Free Radical Biology and Medicine 61 (2013) 197–205

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Epigenetic regulation of extracellular-superoxide dismutase in human monocytes

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ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form
13 March 2013
Accepted 10 April 2013
Available online 18 April 2013

Keywords:
Extracellular-superoxide dismutase
Differentiation
12-*O*-tetradecanoylphorbol-13-acetate
DNA methylation
Histone acetylation

ABSTRACT

Extracellular-superoxide dismutase (EC-SOD) is a major SOD isozyme mainly present in the vascular wall and plays an important role in normal redox homeostasis. We previously showed the significant reduction or induction of EC-SOD during human monocytic U937 or THP-1 cell differentiation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), respectively; however, its cell-specific expression and regulation have not been fully elucidated. It has been reported that epigenetic factors, such as DNA methylation and histone modification, are involved in several kinds of gene regulation. In this study, we investigated the involvement of epigenetic factors in EC-SOD expression and determined high levels of DNA methylation within promoter and coding regions of EC-SOD in THP-1 cells compared to those in U937 cells. Moreover, treatment with a DNA methyltransferase inhibitor, 5-azacytidine, significantly induced the expression of EC-SOD in THP-1 cells, indicating the importance of DNA methylation in the suppression of EC-SOD expression; however, the DNA methylation status did not change during THP-1 cell differentiation induced by TPA. On the other hand, we detected histone H3 and H4 acetylation during differentiation. Further, pretreatment with histone acetyltransferase inhibitors, CPTH2 or garcinol, significantly suppressed the TPA-inducible EC-SOD expression. We also determined the epigenetic suppression of EC-SOD in peripheral blood mononuclear cells. Treatment with granulocyte macrophage colony-stimulating factor (GM-CSF)/granulocyte-CSF induced that expression. Overall, these findings provide novel evidence that cell-specific and TPA-inducible EC-SOD expression are regulated by DNA methylation and histone H3 and H4 acetylation in human monocytic cells.

Introduction

Superoxide dismutase (SOD) plays a pivotal role against oxidative stress induced by reactive oxygen species (ROS) in mammals, and its deficiency may lead to various diseases, such as asthma, atherosclerosis, and type 2 diabetes [1–3]. Extracellular-SOD (EC-SOD) is a major SOD isozyme which protects cells from the damaging effects of superoxide by accelerating the dismutation reaction of superoxide [4]. EC-SOD is especially secreted into the extracellular space and distributed mainly in blood vessel walls, binding to the heparan sulfate proteoglycan in the glycocalyx on the cell surface [5,6]. EC-SOD content is very low compared to other SOD isozymes, but the presence of a high level of EC-SOD throughout the vessel walls might have an important protective role as an anti-inflammatory factor against superoxide in the vascular system [7–9].

Monocytes/macrophages are considered to be important components of blood vessels and to participate in immune systems and arteriosclerosis. Indeed, in artery plaques, the accumulation of monocytes/macrophages is observed, and the events are recognized to be implicated in the exacerbation of atherosclerosis [10,11]. EC-SOD expression is also observed in monocytes/macrophages, and its administration reduces the oxidation of low density lipoprotein, which promotes atherosclerosis [12]. We previously observed significant differences in the cell-specific and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-triggered regulation of EC-SOD in human monocytic U937 and THP-1 cells [13,14]. Treatment with TPA significantly decreased the expression of EC-SOD in U937 cells, but significantly induced its expression in THP-1 cells; however, the

Abbreviations: EC-SOD, extracellular-SOD; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CPTH2, cyclopentylidene-(4-(4′-chlorophenyl)thiazol-2-yl)hydrazone; ROS, reactive oxygen species; AZA, 5-azacytidine; TSA, trichostatin A; PBMCs, peripheral blood mononuclear cells; GM-CSF, granulocyte macrophage colony-stimulating factor; M-CSF, macrophage-CSF; PBS, phosphate-buffered saline; FCS, fetal calf serum; RT-PCR, reverse transcriptional-polymerase chain reaction; MSP, methylation-specific PCR; ChIP, chromatin immunoprecipitation; HAT, histone acetyltransferase; HDAC, histone deacetylase; DNMT, DNA methyltransferase; MBD, methyl-binding proteins.

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.04.013

mechanism of cell-specific and TPA-inducible EC-SOD expression in THP-1 cells remains to be elucidated.

It is well recognized that epigenetics is usually referred to as a mitotically heritable change in gene expression that does not involve any changes of the DNA sequence [15]. DNA methylation, a major epigenetic modification, occurs at the 5′ position of cytosine within CpG. Methylation of CpG within gene promoters plays a pivotal role in tissue- and stage-specific gene regulation [16]. On the other hand, histone modifications of the N-terminal tail, such as methylation and acetylation at lysine or arginine residues, also associate with an active chromatin structure and induce or suppress gene expression [17–19]. Recently, it has been shown that the cell-specific and inducible expression of human EC-SOD is mostly attributed to DNA methylation and histone modification of its promoter region [20]. According to previous reports, in this study, we investigated the involvement of epigenetic factors in the cell-specific and TPA-inducible EC-SOD expression in THP-1 cells. As expected, the cell-specific EC-SOD expression in human monocytes can be attributed to differential DNA methylation of its promoter and coding regions. In addition, we determined the involvement of histone modification, especially histone H3 and H4 acetylation, in TPA-inducible EC-SOD expression in THP-1 cells.

Materials and methods

Reagents

TPA was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 5-Azacytidine (AZA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cyclopentylidene-(4-(4′-chlorophenyl)thiazol-2-yl)hydrazone (CPTH2) was purchased from Calbiochem (San Diego, CA, USA). Garcinol was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Trichostatin A (TSA) was purchased from Cayman Chemical (Ann Arbor, MI, USA). EcoRI was purchased from Boehringer Mannheim (Indianapolis, IN, USA). McrBC was purchased from New England BioLabs (Beverly, MA, USA). Anti-acetyl-histone H3 and H4 rabbit polyclonal antibodies were purchased from Millipore Co. (Billerica, MA, USA). Anti-Sp1 and -Sp3 rabbit monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Biotin-conjugated goat anti-rabbit and -mouse IgG (H+L) antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA). Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage-CSF (M-CSF) were purchased from PeproTech (Rocky Hill, NJ, USA).

PBMC isolation

For preparation of PBMCs, informed consent was obtained from the healthy donors and ethics approval for the study was granted by the Institutional Ethics Committee. Whole blood was collected from healthy donors into EDTA-2Na tubes (Terumo Co., Tokyo, Japan). PBMCs were isolated from whole blood using Histopaque-1077 (Sigma-Aldrich). Briefly, whole blood was diluted 1:1 with phosphate-buffered saline (PBS), layered over Histopaque-1077, and centrifuged at 400 *g* for 30 min. PBMC-rich plasma was collected using a plastic pipette, washed with PBS, and centrifuged at 200 *g* for 10 min. PBMC pellets were again washed with PBS and centrifuged at 200 *g* for 10 min.

Cell culture

Human leukemic cell lines, THP-1 and U937 cells, and PBMCs were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. For differentiation of THP-1 and U937 cells, the cells were seeded at 6×10^5 cells/mL in 3.5 cm dishes and 100 nM or 30 nM TPA was added, respectively. PBMCs were seeded at 1.5×10^6 cells/mL in 3.5 cm dishes and cultured in RPMI supplemented with or without 10 μg/mL GM-CSF and 10 μg/mL M-CSF for 6 days. The culture medium was replaced every 3 days.

Table 1
Primer sequences used in RT-PCR.

Primer	Sequences	Amplicon size
EC-SOD	S; 5′-AGAAAGCTCTCTTGGAGGAG-3′ AS; 5′-ACCGCGAAGTTGCCGAAGTC-3′	496 bp
HDAC1	S; 5′-CCTGAGGAGAGTGGCGATGA-3′ AS; 5′-GTTTGTCAGAGGAGCAGATCGA-3′	69 bp
HDAC2	S; 5′-GCTCTCAACTGGCGGTTCAG-3′ AS; 5′-AGCCCAATTAACAGCCATATCAG-3′	75 bp
HDAC3	S; 5′-CCCAGACTTCACACTTCATCCA-3′ AS; 5′-GGTCCAGATACTGGCGTGAGTT-3′	70 bp
HDAC4	S; 5′-GGGAGAGGATCAAGCTCGTTT-3′ AS; 5′-GGGAGAGGATCAAGCTCGTTT-3′	73 bp
HDAC5	S; 5′-CAACGAGTCGGATGGGATGT-3′ AS; 5′-GGGATGCTGTGCAGAGAAGTC-3′	74 bp
HDAC6	S; 5′-TGCCTCTGGGATGACAGCTT-3′ AS; 5′-CCTGGATCAGTTGCTCCTTGA-3′	69 bp
HDAC7	S; 5′-AGCAGCTTTTTGCCTCCTGTT-3′ AS; 5′-TCTTGCGCAGAGGGAAGTG-3′	66 bp
HDAC8	S; 5′-CGGCCAGACCGCAATG-3′ AS; 5′-CACATGCTTCAGATTCCCTTT-3′	56 bp
HDAC9	S; 5′-AGGCTCTCCTGCAGCATTTATT-3′ AS; 5′-AAGGGAACTCCACCAGCTACAA-3′	75 bp
HDAC10	S; 5′-ATGACCCCAGCGTCCTTTACT-3′ AS; 5′-CGCAGGAAAGGCCAGAAG-3′	66 bp
HDAC11	S; 5′-CCCCTTGGTCATGGGATTT-3′ AS; 5′-CATCCACACCAGTGCCTATAGC-3′	68 bp
18S rRNA	S; 5′-CGGCTACCACATCCAAGGAA-3′ AS; 5′-GCTGGAATTACCGCGGCT-3′	187 bp

Reverse transcriptional-polymerase chain reaction (RT-PCR) analysis

After the cells had been treated, the cells were lysed in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA and RT-PCR were prepared and performed by the methods described in our previous report [21], with minor modifications. The primer sequences used in RT-PCR are presented in Table 1. After amplification, PCR products of EC-SOD and 18S rRNA were loaded onto a 2% (w/v) agarose gel for electrophoresis, and densitometric analysis of the PCR products was performed with Multi Gauge V3.0 (Fuji Film, Tokyo, Japan). For HDAC expression, real-time RT-PCR was carried out by intercalater method with SYBR Premix Ex Taq (Takara Bio Inc., Ootsu, Japan) and Thermal Cycler Dice Real Time System TP800 (Takara) according to the manufacturer's protocol.

Methylation-specific PCR (MSP) analysis

Genomic DNA was isolated using a Puregene Core kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. Bisulfite modification of genomic DNA was carried out using an EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. An aliquot of bisulfite-treated DNA (500 ng) was subjected to MSP amplification. The primer sequences used in MSP of the EC-SOD promoter and coding region were designed for the sodium bisulfite-modified template using MethPrimer software, and these MSP primers are presented in Table 2. After amplification, these PCR products were loaded onto a 2% (w/v) agarose gel for electrophoresis and visualized using FLA5100 (Fuji Film).