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Oxidative Stress Modulates PPAR γ in Vascular Endothelial Cells

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Abstract

The peroxisome proliferator-activated receptor gamma (PPAR γ) plays an important role in vascular regulation. However, the impact of oxidative stress on PPAR γ expression and activity has not been clearly defined. Human umbilical vein endothelial cells (HUVECs) were exposed to graded concentrations of H₂O₂ 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 (Cys)/cystine (CySS) couple. Within 2 h, H₂O₂ reduced HUVEC PPAR γ mRNA and activity and reduced the expression of two PPAR γ -regulated genes without altering PPAR γ protein levels. After 4 h H₂O₂ exposure, mRNA levels remained reduced while PPAR γ activity returned to control levels. PPAR γ mRNA levels remained depressed for up to 72 h after exposure to H₂O₂, without any change in PPAR γ activity. Catalase prevented H₂O₂-induced reductions in PPAR γ mRNA and activity. H₂O₂ 1) reduced luciferase expression in HUVECs transiently transfected with a human PPAR γ promoter reporter, 2) failed to alter PPAR γ mRNA half-life, and 3) transiently increased expression and activity of c-Fos and phospho-c-Jun. Treatment with the AP-1 inhibitor, curcumin, prevented H₂O₂-mediated reductions in PPAR γ expression. In addition, media having an oxidized E_h reduced BAEC PPAR γ mRNA and activity. These findings demonstrate that oxidative stress, potentially through activation of inhibitory redox-regulated transcription factors, attenuates PPAR γ expression and activity in vascular endothelial cells through suppression of PPAR γ transcription.

Keywords

PPAR γ ; endothelial cell; oxidative stress

INTRODUCTION

The ligand-activated peroxisome proliferator-activated receptor gamma (PPAR γ) 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 PPAR γ are employed to improve lipid and glucose metabolism in type 2 diabetes [1]. PPAR γ activation is also mediated by structurally diverse natural lipophilic ligands including fatty acids and their derivatives. Ligand-induced activation of PPAR γ promotes heterodimerization with the retinoid X receptor and binding to PPAR γ response elements in selected target genes, resulting in transcriptional regulation. PPAR γ is expressed in smooth

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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 PPAR γ expression or activity. For example, infusion of insulin and glucose caused endothelial dysfunction, increased reactive oxygen species (ROS) production, and reduced PPAR γ protein levels in rat aorta [4]. These derangements were attenuated in animals treated with the thiazolidinedione PPAR γ ligand, pioglitazone. Thiazolidinedione treatment also attenuated tumor necrosis factor- α (TNF- α) and interleukin-1 α (IL-1 α)-stimulated reductions in PPAR γ mRNA in mature rat adipocytes [5]. These findings suggest that certain pathological stimuli may reduce PPAR γ 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 (H₂O₂), 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]. H₂O₂, 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 PPAR γ and oxidative stress. For example, PPAR γ 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 PPAR γ *in vitro* and *in vivo* reduced the activity and expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [16–18]. In addition to reducing ROS generation, PPAR γ ligands also increased vascular endothelial nitric oxide (NO) production by enhancing the activity of endothelial nitric oxide synthase (eNOS) [19–21]. Collectively, these reports indicate that activation of PPAR γ 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 PPAR γ in cardiovascular disease [21].

While mounting evidence has emphasized that PPAR γ can regulate oxidative stress [11–13], emerging evidence suggests that oxidative stress modulates PPAR γ . For example, H₂O₂-induced oxidative stress significantly reduced PPAR γ activity in renal tubular epithelial cells [14] and osteoblasts [12] and inhibited PPAR β expression in HUVECs [22]. However, the direct effects of oxidative stress on PPAR γ 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 PPAR γ ligands have been shown to favorably modulate inflammatory mediators and vascular function, we hypothesized that oxidative stress would reduce vascular PPAR γ expression and activity thereby contributing to inflammation and redox imbalance in the vascular wall. To explore this hypothesis, the current study investigated the impact of treatment with H₂O₂ on endothelial PPAR γ expression and activity *in vitro*. Furthermore, because intracellular signaling pathways respond not only to ROS but to alterations in the extracellular E_n of the cysteine (Cys)/cystine (CySS) thiol couple [24,25], the current study examined if physiologically relevant alterations in the thiol/disulfide redox state could modulate endothelial PPAR γ expression and activity. Our findings provide novel evidence for direct effects of oxidative stress on PPAR γ expression and activity in vascular endothelial cells.

MATERIALS and METHODS

Cell Culture

Monolayers of human umbilical vein or bovine aortic endothelial cells (HUVECs or BAECs, respectively) from Clonetics (Invitrogen, Carlsbad, CA) were grown and maintained in endothelial growth medium (EGM, Lonza, Conshohocken, PA) 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% CO₂ environment at 37°C as previously reported [16,26]. Forty-eight hours after seeding, the culture medium was changed to 2% FBS EGM medium in HUVECs and 0.5% FBS DMEM medium in BAECs. In all experiments, confluent HUVEC monolayers (passage 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 H₂O₂ (1–1000 µM) in 2% FBS EGM medium for 0.5–72 h. For exposures longer than 8 h, H₂O₂ containing media were refreshed every 8 h. In separate experiments, BAECs were treated with media 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 media was changed to cyst(e)ine-free Dulbecco's Modified Eagle Medium with 0.5% serum. To generate the desired E_h, varied concentrations of Cys and CySS were added to cyst(e)ine-free media to give a constant total amount of Cys equivalents (200 µmol/L) as described previously [24]. E_h for Cys/CySS was calculated with the Nernst equation: $E_h = E_0 + RT/2F \ln([CySS]/[Cys]^2)$, where E₀ = −250 mV at pH 7.4 as previously described [26,27].

Cytotoxicity Assays

Toxilight™ Cytotoxicity assays (Invitrogen, Carlsbad, CA) were employed to select H₂O₂ 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. Media samples above HUVEC monolayers were aspirated periodically as indicated during H₂O₂ treatment. Following addition of AK substrate (provided by the manufacturer), the samples were incubated for 5 minutes and analyzed on a luminometer (Victor 3, 1420 Multilabel Counter, Perkin Elmer, Waltham, MA) 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). 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 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 was accomplished using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) 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) and Superscript II Reverse Transcriptase (Invitrogen). qRT-PCR was performed on a Light Cycler 1.5 PCR Detection System (Roche Diagnostics Corporation, Indianapolis, IN) and I-cycler IQ detection system (Bio-Rad, Hercules, CA). Quantification and melting

curves were analyzed with Light Cycler software (Roche Diagnostics). Human PPAR γ (common mRNA region, see Table 1), PPAR γ 1, PPAR γ 2, PPAR γ 3, and PPAR γ 4 transcript variants (promoter specific region) [28], plasminogen activator inhibitor type-1 (PAI-1), and phosphatase and tensin homolog (PTEN) mRNA levels were normalized to 18S rRNA reference gene and expressed as percent control values as we have previously reported [18]. The primer sequences for qRT-PCR are shown in Table 1. The comparative threshold cycles (C_t) values were normalized for 18S reference gene and compared with a calibrator using the $2^{-\Delta\Delta C_t}$ method [29].

PPAR γ Promoter Activity Assay

HUVECs in 24-well plates were transfected with a construct containing the pGL3 luciferase reporter vector (Promega, Madison, WI) containing a full-length, 3 kb human PPAR γ 1p3000 promoter cDNA (GenBank Accession #: NM_138712) as described (pGL3-PPAR γ 1, 1 μ g/well, provided by Dr. Johan Auwerx, Institut Pasteur) [30] and with the control vector pRL-thymidine kinase driving renilla luciferase expression (0.5 μ g/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) for 24 h according to conditions recommended by the manufacturer. After 2 h incubation with or without H₂O₂, cells were lysed and assayed for PPAR γ and thymidine kinase promoter luciferase activities by luminescence.

PPAR γ mRNA Half-life Determination

PPAR γ 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 H₂O₂ (100 μ M) or sterile PBS (control) for 30 min followed by actinomycin-D (10 μ g/mL). Cells were collected at intervals following actinomycin-D addition, and total RNA was isolated for measurement of PPAR γ mRNA levels using qRT-PCR. The RNA half-life was extrapolated from the PPAR γ mRNA decay curve as the time point following actinomycin D treatment at which there was 50% of the initial RNA level remaining.

Western blot analysis

After treatment with PBS or H₂O₂, HUVEC monolayers were washed with cold Hank's Balanced Salt Solution (HBSS), then solubilized for 10 minutes with cell lysis buffer at 4°C [20 mM Tris pH 7.4, 2.5 mM EDTA, 100 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, 1 tablet/10 ml EDTA-free Complete protease inhibitor cocktail (Roche, Indianapolis, IN), 1 mM β -glycerolphosphate, 2.5 mM Na pyrophosphate]. HUVEC lysates were collected by scraping into microfuge tubes, sonicated on ice (ten 2-second bursts at 2 W), and spun at 16,000 $\times$ g for 10 minutes. The supernatants were transferred to new tubes, and protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein were added to sodium dodecyl sulfate (SDS) sample buffer solution, and proteins (10 μ g) were loaded into 4–12% bis-tris-PAGE precast mini gels (Invitrogen). After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes that were then treated with primary antibody (1:1,000 dilution) in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween, pH 7.4) containing 5% powdered non-fat dry milk. Primary antibodies included c-Fos rabbit polyclonal antibody (Cat # ab7963, 41 kDa, AbCAM Inc., Cambridge, MA), PPAR γ rabbit polyclonal antibody (54.6 and 57.6 kDa, custom made by Bethyl Laboratories, Montgomery, TX), c-Jun and phospho-c-Jun antibodies (Cat #9165, 43 kDa, and #9164, 48 kDa, respectively, Cell Signaling Technologies, Danvers, MA), CDK4 antibody (Cat #SC-260, 34 kDa, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins

were visualized using a peroxidase-coupled anti-goat or rabbit IgG in the presence of LumiGlo reagent. Bands were identified by chemiluminescence, quantified by laser densitometry (Biorad chemidoc XRS/HG densitometer, Hercules, CA), and normalized to CDK4 levels within the same lane. All Western blot gradient (4–12%) gels and 4X NuPAGE Lithium Dodecyl Sulfate Sample Buffer (40 % glycerol, 423 mM Tris HCL, 8 % LDS, 2.1 mM EDTA, 0.075 % Serva Blue G250, 0.025 % Phenol Red) which was diluted to a 1X solution with 50 mM DTT were purchased from Invitrogen. Supersignal chemiluminescence substrate, which detects horseradish peroxidase on immunoblots, was acquired from Pierce (Rockford, IL).

Statistical analysis

For all experiments, statistical analysis was performed by Student's t-test or one-way ANOVA followed by post-hoc analysis with either Dunnet's Multiple Comparison Test or Student-Newman-Keuls test in order to detect differences between and among experimental groups using the software GraphPad Prism, version 4.0 (La Jolla, CA). The level of statistical significance was set at an alpha value of ≤ 0.05 .

RESULTS

H₂O₂-induced cytotoxicity

We employed H₂O₂ treatment as a model system to examine the impact of oxidative stress on endothelial PPAR γ mRNA expression and activity. To select H₂O₂ concentrations that did not cause HUVEC cytotoxicity following prolonged exposure, the release of intracellular adenylate kinase was measured using the Toxilight AssayTM. As shown in Figure 1, compared to treatment with media alone, treatment with H₂O₂ concentrations of 500 and 1000 μ M for 72 h caused significant cytotoxicity indicated by approximately 1.5 fold increases in adenylate kinase release. Exposing HUVECs to H₂O₂ concentrations from 1–100 μ M added to culture media did not cause cytotoxicity.

H₂O₂ inhibits PPAR γ mRNA expression but not PPAR γ protein expression and activity

To examine the effect of oxidative stress on PPAR γ expression, HUVECs were exposed to non-cytotoxic concentrations of H₂O₂, and PPAR γ mRNA levels were measured. Primers recognizing all PPAR γ isoforms as well as those specific for PPAR γ 1–4 were employed in preliminary studies (Table 1). PPAR γ 2 was undetectable in HUVECs whereas PPAR γ 1, 3, and 4 were all detectable and similarly reduced by treatment with H₂O₂ (data not shown). As a result, all subsequent qRT-PCR analyses employed "common" primers detecting all PPAR γ isoforms. As shown in Figure 2A, following treatment with H₂O₂ for 72 h, PPAR γ mRNA levels were slightly diminished by 5 and 25 μ M H₂O₂ and significantly reduced by 50 and 100 μ M H₂O₂. To determine if H₂O₂-induced reductions in endothelial PPAR γ mRNA were associated with comparable alterations in PPAR γ activity, nuclear extracts were prepared and subjected to PPAR γ activity assays. As shown in Figure 2B, only the cytotoxic, 1000 μ M H₂O₂ concentration (positive control) significantly reduced PPAR γ activity, whereas treatment with non-cytotoxic H₂O₂ concentrations for 72 h had no significant effect on PPAR γ activity.

To further explore the temporal onset of H₂O₂-mediated reductions in PPAR γ mRNA levels, HUVECs were examined following exposure to 100 μ M H₂O₂ for 0–8 h. After 1 h, H₂O₂ produced rapid and significant reductions in PPAR γ mRNA levels (Figure 3A), while PPAR γ protein levels remained stable for as long as 8 h following the addition of H₂O₂ (Figure 3B). Compared with untreated HUVECs, H₂O₂ significantly decreased HUVEC PPAR γ activity levels 0.5 and 2 h following the onset of oxidative stress, whereas near complete recovery of PPAR γ activity was observed 4 h following the onset of H₂O₂ exposure (Figure 3C). To explore the biological function of PPAR γ in H₂O₂-treated endothelial cells, PAI-1 and PTEN mRNA levels were examined as representative PPAR γ -target genes in endothelial cells

[3, 18, 32]. Treating HUVECs with H_2O_2 for 2 h significantly decreased PAI-1 and PTEN mRNA levels in a time-dependent manner (Figure 3D and 3E, respectively).

Catalase prevents H_2O_2 -induced reductions in PPAR γ mRNA and activity

To confirm that H_2O_2 was the proximal stimulus for alterations in PPAR γ mRNA levels and activity, studies were performed following the addition of catalase, which degrades H_2O_2 . Catalase prevented reductions in PPAR γ mRNA levels (Figure 4A) and activity (Figure 4B) caused by treatment with 100 μ M H_2O_2 for 30 minutes. To examine the potential role of H_2O_2 -induced generation of oxidized fatty acids from serum lipids in alterations in PPAR γ expression and activity, 2% serum in culture medium was preincubated with 100 μ M H_2O_2 for 2 h at 37 °C, then preincubated with 200 U/mL catalase for an additional 2 h at 37°C in a test tube. HUVECs were then treated with H_2O_2 and catalase preconditioned media for 2 h at 37°C. As shown in Figure 4C, H_2O_2 and catalase preconditioned media did not alter HUVEC PPAR γ mRNA expression, suggesting that H_2O_2 itself and not products of H_2O_2 -induced serum oxidation mediated changes in endothelial PPAR γ expression in this system.

H_2O_2 inhibits PPAR γ promoter activity

To further examine the mechanisms by which H_2O_2 reduced HUVEC PPAR γ mRNA levels, PPAR γ promoter activity was measured in HUVECs transiently transfected with a luciferase reporter construct controlled by the full-length 3 kb PPAR γ promoter cDNA. In pilot experiments, HUVECs in 24-well plates were treated with pGL3 luciferase reporter vectors for 24 h at 37°C in 5% CO_2 /95 % air. Transfection efficiency was measured as the activity of the pRL-thymidine kinase driven Renilla reporter in HUVECs. Transfection efficiency in HUVECs mediated by pGL3 luciferase reporter vector was determined to be 40% (data not shown). H_2O_2 -treated HUVECs demonstrated significant decreases in PPAR γ transcription, with approximately 3-fold lower levels of luciferase activity relative to controls, indicating that H_2O_2 inhibits the activity of the PPAR γ promoter in endothelial cells (Figure 5A). Furthermore, H_2O_2 did not alter PPAR γ mRNA half-life (Figure 5B).

Oxidation of extracellular Cys/CySS redox state reduced PPAR γ activity in BAECs

Although micromolar H_2O_2 concentrations may be generated in microenvironments between adherent leukocytes and vascular endothelial cells, we sought to examine the effects of additional oxidative stimuli to which vascular endothelial cells may be exposed. The redox state of thiol/disulfide couples in plasma has been previously reported to be altered under pathophysiological conditions [33] and to modulate endothelial cell function [26]. Therefore, to determine whether alterations in extracellular redox modulate PPAR γ expression and activity, BAECs were exposed to well characterized concentrations of Cys and CySS in culture media as previously reported [26]. As illustrated in Figure 6A, compared to physiological E_h (−80 mV), treating BAECs with oxidized Cys/CySS redox potential (0 mV) for 2 h reduced PPAR γ activity levels. Interestingly, more oxidized (0 mV) conditions tended to reduce PPAR γ mRNA levels, although this trend did not reach statistical significance (Figure 6B).

H_2O_2 -mediated activation of AP-1 contributes to reductions in HUVEC PPAR γ expression

The PPAR γ promoter contains multiple activator protein 1 (AP-1) sites upstream of the transcriptional start site, and AP-1 was previously reported to reduce PPAR γ expression [34]. AP1 transcription factors consist of homodimers of Jun family members or heterodimers of Jun and Fos. Increased expression of c-Fos or phosphorylation of c-Jun (p-c-Jun) has been shown to contribute to activation of AP-1 [35]. Accordingly, to further explore the mechanisms involved in the regulation of PPAR γ by oxidative stress, we examined the effects of H_2O_2 on the expression and activation of c-Fos, c-Jun, and p-c-Jun. As shown in Figure 7, H_2O_2 stimulated transient increases in c-Jun expression that did not achieve statistical significance

(Figure 7B), as well as significant increases in c-Fos expression (Figure 7C) and c-Jun phosphorylation (Figure 7D) that peaked 1–2 h following the onset of H₂O₂ exposure. To elucidate whether AP-1 mediates alterations in PPAR γ expression caused by H₂O₂, HUVECs were preincubated with curcumin which inhibits H₂O₂-induced AP-1 activation [36]. Curcumin significantly attenuated H₂O₂-mediated reductions in HUVEC PPAR γ expression (Figure 7E).

DISCUSSION

Oxidative stress plays an important role in the pathophysiology of a variety of diseases [36–38]. Mounting evidence demonstrates that PPAR γ regulates oxidative stress and inflammation [7,8]. However, the ability of oxidative stress to regulate PPAR γ is less well defined. Several reports suggested that oxidative stress reduced PPAR γ expression. For example, PPAR γ protein levels were reduced by treatment with 500 μ M H₂O₂ in rat adipocytes [5]. TNF- α , which augments oxidative stress in non-insulin-dependent diabetes mellitus, suppressed PPAR γ expression in rat adipocytes [39]. Chronic glucose feeding in rats also stimulated vascular oxidative stress and decreased aortic and cardiac PPAR γ protein levels [4]. Taken together, these reports provide indirect evidence that oxidative stress may reduce PPAR γ in the vascular wall.

To further examine the impact of oxidative stress on PPAR γ , the current study examined the direct effects of non-cytotoxic H₂O₂ concentrations on endothelial cell PPAR γ expression and activity. Additionally, PPAR γ expression and activity were examined in BAECs after manipulating the extracellular thiol/disulfide E_h of the Cys/CySS couple, which has been suggested to release intracellular H₂O₂ [26,40]. Our results show that prolonged H₂O₂ exposure reduced HUVEC PPAR γ mRNA levels but failed to alter HUVEC PPAR γ activity, whereas acute H₂O₂ treatment reduced PPAR γ mRNA levels, activity, and the expression of two PPAR γ -regulated genes, PAI-1 and PTEN [3,18,32]. Alterations in PPAR γ expression were mediated by H₂O₂ as they were inhibited by catalase and not reproduced by oxidized media components. Although the effects of H₂O₂ occurred in the absence of H₂O₂-induced endothelial cell cytotoxicity, the relevance of these H₂O₂ concentrations to those generated in the vascular wall *in vivo* during physiological or pathological conditions is more difficult to ascertain. H₂O₂ concentrations in the micromolar range have been reported *in vivo* in pathological conditions [41,42]. Furthermore, cultured endothelial cells rapidly metabolize exogenously added H₂O₂ *in vitro* indicating that the average concentration of H₂O₂ to which endothelial cells were subjected in the current study was significantly lower than that initially added to the culture media.

The current results provide direct evidence that H₂O₂ modulates PPAR γ expression in endothelial cells and further clarify the mechanisms by which oxidative stress reduced PPAR γ expression. These findings extend previous reports by coupling the simultaneous examination of PPAR γ mRNA levels with assays of PPAR γ protein, activity, and PPAR γ -regulated gene expression. Our results demonstrate that H₂O₂-induced oxidative stress reduced PPAR γ mRNA levels and activity as well as the expression of PAI-1 and PTEN, two PPAR γ -regulated genes in endothelial cells. Although the mechanisms accounting for sustained PPAR γ protein levels despite reduced PPAR γ mRNA expression remain speculative, our results demonstrate that H₂O₂ reduced not only PPAR γ mRNA levels, but also PPAR γ activity and the expression of PPAR γ -regulated genes. These results emphasize that future investigations should employ caution in drawing conclusions about the function of PPAR γ based on analysis of PPAR γ protein levels alone.

The mechanisms by which oxidative stress reduced PPAR γ mRNA are likely complex, as oxidative stress modulates the activities of numerous signaling pathways and transcription

factors [43–45]. For example, H₂O₂ activates AP1 [46,47], a redox-regulated transcription factor previously reported to reduce PPAR γ expression [34]. The PPAR γ promoter contains multiple AP1 sites upstream of the transcriptional start site [34]. Our results demonstrate increased c-Fos expression and c-Jun phosphorylation following the onset of H₂O₂ exposure. In addition, we found no evidence that H₂O₂ altered PPAR γ mRNA half-life. These findings are consistent with the postulate that H₂O₂ activates AP1 to suppress transcriptional activity of the PPAR γ promoter thereby lowering PPAR γ mRNA levels. This postulate was further supported by evidence that curcumin, an inhibitor of AP1, prevented H₂O₂-induced reductions in PPAR γ mRNA levels. Similar transient reductions in PPAR γ activity with more sustained reductions in PPAR γ mRNA levels were observed after exposing endothelial cells to an oxidative extracellular Cys/CySS redox state (0 mV). These conditions have been previously reported to stimulate H₂O₂ production, activate nuclear factor- κ B, and increase monocyte adhesion to endothelial cells [26]. Taken together, the current results suggest that oxidative stress activates redox-regulated transcription factors in endothelial cells that transcriptionally suppress PPAR γ .

The current study provides novel evidence supporting a role of oxidative signaling in the regulation of PPAR γ expression and function in vascular endothelial cells. It is likely that the cumulative effects of oxidative stress on PPAR γ signaling in vascular endothelial cells are regulated not only by PPAR γ expression but also by oxidative alterations in the generation of endogenous PPAR γ ligands. The nature and potency of these endogenous ligands in a cell or tissue may be influenced by the type and duration of the oxidative stimulus as well as by the endogenous antioxidant capacity of the cell or tissue. Furthermore, the recognition that nitrated lipids serve as potent PPAR γ ligands [48] suggests that alterations in endothelial NO generation may also modulate the spectrum and activity of endogenous PPAR γ ligands. These mechanisms may provide new insights into the growing body of evidence that PPAR γ stimulation promotes vascular protection [21]. Oxidative stress in the vascular wall that promotes reduced NO bioavailability and proinflammatory derangements might be attenuated by activation of PPAR γ which can simultaneously stimulate endothelial NO production and reduce oxidative stress [16,19,20]. The current study provides new evidence that oxidative signals directly regulate the transcription of PPAR γ . These *in vitro* findings can inform future investigations that explore the role of PPAR γ in vascular biology *in vivo* that will be required to better define the role of PPAR γ in vascular inflammation, nitroso-redox balance, and vascular function.

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LIST of ABBREVIATIONS

AK	adenylate kinase
AP1	activator protein 1
BAECs	bovine aortic endothelial cell(s)
Cys	cysteine
CySS	cystine
EGM	endothelial growth medium
E _h	redox potential

eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
GLUT4	glucose transporter type 4
H ₂ O ₂	hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HUVECs	human umbilical vein endothelial cell(s)
IL-1 α	interlukin-1 alpha
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
oxFA	oxidized fatty acid
PAI-1	plasminogen activator inhibitor type-1
PPAR γ	peroxisome proliferator-activated receptor gamma
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-alpha

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Figure 1. The effect of H₂O₂ exposure on HUVEC cytotoxicity

HUVECs were treated with graded H₂O₂ concentrations in EGM containing 2% serum and antimicrobials for 72 h. Cell injury was examined by measuring the release of intracellular adenylate kinase into culture media. Each bar represents the mean $\pm$ SEM adenylate kinase luminescence in relative light units from 3–4 separate experiments. *P < 0.01 vs. control (0 μ M H₂O₂).

Figure 2. Prolonged exposures to non-cytotoxic H₂O₂ concentrations decrease PPAR_γ mRNA expression without altering PPAR_γ activity
 HUVECs were treated with graded concentrations of H₂O₂ three times a day for 72 h. In A, PPAR_γ and 18S mRNA levels were determined by qRT-PCR. Each bar represents the mean PPAR_γ mRNA level $\pm$ SEM from 4 experiments calculated relative to 18S and expressed as fold change vs. control (0 μ M H₂O₂). *P < 0.05 vs. control. In B, PPAR_γ activity assays were performed on nuclear extracts. Each bar represents the mean PPAR_γ activity as arbitrary spectrophotometric optical density readings $\pm$ SEM. *P < 0.01 vs. control (0 μ M H₂O₂).

Figure 3. Acute H_2O_2 exposure reduces PPAR γ mRNA, activity, and biological function

HUVECs were treated with 100 μM H_2O_2 (0.5–8 h) and collected for analysis. In A, each bar represents the mean $\pm$ SEM PPAR γ mRNA levels relative to 18S expressed as fold change vs. control values for HUVECs at the same time point from 3 experiments. * $P < 0.05$ vs control (0 μM H_2O_2). In B, PPAR γ protein levels were determined by western blotting in control and H_2O_2 - treated HUVECs. Each bar represents the mean PPAR γ protein level relative to CDK4 in each sample $\pm$ SEM expressed as fold change vs. control from cells not treated with H_2O_2 , $n=10$. In C, each bar represents the mean PPAR γ activity $\pm$ SEM as a percentage of control cells not treated with H_2O_2 , $n=10$, * $P < 0.05$ vs. controls. In D and E, quantitative real-time PCR was performed for PAI-1 (D), PTEN (E), and 18S. Each bar represents mean $\pm$ SEM PAI-1 or PTEN mRNA levels relative to 18S expressed as fold change from control. $n=3$, * $P < 0.05$ vs. control.

Figure 4. Catalase prevented H₂O₂-mediated decreases in HUVEC PPAR γ mRNA levels and activity

HUVECs were treated with 100 μ M H₂O₂ for 30 min. Selected HUVECs were preincubated with 200 U/mL catalase for 30 min prior to H₂O₂ exposure as indicated. In A, HUVEC PPAR γ mRNA levels determined by qRT-PCR are shown. Each bar represents the mean mRNA level $\pm$ SEM from 8 separate experiments expressed as fold change vs. control. *P < 0.05 vs. control. In B, PPAR γ activity was determined following treatment with or without H₂O₂ $\pm$ catalase treatment. Each bar represents the mean PPAR γ activity level $\pm$ SEM from 8 experiments expressed as % control. *P < 0.05 vs. control. In C, HUVECs were treated for 2 h with culture medium preincubated with 100 μ M H₂O₂ for 2 h followed by preincubation with

200 U/mL catalase for 2 h at 37°C. Each bar represents the mean PPAR γ mRNA levels relative to 18S $\pm$ SEM from 3 experiments expressed as % control. *P < 0.05 vs. control.

Figure 5. Mechanisms of H₂O₂-induced reductions in HUVEC PPAR_γ mRNA levels

In A, luciferase activity was measured in HUVEC transfected with a PPAR_γ promoter reporter construct following treatment with 100 μ M H₂O₂ for 2 h. Each bar represents the mean luciferase activity relative to renilla luciferase $\pm$ SEM. *P < 0.05 vs. control (0 h). In B, PPAR_γ mRNA half-life determination in HUVECs following H₂O₂ exposure was performed. HUVECs were exposed to 100 μ M H₂O₂ for 30 minutes, and cells were harvested at designated time points following actinomycin-D treatment. PPAR_γ mRNA levels were determined by qRT-PCR. Each point represents the mean PPAR_γ mRNA levels in HUVEC $\pm$ SEM.

Figure 6. Impact of extracellular Cys/CySS redox state on PPAR γ mRNA and activity
 Confluent BAECs plated in 100 mm dishes were exposed to oxidized or normal Cys/CySS E_h (0 and -80 mV, respectively) for 2 h and examined for PPAR γ mRNA and activity levels. In A, each bar represents the mean $\pm$ SEM PPAR γ activity in BAECs exposed to defined extracellular Cys/CySS redox states for 2 h, $n=5$, * $P < 0.05$ vs normal (-80 mV) E_h . In B, each bar represents the mean fold changes in PPAR γ mRNA levels relative to normal E_h (-80 mV) $\pm$ SEM.

Figure 7. H_2O_2 increased phospho-c-Jun protein expression levels

HUVECs were treated with control (0 h) or 100 μ M H_2O_2 for 0.5–8 h as indicated. Cell lysates were prepared at each time point and subjected to Western blotting for c-Fos, c-Jun, phospho-c-Jun, and CDK4. In A, representative immunoblots for c-Fos, c-Jun, phospho-c-Jun, and CDK4 are presented. In B–D, bar graphs represent the mean $\pm$ SEM levels of c-Fos, c-Jun and phospho-c-Jun, relative to CDK4 and expressed as fold change from control cells from 6 experiments. * $P < 0.05$ vs. control. In E, HUVECs were preincubated with 10 μ M curcumin for 1 h. HUVECs were then stimulated with or without 100 μ M H_2O_2 for 2 h. PPAR γ mRNA levels were determined by qRT-PCR. Each bar represents the mean PPAR γ mRNA levels

relative to $18S \pm \text{SEM}$ from 2 experiments expressed as % control. * $P < 0.05$ vs. control. + $P < 0.05$ vs. H_2O_2 .

Table 1

Primer information for quantitative real-time PCR (qRT-PCR).

Gene Symbol	mRNA product length(bp)	Annealing temperature(3)	Primer sequences (5'-3')	GenBank Accession #
PPAR γ	Common region *	60	F: TGACAGGAAAGACAACAGACAAAT R: GGGTGATGTGTTTGAACCTTGATT	
PPAR γ 1	162–263 (102 bp)	60	F: GTGGCCGCAGATTGAAAGAAG R: TGTCAACCATGGTCATTTTCG	NM_138712
PPAR γ 2	70–204 (135 bp)	60	F: CAAACCCCTATTCATGCTGTT R: AATGGCATCTCTGTGTCAACC	NM_015869
PPAR γ 3	156–243 (88 bp)	60	F: AGAAGCCTGCATTCTGCAT R: TGGCCTTGTTGTATATTTGTGGTT	NM_138711
PPAR γ 4	162–182 (80 bp)	60	F: GTGGCCGCAGAAATGACCATG R: GAGAGATCCACGGAGCTGAT	NM_005037
PAI-1	53–113 (61 bp)	60	F: AAGGCACCTCTGAGAACTTCA R: CCCAGGACTAGGCAGGTG	M16006
PTEN	1497–1591 (96 bp)	60	F: GGGGAAGTAAGGACCAGAGAC R: TCCAGATGATTCTTTAACAGGTAGC	NM_000314
18S rRNA	1491-1669179 (179 bp)	60	F: GTCTGTGATGCCCTTAGATG R: AGCTTATGACCCGCACTTAC	NR_003286

* PPAR γ 1 (957–1052 bp), PPAR γ 2 (885–980 bp), PPAR γ 3 (984–1079 bp), and PPAR γ 4 (883–978).