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Lack of glutathione peroxidase-1 facilitates a pro-inflammatory and activated vascular endothelium

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ABSTRACT

A critical early event in the pathogenesis of atherosclerosis is vascular inflammation leading to endothelial dysfunction (ED). Reactive oxygen species and inflammation are inextricably linked and declining antioxidant defense is implicated in ED. We have previously shown that Glutathione peroxidase-1 (GPx1) is a crucial antioxidant enzyme in the protection against diabetes-associated atherosclerosis. In this study we aimed to investigate mechanisms by which lack of GPx1 affects pro-inflammatory mediators in primary aortic endothelial cells (PAECs) isolated from GPx1 knockout (GPx1 KO) mice. Herein, we demonstrate that lack of GPx1 prolonged TNF-α induced phosphorylation of P38, ERK and JNK, all of which was reversed upon treatment with the GPx1 mimetic, ebselen. In addition, Akt phosphorylation was reduced in GPx1 KO PAECs, which correlated with decreased nitric oxide (NO) bioavailability as compared to WT PAECs. Furthermore, IκB degradation was prolonged in GPx1 KO PAECS suggesting an augmentation of NF-κB activity. In addition, the expression of vascular cell adhesion molecule (VCAM-1) was significantly increased in GPx1 KO PAECs and aortas. Static and dynamic flow adhesion assays showed significantly increased adhesion of fluorescently labeled leukocytes to GPx1 KO PAECS and aortas respectively, which were significantly reduced by ebselen treatment. Our results suggest that GPx1 plays a critical role in regulating pro-inflammatory pathways, including MAPK and NF-κB, and downstream mediators such as VCAM-1, in vascular endothelial cells. Lack of GPx1, via effects on p-AKT also affects signaling to eNOS-derived NO. We speculate based on these results that declining antioxidant defenses as seen in cardiovascular diseases, by failing to regulate these pro-inflammatory pathways, facilitates an inflammatory and activated endothelium leading to ED and atherogenesis.

1. Introduction

A critical early event in the pathogenesis of cardiovascular diseases such as atherosclerosis is vascular inflammation. This inflammatory process drives a phenotypic change in the vascular endothelium from a protective state, where it maintains a non-adhesive and non-thrombogenic surface, to an activated state, characterized by increased adhesion of leukocytes [1]. The increase in leukocyte-endothelial interactions is driven mainly through the up-regulation of endothelial cell adhesion molecules, in particular vascular cell adhesion molecule-1 (VCAM-1) [2]. Up-regulation of this cellular adhesion molecule is induced by pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and reactive oxygen species (ROS), both of which are prominent in the inflammatory environment. TNF-α and ROS induce adhesion molecule gene expression via the activation of several signaling pathways [3,4]. Most importantly, TNF-α and ROS have been shown to activate the MAPK pathway, in particular extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and P38, as well as the redox-sensitive transcription factor, nuclear factor-κB (NF-κB) [3]. In addition, TNF-α can directly stimulate an increase in ROS production in endothelial cells [5]. Consequently, the activation of these pathways promotes vascular adhesion molecule expression and resultant vascular inflammation and dysfunction.

In addition to vascular inflammation, endothelial dysfunction is driven by compromised nitric oxide (NO) bioavailability, which is a well-recognized contributor to the initiation and progression of atherosclerosis. NO, produced in endothelial cells by constitutively expressed endothelial nitric oxide synthase (eNOS), exerts its protective vascular effects by limiting ROS and adhesion molecule expression. The activity of eNOS is greatly influenced by its phosphorylation status at a critical serine residue at position 1177 by protein kinase B (Akt), which in turn is sensitive to oxidative stress [6,7]. During inflammation, the activation of inflammatory pathways and the increase in ROS result in

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scavenging of NO by superoxide, resulting in the production of another potent ROS, peroxynitrite. This results in the overall downregulation of eNOS function, thereby reducing eNOS-derived NO and its beneficial protective effects [8].

In order to maintain vascular homeostasis, the production of ROS is balanced by the activities of antioxidant enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase, and peroxiredoxins. GPx1 is the most abundant isoform of the selenocysteine-containing GPx protein family, and is responsible for the detoxification of hydrogen peroxide, lipid peroxides and peroxynitrites, ROS species known to damage DNA, lipids and proteins respectively. Mounting evidence now supports an important role for GPx1 in the protection against various cardiovascular pathologies. For example, in humans, GPx1 activity was shown to be a strong and independent risk factor for cardiovascular events, both in diabetes and coronary artery disease settings [9,10]. In experimental models, GPx1 deficiency led to endothelial dysfunction, impaired angiogenesis, and increased infarct size and vascular permeability following ischemia/reperfusion injury [11,12]. Moreover, recent data from our laboratory [13] and others [14] have demonstrated that lack of GPx1 confers susceptibility to atherosclerosis in diabetic and hyperlipidemic settings respectively. In a recent study by Lubos et al., [15] in human endothelial cells silenced for GPx1 expression, it was demonstrated that lowering of GPx-1 activity accelerates oxidative stress and augments NF-κB and JNK activation. Collectively, these studies strengthen the notion that GPx1 plays a major role in vascular homeostasis.

There is a strong clinical need for the development of novel antioxidants to lessen atherosclerotic burden. We have previously suggested that a targeted antioxidant strategy that augments endogenous antioxidant defenses is more likely to succeed clinically in reducing atherogenic burden than vitamins such as vitamins C and E [16]. Our laboratory has focused on the cardiovascular protection afforded by a lipid-soluble low molecular weight seleno-organic GPx mimetic, ebselen (Eb). Indeed, we demonstrate a significant reduction in atherosclerosis and pro-inflammatory mediators after treatment of diabetic mice with ebselen [17,18].

In the current study, we further investigate the role of GPx-1 in modulating inflammatory pathways that contribute to atherogenesis in primary aortic endothelial cells (PAEC) isolated from wild type (WT) and GPx-1 knockout (KO) mice. Additionally, we investigate leukocyte-endothelial cell interactions ex-vivo in aortas from GPx1 KO mice and the effect of ebselen on these pro-inflammatory pathways and interactions. We now demonstrate that lack of GPx-1 results in sustained activation of the MAPK and NF-κB pathways, and increased leukocyte adherence to the vascular endothelium, all of which are rescued with ebselen treatment. Additionally, lack of GPx1 reduces AKT signaling and NO bioavailability. We discuss our findings in light of the impact of reduced antioxidant defense, and in particular, GPx1, on the vascular endothelium and endothelial dysfunction as well as the therapeutic benefits of augmenting GPx1-like defenses.

2. Methods

2.1. Animals

All animal experiments were approved by the Alfred Medical Research and Education Precinct (AMREP) animal ethics committee and investigations conformed to National Health and Medical Research Council (NHMRC; Australia) guidelines. GPx1 KO and WT mice were bred at the AMREP Precinct Animal Centre (PAC). GPx1 KO mice were bred onto the C57/BL6J background (10 generations). WT mice of the same background were generated via Mendelian segregation through the mating of heterozygous C57/BL6J GPx-1 and maintained as a separate line [19]. Male mice between the ages of 8–10 weeks were used for the experiments.

2.2. Isolation of primary aortic endothelial cells

PAECs were isolated from WT and GPx1 KO mice as described previously with modifications [20]. Briefly, aortas were dissected from WT and GPx1 KO mice and cleaned of peripheral fat in saline. A 22-gauge cannula was then inserted into the proximal portion and tied with a silk suture. Vessels were then perfused with Dulbecco's Modified Eagles Medium (DMEM) containing 0.2% collagenase and allowed to incubate at 37 °C for 45 min. Next, vessels were perfused with DMEM containing 20% fetal bovine serum (FBS) and incubated at 37 °C for 2 h. Isolated cells were grown in primary endothelial cell media for 2–3 weeks until confluence. Upon confluency, WT and GPx1 KO PAECs were characterized by the gene expression of cell-specific markers, in particular endothelin A (ETA), endothelin B (ETB), smooth muscle actin (SMA), platelet endothelial cell adhesion molecule (PECAM-1) and GPx1. Additionally, the protein expression of GPx1 was confirmed by Western blot.

2.3. Cell culture

WT and GPx1 KO PAECs were grown in EGM-2 media (Lonza) supplemented with 10% FBS at 37 °C in 5% CO_2. Experiments were performed on cells from passages 3 to 8. Upon 90% confluency, cells were serum starved for 8 h followed by stimulation with TNF-α (20 ng/ml) for 15, 30 and 60 min. For analysis of VCAM-1 expression, cells were not serum starved prior to exposure to 20 ng/ml TNF-α which was increased to 6 h. In some experiments, cells were treated with 0.01 μM ebselen for 15, 30 or 60 min which included pre-treatment for 30 min. In previously published studies in human AECs, we have established that 0.01–0.03 μM ebselen was optimal for the inhibition of JNK and IKK activation, without compromising cell viability [17]. Initial experiments showed similar responses in PAEC (data not shown) and 0.01 μM was chosen for all subsequent experiments.

2.4. Western blotting

PAECs were washed twice with PBS and protein was isolated using RIPA buffer as described previously [17]. Protein concentration was determined using the BCA protein kit. Protein samples (45 μg) was separated on 10% SDS-polyacrylamide gels at 150 V and transferred to Nitrocellulose membranes and blocked as described previously [21]. The membranes were then incubated with either rabbit polyclonal anti-GPx1 antibody (1:100; Abcam Ltd., Cambridge, UK, ab1877), rabbit monoclonal anti-VCAM-1 (1:1000; Abcam, UK, ab29473), rabbit polyclonal anti-phospho JNK (Thr183/Tyr185) (1:1000; Cell Signaling Technology, Inc. #9251), rabbit polyclonal anti-phospho ERK (1:1000; Cell Signaling Technology, Inc. #9101), rabbit polyclonal anti-phospho P38 (1:1000; Cell Signaling Technology, Inc. #9211), rabbit polyclonal anti-phospho Akt (1:1000; Cell Signaling Technology, Inc. #9271) or rabbit monoclonal anti-phospho IKKα/β (Ser176/180) (1:1000; Cell Signaling Technology, Inc. #2697) antibodies overnight at 4 °C. Pierce ImmunoPure Goat anti rabbit IgG, peroxidase conjugated was used as secondary antibody for 1 h. Total MAP kinase signaling was detected with appropriate antibodies (1:1000 Cell Signaling Technology, Inc. #9252 (JNK), #9102 (ERK), #9212(p38)) and Akt (1:1000), #9272)). All membranes were hybridized with a monoclonal α-tubulin antibody (Clone B-5-1-2, Sigma, MO, USA) and visualized using the ECL Advance Western blotting detection kit. Signals were quantitated by densitometry using Quantity One (Bio-Rad, Hercules, CA, USA). Data are expressed relative to α-tubulin and 4 independent experiments were analyzed.

2.5. Cell proliferation assays

WT and GPx1 KO PAECs were seeded in 12 well plates at a concentration of 20,000 cells/well. After overnight attachment, sub confluent monolayers were serum starved in DMEM with 0.2% FBS for 24 h.