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Glutathione Peroxidase-Deficient Smooth Muscle Cells Cause Paracrine Activation of Normal Smooth Muscle Cells via Cyclophilin A

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Abstract

Background/Aims—Reduced activity of the antioxidant glutathione peroxidase-1 (GPx1) correlates with increased risk of cardiovascular events in patients with coronary artery disease. However, it remains unclear whether this imbalance in antioxidant capacity directly contributes to activation of vascular cells. In response to oxidative stress, smooth muscle cells (SMCs) secrete the pro-inflammatory immunomodulator cyclophilin A (CyPA). We hypothesized that reduction in vascular cell GPx1 activity causes secretion of CyPA and paracrine-mediated activation of NF-κB and proliferation of SMCs.

Methods/Results—Using a murine model of GPx1 deficiency (GPx1^{+/-}), we found elevated levels of hydrogen peroxide levels and increased secretion of CyPA in both arterial segments and cultured SMCs as compared to wild type (WT). Conditioned media from GPx1^{+/-} SMCs caused increased NF-κB activation of quiescent WT SMCs, and this was inhibited by the antioxidant N-acetyl-L-cysteine or by cyclosporine A (CsA). In co-culture experiments, SMCs derived from GPx1^{+/-} aorta caused increased proliferation of WT SMCs, which was also inhibited by CsA.

Conclusions—Reduction in vascular cell GPx1 activity and the associated increase in oxidative stress cause CyPA-mediated paracrine activation of SMCs. These findings identify a novel mechanism by which an imbalance in antioxidant capacity may contribute to vascular disease.

Keywords

smooth muscle cells; oxidative stress; atherosclerosis; inflammation

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Disclosures:

None

1. Introduction

Intracellular levels of reactive oxygen species (ROS) in blood vessels influences nitric oxide availability, intracellular signaling, and cellular growth, thereby contributing to the pathobiology of vascular disease (Griendling et al., 2000). Two general mechanisms maintain a redox balance in the blood vessel: 1) production of ROS by electron transfer to oxygen via enzymes such as NADPH oxidases; and 2) reduction in cellular antioxidant activity. In atherosclerotic lesions, expression of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidases (GPx) regulate ROS levels. The role of individual antioxidant enzymes in redox-mediated SMC activation is not fully understood. The K_m of GPx for hydrogen peroxide (H_2O_2) is lower than that of catalase, indicating an important role for GPx in regulation of cellular peroxide metabolism (Makino et al., 1994). Five isoforms of GPx have been identified, GPx1-5, and each are capable of reducing H_2O_2 and lipid peroxide. In contrast to catalase, which is mainly found in the peroxisome, GPx isoforms have distinct subcellular localizations with GPx1 located in the cytosol, mitochondria, and nucleus. The distribution of the isoforms varies among tissues, with GPx1 as the predominant isoform expressed in the vasculature. Deficiency of GPx1 increases vascular oxidative stress (Forgione et al., 2002), causes endothelial cell dysfunction (Chrissobolis et al., 2008), and accelerates atherosclerosis in mice (Lewis et al., 2007; Torzewski et al., 2007). Patients with coronary artery disease have decreased GPx1 activity in plaques and circulating erythrocytes (Blankenberg et al., 2003; Espinola-Klein et al., 2007; Lapenna et al., 1998). In addition, a reduction in GPx1 activity in erythrocytes is associated with an increased risk of cardiovascular events (Blankenberg et al., 2003; Espinola-Klein et al., 2007). However, the direct effects of GPx1 deficiency on vascular cell activation are not known.

In response to oxidative stress, vascular cells can release secretable oxidase factors (SOXF) that activate adjacent cells in a paracrine manner (Satoh et al., 2010). The immunophilin cyclophilin A (CyPA) is secreted by SMCs in response to oxidative stress and promotes proliferation and migration *in vitro* (Jin et al., 2000; Suzuki et al., 2006). Furthermore, CyPA levels are increased in atherosclerotic plaques and carotid arteries following ligation, and transgenic overexpression of CyPA accentuates neointimal formation (Jin et al., 2004; Satoh et al., 2008). These results suggest that secreted CyPA may be a causative factor in the pathogenesis of atherosclerosis.

We hypothesized that reduction in vascular GPx1 activity is sufficient to increase CyPA secretion and cause paracrine activation of smooth muscle cells. Using a murine model of GPx1 deficiency (GPx1^{+/-}), we provide evidence that conditioned media of GPx1-deficient SMCs contains elevated CyPA and is capable of activating NF- κ B and smooth muscle cell proliferation.

2. Material and Methods

2.1 Reagents, Chemicals, and Antibodies

Human recombinant CyPA, cyclosporin A (CsA), and N-acetyl-L-cysteine (NAC) were obtained from Sigma. H_2O_2 was from Fisher Scientific, rabbit CyPA antibody was from BIOMOL Research Laboratories, and anti-rabbit IgG-HRP was from Cell Signaling Technology. Centricon Plus-20 filter tubes were from Millipore and the Luciferase Assay System was from Promega.

2.2 Animals

GPx1^{+/-} (Ho et al., 1997) and control wild-type (WT) littermate mice were used for experiments. Previous studies have shown that, in GPx1^{+/-} tissues, GPx activity was 40–

60% that of WT control (Ho et al., 1997) and genetic deletion of GPx1 does not alter expression of other GPx isoforms (Cheng et al., 1997). It is important to note that there are no compensatory increases in activity of catalase or superoxide dismutases (SODs) with depletion of GPx1 (Ho et al., 1997). These investigations conform to the *Guide for the Care and Use of Laboratory Animals*. The animal protocol was approved by the University of Iowa Institute of Animal Care and Use Committee.

2.3 SMC Cultures

Medial SMC from thoracic aortas from GPx1^{+/-} (Ho et al., 1997) and control wild-type (WT) littermate mice were isolated and cultured as previously described (Miller et al., 1998). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO₂ humidified incubator. Experiments were performed using cells between passages 4–10.

2.4 Preparation of Conditioned Media

Cells were grown in 100 mm dishes for 48 hrs in 0.5% FBS-supplemented DMEM. Conditioned media was collected and centrifuged to remove cell debris. For Western blot analysis, the media from three plates (15 ml total) was concentrated approximately 50-fold using a Centricon Plus-20 filter (Millipore Corporation).

2.5 Detection of ROS in Aorta

Aortic segment (3 mm) were obtained from the thoracic aorta of WT or GPx1^{+/-} mice, rinsed in cold PBS and flash frozen in OCT, sectioned (30 µm) and incubated in 10 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA, Invitrogen) for 30 min at 37°C. CM-H₂DCFDA fluorescence in aortic sections was detected with a Zeiss LSM 510 META confocal microscope (excitation, 488 nm; emission, 505 nm long pass filter). To measure extracellular ROS levels, thoracic aortae were incubated in Amplex Red (20 µM) and HRP (0.2 U/ml) for 30 min, after which the media was collected and fluorescence intensity was determined (excitation and emission wavelengths of 545 and 590 nm, respectively) and normalized to aortic weight.

2.6 Detection of ROS in SMC

To measure extracellular ROS levels, SMCs were incubated with Amplex Red (20 µM) and HRP (0.2 U/ml) in the presence or absence of catalase (500 U/ml) for 30 min, after which the fluorescence intensity of the media was determined (excitation and emission wavelengths of 545 and 590 nm, respectively) and normalized to total protein. Intracellular ROS levels were measured in SMCs as previously described (Stanic et al., 2010). Briefly, cells were incubated in either 10 µM dihydroethidium (DHE, Invitrogen) or 20 µM CM-H₂DCFDA for 45 min. Cells were then rinsed with PBS, collected by trypsinization, centrifuged at 500 × g for 5 min, resuspended in ice-cold PBS and kept on ice until analysis by flow cytometry.

Membrane-enriched fractions were isolated as previously described (Chamseddine and Miller, 2003). Briefly, cells were lysed by sonication in ice-cold protease inhibitor buffer, centrifuged at 500 × g for 10 min at 4°C to remove cell debris, and then the supernatant centrifuged at 100,000 × g for 60 min at 4°C. The pellet was suspended in protease inhibitor buffer and protein concentration measured. NADPH oxidase activity of 20 µg protein was measured by lucigenin-enhanced chemiluminescence (5 × 10⁻⁶ M) after addition of NADPH (10⁻⁴ M). Chemiluminescence was measured in an FB12 luminometer (Zylux Corp) and RLU/sec normalized to protein.

2.7 Quantitative Real-Time PCR

Detection of Nox1 and Nox4 was performed using quantitative real-time PCR (qRT-PCR) as previously described (Stanic et al., 2010) and normalized to ribosomal 18S.

2.8 Western Blot Analysis

Equal volumes of conditioned media and equal amounts of protein of cell lysates were separated by SDS-PAGE, and CyPA expression was assessed by Western blotting using the anti-rabbit CyPA antibody. Signal intensity was quantitated using Scion Image. Conditioned media was normalized to cell equivalent, and lysates were normalized to GAPDH.

2.9 CyPA Immunostaining

Carotids from WT or GPx1^{+/-} mice were paraffin imbedded, sectioned, and immunostained with anti-rabbit CyPA antibody.

2.10 NF-κB Activity

NF-κB mediated transcriptional induction was measured by SMC with replication-deficient adenovirus containing the luciferase reporter gene driven by NF-κB transcriptional activation as previously described (Miller Jr et al., 2007). Luciferase activity (relative light units, RLU) was measured according to the manufacturer's protocol (Promega) and normalized to total protein.

2.11 Smooth Muscle Cell Growth

WT cells were serum-deprived and treated with conditioned media from either WT or GPx1^{+/-} cells as described above. After 48 hrs of incubation, cells were stained with trypan blue and counted using a hemocytometer. In separate experiments, conditioned media collected from WT or GPx1-deficient cells that had been treated with CyPA, CsA, or H₂O₂, was transferred to WT cells. After 48 hours, cells were incubated with 0.05uCi/ml of [methyl-³H]-thymidine for 4 hours and ³H-thymidine incorporation was determined by scintillation counting as described (Stanic et al., 2010).

2.12 Statistical Analyses

Results are expressed as mean $\pm$ standard error of the mean. Statistical comparisons were performed by either Student's two-tailed t-tests or analysis of variance (ANOVA) with Tukey multiple comparison post-test, as appropriate. A value of $p < 0.05$ was considered significant.

3. Results

3.1 Deficiency of GPx1 increases H₂O₂ production and CyPA secretion

We used arterial tissue from the GPx1^{+/-} mice, which have an approximately 50% reduction in GPx activity compared to normal tissues with no significant changes in other antioxidant enzymes (Ho et al., 1997). We first determined if GPx1 deficiency would result in the expected increase in vascular H₂O₂ levels. Both intracellular and extracellular H₂O₂ levels were elevated in aortic sections from GPx1^{+/-} as compared to WT aorta (Fig. 1A, B). We found a similar increase in H₂O₂ levels in SMCs isolated from aorta of GPx1^{+/-} mice (Fig. 1C, D).

Reactive oxygen species (ROS) have been shown to increase CyPA secretion from vascular cells (Suzuki et al., 2006). To determine whether the observed increase in H₂O₂ levels associated with GPx1 deficiency is sufficient to induce secretion of CyPA, we examined CyPA expression in vascular tissue from GPx1^{+/-} mice. As measured by Western blotting

and immunostaining, CyPA levels were increased in GPx1^{+/-} aorta and carotids, respectively, as compared to WT vessels (Fig. 2A, B). We next confirmed that this increase in CyPA was preserved in SMCs cultured from GPx1^{+/-} aorta. As shown in Figure 2C, CyPA levels were increased in the conditioned media of GPx1-deficient SMCs relative to WT conditioned media. As expected, treatment of WT cells with H₂O₂ also resulted in an increase in CyPA levels in the conditioned media. Pretreatment of GPx1-deficient SMCs with the antioxidant NAC (10 mM) decreased CyPA secretion. Expression of intracellular CyPA was also higher in GPx1^{+/-} cells compared to WT (Fig. 2D). In contrast to observations with CyPA secretion, treatment of WT cells with H₂O₂ did not significantly increase CyPA expression in the cell lysates (Fig. 2D). These findings show that a moderate reduction in GPx1 activity in vascular cells is sufficient to increase ROS levels and promote secretion of CyPA.

2.3 CyPA dependent NF- κ B activation and SMC proliferation

The secretion of CyPA is ROS dependent and has been shown to activate signaling pathways in SMCs (Jin et al., 2000). Therefore, we determined whether CyPA treatment of SMCs directly results in an increase in ROS levels and NADPH oxidase expression. Treatment of WT SMCs with recombinant CyPA for 60 minutes did not increase intracellular or extracellular ROS levels (Fig. 3A, B). Similarly, preincubation of SMCs with CyPA for 48 hours did not increase NADPH oxidase activity (Fig. 3C) or the expression of the NADPH oxidase catalytic subunits Nox1 or Nox4 (Fig. 3D).

Several reports have documented a proinflammatory role for CyPA in monocytes/macrophages and endothelial cells, in particular through activation of NF- κ B (Jin et al., 2004; Yuan et al., 2010), but similar studies have not been conducted in SMCs. We found that treatment of WT SMCs with CyPA led to an increase in NF- κ B activation, and the flavoenzyme inhibitor diphenylene iodonium (DPI) prevented this effect (Fig. 4A). We next determined whether the CyPA levels secreted in the conditioned media by GPx1^{+/-} SMCs was sufficient to activate NF- κ B in resting WT cells. The addition of conditioned media collected from GPx1^{+/-} cells, but not WT cells, increased NF- κ B activation as measured by luciferase reporter expression (Fig. 4B). This activation of NF- κ B was inhibited by CsA, confirming the observed stimulation was mediated by CyPA and not another factor in the GPx1^{+/-} conditioned media. Consistent with our earlier results, we also found that DPI and NAC prevented NF- κ B activation.

NF- κ B activation by CyPA has been implicated in cell growth of monocytes/macrophages and glioblastoma (Kim et al., 2005; Sun et al., 2011). Treatment of WT SMCs with CyPA enhanced [³H]-thymidine incorporation, and this effect was inhibited by the treatment of cells with CsA (Fig. 5A). Since GPx1^{+/-} cells had increased levels of extracellular H₂O₂ (Fig. 1), we tested whether H₂O₂ would induce a CyPA dependent increase in cell growth. We found that the conditioned media from cells treated with H₂O₂, when added to WT cells, induced proliferation (Fig. 5B). This effect was inhibited by the addition of CsA to the conditioned media (Fig. 5B), indicating the observed increase in cell growth was mediated by CyPA. Next, conditioned media collected from GPx1-deficient SMCs increased cell number as compared to conditioned media collected from WT cells (Fig. 5C). We extended these findings by using a transwell assay as an alternative experimental approach to collecting conditioned media. WT or GPx1-deficient SMCs were grown in the bottom of the transwell, and WT cells were grown in the insert. The incorporation of [³H]-thymidine by WT cells in the insert was greater when the lower transwell contained GPx1^{+/-} cells as compared to containing WT cells (Fig. 5D). Inhibition of this cell growth by CsA confirmed the mitogenic effect was mediated by CyPA (Fig. 5C, D).

4. Discussion

Reduction in GPx1 activity has been associated with cardiovascular disease in humans, although the significance of this observation is not known. In this study, we show that reduction in GPx1 activity increases intracellular and extracellular H₂O₂ levels both in intact vessels and isolated SMCs, resulting in elevated CyPA expression and secretion. Through a paracrine effect, CyPA activates NF-κB and increases cell proliferation of unstimulated WT SMCs. The effects of CyPA on SMC activation are not associated with changes in NADPH oxidase expression. We propose that reduction in cellular GPx1 may contribute to progression of vascular disease via autocrine and paracrine mechanisms involving CyPA and activation of SMCs (Fig. 6).

Significant recent efforts have focused on defining the mechanisms and effects of ROS in vascular disease; however, these effects may be modified by other cellular-derived factors. It has previously been shown that CyPA has the potential to contribute to atherosclerosis by (1) causing endothelial cell apoptosis and expression of adhesion molecules (Jin et al., 2004), (2) stimulating monocyte/macrophage migration and proliferation (Yuan et al., 2010), and (3) initiating proinflammatory signal transduction pathways and proliferation of SMCs (Jin et al., 2000; Satoh et al., 2008; Satoh et al., 2010). Our findings identify a mechanism by which CyPA expression and secretion by SMCs could be increased in atherosclerosis. In addition, our data provide a link between CyPA and previous observations of GPx1 deficiency in cardiovascular disease.

Oxidative stress has been implicated in the pathogenesis of cardiovascular disease, and increased ROS promotes CyPA secretion. Pharmacologic stimulation of ROS causes secretion of CyPA in SMCs (Liao et al., 2000). This increase in CyPA secretion involves vesicle transport to and docking with the plasma membrane, Rho-GTPase activity, and actin remodeling (Satoh et al., 2010). In contrast to previous studies that have stimulated ROS, we examined a model of decreased antioxidant activity and similarly found the increased production of CyPA. Increased expression of CyPA *in vivo* has been confirmed in our model of GPx1 deficiency as well as following carotid injury and in abdominal aortic aneurysms (Satoh et al., 2008; Satoh et al., 2009). Our data demonstrate that the amount of CyPA secreted from cells with reduced antioxidant activity is sufficient to activate quiescent SMCs. This paracrine effect provides a mechanism for recruitment and amplification of SMC activation in the blood vessel.

We show that the mechanism of activation of SMCs by CyPA involves transcriptional regulation by NF-κB. CyPA induces NF-κB dependent expression of cytokines in monocytes and macrophages (Yuan et al., 2010). However, the current study is the first demonstration of NF-κB activation in vascular cells by CyPA. It has previously been shown that CyPA promotes proliferation of SMCs through ERK phosphorylation (Jin et al., 2000), and our data provide an additional mechanism by which CyPA contributes to SMC activation. NF-κB activation induced by the conditioned media from GPx1-deficient cells is inhibited by pretreatment with either the antioxidant NAC or the flavoenzyme inhibitor DPI. These observations support the hypothesis that CyPA mediates the proinflammatory response initiated by oxidative stress.

Recently some of the results on CyPA have been shown to be due to contamination by lipopolysaccharide (LPS) (Payeli et al., 2008). Whereas there is variability in LPS contamination and CyPA activity, as measured by peptidyl prolyl *cis-trans* isomerase (PPIase) assays, it is important to note that we obtained our CyPA preparations from Sigma, which, unlike other commercial sources, has undetectable LPS contamination (Payeli et al., 2008). It is possible that using higher concentrations of recombinant CyPA would have

yielded more maximal responses. However, a physiologic level of recombinant CyPA was chosen to match the magnitude of NF- κ B activation and growth obtained with the conditioned media. We also acknowledge the potential limitation of studies that use CsA as a pharmacologic inhibitor of CyPA. The effect of CsA on biologic endpoints is well-studied; however, the specific mechanism remains less clear. Nonetheless, CsA is an inhibitor of PPIase activity and is routinely used as an inhibitor of CyPA activity.

5. Conclusions

A number of recent studies using genetically modified mice have confirmed a pathologic role for CyPA in the development of atherosclerosis (Nigro et al., 2010), aortic aneurysms (Sato et al., 2009), and neointimal hyperplasia (Sato et al., 2008). We provide evidence that oxidative stress increases CyPA secretion and activates proinflammatory pathways and cell proliferation in a paracrine manner. Together, these observations identify CyPA as a biomarker and potential therapeutic target in cardiovascular disease.

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Figure 1. Hydrogen peroxide levels are increased in GPx1-deficient aorta and SMCs
 (A) Aortae were isolated from GPx1^{+/-} and WT mice and H₂O₂ levels measured by *in situ* CM-H₂DCFDA fluorescence. Arrow indicates endothelium. (B) Aortae were incubated with Amplex Red and the fluorescence of the media measured. Relative fluorescent units (RFU) were normalized to aortic weight; n=6. (C) and (D) SMCs were isolated from aortas of GPx1-deficient and WT mice, grown in culture and serum starved for 48 hours. (C) Intracellular H₂O₂ levels were measured by CM-H₂DCFDA fluorescence and FACS analysis. (D) Extracellular H₂O₂ levels are reported as catalase-inhibitable Amplex Red fluorescence and normalized to total protein. For (C) and (D), relative fluorescence was normalized to WT. * p<0.05 compared with WT; n=5.

Figure 2. Cyclophilin A levels are increased in GPx1-deficient vessels and SMCs

(A) Aorta were collected from WT and GPx1^{+/-} mice and processed for Western blotting for CyPA levels; n=3. (B) Carotid arteries were immunostained for CyPA. Arrow indicates endothelial layer. Scale bar=100 μm. (C) WT SMCs were grown in 0.5% serum for 48 hrs in the presence or absence of 25 μM H₂O₂ whereas GPx1^{+/-} SMCs were grown in the presence or absence of NAC (10 mM). Conditioned media (CM) was collected from all samples and immunoblotted for CyPA; n=5. (D) Cell lysates from untreated GPx1^{+/-} and WT SMCs with and without 25 μM H₂O₂ were immunoblotted with CyPA; n=5. All bands were quantitated and normalized to WT levels. * p<0.05 compared with WT cells.

Figure 3. Effects of cyclophilin A on ROS and NADPH oxidase levels

WT SMCs were growth arrested in 0.5% serum and then treated with CyPA at the indicated concentrations for 1 hr. (A) Intracellular ROS levels were assessed by dihydroethidium (DHE) and flow cytometry; n=5. (B) Extracellular ROS were measured by Amplex Red fluorescence; n=5. (C) WT SMCs were treated with 10 nM CyPA. Membrane-enriched fractions were isolated and treated with NADPH (100 μ M) and superoxide levels measured by lucigenin-enhanced chemiluminescence; n=3. (D) WT SMCs were treated with 10 nM CyPA for 48 hrs, then RNA was collected and Nox1 and Nox4 expression assessed by real-time PCR. n=3.

Figure 4. Conditioned media from GPx1-deficient SMCs activates NF-κB in WT SMCs
 (A) WT SMCs were transfected with an NF-κB luciferase reporter as described in *Materials & Methods*. Cells were treated with 10 nM CyPA with or without 10 μM DPI for 48 hrs, then NF-κB activity was measured 6 hrs later and normalized to total protein; n=4. (B) Conditioned media were collected from WT and GPx1^{+/-} SMCs after 48 hrs and transferred to WT SMCs transfected with the reporter construct as in (A). Where indicated, GPx1^{+/-} SMCs were pretreated with 10 μM DPI, 10 mM NAC, or 2.5 μg/ml cyclosporine A (CsA) prior to collection of conditioned media. NF-κB luciferase activity was estimated as in (A); n=4. * p<0.05 compared with WT cells.

Figure 5. Cyclophilin A increases proliferation of SMCs

(A) WT SMCs were serum-starved for 48 hrs, then 10 nM CyPA with or without 2.5 μ g/ml CsA added for an additional 24 hrs. Proliferation measured by [³H]-thymidine uptake. (B) WT SMCs were grown in 0.5% serum with 25 μ M H₂O₂ in the presence or absence of 2.5 μ g/ml CsA for 24 hrs, then conditioned media collected and added to growth-arrested WT SMCs. [³H]-thymidine incorporation was measured 24 hrs later. (C) Conditioned media from WT or GPx1^{+/-} SMCs grown with or without 2.5 μ g/ml CsA were added to growth-arrested WT SMCs. Cells were counted 24 hrs later. (D) [³H]-thymidine incorporation by WT SMCs grown in a transwell insert with either WT or GPx1^{+/-} SMCs was measured. * $p < 0.05$ compared with WT cells; $n = 5$.

Figure 6. Relationship between GPx1 deficiency and cyclophilin A in activation of vascular cells
Reduction in GPx1 activity leads to increased H₂O₂ levels, contributing to secretion of CyPA and subsequent paracrine activation of NF-κB and cell proliferation.