OVEREXPRESSION OF Prdx6 REDUCES H2O2 BUT DOES NOT PREVENT DIET-INDUCED ATHEROSCLEROSIS IN THE AORTIC ROOT

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Abstract—The mammalian 1-Cys peroxiredoxin (Prdx6) is a unique member of the peroxiredoxin family of proteins capable of protecting cells from metal-catalyzed oxidative damage. We recently identified Prdx6 as a candidate for the quantitative trait locus Ath1, a gene responsible for a difference in diet-induced atherosclerosis susceptibility in mice. To investigate the role of Prdx6 in atherosclerosis, we generated transgenic mice that overexpress the Prdx6 allele from the Ath1-resistant 129/SvJ strain on an Ath1-susceptible C57BL/6J background. These mice expressed significantly elevated levels of Prdx6 mRNA and protein in multiple tissues including liver, aorta, and peritoneal macrophages, which accumulated significantly lower levels of hydrogen peroxide, revealing an enhanced antioxidant activity in these mice. However, overexpression of Prdx6 had no protective effect on LDL oxidation in vitro, and transgenic mice fed an atherogenic diet for 10 weeks did not possess an increased resistance to atherosclerosis nor did they maintain the high prediet plasma HDL levels consistent with the Ath1-resistant phenotype. In addition, the Prdx6 allele from the susceptible strain was shown to have a higher antioxidant activity than that of the resistant strains. These data suggest that the increased peroxidase activity attributable to Prdx6 overexpression in transgenic mice is not sufficient to protect mice from atherosclerosis, and that Prdx6 is not likely to be the gene underlying Ath1. © 2003 Elsevier Inc.

Keywords—Atherosclerosis, Prdx6, Antioxidant, Ath1, Transgenic, Mice, Aop2, Free radicals

INTRODUCTION

Research in the last few decades has led to the recognition of atherosclerosis as a chronic inflammatory disease [1,2]. One of the earliest molecular events that drives monocyte recruitment into the arterial wall is thought to be the entrapment and subsequent oxidative modification of LDL particles in the subendothelial space. Several studies have suggested that cells of the artery wall secrete oxidative products that initiate this process through lipid oxidation and that oxidative stress in this microenvironment enhances the expression of specific chemotactic and adhesion molecules, which promote attachment of monocytes to the endothelium as well as the subsequent differentiation into macrophages and uptake of oxidized LDL to form foam cells [3–8]. Oxidative stress has also been implicated in later stages of atherosclerosis, including during activation of smooth muscle cells and platelets, plaque rupture, and thrombus formation [9].

Despite the apparent role of oxidation in atherosclerosis, little is known about the endogenous proteins that control this process in vivo. We have used genetic approaches to try to identify genes associated with atherosclerosis in mice. The Ath1 locus was a quantitative trait locus (QTL) found to be associated with reduced plasma HDL levels and increased atherosclerotic lesions in the aortic root of susceptible female mice of the C57BL/6J strain when fed a high-fat diet, as compared to the resistant strains C3H/HeJ and BALB/cJ, which exhibit no change in plasma HDL levels and the absence of atherosclerotic lesions [10]. This locus was originally mapped to a 3 cM region on chromosome 1 in mouse, and a QTL mapping to the same location in the resistant strain SPRETUS/EiJ was recently narrowed to a 0.54 cM interval (cM position 83.6) [11]. This region colocalizes with the peroxiredoxin 6 (Prdx6) gene (previously called antioxidant protein 2) [12,13], a unique member of the thiol-specific antioxidant (TSA) family of proteins [14].
These proteins possess peroxidase activity that protects cells from oxidative damage resulting from metal-catalyzed oxidation systems [15–17]. In addition to fine map location, evidence to support Prdx6 as the gene underlying the Athl QTL includes an amino acid variation and Prdx6 mRNA expression differences between resistant and susceptible strains of mice [11]. The notion that an antioxidant may be responsible for the susceptibility difference between the Athl strains is also intriguing since earlier studies have demonstrated that the induction of inflammatory genes and the accumulation of lipid peroxidation products cosegregate with aortic atherosclerotic lesion formation in Athl recombinant inbred mouse strains [18,19].

Several reports have suggested a protective role for antioxidants against atherosclerosis [20,21]. Since LDL oxidation has long been implicated in foam cell formation and atherosclerotic lesion development in the arterial wall, and several studies suggest that antioxidants possess antiatherogenic functions, we were interested in the possibility that overexpression of an antioxidant protein might protect against the disease in mice. The present study investigated the in vitro and in vivo activity of the Prdx6 protein and used transgenic mice to test the ability of overexpression of the Prdx6 allele from the resistant strain to rescue the atherosclerosis-susceptible phenotype of the C57BL/6J background strain.

MATERIALS AND METHODS

Transgene construct

A genomic clone containing the entire Prdx6 gene (spanning 10,182 bp) and 6644 bp of upstream sequence was isolated from a LambdaFix II genomic library (Stratagene, La Jolla, CA, USA) derived from the Athl-resistant 129/SvJ mouse strain, as described previously [13]. (The coding region of Prdx6 from 129/SvJ was previously reported to contain the same nucleotide variant found in the resistant C3H/HeJ and BALB/cJ strains, which corresponds to a single amino acid difference in position 124 encoding alanine in the susceptible B6 strain and aspartic acid in the inbred resistant strains.) The insert was sequenced and was excised from the clone using NcoI, which cuts in the 5’ and 3’ flanking vector sequence. The isolated fragment retained 43 bp of vector sequence at each end of the insert, containing the T3 promoter adjacent to the 5’ end and the T7 promoter adjacent to the 3’ end. The fragment was purified and microinjected into oocytes to produce a transgene designated Prdx6\(^{129/SvJ-Tg}\). The alleles from the C57BL/6J, C3H/HeJ, BALB/cJ, and 129/SvJ strains are designated as Prdx6\(^{B6}\), Prdx6\(^{C3H}\), Prdx6\(^{Balb}\), and Prdx6\(^{129/SvJ}\), respectively.

Mice

C57BL/6J (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Three days before they were mated, 20 females were induced to superovulate by injecting them with 5 international units (IU) of pregnant mare serum gonadotrophin on day 1, and 5 IU of human chorionic gonadotrophin on day 3. The females were mated on the evening of day 3 and checked for vaginal plugs the next morning. Those with vaginal plugs were euthanized by cervical dislocation, and their one-celled embryos were harvested. The Prdx6\(^{129/SvJ-Tg}\) construct was microinjected into 382 of these embryos at a concentration of 4 ng/μl and into another 420 embryos at a concentration of 2 ng/μl. About 30–40 injected embryos were implanted into each of 20 pseudopregnant females.

The 58 pups born from these females were genotyped for the transgene as described below, and transgenic mice were used as founders to generate independent lines. They were bred to B6 mice, and F1 progeny heterozygous for the transgene were mated to each other to produce F2 mice. F2 females carrying the transgene, along with negative littermates, were phenotyped for susceptibility to atherosclerosis according to the following protocol. At 6–8 weeks old, F2 mice were fed an atherogenic diet for 10 weeks. The atherogenic diet [20] contained 15% diabetic fat, 50% sucrose, 20% casein, 0.5% cholic acid, 1.0% cholesterol, corn oil, cellulose, vitamins, and minerals. At the end of the 10 weeks consumption of the atherogenic diet, mice were euthanized and their hearts with ascending aortas were removed. The aortic roots were sectioned and analyzed for atherosclerotic lesions in the arterial root, as described for the Athl phenotype [10,11]. Their livers were frozen for subsequent RNA and protein analysis and spleens were frozen for DNA isolation. Animal protocols were approved by the institutional Animal Care and Use Committee at The Jackson Laboratory.

Genotyping

Approximately 1 cm of each tail tip was placed in 500 μl of tail buffer [50 mM Tris-Cl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, and 1% SDS] and digested overnight with 1 mg/ml protease K at 55°C. DNA was extracted with phenol:chloroform:isoamyl alcohol and precipitated from the upper aqueous phase with 2 vol of 100% ethanol. The precipitated DNA pellets were dried in a 65°C heat block for 15 min and resuspended in 1 ml TE (pH 7.5–8.0). PCR genotyping was performed using a Prdx6 forward primer designed to the 3’ UTR of the Prdx6 gene (5’-CAAGGATGTCAGTGTTGG-3’) and a transgene-specific reverse primer designed specifically to the 3’ flanking T7 vector sequence (5’-GTAATACGACTCAGATTTTGAGGCGC-3’). PCR was carried out for 30 cycles under the conditions described above.

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following conditions: 95°C for 30 s; 55°C for 1 min; and 72°C for 1 min.

Southern blots

Tail DNA (10 μg) from each F1 or F2 transgenic mouse to be tested was digested with HincII, electrophoresed on a 0.7% agarose gel, and transferred to a Hybond nylon membrane (Amersham Corp., Arlington Heights, IL, USA). A Prdx6-specific probe was generated by PCR amplification of Exon V of Prdx6 using the genomic Prdx6129SvJ-Tg clone as a template with the following primers: Exon V forward (5’-AGGGAGAGGCGT-GATGG) and 3’UTR reverse (5’-CCTCAAAAGCA-GAA TTCAG-3’), under the PCR conditions stated above. The 811 bp probe was 32P-labeled by random priming using the Redi-Prime Kit (Amersham Corp.), and the blots were hybridized using the Rapid-Hybe solution and protocol (Amersham Corp.).

Real-time PCR analysis

Total RNA was extracted by using RNeasy Mini Kit (Qiagen, Santa Clarita, CA, USA), following the manufacturer’s instructions. RNA concentrations were measured with RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA). cDNA was synthesized by reverse-transcribing 2 μg of total RNA with Omniscript RT kit (Qiagen), using oligo(dT)15 primer and random hexamers (Promega, Madison, WI, USA). Real-time PCR was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Inc., Foster City, CA, USA), as described previously [21]. The primers for Prdx6 cDNA were: forward, 5’-TTGATGATAAGGGCAGGGAC-3’ and reverse, 5’-CTACCATACGCTCCTCCC-3’. Quantitative real-time PCR was normalized to the copies of Gapd mRNA from the same sample. Data were analyzed by Sequence Detector software (Applied Biosystems, Inc.). All PCR assays were performed in triplicate.

Generation of Prdx6-specific antibodies

To generate the Prdx6-specific antibodies used in ELISA and Western blot assays, two synthetic peptides were generated. Peptide five, with amino acid sequence FPKGVFTKELPSGKKYLRC, corresponded to amino acid 202–220 of murine Prdx6. Peptide three, with amino acid sequence SKDINAYGETPTEKLPC, corresponded to amino acid 83–99. Both peptides were chosen based on an antigenicity calculation (Jameson-Wolf, based on hydrophilicity, surface probability, flexibility, and secondary structure predictions). In addition, peptide five has been previously shown to be an antigen determinant [22]. A cysteine was added to the C-terminal end of the peptide so that it would specifically couple to bovine serum albumin (BSA) and affinity gel matrices.

Rabbits were immunized with BSA-conjugated peptides, and repeated boosters were performed. The IgG antibody fraction was purified from the antisera obtained after four immunizations using a Protein G Sepharose 4 Fast Flow column (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 20 mM Tris-Cl (pH 8.0), 2 mM EDTA, and 1 mM dithiothreitol (DTT), and further purified on SulfoLink (Pierce, Rockford, IL, USA) affinity columns, to which the synthetic peptides had been coupled by their cysteine residues.

ELISA

ELISAs were used to compare the amount of Prdx6 protein produced in mice from transgenic lines to that produced in controls. Prdx6 protein was extracted from approximately 40 mg of liver from each mouse by homogenizing in an ice bath in 1 ml of H2O-containing protease inhibitors (catalog number 1836153, Boehringer Mannheim, Mannheim, Germany). Homogenates were centrifuged for 30 min at 20,000 × g, and the protein-containing supernatants were collected. Protein concentrations in each supernatant were determined by the Bio-Rad dye-binding protein assay (Bio-Rad, Richmond, CA, USA). Next, 1 μg of rabbit antipeptide 3 antibody (Pab 3) in 100 μl of 0.1 M Na-carbonate/bicarbonate [0.02% (w/v) Na3] pH 9.6] was added to each well of a 96-well plate. The plates were incubated overnight at room temperature.

The next morning, wells were washed with PBS and blocked with 200 μl of blocking buffer [2% (w/v) fat-free dry milk in PBS] for 1 h at 37°C. After 1 h, the wells were washed six times in wash buffer [0.05% (v/v) Tween 20 in PBS]. The samples were diluted 1:100 for both controls and 1:200 for high-fat samples in dilution buffer [0.2% (w/v) fat-free dry milk, 0.05% (v/v) Tween 20 in PBS], and 100 μl were added to each well and incubated at 37°C for 3 h. The wells were again washed six times in wash buffer, and 100 μl of a 1:1000 dilution of alkaline phosphatase-labeled streptavidin (in dilution buffer) were added to each well and incubated at 37°C for 2 h. The plate wells were washed six more times, and 100 μl of a 1:1000 dilution of alkaline phosphatase-labeled streptavidin (in dilution buffer) were added to each well and incubated at 37°C for 2 h. The plate wells were washed six more times, and 100 μl of a 1:1000 dilution of alkaline phosphatase-labeled streptavidin (in dilution buffer) were added to each well and incubated at 37°C for 1 h. The wells were washed five times in wash buffer and once in PBS. Two tablets of phosphatase substrate (Sigma 104 phosphatase substrate tablets, Sigma Chemical Co., St. Louis, MO, USA) were dissolved in 10 ml of diluted substrate solution (0.1 M diethanol amine-HCl, 0.5 mM MgCl2, pH 9.8). Immediately, 100 μl of this substrate was added to each well and plates were incubated in the dark at room temperature for 20 min. Reactions were stopped with 25 μl of 0.5 M NaOH.
Western blots

Western blots were used to compare Prdx6 protein levels in liver and macrophages of mice from transgenic lines and their controls. Livers were cut into small pieces (1 to 2 mm) and lysed in 0.25 M sucrose, 25 mM KCl, 50 mM triethanolamine-HCl, 5 mM M&Cl2, 0.5 mM PMSF, 1 mM DTT, and 1:100 dilution of protease inhibitors using a Potter-Elvhjelm homogenizer (Wheaton Science Products, Millville, NJ, USA). Lysates were filtered through four layers of cheesecloth and centrifuged at 800 × g for 20 min. Supernatants were collected and quantified for protein concentration with the Bio-Rad protein assay. Resident peritoneal macrophages from transgenic mice and littermate controls were lysed in a lysis buffer [20 mM HEPES, 1% Triton X-100, 10% glycerol, protease inhibitors, pH 7.2 (Boehringer Mannheim)] and centrifuged for 10 min at 20,000 × g at 4°C. Supernatant was collected and the protein concentrations were measured with the Bio-Rad protein assay. Samples were subjected to SDS-PAGE and subsequently transferred to PVDF membrane (Bio-Rad) in a semidy transfer system (Bio-Rad). The membranes were blocked with 5% skim milk in Tris-HCl buffer, and Prdx6 protein was detected with the rabbit anti-Prdx6 peptide antibody (Pab 5), followed by horseradish peroxidase-labeled secondary antibody and ECL technique (Amersham Corp.). When tested with purified recombinant protein made in E. coli, Pab 5 reacts with both Prdx6 and the highly related protein Aop2-R51 [13]. However, the Pab 5 antibody does not detect any protein in liver or macrophages of Prdx6 knockout mice (data not shown), indicating that it is specific for Prdx6.

Immunohistochemistry

Immunohistochemical staining was used to compare the distribution of Prdx6 expression in mice from transgenic lines to that in controls. Mouse tissues were paraffin embedded and sectioned after being fixed in 4% paraformaldehyde. Endogenous peroxidase activity was blocked with 0.3% H2O2, and nonspecific binding sites were blocked with nonimmune goat serum. Subsequently, a 1:1000 dilution of rabbit anti-Prdx6 peptide 5 antibody (Pab 5) was applied. Specific binding was detected by using biotinylated secondary antibody and avidin-biotin-horseradish peroxidase complex and adding diaminobenzidine as a substrate. Nuclei were counterstained with hematoxylin.

Recombinant Prdx6 expression construct

Recombinant Prdx6 was produced and purified mainly as described [22]. The full-length coding region from the Prdx6B6 and Prdx6C3H allele was PCR amplified and subsequently used for cloning. Previously isolated PCR fragments spanning this region were used as a template for PCR with the following primers: forward primer (5’-CGGCATATGCCGAGGTTGCTTC-3’), which contained nucleotide 1–21 of the mouse Prdx6 coding sequence, the initiation codon, and a NdeI cleavage site, and reverse primer (5’-CGCGAATTCTTATTAAGGCTGG GTGTAT AACGG-3’), which contained nucleotide 653–671 of the mouse Prdx6 coding sequence, two stop codons, and an EcoRI cleavage site. PCR was carried out for 30 cycles under the following conditions: 94°C for 30 s; 60°C for 30 s; and 72°C for 45 s, with a 7 min extension at 72°C. The resulting PCR products were cloned into the pGEM-T vector (Promega). A Ndel-EcoRI fragment from pGEM-T, containing the cDNA encoding Prdx6, was subcloned into pET-17b (Novagen, Madison, WI, USA), generating pETPRDX6B6 and pETPRDX6C3H. The nucleotide sequence of both were verified by DNA sequencing.

Production and purification of recombinant Prdx6

The BL21(DE3)pLysS strain of E. coli was transformed with the two expression constructs described above. Transformed cells were grown overnight in a small volume of LB supplemented with carbenicillin and chloramphenicol, and thereafter transferred to new medium. Production of recombinant protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested, disrupted, and recombinant protein was purified from the soluble fraction of the bacterial cells, mainly as described previously [22]. The purification method included streptomycin sulfate precipitation, ammonium sulfate precipitation, hydrophobic chromatography, and anion exchange chromatography. However, in contrast to what was described by Kang and coworkers, the sample was applied to a Q-Sepharose column (Pharmacia LKB Biotechnology) equilibrated with 20 mM Tris-Cl (pH 8.0), 2 mM DTT, and 1 mM EDTA. At this pH, recombinant Prdx6 was bound to the column matrix. Thereafter, bound material was eluted with a linear gradient of 0–0.5 M NaCl in 20 mM Tris-Cl (pH 8.0), 2 mM DTT, and 1 mM EDTA; the comparable peaks were isolated and purified; and peroxidase activity was measured as described below.

Glutamine synthetase (GS) protection assay

A glutamine synthetase (GS) assay was used to determine the antioxidant property of the recombinant Prdx6 proteins [23]. Briefly, the assay was performed at 37°C in a 100 μl reaction mixture containing 0.6 units of E. coli glutamine synthetase (Sigma Chemical Co.), 3 μM FeCl3, 10 mM dithiothreitol (DTT), and various amounts of recombinant Prdx6 or catalase, at pH 7.0. Aliquots were removed at different time intervals and
measured for the remaining GS activity by the γ-glutamyl transferase assay.

*Intracellular hydrogen peroxide assay*

An intracellular hydrogen peroxide assay was used to compare Prdx6 antioxidant properties in cultures of macrophage from transgenic mice and their controls. Peritoneal macrophages from transgenic mice overexpressing Prdx6 (line 153) and their littermate controls were cultivated in chambered tissue culture slides (Falcon, Macalaster Bicknell Co., Millville, NJ, USA) in DMEM without phenol red (Gibco BRL, Grand Island, NY, USA). The intracellular H$_2$O$_2$ levels in these cells were measured as described previously [24]. Before the cells were assayed, they were washed with Krebs-Ringer buffer and then incubated in this buffer containing 5 μg/ml of 2',7'-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 5 min. Subsequently, the cells were observed and imaged by using a fluorescence confocal microscope, Model TCS NT (Leica Lasertechnik GmbH, Heidelberg, Germany), and one random image from each slide was chosen for image analysis using the software MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA). For quantitation, 33 cells from the Tg− and 39 cells from Tg+ were counted and average fluorescence is reported as mean ± SEM. Significance was determined using a Student’s t-test, with the level of statistical significance defined as $p < .05$.

*Plasma cholesterol*

After they had been fed the atherogenic diet for 0, 6, and 10 weeks, mice were fasted for 4 h and their blood was sampled into microcentrifuge tubes containing 2 μl of 500 mg/ml EDTA. The plasma was separated by centrifuging at 1500 rpm for 5 min at 4°C and analyzed for total and HDL cholesterol, as described previously [25]. Total cholesterol in each sample was measured from 5 μl of plasma. Before measuring the HDL cholesterol, the apoB-containing lipoproteins in each sample were selectively precipitated by mixing 25 μl of plasma with an equal volume of polyethylene glycol (200 mg/ml) and centrifuging the samples at 12,000 × g for 5 min [26]. HDL cholesterol was measured by mixing 10 μl supernatant with 150 μl cholesterol/HP enzymatic cholesterol reagent (Boehringer Mannheim) and measuring its absorbance on a MR5000 Microplate reader (DyneTech Laboratories, Inc., Chantilly, VA, USA).

*Measurement of oxidation products*

Thiobarbituric acid-reactive substance (TBARS) [27], relative electrophoretic mobility (REM) [28], and oxidation-specific immuno-epitopes detected with antibody against oxLDL (OBO4) in LDL oxidized by macrophages [28, 29] were measured in transgenic and control mice. Macrophages from Prdx6-Tg and control mice were incubated with LDL for different periods of time, and TBARS, REM, and oxidation-specific epitopes in the medium containing LDL were measured, as described previously.

*Measurement of atherosclerotic lesions*

Atherosclerotic lesions were measured as described previously [30]. Briefly, hearts were excised, rinsed in 0.9% saline, fixed in formalin, and embedded in 25% gelatin. The aortic root of each heart was sectioned every 10 microns on a cryostat (Bright Instrument Company, Ltd, Huntingdon, England). Slides were stained with Oil Red O (Sigma Chemical Co.) to visualize the neutral lipids, with hematoxylin to visualize the nuclei and basophilic tissue, and counterstained with light green. The cross-sectional lesion areas of five aortic sections per mouse were determined with computer-assisted imaging. Lesion size is expressed as mean μm$^2$ ± SEM. Differences were evaluated by Student’s t-test, and significance level was $p < .05$.

**RESULTS**

*Production of transgenic mice*

We derived the transgene construct from the atherosclerosis-resistant 129/SvJ strain. The variant amino acid found in the susceptible B6 strain causes a change in net charge at this position that may account for the difference in 2D gel migration pattern of the Prdx6 protein previously reported between C57BL/6J and DBA [12]. The variant residue may, therefore, impact the function of the encoded protein. Based on the crystal structure of the human homologue, this amino acid falls between an α helix and β sheet in the thioredoxin-fold region of the native protein [31], suggesting that it may be involved specifically in the peroxidase activity of the protein.

The Prdx6$^{129/SvJ-Tg}$ transgene construct contains the entire Prdx6 gene, composed of five exons and four introns spanning around 10 kb, approximately 6.6 kb of upstream sequence, and flanking vector sequence from the Lambda Fix II vector (Fig. 1A). This transgene was injected into oocytes, which were subsequently implanted into female mice as described in Materials and Methods. We genotyped the 58 pups born from the implanted females with a transgene-specific PCR assay that used a Prdx63′ UTR forward primer and a T3 reverse primer designed to the 3′ flanking vector sequence. Four of the pups, numbers 105, 139, 146, and 153, were shown to carry the transgene (Fig. 1B). Mouse 146 died shortly after it was genotyped, and the other three were used as founders to generate three independent transgenic lines. They were bred to B6 mice, and F1
progeny heterozygous for the transgene were mated to generate F2 mice. The F1 and F2 mice were all genotyped with the same transgene-specific PCR assay described above.

By using Southern blotting with HindII-digested DNA and an Exon V-specific probe (Fig. 1A), we were able to distinguish endogenous and transgenic Prdx6 genes in the mice. Each gene was detectable as unique size fragments as expected: the Prdx6129/SvJ-Tg transgene construct generated the expected 2587 bp band, whereas the endogenous Prdx6 gene generated a larger restriction fragment (Fig. 1C). Although all three founder lines possessed the transgene-specific band, the relative band intensity for each line suggested that only a single copy of the transgene was present in mice from line 139, whereas multiple copies were present in mice from lines 153 and 105. These two lines were selected for subsequent analysis.

Expression of the Prdx6 transgene in transgenic lines

To ensure that transgenic mice overexpressed the transgene, Prdx6 mRNA expression in liver and aorta of B6 and Prdx6 transgenic mice was compared (Fig. 2). In the liver, Prdx6 mRNA expression levels were 27 and 18 times higher in the Prdx6 transgenic mice on chow and atherogenic diet, respectively, compared with those in B6 mice. In the aorta, Prdx6 mRNA expression levels were 16 and 17 times higher in Prdx6 transgenic mice on chow and atherogenic diet, respectively, compared with those in B6 mice.

Expression of the Prdx6 transgene protein

Affinity-purified antibodies generated from the Prdx6 peptide were first characterized by ELISA and Western blot analysis. The Prdx6 antibody recognized a polypeptide with a molecular weight of approximately 32 kDa in both human and mouse liver homogenates, which was subsequently identified as Prdx6 by N-terminal amino acid sequencing.

A combination of Western blotting, ELISA, and immunohistochemical analyses revealed that Prdx6 protein was expressed in several tissues of transgenic mice, with highest levels found in the lungs, liver, kidneys, intestines, and epithelial cells of all tissues. Immunohistochemical analyses further revealed a similar tissue distribution in Prdx6 between transgenic mice and controls, though higher levels of Prdx6 were detected in most tissues of transgenic mice (data not shown). Specifically,
Western blot analysis of liver homogenates demonstrated that transgenic mice from lines 105 and 153 expressed significantly more Prdx6 protein than did littermate controls (Fig. 3A). In aorta, Prdx6 was expressed in the media adjacent to internal elastic lamina and in some smooth muscle cells (Fig. 3B). Prdx6 was also found in cell lysates from macrophages, another important cell type in atherogenesis. Western blot analysis of macrophages from line 153 transgenic mice also confirmed overexpression of Prdx6 in this cell type (Fig. 3C).

**Analysis of Prdx6 function**

In the presence of an electron donor, Fe$^{3+}$ catalyzes the reduction of O$_2$ to H$_2$O$_2$, which is then converted to hydroxyl radical (OH') by the Fenton reaction [32]. This oxidation system damages proteins, but this damage can be prevented by enzymes that eliminate H$_2$O$_2$. Several studies have used the inactivation of glutamine synthetase (GS) as a measure of oxidative damage in such systems. To determine the activity of Prdx6 protein in our transgenic mice, we first tested the ability of recombinant Prdx6 to protect GS from inactivation by an iron-catalyzed oxidation system. The recombinant Prdx6$^{B6}$ protein was purified to over 95% with a yield of 50%, and was detected by using an immunoblot analysis involving specific polyclonal antibodies. The N-terminal amino acid sequence, purity, and accurate molecular mass of recombinant Prdx6 was verified by NH$_2$-terminal amino acid sequencing and electrospray mass spectroscopy (data not shown). We found that Prdx6 protected GS from inactivation when DTT was used as an electron donor (Fig. 4). The protective activity of recombinant Prdx6 was confirmed by real-time PCR and Western blot analysis (Fig. 2). In the liver, Prdx6 mRNA expression was higher in the chow group compared to the atherogenic diet group (Fig. 2A). Similarly, in the aorta, Prdx6 mRNA expression was higher in the chow group compared to the atherogenic diet group (Fig. 2B).

**Fig. 2.** Prdx6 mRNA expression is higher in the (A) liver and (B) aorta from Prdx6 transgenic mice on both chow and atherogenic diet. Real-time PCR was performed on cDNA reversed transcribed from total RNA extracted from B6 and Prdx6 transgenic females. Prdx6 mRNA expression was adjusted to 1000 mRNA copies of Gapdh. Data are expressed as mean ± SEM from three mice in each group. *p < .05; **p < .01 compared with B6 on the same diet.

**Fig. 3.** (A) Overexpression of Prdx6 protein in liver lysates from transgenic and littermate control mice. Liver lysates were prepared from transgenic mice from lines 105 and 153 and nontransgenic littersmates. The indicated amounts of proteins from each sample were separated by SDS-PAGE along with the different amounts of recombinant purified Prdx6. Protein (5 ng) was applied to each lane in SDS-PAGE, and Prdx6 protein was detected with the polyclonal anti-Prdx6 peptide antibody 5. (B) Prdx6 protein expression in the aorta from transgenic and littermate control mice. Distribution of Prdx6 in the aorta from a Prdx6$^{129/SvJ-Tg}$ transgenic mouse (line 153), detected with immunohistochemical staining using Prdx6 peptide antibody 5. (C) Prdx6 expression in the lysate of macrophages from a Prdx6 transgenic mouse (Tg$^+$) (line 153) and a littermate control (Tg$^-$). Protein (5 μg) was applied to each lane in SDS-PAGE, and Prdx6 protein was detected with the polyclonal anti-Prdx6 peptide antibody 5 after transblot using ECL technique.
binant Prdx6, which was comparable to that of the positive catalase control, confirmed its antioxidant function. The negative control contained no protein, and glutamine synthetase was slowly oxidized over time.

To explore the antioxidant activity of Prdx6 in vivo, we compared H₂O₂ levels in peritoneal macrophages from transgenic mice of line 105, which overexpress Prdx6, and those from the littermate controls. Macrophages are the progenitors of foam cells in atherosclerotic lesions. As shown in Fig. 5A, and quantified in Fig. 5B, peritoneal macrophages of transgenic mice had significantly lower levels of H₂O₂ than did those from littermate controls (p = .00017), demonstrating that cellular Prdx6 can eliminate H₂O₂ in macrophages and that overexpression of Prdx6 in transgenic mice increases its peroxidase activity in these cells. These results were reproduced in multiple experiments.

To determine if Prdx6 overexpression has an affect on oxidized LDL, we analyzed transgenic and control mice using the TBARS assay, REM, and oxidation-specific immuno-epitopes detected with antibody against oxLDL (OB04) in LDL oxidized by macrophages. As shown in Table 1, there is no significant difference in oxLDL by any measurement between transgenic and control mice after 2 and 4 h incubation. By 16 h, a small but significant difference is observed by TBARS, suggesting that transgenic mice have slightly less oxLDL as assayed by TBARS; no difference is seen by the other two methods. Together, the
data suggest that overexpression of \textit{Prdx6} in macrophages has little or no effect on LDL oxidation in vitro.

\textbf{Measurement of atherosclerosis in transgenic mice}

To determine if the high peroxidase activity in \textit{Prdx6}129/SvJ-Tg transgenic mice could rescue the \textit{Ath1}-susceptible phenotype of C57BL/6J mice, we placed transgenic F2 mice from each line and their littermate controls on a high-fat diet for 10 weeks. We compared their total cholesterol and HDL levels at 0 and 10 weeks and the size of their atherosclerotic lesions in the aortic root at 10 weeks. As shown in Table 2, the size of lesions did not differ between control B6 mice and the two transgenic lines overexpressing \textit{Prdx6}. Furthermore, HDL-C concentrations were consistent with levels previously reported for the susceptible C57BL/6J strain as compared to the resistant strains [20].

\textbf{Comparison of two \textit{Prdx6} alleles}

Finally, we sought to determine if the resistant and susceptible strains produce functionally different \textit{Prdx6} protein, which might be responsible for the phenotypic differences characterized by \textit{Ath1}. We previously identified a single amino acid variation at position 124 in the encoded \textit{Prdx6} protein between the \textit{Ath1}-resistant and -susceptible strains: aspartic acid in the resistant C3H and BALB/cJ and alanine in the susceptible C57BL/6J. To determine if this variation alters the peroxidase function of the protein, the peroxidase activity of both recombinant proteins were measured by the glutamine synthetase protection assay. Interestingly, we found that protein encoded by the B6 allele protected GS from damage by the DTT oxidation system to a significantly higher extent than the C3H allele (Fig. 6). While both recombinant proteins were equally capable of protecting GS from inactivation at a concentration of 50 \textmu M, only the B6 allele showed protection at 10 \textmu M, and this was comparable to the efficacy of the higher concentration of the other allele.

\textbf{DISCUSSION}

We have previously reported evidence implicating peroxiredoxin 6 (\textit{Aop2}) as an atherosclerosis-resistant candi-

![Graph](image-url)
Prdx6 overexpression in transgenic mice

Table 2. HDL and Total Cholesterol and Lesion Levels in Transgenic Mice

<table>
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<tr>
<th>Strain</th>
<th>Transgene</th>
<th>Lesions ± SEM</th>
<th>HDL at 0 weeks</th>
<th>HDL at 10 weeks</th>
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<tbody>
<tr>
<td>DBA/2</td>
<td></td>
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<tr>
<td>129/SvJ</td>
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<tr>
<td>Line 105</td>
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<td>Line 153</td>
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date gene in mice [11]. The mapping data that colocalizes Prdx6 with Ath1, as well as the observation that Prdx6 differs between atherosclerosis-resistant and -susceptible strains in either expression or amino acid sequence, prompted further investigation into its antioxidant function and potential antiatherogenic activity. In the present study, we have successfully generated transgenic mice carrying the Prdx6 allele found in the Ath1-resistant inbred strains BALB/cJ, C3H/HeJ, DBA, and 129/SvJ, on an Ath1-susceptible (B6) background. We found that recombinant Prdx6 is capable of antioxidant activity in vitro, and that overexpression of this allele in transgenic mice results in decreased H$_2$O$_2$ accumulation and has no effect on LDL oxidation. However, the enhanced antioxidant activity was not sufficient to protect these mice from the Ath1-susceptible phenotype when fed a high-fat diet.

The transgenic lines carrying multiple copies of the transgene also showed a marked increase in Prdx6 mRNA in the liver and aorta, and these elevated levels were maintained after 6 weeks on an atherogenic diet. Furthermore, Western blotting and immunohistochemistry confirmed elevated expression of the Prdx6 protein in multiple tissues from both lines 105 and 153, demonstrating that higher levels of protein were achieved in all relevant tissues.

The ability of recombinant human Prdx6 to protect glutamine synthetase against inactivation in an oxidative system has been demonstrated previously in vitro [22], and expression of this protein in NIH3T3 cells removes intracellular H$_2$O$_2$. In addition, we have recently shown that Prdx6-null mice have an increased accumulation of hydrogen peroxide, higher protein oxidation levels, more severe oxidant-induced tissue damage, and mortality [21]. Our results demonstrate that murine Prdx6 possesses the same function as the human protein in an in vitro assay, and this antioxidant activity is comparable to that of catalase, a well-characterized and potent antioxidant. This study has further demonstrated that overexpression of Prdx6 in mice leads to reduced intracellular H$_2$O$_2$ in macrophages, suggesting that the level of this activity in vivo can be physiologically upregulated. It is also important to note that other targets for Prdx6 besides hydrogen peroxide are likely to exist, including lipid peroxides [33–35], which have already been demonstrated as substrates for Prdx6 and suggest that its real function may rely on multiple specificities. However, our results demonstrating that Prdx6 overexpression does not protect LDL from oxidation suggest that this may not be an in vivo target of this protein and may not be responsible for atherosclerosis protection in Ath1-resistant mice.

Despite the ability of Prdx6 to reduce intracellular H$_2$O$_2$ levels in transgenic mice, it also did not protect these mice from atherosclerosis when fed a high-fat diet. One possible explanation is that there are critical regulatory sequences, which are either different in the 129/SvJ or not included in the Prdx6$^{129/SvJ-Tg}$ transgene construct, that allow for proper regulation of the gene under high-fat conditions. It is also possible that the presence of the susceptible Prdx6 allele has a dominant negative effect, and the phenotype cannot be rescued by the resistant allele unless the B6 allele is eliminated. However, our additional observation that the B6 allele appears to have a greater antioxidant activity in our in vitro glutamine synthetase protection assay than the C3H allele more strongly suggests that Prdx6 is not the gene underlying Ath1. This is also consistent with the inability of this protein to protect LDL from oxidation in vivo and with other data from our laboratory showing that overexpression of Prdx6 on an apoE KO background had no effect (data not shown). Together, these data strongly suggest that Prdx6 is not the gene underlying Ath1 in mice.

What is clear, however, is that a decrease in hydrogen peroxide levels due to enhanced Prdx6 activity in critical tissues is not sufficient to rescue the Ath1 phenotype.

While we doubt Prdx6 is responsible for the Ath1 phenotype, its activity as an antioxidant does appear to be important for the oxidative stress response and inflammation. Previous studies have identified Prdx6 as a gene highly inducible by KGF in keratinocytes [36]. In addition, its induction is associated with wound repair in the skin in mice [36], and the human homolog has been associated with the inflammatory disorder psoriasis [37]. We have recently demonstrated that this gene can respond to growth factors and oxidative stress stimuli in hepatocytes [38]. Further understanding of the role of this protein will also come from study of the specificity of this member of the peroxiredoxin family. All mammalian peroxiredoxins besides Prdx6 use thioredoxin as the immediate electron donor, and previous studies have shown that recombinant human Prdx6 isolated from bacteria can reduce H$_2$O$_2$, using dithiothreitol as an electron donor in vitro [22]; but, the in vivo electron donor has not yet been identified. In addition, H$_2$O$_2$ is known to play an important role in cell signaling [39,40], and several peroxiredoxin proteins are capable of regulating distinct cellular processes through their ability to reduce the accumulation of H$_2$O$_2$ [16,17,41]. Further studies into the regulation and specificity of Prdx6 will help to
elucidate its function as an antioxidant and its potential anti-inflammatory properties.

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