Free Radical Biology & Medicine 48 (2010) 1618–1625

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Oxidative stress modulates PPARγ in vascular endothelial cells

Carmelo Blanquicett, Bum-Yong Kang, Jeffrey D. Ritzenthaler, Dean P. Jones, C. Michael Hart *

Department of Medicine, Emory University, and Atlanta VA Medical Centers, Atlanta, GA 30033, USA

ARTICLE INFO

Article history:
Received 11 September 2009
Revised 5 March 2010
Accepted 9 March 2010
Available online 17 March 2010

Keywords:
PPARγ
Endothelial cell
Oxidative stress
Free radicals

ABSTRACT

The peroxisome proliferator-activated receptor γ (PPARγ) plays an important role in vascular regulation. However, the impact of oxidative stress on PPARγ expression and activity has not been clearly defined. Human umbilical vein endothelial cells (HUVECs) were exposed to graded concentrations of H_2O_2 for 0.5–72 h, or bovine aortic endothelial cells (BAECs) were exposed to alterations in extracellular thiol/disulfide redox potential (E_h) of the cysteine/cystine couple. Within 2 h, H_2O_2 reduced HUVEC PPARγ mRNA and activity and reduced the expression of two PPARγ-regulated genes without altering PPARγ protein levels. After 4 h H_2O_2 exposure, mRNA levels remained reduced, whereas PPARγ activity returned to control levels. PPARγ mRNA levels remained depressed for up to 72 h after exposure to H_2O_2, without any change in PPARγ activity. Catalase prevented H_2O_2-induced reductions in PPARγ mRNA and activity. H_2O_2 (1) reduced luciferase expression in HUVECs transiently transfected with a human PPARγ promoter reporter, (2) failed to alter PPARγ mRNA half-life, and (3) transiently increased expression and activity of c-Fos and phospho-c-Jun. Treatment with the AP1 inhibitor curcumin prevented H_2O_2-mediated reductions in PPARγ expression. In addition, medium having an oxidized E_h reduced BAEC PPARγ mRNA and activity. These findings demonstrate that oxidative stress, potentially through activation of inhibitory redox-regulated transcription factors, attenuates PPARγ expression and activity in vascular endothelial cells through suppression of PPARγ transcription.

Published by Elsevier Inc.

The ligand-activated peroxisome proliferator-activated receptor γ (PPARγ)[1] is a member of the nuclear hormone receptor superfamily of transcription factors that regulates genes involved in lipid and glucose metabolism. Clinically, synthetic thiazolidinediones that activate PPARγ are employed to improve lipid and glucose metabolism in type 2 diabetes [1]. PPARγ activation is also mediated by structurally diverse natural lipophilic ligands including fatty acids and their derivatives. Ligand-induced activation of PPARγ promotes heterodimerization with the retinoid X receptor and binding to PPAR response elements in selected target genes, resulting in transcriptional regulation. PPARγ is expressed in smooth muscle [2] and endothelial cells [3] of the vascular wall and exerts pleiotropic effects on metabolism and inflammation in vascular biology [1]. Limited evidence suggests that factors promoting vascular dysfunction may reduce PPARγ expression or activity. For example, infusion of insulin and glucose caused endothelial dysfunction, increased reactive oxygen species (ROS) production, and reduced PPARγ protein levels in rat aorta [4]. These derangements were attenuated in animals treated with the thiazolidinedione PPARγ ligand, pioglitazone. Thiazolidinedione treatment also attenuated tumor necrosis factor-α (TNF-α)- and interleukin-1α-stimulated reductions in PPARγ mRNA in mature rat adipocytes [5]. These findings suggest that certain pathological stimuli may reduce PPARγ expression.

ROS and oxidative stress play critical roles in the pathogenesis of cardiovascular disease and can modulate vascular function through a variety of mechanisms [6]. For example, hydrogen peroxide (H_2O_2), a reactive species that permeates lipid membranes, is capable of causing damage to multiple cellular components including lipids, proteins, and DNA at high concentrations [7,8]. H_2O_2, produced endogenously by inflammatory or vascular cells, can induce oxidative stress, which may contribute to vascular disease and endothelial cell dysfunction [9]. In addition to directly damaging vascular wall cells, lower ROS concentrations stimulate alterations in signaling and gene expression that modulate vascular function [10]. Recent studies have suggested mechanistic links between PPARγ and oxidative stress. For example, PPARγ activation regulated oxidative stress in colon tumor cells [11], osteoblasts [12], macrophages [13], renal tubular epithelial cells [14], cardiomyocytes [15], and vascular endothelial cells [16]. Stimulation of PPARγ in vitro and in vivo reduced the activity and expression of nicotinamide adenine dinucleotide phosphate oxidase [16–18]. In addition to reducing ROS generation, PPARγ ligands also increased vascular endothelial nitric oxide (NO) production by enhancing the activity of endothelial nitric oxide synthase [19–21]. Collectively, these

Abbreviations: AK, adenylate kinase; AP1, activator protein 1; BAEC, bovine aortic endothelial cell; Cys, cysteine; CySS, cystine; EGM, endothelial growth medium; E_h, redox potential; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; PAI-1, plasminogen activator inhibitor type-1; PPARγ, peroxisome proliferator-activated receptor γ; PTEN, phosphatase and tensin homolog; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α.

* Corresponding author. Fax: +1 404 728 7750.
E-mail address: michael.hart3@va.gov (C.M. Hart).

0891-5849/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2010.03.007

reports indicate that activation of PPARγ in vascular wall cells has the potential to reduce oxidative stress, enhance NO bioavailability, and decrease endothelial dysfunction. These reports also suggest the therapeutic potential for targeting PPARγ in cardiovascular disease [21].

Although mounting evidence has emphasized that PPARγ can regulate oxidative stress [11–13], emerging evidence suggests that oxidative stress modulates PPARγ. For example, H_2O_2-induced oxidative stress significantly reduced PPARγ activity in renal tubular epithelial cells [14] and osteoblasts [12] and inhibited PPARβ expression in human umbilical vein endothelial cells (HUVECs) [22]. However, the direct effects of oxidative stress on PPARγ expression and activity in endothelial cells have not been examined. Because oxidative stress contributes to vascular pathology in patients with hypertension, diabetes, and atherosclerosis [23], and because PPARγ ligands have been shown to favorably modulate inflammatory mediators and vascular function, we hypothesized that oxidative stress would reduce vascular PPARγ expression and activity, thereby contributing to inflammation and redox imbalance in the vascular wall. To explore this hypothesis, this study investigated the impact of treatment with H_2O_2 on endothelial PPARγ expression and activity in vitro. Furthermore, because intracellular signaling pathways respond not only to ROS but also to alterations in the extracellular E_h of the cysteine (Cys)/cystine (CySS) thiol couple [24,25], this study examined whether physiologically relevant alterations in the thiol/disulfide redox state could modulate endothelial PPARγ expression and activity. Our findings provide novel evidence for direct effects of oxidative stress on PPARγ expression and activity in vascular endothelial cells.

Materials and methods

Cell culture

Monolayers of HUVECs or bovine aortic endothelial cells (BAECs) from Clonetics (Invitrogen, Carlsbad, CA, USA) were grown and maintained in endothelial growth medium (EGM; Lonza, Conshohocken, PA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor, 1.0 μg/ml hydrocortisone, 12 μg/ml bovine brain extract, 50 μg/ml gentamicin, and 50 ng/ml amphotericin-B in a 5% CO_2 environment at 37 °C as previously reported [16,26]. Forty-eight hours after seeding, the culture medium was changed to 2% FBS EGM in HUVECs and 0.5% FBS Dulbecco's modified Eagle medium (DMEM) in BAECs. In all experiments, confluent HUVEC monolayers (passages 2–6), plated on 0.2% gelatin-coated 100-mm plastic tissue culture dishes, were treated with vehicle (phosphate-buffered saline; PBS) or with graded concentrations of H_2O_2 (1–1000 μM) in 2% FBS EGM for 0.5–72 h. For exposures longer than 8 h, H_2O_2-containing media were refreshed every 8 h. In separate experiments, BAECs were treated with medium that was manipulated to generate clinically relevant alterations in the Cys:CySS redox potential as we have previously reported [26]. BAECs rather than HUVECs were employed in these studies to precisely replicate the carefully characterized system in which alterations in E_h caused changes in endothelial gene expression and function. In brief, the medium was changed to cyst(e)ine-free DMEM with 0.5% serum. To generate the desired E_h, varied concentrations of Cys and CySS were added to cyst(e)ine-free medium to give a constant total amount of Cys equivalents (200 μmol/L) as described previously [24]. The E_h for Cys/CySS was calculated using the Nernst equation, $E_h = E_0 + RT/2F \ln([\mathrm{CySS}]/[\mathrm{Cys}]^2)$, where $E_0 = -250$ mV at pH 7.4 as previously described [26,27].

Cytotoxicity assays

Toxilight cytotoxicity assays (Invitrogen) were employed to select H_2O_2 exposure conditions that were not associated with HUVEC cytotoxicity. In brief, the Toxilight assay monitors adenylate kinase (AK) release from cells as an indicator of cell viability. Samples of the medium above HUVEC monolayers were aspirated periodically as indicated during H_2O_2 treatment. After addition of AK substrate (provided by the manufacturer), the samples were incubated for 5 min and analyzed on a luminometer (Victor 3, 1420 Multilabel Counter; Perkin–Elmer, Waltham, MA, USA) for a period of 1 s.

PPARγ activity assay

Nuclear extracts of selected samples were prepared in complete lysis buffer using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA). PPARγ activity was then quantified using the TransAM PPARγ activity kit (Active Motif). Briefly, this kit employs an ELISA-type format with an immobilized oligonucleotide containing PPARγ response elements. A primary antibody recognizes an accessible epitope on the PPARγ protein upon DNA binding. A secondary HRP-conjugated antibody is added, and colorimetric readouts are obtained using spectrophotometry to estimate relative changes in PPARγ nuclear binding following designated interventions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA isolation and purification were accomplished using the TRIzol reagent (Invitrogen) and RNeasy mini-kit (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using random nanomer primers (BD Biosciences, San Jose, CA, USA) and Superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed on a LightCycler 1.5 PCR detection system (Roche Diagnostics, Indianapolis, IN, USA) and I-cycler IQ detection system (Bio-Rad, Hercules, CA, USA). Quantification and melting curves were analyzed with LightCycler software (Roche Diagnostics). Human PPARγ (common mRNA region, see Table 1), PPARγ1, PPARγ2, PPARγ3, and PPARγ4 transcript variants (promoter-specific region) [28]; plasminogen activator inhibitor type-1 (PAI-1); and phosphatase and tensin homolog (PTEN) mRNA levels were normalized to the 18 S rRNA reference gene and expressed as percentage control values as we have previously reported [18]. The primer sequences for qRT-PCR are shown in Table 1. The comparative threshold cycle (C_t) values were normalized for the 18 S reference gene and compared with a calibrator using the $2^{-\Delta\Delta Ct}$ method [29].

PPARγ promoter activity assay

HUVECs in 24-well plates were transfected with a construct containing the pGL3 luciferase reporter vector (Promega, Madison, WI, USA) containing a full-length, 3-kb human PPARγ1p3000 promoter cDNA (GenBank Accession No. NM_138712), as described (pGL3-PPARγ1, 1 μg/well, provided by Dr. Johan Auwerx, Institut Pasteur) [30], and with the control vector phRL-thymidine kinase driving *Renilla* luciferase expression (0.5 μg/well, used as an internal reporter to normalize for variations in transfection efficiency; Promega). HUVECs were transfected with these reporters using JetPEI-HUVECs (Polyplus-Transfection, Inc., New York, NY, USA) for 24 h according to conditions recommended by the manufacturer. After 2 h incubation with or without H_2O_2, the cells were lysed and assayed for PPARγ and thymidine kinase promoter luciferase activities by luminescence.

PPARγ mRNA half-life determination

PPARγ mRNA half-life determinations were performed in HUVECs as previously described [31]. Briefly, 1×10^6 cells were split into dishes and incubated for 24 h to allow the cells to adhere and reach 75% confluence. The cells were then incubated in EGM without FBS overnight. Each monolayer was then treated with H_2O_2 (100 μM) or sterile PBS (control) for 30 min followed by actinomycin-D (10 μg/ml). Cells were collected at intervals after actinomycin-D addition, and total RNA was isolated for