

Commentary

AOP2 (Antioxidant Protein 2): Structure and Function of a Unique Thiol-Specific Antioxidant

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ABSTRACT

The accumulation of reactive oxygen species (ROS) in response to extracellular signals or intracellular biochemical processes can be regulated by the coordinate action of many antioxidant proteins. Because moderate levels of ROS can act as intracellular messengers in many of these processes, this modulation is critical for the transduction of specific signals. The thiol-specific antioxidant (TSA) family is a highly conserved group of enzymes that can reduce hydroperoxides in the presence of a thiol-containing electron donor. AOP2 (antioxidant protein 2) is a newly described member that shows significant evolutionary conservation between many different organisms. The protein contains three motifs that are highly conserved within the TSA family, including a cysteine residue that is the active site of oxidation for this class of proteins. Although AOP2 possesses TSA activity, it has several unique characteristics, including the absence of a second cysteine residue that is conserved in all other TSA proteins, the presence of a unique carboxy-terminal domain, and a demonstrated phospholipase activity. Furthermore, AOP2 shows conservation of several amino acids important in dimer formation and active site configuration that are not found in the other family members. Together, these data strongly suggest that AOP2 is a novel thiol-dependent antioxidant that functions to scavenge particular hydroperoxides in the cell and mediate specific signals. There is also evidence supporting a role for AOP2 in certain disease processes including atherosclerosis. Further evaluation of this protein and its substrate specificity will likely shed light on its precise role in cellular oxidant defense, signal transduction and pathogenesis. *Antiox. Redox Signal.* 1, 571–584.

INTRODUCTION

OVER THE PAST SEVERAL YEARS, many investigations have focused on understanding how cells respond to their environment and cope with a wide variety of environmental insults. These stress-induced responses are quite diverse in nature, including the typical inflammatory response following infection and wound repair after cellular injury. Although these events are involved in different physiological processes, they share many of the same biochemical mechanisms. One common occur-

rence is the generation of reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (HO^{\bullet}), nitric oxide (NO^{\bullet}), and hydrogen peroxide (H_2O_2). These are highly reactive molecules that can directly damage membranes, proteins, and DNA in the cell and, at certain concentrations, can regulate specific signal transduction pathways by acting as intracellular messengers. In addition to their known role in immunological host defense, ROS are generated as a by-product during normal metabolic activity, such as cellular respiration, and are also thought to play an integral

role in many pathophysiological processes including aging, heart disease, and cancer. In an effort to combat the accumulation of ROS, particular defense mechanisms have evolved to aid in the cell's response to oxidative stress. These include a diverse group of antioxidant molecules, each of which is thought to have a specific function in the stress response. The continual balance between oxidants and antioxidants in the cell is believed to be a critical factor in the regulation of many biological processes.

Introduction to the thiol-specific antioxidant family

Several antioxidant proteins have been identified that play essential roles in "detoxifying" free radicals. The most well-characterized groups include catalases, glutathione peroxidases, and superoxide dismutases. However, other antioxidant proteins have recently emerged, although their specific functions in the cell remain undetermined. One newly described family is the thiol-specific antioxidant (TSA) family, which shows no significant homology with previously identified antioxidant proteins. These proteins, also named rehydrins,

thioredoxin-dependent peroxide reductases (TPx), and recently renamed peroxiredoxins (PRx) (Chae *et al.*, 1994b; Rhee and Chae, 1994), comprise a family of enzymes that reduce hydroperoxides using thiol-containing electron donors. The family was first described in yeast with the isolation and characterization of yeast TSA (TPx), a protein capable of protecting cells from oxidative damage induced by metal catalyzed oxidative systems in the presence of dithiothreitol (DTT), but not in the presence of ascorbate (Kim *et al.*, 1988). This finding suggested a thiol-specific function for this protein. Since then, many homologous proteins have been identified that are highly conserved in prokaryotes and eukaryotes, and possess TSA activity (Chae *et al.*, 1994b). Thus, this group of proteins likely represents a new family of important antioxidant molecules.

Despite apparent sequence similarities, current literature on this family is confusing due to nonconsensus of protein function, inconsistencies in nomenclature, and ambiguities in subfamily classification. Assortment of the mammalian TSA family members by nucleotide and protein sequence comparison was performed, and is shown in Table 1 along with

TABLE 1. ORGANIZATION OF THE MAMMALIAN THIOL-SPECIFIC ANTIOXIDANT FAMILY

	Group I	Group II	Group III	Group IV	Group V
Human	PAG, TDX2 (Prosperi <i>et al.</i> , 1993) NKEFA (Shau <i>et al.</i> , 1994)	TSA, TDX1 (Lim <i>et al.</i> , 1994) NKEFB (Shau <i>et al.</i> , 1994) TPXII (Zhang <i>et al.</i> , 1997)	MER5 (Tsuji <i>et al.</i> , 1995)	AOE372, TDXN (Jin <i>et al.</i> , 1997)	KRG-1 (Frank <i>et al.</i> , 1997) aiPLA ₂ (Kim <i>et al.</i> , 1997) AOP2 (Iakoubova <i>et al.</i> , 1997)
Mouse	MSP23 (Ishii <i>et al.</i> , 1993)	TSA, TDPX1 (Ichimiya <i>et al.</i> , 1997)	MER5, TDXM (Yamamoto <i>et al.</i> , 1999)	AOE372, TDXN (Jin <i>et al.</i> , direct GenBank submission)	AOP2 (Iakoubova <i>et al.</i> , 1997)
Rat	OSF3 (Kawai <i>et al.</i> , 1994) HBP23 (Iwahara <i>et al.</i> , 1995) PrxI (Matsumoto <i>et al.</i> , 1999)	TSA, TDX1 (Chae <i>et al.</i> , 1994b) PrxII (Matsumoto <i>et al.</i> , 1999)	AOP1 (Tsuji <i>et al.</i> , 1995) PRx III (Matsumoto <i>et al.</i> , 1999)	PRx IV (Matsumoto <i>et al.</i> , 1999)	GPx (Munz <i>et al.</i> , 1997) aiPLA ₂ (Peshenko <i>et al.</i> , 1996)
Yeast	?	TSA1, TPx (Chae <i>et al.</i> , 1993)	?	?	YBG4 (Scherens <i>et al.</i> , 1993)

Based on amino acid sequence comparison, TSA proteins from human, mouse, and rat have been assorted into five groups. Corresponding yeast proteins that have been identified are shown. References for reported proteins are also included.

the corresponding references. The first family member was named *Osf3* and *Msp23* in mouse, and *PAGA* and *NKEFA* in human. It encodes a 199-amino-acid cytoplasmic protein that is induced by stress in macrophages, and is thought to be involved in cell proliferation and differentiation. The second member is named *Tsa* and *Tdpx1* in mouse, and *TSA*, *TDX1*, *TPxII*, and *NKEFB* in human. It encodes a 198-amino-acid protein localized to the cytosol that is also thought to be associated with cellular stress response. The third family member is *Mer5* (renamed *Aop1*), first cloned from mouse and later from human as *TDXM*. It encodes a 257-amino-acid protein expressed in mitochondria that may function, along with other mitochondrial antioxidants, to scavenge ROS from the electron transport chain. The newest family member is *AOE372*, encoding a 271-amino-acid protein that was isolated as a proliferation-associated gene (PAG)-associated protein from human and was recently cloned from mouse and rats. There are conflicting reports on the cellular localization of *AOE372*, which was first identified as a cytosolic heterodimer (Jin *et al.*, 1997), but was also detected in conditioned medium from Jurkat and HL60 cells (Haridas *et al.*, 1998) and recently shown to be a secreted protein (Matsumoto *et al.*, 1999). Due to the demonstrated ability of these four proteins to reduce H_2O_2 using thioredoxin as the electron donor (Jin *et al.*, 1997; Kang *et al.*, 1998b), they have been renamed PRx I–IV, respectively. Finally, the most divergent member of the TSA family is *Aop2* (antioxidant protein 2) (Iakoubova *et al.*, 1997), also named *GPx* in mouse (Munz *et al.*, 1997) and *aiPLA₂* (Kim *et al.*, 1997) and *KRG-1* (Frank *et al.*, 1997) in human. This 224-amino-acid protein shares only 31% identity and 44% homolog with its closest family member. Although different in amino acid composition, one unifying feature of the family is the presence of a cysteine residue in the amino terminus that is conserved in all TSA proteins and has been shown to be the active

site for enzymatic activity (Chae *et al.*, 1994c). In addition, all members except AOP2 have a second conserved cysteine in their carboxyl terminus that corresponds to Cys170 in yeast TSA, dividing the TSA family into the 2-Cys and 1-Cys subfamilies. Consequently, AOP2 has also been named 1-Cys PRx to distinguish it from other peroxiredoxins (Kang *et al.*, 1998a). Although the function of 2-Cys antioxidants has begun to be elucidated, the role of AOP2 is not yet understood and it will be the focus of this commentary.

Aop2: gene and protein

Murine *Aop2* was first isolated from liver and kidney as a cDNA corresponding to a protein variant between the C57BL/6J and DBA/2J strains of mice (Iakoubova *et al.*, 1997). *Aop2* has also been identified in many different species, including human, pig, mouse, rat, nematode, barley, rice, moss, arabidopsis, yeast, and bacteria.* Comparison of the encoded protein sequences reveals a high degree of identity, with the human protein sharing 89% identity and 93% similarity with murine APO2, and 48% identity and 63% similarity with yeast AOP2. We cloned the corresponding gene in mouse and determined it to have five exons and four introns, spanning approximately 11 kb (Phelan *et al.*, 1998). Analysis of the proximal promoter reveals several putative binding sites for SP1, and consensus sequences for other transcription factors including SREBP, HSF, Pit1, c-Jun, and c-Myc (Phelan *et al.*, 1998; Lee *et al.*, 1999). Lee *et al.* have shown specific binding of Sp1 and Pit-1a to these elements by gel-shift assays, suggesting a possible role in *Aop2* regulation. The murine *Aop2* gene was localized to chromosome 1 at 83.6 cM position on the MGD/Chromosome Committee maps (Iakoubova *et al.*, 1997), and we have since used a large backcross to fine map the gene to a 0.05 cM interval between D1Mit266 and D1Mit424 (Beier, Phelan, and Paigen, manuscript in prepara-

*Accession numbers for the various AOP2 protein sequences used for consensus determination are: *Homo sapiens* (human) (D14662); *Bos taurus* (pig) (AAC84043); *Mus musculus* (mouse) (Y12883); *Rattus norvegicus* (rat) (AAB66341); *Onchocerca volvulus* (nematode) (U31052); *Hordeum vulgare* (barley) (X76605); *Oryza sativa* (rice) (D3917); *Arabidopsis thaliana* (thale cress) (O04005); *Bromus secalinus* (Seed plant) (P52571); *Tortula ruralis* (moss) (U40818); *Saccharomyces cerevisiae* (yeast) (P34227); *Proteobacteria* (AF075709); and *Archaeobacteria* (U36479).

tion). This region corresponds to chromosome 1 in humans, yet neither the human gene nor any other *Aop2* gene has been mapped to date.

The full-length mouse cDNA is approximately 1.45 kb (Munz *et al.*, 1997), which corresponds to the transcript size observed by our group and others by Northern blot (Munz *et al.*, 1997; Lee *et al.*, 1999). In contrast, the full-length cDNA for rat, bovine, and human is 1.4 kb (Andreeva *et al.*, 1998), 1.68 kb (Fisher *et al.*, 1999), and 1.65 kb (Kim *et al.*, 1997), respectively. The difference between rat/mouse and bovine/human sequences is mostly in the length and content of the 3' untranslated region (UTR). The transcript size detected by Northern blot is 1.7 kb in rat and 1.9 kb in human, suggesting either that the published cDNAs are incomplete, or there may be possible alternative splicing or processing modifications of the transcript. Analysis of the EST Database suggests that *Aop2* may be expressed ubiquitously, although its tissue distribution is variable. In mouse and rat, the highest levels are found in lung, moderate levels in heart, liver, and kidney, lower levels in brain, spleen, and stomach, and lowest levels in intestine (Munz *et al.*, 1997; Kim *et al.*, 1998; Lee *et al.*, 1999). The encoded protein is 224 amino acids in length, with a predicted size of 25 kD, corresponding to the size observed by two-dimensional gel electrophoresis reported by Iakoubova *et al.* *In vitro* transcription/translation of AOP2 produces a protein of approximately the predicted size, as well as a 32-kD band in mouse and rat. The discrepancy is likely due to an *in vitro* artifact, since immunoblots of endogenous AOP2 in rat tissues reveal only a 26-kD product (Kim *et al.*, 1998). The protein expression pattern has only been reported for the rat thus far, in which AOP2 was detected exclusively in the lung (consistent with its highest mRNA expression) (Kim *et al.*, 1998). However, the lack of detectable AOP2 expression in other tissues is likely due to insensitivity of their assay, since the mRNA tissue distribution indicates widespread expression, and we have detected AOP2 protein in the liver (data not shown). Finally, the cellular localization of the human protein was determined to be cytoplasmic in COS cells transiently transfected with a recombinant influenza virus

hemagglutinin (IVH)-tagged construct (Frank *et al.*, 1997), and by subcellular localization of recombinant human AOP2 (Kang *et al.*, 1998a). Together, these data strongly suggest an important cytosolic role for AOP2 in many different tissues.

It is clear that *Aop2* belongs to a multigene family, since many studies have reported multiple bands detected by Southern blot (Frank *et al.*, 1997; Munz *et al.*, 1997; Phelan *et al.*, 1998). We identified two other murine genes that are highly related to *Aop2*: *Aop2-rs1* and *Aop2-rs2*, which share 93% and 80% nucleotide identity with *Aop2*, respectively (Phelan *et al.*, 1998). Both related genes lack introns, and are regulated by different promoters. Despite their appearance as pseudogenes, both appear to be expressed in a tissue-specific manner, and *Aop2-rs1* may encode a full-length protein. *Aop2-rs1* has been identified by reverse transcriptase polymerase chain reaction (RT-PCR) from liver and kidney (Phelan *et al.*, 1998), and expression of *Aop2-rs2* has been demonstrated by RNase protection assay, revealing much lower levels in general, with highest levels in liver (Munz *et al.*, 1997). Therefore, one or both of these genes may encode a functional protein. Another lab claimed to find two *Aop2*-related intronless genes, which they named CP2 and CP5 (Lee *et al.*, 1999). However, their relationship to *Aop2-rs1* and *Aop2-rs2* is unclear. First, the authors report only 62 bp of open reading frame sequence for the two genes and the sequences are identical to each other. The evidence that they represent two distinct genes is based on a southern blot, which is not shown. The reported sequence does differ from *Aop2-rs2*, and is identical to *Aop2-rs1*, but since the sequence is very short and is in a highly conserved region, we cannot determine if it is *Aop2-rs1*. The isolation of pseudogene-like sequences for other TSA proteins has also been reported, including a highly related pseudogene of PAG (Prosperi *et al.*, 1994), and two recently identified PRx II-like genes (Lim *et al.*, 1998). However, none of these have yet been shown to be expressed. Therefore, the existence of these *Aop2*-related genes may be physiologically relevant, and their antioxidant activity will need to be measured to determine their functional significance.

AOP2 structure/function

To begin to understand the role of AOP2 as a cellular antioxidant, we can examine the activity of other TSA proteins. The original yeast TSA protein was given the name by virtue of the fact that it could protect cellular enzymes from inactivation mediated by metal-catalyzed oxidative systems containing sulfur, but not those containing oxygen (Kim *et al.*, 1988). This was further supported by evidence that yeast TSA uses thiol-containing electron donors to reduce hydroperoxides (Chae *et al.*, 1994a; Kwon *et al.*, 1994; Netto *et al.*, 1996). Several family members have since been shown to possess this activity, and the ability of an enzyme to protect glutamine synthetase from inactivation mediated by such systems has become the standard assay for TSA classification (Chae *et al.*, 1999). Like other TSA proteins, AOP2 has been shown *in vitro* and *in vivo* to possess peroxidase activity (Kang *et al.*, 1998a). However, evidence suggests that the endogenous electron donor used by AOP2 may differ. Although all five TSA proteins can reduce H_2O_2 *in vitro* in the presence of DTT, recent studies have shown that AOP2 is the only one that is unable to reduce H_2O_2 in the presence of thioredoxin (Fisher *et al.*, 1999; Kang *et al.*, 1998a). This suggests an alternative electron donor for AOP2, which remains unknown.

Since AOP2 may have a novel role as a cellular antioxidant, it is important to elucidate its specific function. However, little is known about its specific substrates or the regions required for its TSA activity. Due to the high degree of evolutionary conservation in amino acid sequence within this family, we can gain some insight into TSA function and specificity by comparison of mammalian TSA proteins (Fig. 1). Alignment of the mammalian consensus sequences reveals three highly conserved motifs. The first motif exhibits striking conservation throughout evolution, and contains the active site cysteine present in all family members from all species. This conserved cysteine residue (corresponding to Cys47 in yeast TSA) has been shown to be the active site of oxidation in a DTT/ Fe^{3+} / O_2 oxidation system (Chae *et al.*, 1994c). Eight other amino acids in this 14-amino-acid region are 100% conserved be-

tween mammalian TSA proteins, suggesting an essential role in TSA function. A second motif spans 11 amino acids and contains six conserved residues, and the third motif contains 6 conserved residues out of 15. Although highly conserved, the function of these two motifs is unclear because they are not found in other proteins and are not associated with the active site. In addition to these homologous regions, all TSA proteins except AOP2 have a second conserved cysteine residue, corresponding to Cys170 in yeast TSA. The lack of this second conserved cysteine in AOP2 and its inability to use thioredoxin as an electron donor indicates a unique substrate specificity, and possibly, a novel antioxidant function.

A better understanding of the relationship between the sequence and function of AOP2 can come from analysis of the protein structure. Previous attempts to crystallize native TSA proteins have been complicated by the heterogeneity of intermolecular disulfide interactions (Choi *et al.*, 1998). However, in May of last year the crystal structure of the human AOP2 protein (hORF6) was reported (Choi *et al.*, 1998), the first of the TSA family. The crystallized protein contains two discrete domains and exists as a dimer. The larger amino-terminal domain spanning amino acids 1–174 contains a thioredoxin fold, which encompasses the active cysteine residue. Thioredoxin folds have been found in other antioxidants including glutathione peroxidase and glutathione S-transferase and have been shown to be the active site for thioredoxin oxidation (Martin, 1995). The location of the active site cysteine is at the bottom of a narrow pocket and exists as Cys-SOH (cysteine-sulfenic acid) in the crystal. The positioning of two positively charged residues (His 39 and Arg 132) in close proximity (3.76 Å) to the active site is thought to lower the pKa of the thiol by stabilizing its ionic state through hydrogen bonding. Due to the significant distance between two active sites in the dimer, it has been suggested that they would probably function independently (Choi *et al.*, 1998). All three highly conserved TSA motifs are contained within this amino-terminal domain. The second domain (spanning amino acids 175–224) contains five residues that are responsible for dimerization through interactions

PRxI	-----
PRxII	-----
PRxIII	MAAAsgRLLhu---SVARxsSsIxhuISAssLRPsAst.....RTsLTs.hLhSu
PRxIV	Meshs.hLhssTsptsRap+xxxLLLxPLLLFLLxstulQGhEo--RxRTREpECHFYAG
Aop2	-----
consensus
PRxI	-----MSSG..NAKIGaPAPsFKATAVMPDGQFKDISLS-YKGKYVVF
PRxII	-----MASG..NAPIGKsAPDFpuTAVVD.GAFKELKLSDY+GKYVVL
PRxIII	-----ssQu.KxhFSTSSShsPAVTQHAPaFKGTAVVN.GEFK-LSLDDFKGKYVL
PRxIV	GQVYPGEsSRVSVADHSLHLS..KAKISKAPYWEGTAVIs.GEFKELKLTDIRGKYLVF
Aop2	-----MPGG...LLLGDxAPNFEANTTlG+IRFHDFLGD..S..WGIL
consensush..u....lst. AP .a..ssshs.ht F +.h.hs....ahlh
PRxI	FFYP LDFTFVCPTEIIAFSDRA-EFKKLNCQVIGAS VD SH FCH LAW...INTPKKQGGGLG
PRxII	FFYP LDFTFVCPTEIIAFSS+AEDFRKLGCVELGV SVDS Q Fs HLAW...INTPRKEGGLG
PRxIII	FFYP LDFTFVCPTEIVAFSDKANEFDVNCEVVAV SVDS SH Fs HLAW...INTPRKNGGLG
PRxIV	FFYP LDFTFVCPTEIIAFGDRLEEF+SINTEVVAC SVDS Q F THLAW...INTPRRQGGGLG
Aop2	FSHPR DFTFVCTTELGRAAKLAPEFAKRNVKLIALL SIDS VED Hh AWSKDINAYNsttPTE
consensus	F .a PhDFT .VC s TElhthup.h.- F .phssplluh SIDS .. SHh AW...lNs.pppssht
PRxI	PMNIPLlSDPKRTIAQDYGVLK...AD...EGISFRGLFIIDDKGILRQITlNDLVP GRS
PRxII	PLNIPLLuDVT+pLSpsYGVLK...sD...EGIAYRGLFIIDuKGVLrQITVNDLVP GRS
PRxIII	HMNIslLSDlTKQISRdyGVLL...Eu...uGlALRGLFIIDPngVlKHLsvNDLVP GRS
PRxIV	PIRIPLlSDLSHQISKdyGVYL...ED...SGHTLRGLFIIDDKGILRQITlNDLVP GRS
Aop2	KLFPFIIDD+sR-LAILLGMLDPsEKD-psMPVTARVVFlFGPDKKLKLsILYPATT GRN
consensus	.h.hsll.D.p.lu..hGhh....t. ...shsh RslFl hs.pth L +...l.shss GRS
PRxI	VDEH lRLVQAFQFTDKHGVECPAGWKPG.SDTIKPDVpKS.....KE
PRxII	VDEAL RLVQAFQYTDHGVECPAGWKPG.SDTIKPNVDDS.....KE
PRxIII	VEEs LRlVKAfQaVETHGVECPsNWTP.-SPTIKPSPsAS.....KE
PRxIV	VDETL RLVQAFQYTDKGVECPAGWKPG.SETIIPDPAGK.....LK
Aop2	FDEIL RVVxSLQLTuppXVATPVDWKcG-SVMVlPTlsEEEAKphFPKGVFTKELPSGKK
consensus	h- E .LRlV.uhQhs.....ssPss Wps .. S .hlh Ps .stp.....hc
PRxI	YFSKQK..
PRxII	YFSKHN..
PRxIII	YFpKVpQ.
PRxIV	YFD.....
Aop2	YLRYPQP
consensus	Yh

FIG. 1. Amino acid sequence alignment of the TSA family. The human, mouse, and rat homologs for each TSA protein were aligned using ClustalW (1.7) to determine a mammalian consensus sequence for each family member. [The ClustalW default grouping of amino acids issued for amino acid codes: o (alcohol); l (aliphatic); a (aromatic); c (charged); h (hydrophobic); p (polar); s (small); u (tiny); t (turnlike); - (negative); + (positive).]. These consensus sequences were aligned and a consensus sequence for the entire TSA family was generated using the Consensus program. Amino acid identities are shown in uppercase boldface in the consensus sequence.

with residues surrounding the active site of the other subunit. These include Pro191, which resides in the entrance to the active site of the opposing monomer. While the intramolecular contacts appear to be minimal for dimerization, these intermolecular bonds allow for close proximity between domain I of one monomer and domain II of the other. Dimerization of

other TSA proteins has also been demonstrated. Yeast TSA exists as a homodimer, and the oxidized Cys47 reacts rapidly with the carboxy-terminal cysteine (#170) (conserved in 2-Cys TSA proteins) to form a disulfide bond, which subsequently can be reduced by interaction with the redox active cysteines of thioredoxin (Chae *et al.*, 1994c). This type of interac-

tion has also been shown for other 2-Cys peroxidases, suggesting a common mechanism for peroxidase function. However, the absence of the second conserved cysteine in the AOP2 dimer suggests the importance of other residues in mediating its activity, and raises the possibility that the distinct dimer-stabilization mechanism specifies a unique peroxidase activity.

In an effort to elucidate its unique function further, all identified AOP2 protein sequences from a range of different organisms were compared. The human AOP2 sequence, with evolutionarily conserved residues, is shown in Fig. 2. Forty-eight amino acids in the AOP2 protein have been conserved throughout evolution, 21% of the entire protein. Half of these are also conserved among the other TSA family members. The motif containing the active site cysteine, which shows the highest identity among TSA proteins, is also most highly conserved between AOP2 proteins from divergent species (13 out of 14 residues). This is in contrast to motif 2, which contains 4 conserved residues out of 11, and motif 3, which contains 6 conserved residues out of 15. Other amino acids outside of these motifs are also conserved throughout the entire family, including Arg132 and Pro191, which have both been localized around the active site pocket of the AOP2 dimer (Choi *et al.*, 1998). Interestingly, there are also amino acids that are unique to the AOP2 lineage, yet conserved in other TSA family members, suggesting some role in AOP2 specificity. These in-

clude His39 and Thr48, both of which are located in the first highly conserved motif. His39, replaced by either tyrosine or tryptophan in the thioredoxin-peroxidases, was shown to be critical for establishing a positively charged environment surrounding the active site for cysteine oxidation (Choi *et al.*, 1998). Thr48, which corresponds to a conserved proline in all other family members, is adjacent to the active cysteine and is important in dimer stabilization (Choi *et al.*, 1998). Other AOP2 amino acids that are nonvariant between prokaryotes and eukaryotes, yet different in the other family members, are Asn156 and Leu167 in motif 3, and Lys199 and Phe202 within the AOP2-specific carboxy-terminal region. Analysis of the sequence also exposes the absence of a secretion signal suggesting an intracellular function for the protein, which is consistent with the subcellular localization. Finally, it is worth noting the presence of putative post-translational modification sites, including four PKC phosphorylation sites, three CK-2 phosphorylation sites, and three potential *N*-myristylation sites within the human protein (see Fig. 2). At least two putative PKC phosphorylation sites involve residues that are unique to AOP2, and may be important in the regulation of AOP2 activity.

Comparison of the structure and function of AOP2 creates many possibilities about the unique function of AOP2. The amino-terminal domain likely includes those residues that are critical for peroxidase activity. The conser-

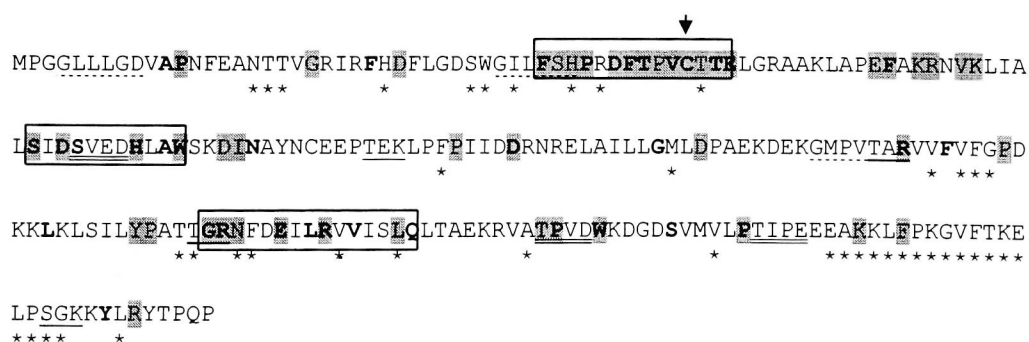


FIG. 2. Human AOP2 amino acid sequence and evolutionarily conserved residues. The boxes designate conserved motifs between the TSA family. Amino acids in boldface are 100% conserved between all mammalian TSA proteins. Amino acids that show 100% identity between Aop2 homologs are shaded, and the active site cysteine common to all TSA proteins is indicated with an arrow. Amino acids denoted with an asterisk are unique to AOP2, yet conserved between all other mammalian TSA proteins. Putative sites for (PKC) phosphorylation (underlined), CK2 phosphorylation (double underlined), and *N*-myristoylation (dotted underlined) are indicated.

vation of Arg132 and Pro191 in all TSA proteins suggests basic roles in dimer stabilization and active site configuration. Whereas Arg132 likely plays a basic role in dimerization, Pro191, which is localized near the entrance to the active site pocket of the other monomer, may be an important factor in regulating access to the active site in all TSA proteins. Although the active site of AOP2 may possess a thiol-specific antioxidant activity similar to that of other family members, it may specify a different electron donor due to specific residues within and surrounding this domain. The striking conservation of His39 and Thr48 between divergent species may suggest its importance in the specificity of AOP2 for particular electron donors, since these residues are uniquely conserved in AOP2. It has also been suggested that the role of His39 in the dimer may mimic that of Cys170 in 2-Cys proteins (Choi *et al.*, 1998). The substitution of threonine for proline at residue 48 may instead be critical in substrate specificity due to its nearby contacts within the dimer. This may also be true for the specific amino acids found in motif 3, which differ considerably from the other TSA proteins. In contrast, the internal highly conserved motif shows little uniqueness in AOP2 proteins, and thus, may mediate a function common to all TSA proteins, although its significance is unclear. Because this family is capable of forming heterodimers as well as homodimers, this interaction may also influence substrate specificity. It is also important to note that 18 amino acids near the carboxyl terminus of AOP2 show no homology with other TSA family members. Interestingly, this region does share limited homology with a portion of the cofactor-binding site of porphobilinogen deaminase proteins (Q57989), all of which possess the following motif: KG(V/L)F(T/V)KE(L/I). However, the specific function of this region is unknown.

Despite the demonstrated thiol-specific antioxidant activity of AOP2, and its obvious sequence homology with the TSA family, other functions for AOP2 have been suggested. The *Aop2* cDNA was also isolated from human (Frank *et al.*, 1997), and then mouse (Munz *et al.*, 1997), as a glutathione peroxidase (GPx) for its high amino-terminal amino acid homology with a bovine nonselenium glutathione perox-

idase, of which only the amino-terminal 24 amino acids were known. While the human protein demonstrates no glutathione peroxidase activity as measured by its inability to protect glutamine synthetase from inactivation in the presence of glutathione (Kang *et al.*, 1998a), bovine AOP2 was able to use glutathione as the electron donor in experiments measuring NADPH consumption (Fisher *et al.*, 1999). Although these results may reflect a species difference in function, it is unlikely due to their 98% sequence similarity. Thus, the *in vivo* electron donor is still unknown. Furthermore, the question as to what is the best assay for AOP2 activity is raised. This is reiterated by the demonstration that AOP2 has calcium-independent phospholipase activity with maximal activity at pH 4, as determined by measured enzyme activity *in vitro* after enzyme purification (Kim *et al.*, 1997, 1998). The presence of a lipase motif (GX SXG) in the amino-terminal portion of the mammalian AOP2 proteins (Kim *et al.*, 1997) was an additional piece of suggestive evidence for this activity. A low level of phospholipase activity has, in fact, been reported by another group (Kang *et al.*, 1998a), but it may be orders of magnitude less than its peroxidase activity and does not require the GX SXG motif. In addition, the acid-dependent nature of this lipase activity means it is probably not functional in the neutral pH of the cytosol. Although AOP2 shows little sequence homology with the few members of the calcium-independent PLA₂ family that have been cloned and sequenced, the fact that phospholipase activity has been demonstrated by independent investigations may reveal a dual function for this protein. On the other hand, the observation that this activity is significantly less than its antioxidant activity may instead simply reflect a specificity of AOP2 for phospholipids. Consistent with this idea, a recent study has shown that phospholipid hydroperoxides can act as substrates for recombinant bovine and human AOP2 expressed and purified from *E. coli* (Fisher *et al.*, 1999). In contrast, glutathione peroxidase could not reduce phospholipid hydroperoxides. Maximal enzymatic activity for AOP2 was shown to occur between pH 7 and 8, concordant with the cytosolic localization of AOP2. Nevertheless, the ability of

AOP2 to reduce PLPC hydroperoxides, and its phospholipase activity, presents a unique potential function for a cellular antioxidant.

Thus, the true *in vivo* function of AOP2 remains to be determined. Given the above-described evidence, three models for AOP2 activity can be postulated. These are shown in Fig. 3, A–C. It is known that AOP2 can exist in a homodimeric form. Thus, model A proposes that AOP2 exists as a redox-sensitive dimer in the cytosol, which can be changed from a reduced to oxidized state without additional modification. In this scenario, the mechanism of AOP2 regulation might be at the level of *de novo* transcription/translation. This is supported by evidence that *Aop2* transcription is induced in response to KGF (Frank *et al.*, 1997). However, evidence from other TSA proteins suggests that it may also participate in hetero-

dimeric interactions, and may exist in monomeric form under some circumstances. Thus, model B postulates that AOP2 may be reduced in its monomeric state, and can form either a homo- or heterodimer upon activation by an unidentified signal. In this model, the activation event may be phosphorylation mediated by signaling from the external stimulus, which could result in a conformational change in the protein. Dimerization may, in turn, expose the active cysteine to be oxidized by some hydroperoxide, ultimately leading to dissociation of the complex and reduction of the monomers by the thiol-containing electron donor. In contrast, model C postulates that oxidation of the AOP2 monomers by H_2O_2 might lead to its dimerization, and subsequent reduction by the unknown electron donor. While the actual mechanism of AOP2 activity remains to be de-

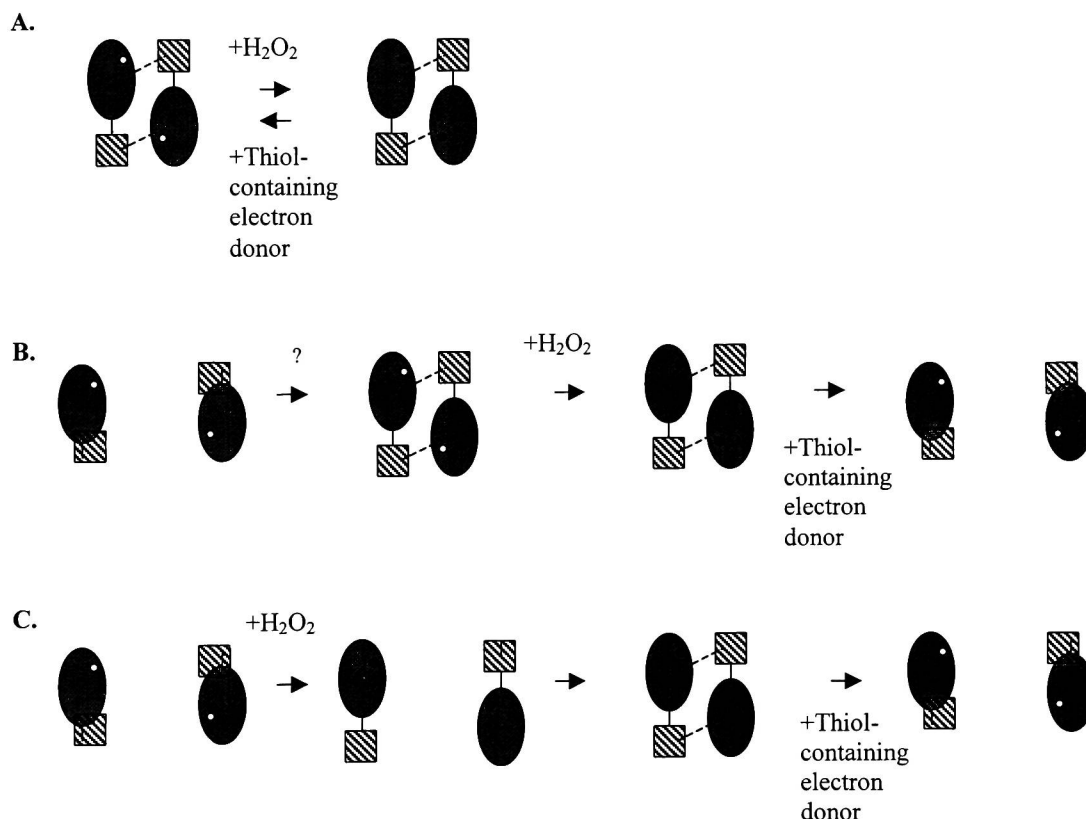


FIG. 3. Models for AOP2 activity modulation. The purple region indicates domain I and the blue region indicates domain II. The active site cysteine is indicated by the small circle in domain I, which can exist in a reduced state (white) or an oxidized state (black). **A:** AOP2 exists as a dimer in the cytosol, and its activity is modulated by the redox-state of the protein. **B:** AOP2 exists in an inactive monomeric state in the cytosol and, upon stimulation by a specific signal (*i.e.*, phosphorylation), forms a dimer. The active site cysteines of the dimer can then be oxidized, leading to dissociation of the complex, and subsequent reduction of the active site. **C:** AOP2 is oxidized in its monomeric form in the cytosol, leading to dimerization. Formation of the dimer allows for reduction to occur and subsequent disassembly of the complex.

terminated, significant progress will come from identification of the *in vivo* electron donor and the hydroperoxide substrate specificity. For example, the abundance of the electron donor and the nature of the hydroperoxide may specify AOP2 activity. Furthermore, it is important to remember that the two highly related genes *Aop2-rs1* and *Aop2-rs2* may encode proteins with similar activity as AOP2. In fact, a 15-amino-acid region containing the active site cysteine residue is conserved between all three genes (Phelan *et al.*, 1998), including His39, which is conserved in all 1-Cys peroxiredoxin proteins. Because these amino acids are thought to create and stabilize the active site of the enzyme, these related proteins may also be important in the cellular stress response.

Potential role for AOP2 in cell signaling and pathogenesis

Due to the known involvement of redox proteins in cell communication, several labs are currently investigating the function of the TSA family in signal transduction. Many studies have reported the generation of ROS during cytokine and growth factor action, hormone secretion, ion transport, gene transcription, and apoptosis (Lander, 1997). Evidence suggests that these stimuli use reactive intermediates as mediators of intracellular signals. Although the mechanism of action for this group of messengers is unclear, one suggestion has been the inactivation of protein tyrosine phosphatases by ROS, thereby allowing necessary protein phosphorylation during transduction of a signal (Sundaresan *et al.*, 1995). This has been shown for transmission of the platelet-derived growth factor (PDGF) signal by H_2O_2 . While the role of ROS in such pathways is becoming increasingly supported, the role of antioxidant proteins as regulators of such messengers has just recently been proposed. The function of antioxidants as ROS scavengers may result in sequestering of ROS and subsequent suppression of phosphatase inactivation, leading to signal termination. Thiol-specific antioxidants may have a specific role in this process by sequestering specific ROS only in the presence of a thiol-containing electron donor generated by specific signals. Thiol-specific antioxidants

have, in fact, been shown to mediate certain responses to growth factors. Overexpression of PRx I and Prx II in cultured cells resulted in the elimination of intracellular H_2O_2 induced by growth factor stimulation (Kang *et al.*, 1998b). PRx II was also able to block NF- κ B activation by H_2O_2 or tumor necrosis factor- α (TNF- α), suggesting an important role in cell signaling by sequestering hydroperoxides. Furthermore, antioxidant enzyme 372 (AOE372) was able to inhibit NF- κ B activation by regulating I- κ B phosphorylation in the cytoplasm (Jin *et al.*, 1997). This is consistent with the idea that antioxidants can participate in signaling by sequestering hydroperoxides, thereby relieving suppression of critical phosphatases. There is growing evidence that AOP2 is also involved in these processes. First, the human *Aop2* was cloned (as KRG-1) by one lab on the basis of its super-induction in keratinocytes by keratinocyte growth factor (KGF) (Frank *et al.*, 1997). Munz *et al.* went on to demonstrate over a 100-fold increase in murine *Aop2* expression during the first 24 hr of cutaneous wound repair, when KGF levels are very significantly elevated (Munz *et al.*, 1997). This effect was specific for KGF, and was localized to the hyperproliferative epithelial edge, suggesting a role in cell proliferation. We have also shown that *Aop2* expression is induced in response to oxidative stress in a mouse hepatocyte cell line (data not shown). More directly, Kang *et al.* have shown that while PDGF stimulation of NIH-3T3 cells results in elevated intracellular H_2O_2 , overexpression of *Aop2* inhibits this increase, and this inhibition depends on the active site Cys47 (Kang *et al.*, 1998b). Thus, the unique specificity of AOP2 for particular cellular substrates may allow for the transmission of a defined set of signals through sequestering of specific hydroperoxides.

A model for the role of AOP2 in cellular stress response and signal transduction is shown in Fig. 4. It is clear that H_2O_2 can be induced in response to a wide variety of stimuli, including growth factors, cytokines, and hormones, all of which utilize similar signaling pathways. Although specific kinases are activated in response to these stimuli, H_2O_2 accumulation may lead to the inactivation of protein tyrosine phosphatases. This event would

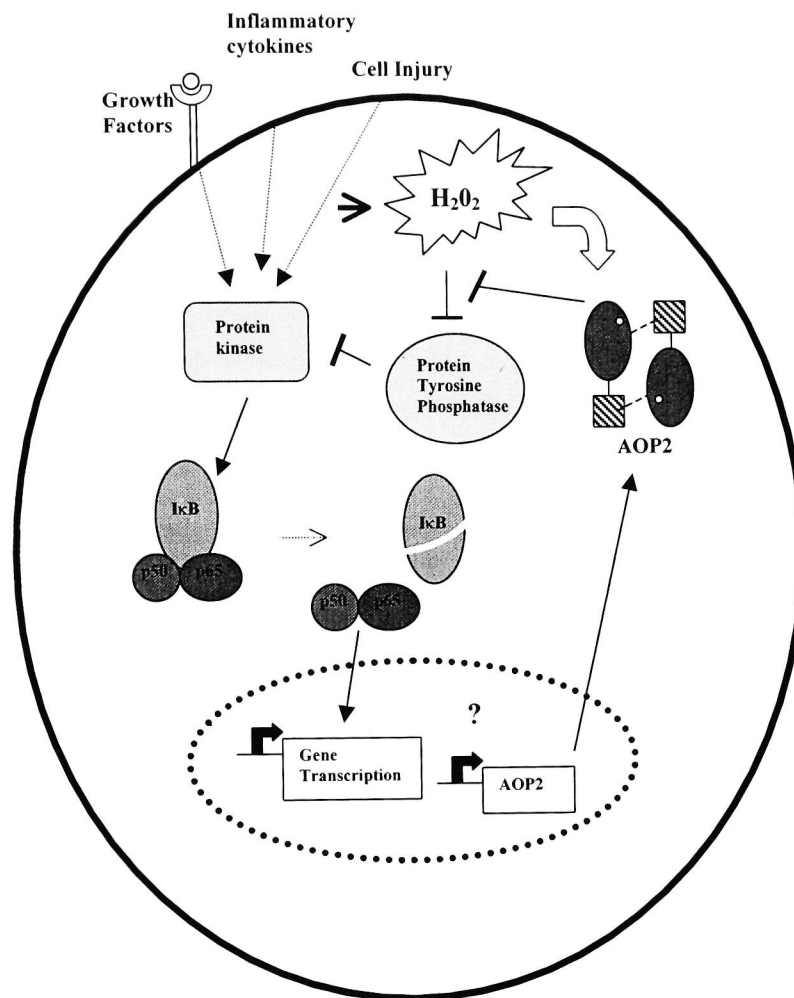


FIG. 4. Hypothetical model for AOP2 action in signal transduction. Extracellular signals such as growth factors and cytokines activated specific protein kinases in the cell that lead to activation of transcription factors such as NF- κ B. Generation of H_2O_2 by these signals results in the inactivation of protein tyrosine phosphatases, allowing transmission of the signal. Transcription of AOP2 is induced by an unknown mechanism, and AOP2 protein sequesters H_2O_2 by an unknown electron donor, inhibiting phosphatase inactivation and facilitating regulation of the signal.

allow for propagation of the signal via phosphorylation of critical factors such as NF- κ B, leading to specific gene transcription. Because thioredoxin-dependent antioxidant defense systems have shown greatest selectivity for NF- κ B activation, this pathway may likely serve as a target for AOP2 regulation. We know that Aop2 expression is highly induced in keratinocytes in response to KGF. We also know that Aop2 overexpression leads to reduction in the intracellular pool of H_2O_2 generated by PDGF stimulation. Thus, some unknown mediator may lead to induction of Aop2 expression (and/or activation of existing AOP2 protein) resulting in the accumulation of AOP2 in the cytosol and the sequestering of H_2O_2 . The presence of multiple heat shock elements and

Sp1 binding sites in the proximal promoter suggest that these factors may be involved in its induction. This reduction in H_2O_2 may inhibit phosphatase inactivation, and allow for regulation of the signal. Although speculative at this point, this model proposes an efficient method for reduction of hydroperoxides and regulation of intracellular signals by AOP2.

As more antioxidants have emerged over the past decade, there have been many reports linking them with pathogenesis. Diseases ranging from diabetes to cancer and heart disease are associated in some way with the production of free radicals and the involvement of in-born defense mechanisms. Recent studies have implicated TSA proteins in such pathophysiological processes. Studies have proposed a role

for TSA proteins in inhibition of apoptosis, as shown by the ability of stably transfected human TPx II to inhibit apoptosis induced by chemicals or serum deprivation (Zhang *et al.*, 1997). TPx II overexpression was found to inhibit lipid peroxidation and H₂O₂ accumulation in cells, suggesting a role upstream of Bcl-2 in apoptosis suppression (Zhang, *et al.*, 1997). Furthermore, expression of mouse TPx was able to protect neuronal cells from apoptosis in the absence of nerve growth factor (NGF) and serum, suggesting a similar role (Ichimiya *et al.*, 1997).

There is some evidence to suggest that *Aop2* plays a role in certain disease processes, as well. Frank *et al.* have shown significantly elevated levels of the human gene in psoriasis-affected skin (Frank *et al.*, 1997). This condition is an inflammatory disorder and involves elevations in cellular stress responses genes. Thus, overexpression of *Aop2* in these cells may provide added protection in the face of increased oxidative stress. We also have evidence to suggest that AOP2 may play a role in atherosclerosis resistance in mice. Genetic studies from our laboratory have identified *Aop2* as a candidate for a quantitative trait locus (QTL) underlying an atherosclerosis susceptibility difference between certain inbred strains of mice. This locus, originally named *Ath1*, is responsible for resistance to high-density lipoprotein (HDL) reduction and atherosclerosis in C3H/HeJ and BALB/cJ mice fed a high-fat diet, as compared to susceptible C57BL/6J mice (Paigen *et al.*, 1987). Our evidence for involvement of AOP2 in atherosclerosis includes colocalization of *Aop2* to the same 0.05 cM interval as *Ath1*, and differences in amino acid sequence and mRNA expression of *Aop2* between resistant and susceptible strains (Beier *et al.*, in preparation). In addition, the role of lipid oxidation in atherosclerosis progression has been well established, implicating a role for antioxidant proteins in this process. Thus, AOP2 may play a role in the liver by reducing exposure of lipoproteins to oxidative modification, or it may protect cells in the artery wall from oxidative damage initiated by low-density lipoprotein (LDL) oxidation. The presence of putative binding sites for heat shock factor (HSF), implicated in atherosclerosis, and sterol

response element binding protein (SREBP), a known regulator of lipid metabolism, in the *Aop2* promoter suggests possible transcriptional regulation of *Aop2* in these processes. We are now testing the role of AOP2 in atherosclerosis by a transgenic rescue experiment. Therefore, mounting evidence suggests that AOP2 is likely an important cellular antioxidant that may play a role in basic biochemical defense mechanisms as well as clinical disease. Further investigation of the substrate specificity for this protein, and identification of its *in vivo* electron donor will help to uncover its actual function and specificity. In addition, we hope to elucidate the pathways that lead to transcriptional regulation of *Aop2* and modulation of its activity. Together, significant progress can be made in understanding the role of this novel antioxidant in cellular stress response and signal transduction.

ACKNOWLEDGMENTS

The communicated work was performed in the laboratory of Beverly Paigen at The Jackson Laboratory, Bar Harbor, ME. The work was supported by ASTRA Pharmaceuticals and NHLBI 1-F32-HL10124-01. I would like to thank Mathew A. Merrill for excellent technical assistance, and Beverly Paigen for support.

ABBREVIATIONS

aiPLA₂, acidic calcium-independent phospholipase A₂; AOE372, antioxidant enzyme 372; *Aop2*, antioxidant protein 2; *Aop2*-rs, antioxidant protein 2-related sequence; *Ath*, atherosclerosis susceptibility locus; cM, centimorgan; CK, casein kinase; CP, genomic fragment of 1-Cys peroxiredoxin; DTT, dithiothreitol; EST, expressed sequence tag; GPx, glutathione peroxidase; H₂O₂, hydrogen peroxide; HBP, heme binding protein; HDL, high-density lipoprotein; HO·, hydroxyl radical; hORF, human open reading frame; HSF, heat shock factor; IVH, influenza virus hemagglutinin; KGF, keratinocyte growth factor; KRG, keratinocyte-regulated gene; LDL, low-density lipoprotein; MGD, mouse genome database; MSP, macro-

phage stress protein; NF- κ B (nuclear factor kappa B; NGF, nerve growth factor; NKEF, natural killer cell enhancing factor; NO \cdot , nitric oxide; O $_2$ \cdot^- , superoxide anion; OSF, osteoblast-specific factor; PAG, proliferation-associated gene; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLA $_2$, phospholipase A $_2$; PRx, peroxiredoxin; QTL, quantitative trait locus; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase, chain reaction; SREBP, sterol response element binding protein; TDPX, thioredoxin peroxidase; TDx, thioredoxin peroxidase; TDXM, mitochondrial thioredoxin peroxidase; TNF- α , tumor necrosis factor- α ; TPx, thioredoxin-dependent peroxide reductase; TSA, thiol-specific antioxidant.

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