1α mRNA stabilization. Degradation of the HIF-1α subunit at normal oxygen concentration is regulated by prolyl hydroxylases (PHDs 1–3) and oxygen-, iron-, and 2-oxoglutarate-dependent enzymes (for reviews see Pugh et al., 1999; Willam et al., 2004). Accordingly, the activity of PHDs can be diminished not only by hypoxia, but also by iron chelators or 2-oxoglutarate analogues, like dimethyloxaloylglycine (DMOG).

Ref-1 was shown to be regulated by hypoxia in endothelial (Hall et al., 2001; Ziel et al., 2004) and cancer cells (Hedley et al., 2004). In human umbilical vein (HUVEC) and calf pulmonary artery (CPAE) endothelial cells kept for 18 h in hypoxic conditions, a decrease in Ref-1 expression was noted (Hall et al., 2001). In contrast, in the hypoxic microenvironment of invasive cervical carcinomas (Hedley et al., 2004) and in rat pulmonary artery endothelial cells (PAEC) (Ziel et al., 2004), a significant increase of Ref-1 expression has been observed.

We previously showed that, in HMEC-1 cells, hypoxia differently influences angiogenic gene expression, up-regulating vascular

Abbreviations: AdGFP, adenoviral vectors containing GFP cDNA; AdHIF-1α, adenoviral vectors containing HIF-1α cDNA; BER, base excision repair; DMOG, dimethyloxaloylglycine; FTI-277, farnesyltransferase inhibitor; GGTI-298, geranylgeranyl transferase inhibitor; HIF-1, hypoxia inducible factor-1; HMEC-1, human microvascular endothelial cells; PHDs, prolyl hydroxylases; Ref-1, redox factor-1; VEGF, vascular endothelial growth factor; 3-HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

* Corresponding author. Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland. Tel.: +48 12 664 63 75; fax: +48 12 664 69 18.
E-mail address: jozef.dulak@uj.edu.pl (J. Dulak).

1537-1891/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.vph.2009.05.005

endothelial growth factor (VEGF), having no influence on heme oxygenase-1 (HO-1) protein level or diminishing IL-8 or endothelial nitric oxide synthase (eNOS) production (Loboda et al., 2006a,b). The latter effect was reverted by atorvastatin, a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor (Loboda et al., 2006a). Atorvastatin, a member of the statin family, inhibits the synthesis of mevalonate, which also prevents the synthesis of its downstream intermediates, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). However, the protective function of statins in cardiovascular diseases is not only dependent on lowering cholesterol level, but is also mediated by the pleiotropic effects of these drugs on regulating the expression of many genes in the vascular system (Liao and Laufs, 2005; Loboda et al., 2006a).

Therefore, the aim of this study was to determine the effect of HIF-1 activation on Ref-1 expression in HMEC-1 cells and to examine whether inhibition of geranylgeranylation and farnesylation by the use of atorvastatin and specific inhibitors of these processes is able to reverse the inhibitory effect of hypoxia on Ref-1 expression.

2. Materials and methods

2.1. Reagents

Dimethyloxaloylglycine (DMOG) was obtained from Alexis Biochemicals, atorvastatin was purchased from Calbiochem and kindly provided by Pfizer. Prenylation inhibitors GGTI-298 and FTI-277 were from Sigma-Aldrich. The cell culture medium (MCDB 131) was purchased from Gibco. Oligo(dT) primers, dNTPs and MMLV reverse transcriptase were obtained from Promega. Rabbit polyclonal anti-Ref-1 antibody was obtained from Santa Cruz, mouse monoclonal anti-α-tubulin was from Calbiochem, anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was bought from Cell Signalling whereas anti-mouse IgG conjugated with horseradish peroxidase (HRP) was from Pierce. Nuclear Extract Kit used for cytosolic and nuclear fraction isolation was purchased from Active Motif. Lipofectamine used for siRNA delivery was obtained from Invitrogen, and chemically synthesized scrambled and HIF-1α siRNA were from Dharmacon.

2.2. Cell culture and incubation experiments

Human microvascular endothelial cells (HMEC-1) were kindly provided by Dr. Francis Candal (Center for Disease Control and Prevention, Atlanta) and they were cultured as described previously (Loboda et al., 2005). Cells were kept either under normoxic (21% O_2) or hypoxic conditions (2% O_2) for 24 h. DMOG (250 μM–1 mM) was dissolved in PBS and added to the cells for the indicated time for each experiment. Atorvastatin was dissolved in DMSO. Solvent at appropriate concentration was included in the control.

2.3. Transfection of cells with siRNA

The cells were transfected with 50 nM of chemically synthesized siRNA targeting human HIF-1α mRNA and scrambled siRNA was used as a control. siRNA and lipofectamine 2000 were separately diluted in OptiMEM medium without serum, incubated 5 min at room temperature, combined and then incubated for the next 20 min followed by adding it to the cells for 24 h.

2.4. Transduction of the cells with adenoviral vectors

Adenoviral vector containing HIF-1α cDNA (AdHIF-1α) was a kind gift from Prof. Seppo Yla-Herttuala (University of Kuopio, Kuopio, Finland) and Prof. Lorenz Poellinger (Stockholm, Sweden). pAdHIF-1α was generated as described (Pajusola et al., 2005). Briefly, the construct was stabilized against prolyl hydroxylation and subsequent ubiquitin-mediated proteolytic degradation in normoxic conditions by point mutations (P402A/P563A). A control vector harboring green fluorescent protein (GFP) cDNA (AdGFP) was produced using the Adeno-X system. Vectors were propagated in HEK 293 cells and then titered by detection of the hexon protein with Adeno-X rapid titer ELISA kit according to the vendor's protocol.

2.5. Immunocytochemistry

24 h after seeding 10^5 cells on a BD-Falcon two-chamber culture slide, the cells were stimulated with DMOG for 3 h. Afterwards, cells

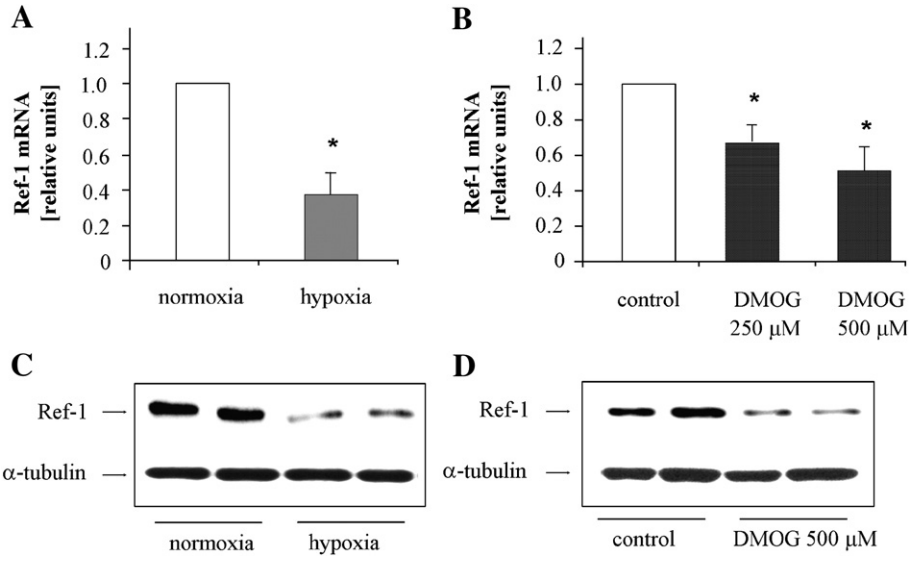

Fig. 1. HIF-1 activation down-regulates Ref-1 activity. Attenuation of Ref-1 mRNA level after stimulation with hypoxia (2% oxygen) (A) or with DMOG (B) for 24 h detected with reverse transcription real-time RT-PCR. Results are calculated with the $2^{-\Delta\Delta Ct}$ method and data are expressed as mean ± SD of three independent experiments performed in duplicates, $^*p<0.05$ vs normoxia (A) and vs control (B). Decrease in Ref-1 levels in relation to α-tubulin in hypoxia (C) and after DMOG treatment (D) determined by Western blotting. Representative of three different experiments.