OXIDATION OF 2-CYS-PEROXIREDOXINS BY ARACHIDONIC ACID PEROXIDE METABOLITES OF LIPOXYGENASES AND CYCLOOXYGENASE-2

Pauline Cordray4, Kelly Doyle3, Kornelia Edes4, Philip J. Moos1 and F. A. Fitzpatrick2, 3, 4

From Department of Pharmacology and Toxicology 1, Department of Oncological Sciences2, Department of Medicinal Chemistry3, and Huntsman Cancer Institute4, University of Utah, Salt Lake City, Utah 84112

Running head: Cellular lipid dioxygenases co-oxidize peroxiredoxins

Address correspondence to: F.A. Fitzpatrick, Ph.D., Huntsman Cancer Institute, 2000 Circle of Hope, University of Utah, Salt Lake City, Utah 84112-5550, Tel. 801-581-6204; Fax. 801-585-0011; Email: frank.fitzpatrick@hci.utah.edu

Human peroxiredoxins serve dual roles as antioxidants and regulators of H2O2 mediated cell signaling. The functional versatility of peroxiredoxins depends on progressive oxidation of key cysteine residues. The sulfenic or sulfonic forms of peroxiredoxin lose their peroxidase activity, which allows cells to accumulate H2O2 for signaling, or pathogenesis in inflammation, cancer and other disorders. We report that arachidonic acid lipid hydroperoxide metabolites of 5-, 12-, 15-lipoxygenase-1 and cyclooxygenase-2 oxidize the 2-Cys-peroxiredoxins 1, 2 and 3 to their sulfenic and sulfonic forms. When added exogenously to cells, 5-, 12- and 15-hydroperoxy-eicosatetraenoic acids also over-oxidized peroxiredoxins. Our results suggest that lipoxygenases and cyclooxygenases may affect 2-Cys peroxiredoxin signaling, analogous to NADPH oxidase (Wood, Z.A., Poole, L.B and Karplus P.A. (2003) Science 300, 600-653). Peroxiredoxin-dependent mechanisms may modulate the receptor-dependent actions of autacoids derived from cellular lipoxygenase and cyclooxygenase catalysis.

EXPERIMENTAL PROCEDURES

Materials — The COX inhibitor, aspirin, the LOX inhibitor, nordihydroguaiaretic acid (NDGA), and porcine pancreatic phospholipase A2 (PLA2) were from Sigma (St. Louis, MO). Arachidonic acid (AA) (5, 8, 11, 14-cis-eicosatetraenoic acid) was from NuChek Prep (Elysian, MN). Biotin conjugated to polyethylene oxide-maleimide was from Pierce Chemical (Rockford, IL). The COX-2 inhibitor, NS-398; the LOX inhibitor, baicalein; the lipid hydroperoxide metabolites 5-, 12-, and 15-hydroperoxy-eicosatetraenoic acid (HpETE); anti-12-LOX, anti-COX-2 and Express™ enzyme immunoassay (EIA) kits for measurement of 15(S)-hydroxy-eicosatetraenoic acid (15-HETE) and leukotriene B4 (LTB4) were all from Cayman Chemical (Ann Arbor, MI). Correlate™ EIA kits...
for measurement of 12(S)-HETE were from Assay Designs (Ann Arbor, MI). Anti-5-LOX antibodies were from Transduction Laboratories (Lexington, KY). Anti-15-LOX was from Dr. Conrad (UCSD School of Medicine, San Diego, CA) and anti-platelet 12-LOX antibody (25) was from Dr. Colin Funk (Queen's University, Kingston, Ontario, Canada). Rabbit polyclonal antibodies for Prx 1, 2, 3, 4 and PrxSO3 were from Lab Frontier (Seoul, Korea http://bio.labfrontier.com/index.asp). The commercially available anti-Prx1 (LF-PA0001) and anti-Prx2 (LF-PA0007) antibodies were raised against full length recombinant human Prx1 or Prx2 antigen purified from E coli. Anti-Prx3 (LF-PA0030) was raised against purified, recombinant human Prx3 protein antigen without the mitochondrial leader sequence. Anti-Prx4 (LF-PA0009) was raised against purified, recombinant human Prx4 protein antigen without the secretion leader sequence. Anti-PrxSO3 was raised against a sulfonlated peptide corresponding to the active site sequence common to human Prx1-4. The anti-PrxSO3 antibody (LF-PA0004) recognizes both the sulfinic and sulfonic forms of Prx and detects over-oxidized Prx enzymes with high sensitivity and specificity, as first described by Woo et al. (10, 11). Antibodies to PrxSO3 are not selective for individual Prx – they recognize PrxSO3 derived from oxidation of Prx1, 2, 3, or 4. Conversely, antibodies against the separate Prx isoforms, anti-Prx1, anti-Prx2, anti-Prx3 and anti-Prx4, are not selective for the valence state of Prx – they recognize oxidized and reduced forms of Prx1-4. HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PVDF membranes and Western Lighting™ chemiluminescence reagents were from PerkinElmer Life Sciences (Boston, MA). The cell lysis buffer was 50 mM Tris, pH 7.4, 0.1M NaCl, 2 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, and 1 x Complete™ protease inhibitor (Roche Applied Science). Dulbecco’s modified essential medium (DMEM) and supplements were from Invitrogen (Carlsbad, CA) and fetal calf serum (FCS) was from Hyclone (Logan, UT).

Cell Lines for Inducible, Selective Expression of 5-, 12-, 15- Lipooxygenase and Cyclooxygenase-2 Enzymes — 293 EcR cell lines that conditionally express wild type 5-LOX, platelet-type 12-LOX, 15-LOX-1, COX-2 or mutant, inactive ΔIle662 15-LOX-1 under the control of a ponasterone-responsive, heterologous transcription complex (26) were used as described (27). LOX and COX-2 enzymes were stably inserted 3’ to an edcsyne response element (26, 27). Cells were grown in medium containing 10% fetal calf serum, 1x DMEM, 100 units of penicillin / streptomycin, 2mM L-glutamine, and 1 mM pyruvate. At ~ 80% confluence 10 μM ponasterone was added to the medium and cells were incubated 48 h at 37°C to induce expression of 5-LOX, 12-LOX, 15-LOX-1, or ΔIle662 15-LOX-1. 293 EcR cells harboring a wild type COX-2 gene were incubated with 10 μM ponasterone for 24 h at 37°C to induce COX-2 protein expression. LOX and COX-2 protein levels were measured by immunoblotting.

Oxidation of Prx to PrxSO3 by LOX and COX-2 Catalysis of Exogenous AA — After 48 h, the medium consisting of 10% v/v FCS, 1x DMEM with 10 μM ponasterone was removed from cells expressing 5-LOX, 12-LOX, 15-LOX-1 or ΔIle662 15-LOX-1 and replaced with 1% FCS, DMEM with 10 μM ponasterone. These cells in low serum medium were then treated for 0.5 - 4 h with 60 μM AA to initiate LOX catalysis, or 60 μM AA plus 1-10 μM baicalein or NDGA to inhibit LOX catalysis. Likewise, after 24 h the DMEM with 10% v/v FCS and 10 μM ponasterone was removed from cells expressing COX-2 and replaced with medium containing 1% FCS and 10 μM ponasterone. These cells in low serum medium were then treated for 0.5 - 4 h with 60 μM AA to initiate COX-2 catalysis, or 60 μM AA plus 10 μM NS-398 or 100 μM aspirin to inhibit COX-2. Non-induced 293 EcR cells were also exposed directly to 0-30 μM of 5-HpETE, 12-HpETE, 15-HpETE or H2O2 for 30 min at 37°C. All enzymatic reactions were quenched by freezing cells in lysis buffer (Complete protease inhibitor, 0.05M Tris pH 7.4, 0.1M NaCl, 2mM EDTA, 1mM NaF, 1mM Na orthovanadate). Cells (~1 x 10^6) were lysed, sonicated 15 s at 4°C and centrifuged for 5 min at 10,000 ×g. Samples (15 μg protein) were fractionated by PAGE. Fig. 1-5 and 7 are based on SDS-PAGE with a Tris glycine 10-20% polyacrylamide gradient gel. Prx1 and 3 isozymes (oxidized and reduced) with molecular weights 26.5 and 27 kDa co-migrate under these conditions.
conditions. Different 2-Cys Prx isoforms or PrxSO₃ can be detected by successive blotting, stripping and re-blotting with antibodies selective for PrxSO₃, Prx1, 2, 3 and 4. Fig. 6B is based on electrophoresis with native, non-reducing 3-12% gradient polyacrylamide gels.

Measurement of Cellular 5-, 12-, and 15-Lipoxygenase Activity — 15(S)-HETE, 12(S)-HETE and LTB₄ in the cell culture media were quantified by enzyme immunoassay. Levels of these metabolites reflect cellular LOX activity. 293 EcR cells expressing 15-, 12- and 5-LOX enzymes were incubated 37°C for 30 min with 60 μM AA, 60 μM AA plus 10 μM NDGA, 60 μM AA plus 3 μM baicalein, or vehicle. Cell culture media was removed and diluted 1:5, 1:10, 1:20, and 1:50 in 0.05 M pH 7.4 phosphate buffer (PBS) for analysis according to the protocol supplied with the EIA kits. Results are the mean ± S.E., n = 3-4.

Oxidation of Prx to PrxSO₃ in Cells Treated with Phospholipase A2 — 293 EcR cells expressing 15-LOX-1 or 12-LOX were incubated for 30, 60 and 90 min in 1% v/v FCS and DMEM with 20 units/ml pancreatic PLA₂, to determine whether metabolism of endogenous fatty acids released from cellular phospholipids leads to PrxSO₃ formation. Cells were also incubated for 60 min with 60 μM AA to compare PrxSO₃ formation via oxidation of endogenous, versus exogenous fatty acids. Cells were lysed at times specified; PrxSO₃ and Prx1 were determined by western blotting.

Oxidation of Prx to PrxSO₃ in Human Cells That Constitutively Express LOX — Undifferentiated HL-60 human leukemia cells (ATCC, Manassas, VA) were grown in RPMI medium, 10% FCS with 2 mM glutamine. Human platelets were suspended in Ca²⁺ free 1% v/v FCS and DMEM. HL-60 cells (1 x 10⁶ cells/ml) and human platelets (6 x 10⁷ cells/ml) constitutively express 5-LOX and human platelets (6 x 10⁷ cells/ml) constitutively express 12-LOX. Cell suspensions were incubated for 30 min, 37°C in 1% v/v FCS and DMEM with 0 or 30 μM AA. Cells were lysed and 5-LOX, 12-LOX, PrxSO₃ and Prx1 were determined by western blotting.

Detection of Prx Oxidation by Alkylation with Biotin-Conjugated Maleimide — We adapted an independent method (28) to determine whether, and which, 2-Cys-Prx were co-oxidized during LOX catalysis. Briefly, 293 EcR cells expressing 15-LOX-1 were incubated for 30 min with vehicle or 60 μM AA in 1% FCS 1× DMEM. In step 1, cells were washed, lysed, frozen and reacted under vacuum for 1 h at 25°C with 10 mM N-ethylmaleimide (NEM) and 10 mM iodoacetic acid (IAA) in 1 ml of O₂-depleted 50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1% Triton X-100, 5 mM NaF, 50 μg/ml leupeptin and 50 μg/ml aprotinin. The cell lysate was mixed in 1% SDS for 2 h at 25°C in the dark. In step 2 proteins were precipitated with 10% v/v TCA for 1 h, 4°C. The protein precipitate was washed twice with acetone, then dissolved and reduced for 0.5 h at 50°C with 4 mM DTT in 0.1 ml of O₂-free 50 mM Hepes, pH 7.7, 1 mM EDTA, and 2% v/v SDS. In step 3 the reduced proteins in the sample were reacted for 0.5 h at 50°C with 1 mM biotin conjugated to polyethylene oxide-maleimide in 0.9 ml of 50 mM sodium phosphate, pH 7.0, 1 mM EDTA. Proteins were precipitated in 0.3 ml of buffer without SDS. A portion of sample containing 200 μg protein was added to 100 μL Immobilized NeutrAvidin in PBS 0.1% Tween (1.0 ml final volume). Samples were rotated 12h at 4°C, centrifuged and NeutrAvidin beads were washed 2x with PBS/0.1% Tween. Laemmli loading buffer (50 μL) with 5% BME was added to the beads, boiled for 10 min to release biotinylated proteins and then fractionated by SDS-PAGE. Proteins were transferred to PVDF membranes for western blot analysis. The membrane was probed with an anti-Prx1, 2, 3 and 4 antibodies.

Two-dimensional PAGE Analysis—293 EcR cells expressing 15-LOX-1 were treated with AA as described above to oxidize Prx to PrxSO₃. We used a modified method to prepare protein samples for two-dimensional PAGE (9). Briefly, cells (~1 x 10⁸) were washed once in PBS and lysed in 2D lysis buffer (8M urea, 4% CHAPS, 40mM Tris (pH 8.4), and 1X protease inhibitor cocktail) by sonication (2 x 15s at 4°C). The lysates were centrifuged at 14,000 x g for 10 min and protein concentration was determined using a fluorometric method (Quant-iT kit, Invitrogen). Immobilized pH gradient strips (pH 3-10 NL)
(Invitrogen) were soaked for 75 min in rehydration buffer (8M urea, 2% CHAPS, 20 mM DTT, 0.5% (v/v) carrier ampholyte solution (Invitrogen), and 0.005% bromophenol blue) containing sample (5 ug protein). A ZOOM IPGRunner System (Invitrogen) was used to perform isoelectric focusing (~2800Vh). The pH strips were equilibrated for second dimension SDS-PAGE according to the manufacture’s protocol, which were then run on ZOOM Tris-glycine 4-20% polyacrylamide gradient gels (Invitrogen). Proteins were transferred to PVDF membrane to be visualized by immunoblot with anti-Prx 1, 2, 3 and 4 antibodies.

Western Immunoblot— Proteins were measured using mouse anti-5-LOX (1:500), rabbit anti-platelet 12-LOX (1:1000), rabbit anti-15-LOX (1:1000), rabbit anti-COX-2 (1: 450), rabbit anti-Prx1, 2, 3 or 4 (1:2000) and rabbit anti-PrxSO3 (1:2000), followed by HRP-conjugated secondary antibody (1:4000). Chemiluminescent protein-antibody complexes were quantified with a Kodak Image Station 440™. The ratio of PrxSO3 / Prx1 was calculated from the image density. PrxSO3 / Prx1 ratios were expressed in arbitrary units. Bar graphs depict the mean ± S.E., for n = 3- 6 separate experiments.

Statistics— Statistical significance at 95% confidence was determined by analysis of variance with Bonferroni’s post-hoc test.

RESULTS

Oxidation of 2-Cys Prx to PrxSO3 by Cellular LOX and COX-2 Catalysis —Immunoblots with isoform specific anti-Prx antibodies show that 293 EcR cells constitutively expressed Prx1, 2, 3 and 4; their levels did not change due to treatment with ponasterone or AA (Fig. 1A, lanes 1-4). The panels for Prx1, 2, 3 and 4 in Fig. 1A represent the chemiluminescence signal at different exposure times for each separate Prx isoform, whose relative expression varies. Separate experiments showed that these proportions were ~ 30% Prx1, ~10% Prx2, ~ 60% Prx3, and ~2% Prx4.

15-LOX-1 protein was induced in EcR cells treated with ponasterone, as expected (27) (Fig. 1A, lane 1 and 2). Prx was oxidized to PrxSO3 during 15-LOX-1 catalysis. Quantification of PrxSO3 is simplified by the fact that it migrates as a single band on 10-20% polyacrylamide gradient gels. Likewise, Prx1 ~ 26.5 kDa, Prx2 ~ 24 kDa, Prx3 ~ 27 kDa and Prx4 ~ 28 kDa all migrate closely and are not fully resolved on a 10-20% gradient polyacrylamide gel. Prx1 was used as a loading control to normalize quantitation of PrxSO3. Results were comparable if PrxSO3 levels were normalized against tubulin, Prx2 or Prx3. The PrxSO3 / Prx1 ratio rose 7-8 fold when 293 EcR cells expressing wild type 15-LOX-1 were treated with 60 μM AA (Fig. 1A, lane 2). This increase was significant (bar graph, p<0.05) compared to cells treated with vehicle (Fig. 1A, lane 1 and 3), or to cells expressing mutant, inactive Δ ile^62 15-LOX-1 (Fig. 1B). The LOX inhibitors, NDGA and baicalein, each blocked PrxSO3 formation with IC50 ~10 μM and 3 μM respectively (Fig. 1B lane 5-10). Cells expressing mutant Δ ile^62 15-LOX-1 had undetectable levels of 15-HETE in their medium (<0.10 ng/ml), consistent with loss of its catalytic function. Results were similar in 293 EcR cells expressing 12-LOX or 5-LOX. The PrxSO3 / Prx1 ratio rose 6-9 fold when cells with 12-LOX were treated with 60 μM AA, compared to vehicle or cells not expressing 12-LOX (p<0.05, Fig. 2A, lanes 1-4). NDGA and baicalein each inhibited PrxSO3 formation by 12-LOX catalysis, with IC50 ~5 μM and 3 μM, respectively (Fig. 2A, lanes 5-10). The PrxSO3 / Prx1 ratio rose 5-12 fold in cells expressing 5-LOX treated with AA, compared to vehicle or control cells not expressing 5-LOX (p<0.05 Fig. 2B, lanes 1-4). NDGA and baicalein inhibited PrxSO3 formation by 5-LOX catalysis with IC50 ~5 μM and 6 μM, respectively (Fig. 2B, lanes 5-10). We verified that NDGA and baicalein inhibited 15-LOX, 12-LOX and 5-LOX activity induced by ponasterone in 293 EcR cells [Table 1].

The PrxSO3 / Prx1 ratio rose 2-4 fold when cells expressing COX-2 were treated with 60 μM AA, compared to vehicle or cells that did not express COX-2 (p<0.05, Fig. 3, lanes 1-4). NS-398 a selective COX-2 inhibitor blocked PrxSO3 formation; however, aspirin (acetylsalicylic acid) did not. This result is consistent with the ability of acetylated Ser^516 COX-2 to metabolize AA into a
lipid hydroperoxide, 15(R)-HpETE, but not prostaglandins (29).

Prx was also oxidized to PrxSO₃ when endogenous fatty acids were liberated from cellular phospholipids by treatment with pancreatic PLA₂. The PrxSO₃ / Prx1 rose ~2.5 fold within 30 min of exposing 15-LOX-1 cells to PLA₂ (20 units/ml) at pH 7.0 (Fig. 4A, lane 1 vs. 4). This was comparable to a ~3.6 fold increase in PrxSO₃ / Prx1 when these cells were incubated with 60 μM exogenous AA for 60 min (Fig. 4A, lane 4 vs. 5). Results were similar in cells expressing 12-LOX. The PrxSO₃ /Prx1 ratio rose ~2 fold after 90 min when endogenous fatty acids were released by PLA₂, compared to a 3.2 fold increase after 60 min treatment with 60 μM exogenous AA (Fig. 4A, lane 8 and 10 vs. lane 9). When added exogenously to cells 3-30 μM each of 5-, 12- and 15-HpETEs oxidized Prx more than did 3-30 μM H₂O₂ (Fig 4B).

Composition, Reversibility and Oligomerization of PrxSO₃ Formed by Cellular LOX Catalysis — We used an independent method to determine which 2-Cys Prx isoforms were co-oxidized by LOX catalysis. Treatment of samples with excess N-ethyl maleimide and iodoacetic acid alkylates the nucleophile, PrxSH, but not the electrophile, PrxSOH. Subsequent reduction of PrxSOH to PrxSH, then reaction with maleimide-biotin converts PrxSOH into PrxS-biotin (Fig. 5A). 293 EcR cells expressing 15-LOX-1, treated 30 min with 60 μM AA, contained about 4-5 fold more S-biotinylated Prx2 and Prx3 than cells treated with vehicle (Fig. 5A, NA-pulldown). This represents oxidation of ~20-30 % of Prx2 and Prx3 to the corresponding PrxSOH under these conditions. Data shown in Fig. 5A represent the reversible oxidation of Prx cysteinyl thiol to sulfenic acid, which occurs through normal catalytic cycling.

We also used two dimensional gel electrophoresis and immunoblotting for individual Prx isoforms to measure Prx sulfenic and sulfonic acids, which occur from over-oxidation and inactivation of PrxSO (Fig 5B). Vehicle-treated 293 EcR cells harboring 15-LOX-1 contained Prx1, Prx2, Prx3 and Prx4 that migrated as a single main species, with a small amount, < 5% of Prx1 and Prx3 as more acidic species. Cells treated with 10 μM ponasterone for 48 h to induce 15-LOX-1 expression, followed by incubation with 60 μM AA for 30 min contained appreciable amounts of a Prx1, Prx2 and Prx3 migrating at a more acidic position, consistent with formation of the corresponding PrxSO₂ (9). Densitometry of the oxidized Prx species, relative to the total of both Prx species, showed formation of ~13% Prx1SO₂, ~31% Prx2SO₂ and ~36 % Prx3SO₂ (Fig 5B).

PrxSO₃ protein levels declined by ~ 65 % via first-order kinetics with t ½ = 0.5 h, 0.5 h and 0.6 h in 293 EcR cells expressing, 15-LOX-1, 12-LOX and 5-LOX, respectively (Fig. 6A). PrxSO₃ overoxidized by lipid hydroperoxide metabolites migrated as a high molecular weight complex, ~400 kDa, analogous to PrxSO₃ oxidized by 1 mM H₂O₂ (Fig. 6B).

Oxidation of Prx to PrxSO₃ in Human Cells That Constitutively Express LOX —Prx was also oxidized to PrxSO₃ in other human cell types that express LOX enzymes constitutively, not just in 293 EcR cells engineered for inducible expression of LOX. When incubated with 30μM AA for 30 min, the PrxSO₃/Prx1 ratio rose ~3-fold in 1 x 10⁶ undifferentiated HL-60 human leukemia cells, which express 5-LOX; PrxSO₃/Prx1 rose ~10-fold in 6 x10⁷ human platelets, which express 12-LOX (Fig. 7).

DISCUSSION

HpETEs, the lipid hydroperoxide metabolites of 5-LOX, 12-LOX, 15-LOX-1 or COX-2, oxidized 2-Cys Prx. PrxSO₃ formation required catalysis as shown by genetic inactivation of 15-LOX-1 (Fig. 1), by the effects of inducer (ponasterone), substrate (AA) and inhibitor combinations on EcR 293 cells that conditionally expressed LOX enzymes (Fig. 1 and 2) and by the dioxygenase activity of COX-2 acetylated by aspirin (29) (Fig. 3). 2-Cys Prx was also overoxidized by lipid hydroperoxides added exogenously to cells (Fig. 4B). This is consistent with the affinity of alkyl hydroperoxides (4, 8) for Prx homologs in E. coli, and the proximity of hydrophobic domains and vulnerable cysteine residues in 2-Cys Prx. There may be other reasons for the apparent differential sensitivity of Prx toward exogenously added HpETE metabolites versus H₂O₂. This includes factors that affect the amount and/or stability of
the added compounds reaching the Prx. For instance, catalase present in the FCS would efficiently scavenge the hydrogen peroxide. Additionally, these oxidants might trigger other cellular processes that modulate Prx oxidation and reduction. It is important to note that Yang et al (9) have shown that steady-state levels of < 1 μM H₂O₂ can overoxidize Prx1, when the H₂O₂ is generated by glucose oxidase in situ.

For simplicity we designated oxidized Prx as PrxSO₃; however, it was likely a mixture of PrxSO₂ and PrxSO₃, based on the specificity of the antibodies we used (10), and previous results by others (9, 42). The oxidized Prx measured in our experiments consisted mainly of Prx2 and Prx3 isoforms, with some contribution by Prx1. Cellular Prx oxidation by lipid hydroperoxides was ~ 65% reversible, analogous to its oxidation by H₂O₂ (10, 11), and PrxSO₃ was found as an oligomer whether it was formed by endogenous lipid hydroperoxides or by 1 mM H₂O₂ administered exogenously (Fig. 6). Recently, Phalen et al (30) reported that the oxidation state governs structural transitions in Prx2 that correlate with cell cycle arrest and recovery. Although speculative, our results suggest that LOX and COX enzymes can modulate cellular processes by a peroxiredoxin-dependent mechanism, which is coupled to their biosynthesis of lipid mediators (23, 24). In other words, redox mechanisms of action by LOX and COX might be inseparable from autacoid receptor-mediated mechanisms, as first proposed by Brash in 1985 (23).

According to the “floodgate” model the destruction of Prx peroxidase activity, by its oxidation to PrxSO₂ / SO₃, regulates H₂O₂ mediated signal transduction in eukaryotes (6, 7, 13, 14). By oxidizing Prx1, 2 and 3 the hydroperoxide metabolites of mammalian LOX enzymes might regulate PDGF and TNFα signaling, analogous to H₂O₂ and NOX (12-15, 31). Importantly, LOX enzymes might exert localized effects by redistributing between the cytosol, nucleus and plasma membrane of cells stimulated with various agonists (19-21, 32,33), including AA (33). In particular, 5-LOX has an SH3 binding motif for association with the GRB2 adaptor protein involved in PDGF-Ras signaling (18). In addition, tyrosine kinases (19) and serine / threonine kinases (35-37) can modulate the catalytic activity and sub-cellular redistribution of 5-LOX and, hypothetically, any novel functions derived from specific protein-protein partnerships.

In summary, LOXs, COXs and HpETEs should be integrated with NOXs and H₂O₂ in the Prx “floodgate model” (6, 7). Oxidation of Prx might explain how cells modulate the activation of LOX and COX by H₂O₂ (38, 39) and the activation of NOX activity by AA (40, 41).

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REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** Oxidation of Prx to PrxSO$_3$ by cellular 15-LOX-1 catalysis. Panel A Immunoblots of 15-LOX-1, Prx1, Prx2, Prx3, Prx4 and PrxSO$_3$ proteins (arrows) from 293 EcR cells harboring inducible, wild type *15-LOX-1* treated with 10 μM ponasterone for 48 h (panel A, lane 1 & 2), then incubated with 60 μM AA (panel A, lane 2 & 4). Chemiluminescence exposure times for the Prx panels varied as follows: 30s PrxSO$_3$, 5s Prx1, 30s Prx2, 1s Prx3, 60s Prx4. The ratio of PrxSO$_3$/Prx1 rose (bar graph, p<0.05, n = 6) in cells expressing wild type15-LOX-1 treated with 60 μM AA (panel A lane 2), compared to vehicle (panel A lane 1), or to cells with no 15-LOX-1 induction (panel A, lane 3 & 4). Panel B. Immunoblots of 15-LOX-1, Prx1 and PrxSO$_3$ protein (arrows) in 293 EcR cells harboring inducible inactive mutant Δile$^{662}$ 15-LOX-1 (panel B, lane 1-4) or wild type 15-LOX-1 (panel B, lanes 5-10) treated with 10 μM ponasterone for 48 h (panel B, lane 1, 2, and 5-10), then incubated with 60 μM AA (panel B, lane 2, 4 and 5-10). The ratio of PrxSO$_3$/Prx1 was unchanged in cells expressing Δile$^{662}$ 15-LOX-1 treated with or without ponasterone or AA (bar graph, panel B lane 1-4). LOX inhibitors, NDGA (bar graph, panel B lanes 5-7) and baicalein (bar graph, panel B lane 8-10), blocked the oxidation of Prx to PrxSO$_3$.

**Fig. 2.** Oxidation of Prx to PrxSO$_3$ by cellular 12-LOX and 5-LOX catalysis. Immunoblots of 12-LOX, 5-LOX, Prx1 and PrxSO$_3$ protein (arrows) in 293 EcR cells harboring inducible, wild type 12-LOX (panel A) or 5-LOX (panel B) treated with 10 μM ponasterone for 48 h (panels A and B, lanes 1, 2, 5-10). The ratio of PrxSO$_3$/Prx1 rose (bar graph, p<0.05, n = 3-5) in cells expressing 12-LOX or 5-LOX treated with 60 μM AA (lane 2) compared to vehicle (lane 1) or cells not expressing 5-LOX or 12-LOX (lane 3, 4 and 5-10). The ratio of PrxSO$_3$/Prx1 was unchanged in cells expressing Δile$^{662}$ 15-LOX-1 treated with or without ponasterone or AA (bar graph, panel B lane 1-4). LOX inhibitors NDGA (lanes 5-7) and baicalein (lanes 8-10) blocked the oxidation of Prx to PrxSO$_3$ in cells with 12-LOX (panel A) or 5-LOX (panel B) plus AA.

**Fig. 3.** Oxidation of Prx to PrxSO$_3$ by cellular COX-2 catalysis. Immunoblots of PrxSO$_3$, Prx1 and COX-2 protein (arrows) in 293 EcR cells harboring wild type COX-2 induced by 10 μM ponasterone for 24 h (lane 1, 2, 5, 6, 9, and 10). The ratio of PrxSO$_3$/Prx1 rose (bar graph, p<0.05, n = 4) in cells expressing COX-2 treated with 60 μM AA (lane 2) compared to vehicle (lanes 1) or cells not expressing COX-2 (lane 3, 4, 7, 8, 11, 12). The selective COX-2 inhibitor NS-398 blocked oxidation of Prx to PrxSO$_3$ (lane 10 vs. lane 2); aspirin did not (lane 6 vs. lane 2). COX-2 acetylated at Ser$^{516}$ by aspirin metabolizes AA into the lipid hydroperoxide 15(R)-HpETE (29).
Fig. 4. Oxidation of Prx to PrxSO₃ in EcR 293 cells treated with phospholipase A₂ or lipid hydroperoxides. Panel A. Immunoblots of PrxSO₃ and Prx1 (arrows) in 293 EcR cells with wild type, inducible 15-LOX-1 (panel A, lane 1-5) or 12-LOX (panel A, lane 6-10) treated with 10 μM ponasterone for 48 h. The ratio of PrxSO₃/Prx1 (bar graph) rose when cells were incubated with pancreatic PLA₂ (20 units/ml) at pH 7.0 for 30-90 min (panel A lane 1-3 vs. lane 4, and lane 8 vs. lane 9). The increase in PrxSO₃ due to PLA₂ treatment was comparable to that seen with 60 μM AA (panel A lane 5 and 10). Panel B. Immunoblots of PrxSO₃ and Prx1 (arrows) in 293 EcR cells treated with 0-30 μM 15-HpETE, 12-HpETE, 5-HpETE or H₂O₂.

Fig. 5. Prx1, Prx2 and Prx3 are the main 2-Cys Prx oxidized by LOX catalysis in 293 EcR cells. Panel A. Scheme for detecting and isolating oxidized Prx isoforms from EcR 293 cells expressing 15-LOX-1, treated with 60 μM AA for 30 min. PrxSOH is proportional to the amount of biotinylated Prx isolated by neutravidin (NA) pulldown. Immunoblots of Prx2 and Prx3 in NA pulldown and whole cell lysate. Panel B. 2-Dimensional gel electrophoresis and immunoblot analysis of Prx1-4 in comparable samples. Arrows indicate the relative proportions of a more acidic PrxSO₂ species in cells treated with ponasterone plus AA, compared to vehicle.

Fig. 6. Reversibility of Prx oxidation and oligomerization of PrxSO₃. Panel A. Cellular content of PrxSO₃ as a function of time in 293 EcR cells expressing 5-LOX (-), 12-LOX (-♦-) or 15-LOX-1 (-○-). The half-life (t₁/₂) for PrxSO₃ was 0.6 h, 0.5 h and 0.5 h, respectively. Panel B. Immunoblot of PrxSO₃ fractionated on a native gel. The sample was from 293 EcR cells expressing 12-LOX treated with 60 μM AA or 1 mM H₂O₂.

Fig. 7. Oxidation of Prx to PrxSO₃ in human cells that constitutively express LOX. Immunoblots of PrxSO₃, Prx1, 5-LOX and 12-LOX in undifferentiated HL-60 human leukemia cells and human platelets incubated with 30 μM AA for 30 min. The protein content in the immunoblots derives from 1 x 10⁶ HL-60 cells per lane and 6 x 10⁷ platelets per lane.

The abbreviations used are: AA, arachidonic acid; COX, cyclooxygenase; DMEM, Dulbecco’s modified essential medium; HRP, horse radish peroxidase; HpETE, hydroperoxy-eicosatetraenoic acid; HETE, hydroxy-eicosatetraenoic acid; IAA, iodoacetic acid; LOX, lipoxygenase; NEM, N-ethyl maleimide; NOX, NADPH oxidase; NDGA, nordihydroguaretic acid; PBS, phosphate buffered saline; PG, prostaglandin; Prx, peroxiredoxins; PrxSOH, peroxiredoxin sulfenic acid; PrxSO₂, peroxiredoxin sulfinate; PrxSO₃, peroxiredoxin sulfonate.

Table 1 NDGA and Baicalein Inhibit Cellular Lipoxygenase Activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>15-LOX Activity</th>
<th>12-LOX Activity</th>
<th>5-LOX Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15(S)-HETE ng/ml</td>
<td>12(S)-HETE ng/ml</td>
<td>LTB₄ ng/ml</td>
</tr>
<tr>
<td>60 μM AA</td>
<td>120.0 ± 6.1</td>
<td>85.8 ± 9.1</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>60 μM AA plus 10 μM NDGA</td>
<td>16.0 ± 4.3 (87 ± 4% inhibition)**</td>
<td>5.8 ± 0.4 (93 ± 1% inhibition)**</td>
<td>0.2 (98% inhibition)</td>
</tr>
<tr>
<td>60 μM AA plus 3 μM baicalein</td>
<td>65.7 ± 25.6 (45 ± 21% inhibition)*</td>
<td>41.8 ± 10.9 (51 ± 13% inhibition)*</td>
<td>3.2 (61% inhibition)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.4 ± 0.1</td>
<td>2.0 ± 0.7</td>
<td>0.7 ± 0.05</td>
</tr>
</tbody>
</table>
Figure 1

** p<0.01, * p<0.05
Figure 2

A

PrxSO₃ / Prx1
Arbitrary Units

PrxSO₃
Prx1
12-LOX

B

PrxSO₃ / Prx1
Arbitrary Units

PrxSO₃
Prx1
5-LOX

AA
− + − +
Ponasterone
+ + − −
Lane
1 2 3 4

NDGA
0 1 10 μM
Baicalin
0 1 3 μM

Lane
1 2 3 4 5 6 7 8 9 10
Figure 3

![Graph showing PrxSO₃ / Prx1 Arbitrary Units with labels for PrxSO₃, Prx1, and COX-2. AA and Ponasterone concentrations are indicated in lanes 1 to 8, with 100 μM Aspirin and 10 μM NS-398 concentrations in lanes 9 to 12.](image-url)
Figure 4

A

PrxSO₃/Prx1
Arbitrary units

PrxSO₃
Prx1
PLA2 + + + − −
AA − − − + +
Minutes 30 60 90 0 60
Lane 1 2 3 4 5
15-LOX-1

12-LOX

B

15-HpETE 12-HpETE 5-HpETE H₂O₂

PrxSO₃
Prx 1

0 3 10 30 3 10 30 0 3 10 30
µM
Figure 5

A

Prx reduced  SH  Prx alkylated  S-R  Prx alkylated  S-R
Prx oxidized  SOH  Prx oxidized  SOH  Prx biotin  S-R-biotin

NA Pulldown  Prx3
Cell Lysate  Prx2
+
-

Neutravidin (NA) beads
SDS-PAGE
Immunoblot anti-Prx

B

Vehicle

Prx1  Prx2  Prx3  Prx4

Ponasterone + AA

% PrxSO2

15%  31%  36%

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Figure 6

A

PrxSO₃ / Prx1
% Maximum

0        2        4                   8                 12

100
50

B

PrxSO₃ - Ab

660 kD
440 kD

H₂O₂                       –  +  –  –
Ponasterone                 –  –  +  +
AA                          –  –  –  +
Figure 7

Undifferentiated HL-60 Cells

PrxSO₃ →
Prx1 →
5-LOX →

AA 0 30 μM

1 × 10⁶ cells per lane

Platelets

PrxSO₃ →
Prx1 →
12-LOX →

AA 0 30 μM

6 × 10⁷ cells per lane
Oxidation of 2-cys-peroxiredoxins by arachidonic acid peroxide metabolites of lipoxygenases and cyclooxygenase-2
Pauline Cordray, Kelly Doyle, Kornelia Edes, Philip J. Moos and F. A. Fitzpatrick

J. Biol. Chem. published online September 13, 2007

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