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Epo-C12 inhibits peroxiredoxin 1 peroxidase activity

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ABSTRACT

Epo-C12 is a synthetic derivative of epolactaene, isolated from *Penicillium* sp. BM 1689-P. Epo-C12 induces apoptosis in human acute lymphoblastoid leukemia BALL-1 cells. In our previous studies, seven proteins that bind to Epo-C12 were identified by a combination of pull-down experiments using biotinylated Epo-C12 (Bio-Epo-C12) and mass spectrometry. In the present study, the effect of Epo-C12 on peroxiredoxin 1 (Prx 1), one of the proteins that binds to Epo-C12, was investigated. Epo-C12 inhibited Prx 1 peroxidase activity. However, it did not suppress its chaperone activity. Binding experiments between Bio-Epo-C12 and point-mutated Prx 1s suggest that Epo-C12 binds to Cys^{52} and Cys^{83} in Prx 1. The present study revealed that Prx 1 is one of the target proteins through which Epo-C12 exerts an apoptotic effect in BALL-1 cells.

1. Introduction

Epolactaene is a natural product, isolated from *Penicillium* sp. BM 1689-P (Fig. 1**A**).[1] This compound induces neurite outgrowth and arrests human neuroblastoma cells SH-SY5Y in the G1 phase of the cell cycle.[1,2] Nagumo and coworkers identified heat shock protein 60 (HSP60) as a ligand for epolactaene.[3,4] They reported that epolactaene binds Cys^{442} in human Hsp60 at the α,β-unsaturated ketone moiety inhibiting its chaperone activity.[4] Our group synthesized an artificial epolactaene derivative Epo-C12, which has a saturated *n*-dodecanoyl group at the side-chain (Fig. 1**B**).[5] Our previous study revealed that Epo-C12 induces apoptosis in human acute lymphoblastoid leukemia BALL-1 cells more potently than epolactaene.[6,7]Bio-Epo-C12, a biotinylated Epo-C12 derivative, was prepared to explore proteins that can bind Epo-C12 from BALL-1 cell lysate (Fig. 1**C**).[8] Fatty acid synthase, ATP citrate lyase, elongation factor 2, HSPs 90β and 60, adenine nucleotide translocator 2, and peroxiredoxin 1 (Prx 1) were identified as candidate binding proteins for Epo-C12 through a combination of pull-down experiments using Bio-Epo-C12 and mass spectrometry. Epo-C12 has an interesting reactivity with thiols. The reaction of Epo-C12 with 2.0 equiv of *N*-acetylcysteine methyl ester (**1**) gave disulfide **2**, dodecanoic acid (**3**), and adduct **4** (Scheme 1).[9] Peroxiredoxin 1 (Prx 1), one of the candidate binding proteins, has four cysteine residues (Cys^{52}, Cys^{71}, Cys^{83}, and Cys^{173}) that play crucial roles in its physiological activities. Prx 1 functions as a peroxidase reducing H_2O_2 mediated by the oxidation of the *N*-terminal peroxidatic Cys^{52} (C_P-SH). The resulting cysteine sulfenic acid (C_P-SOH) subsequently forms an intermolecular disulfide bond with the C-terminal resolving Cys^{173} (C_R-SH) in the adjacent Prx 1 monomer to form a Prx 1 homodimer (Fig. 2**A**).[10–13] The disulfide linkage is reduced by NADPH-dependent thioredoxin (Trx)/thioredoxin reductase (TrxR) cycles to complete the intracellular Prx 1 catalytic cycle.[13] Besides its peroxidase activity, Prx 1 can form high molecular weight (HMW) species, with chaperone activity (Fig. 2**B**).[14–18] The redox status of Cys^{83} influences the oligomeric structures and the chaperone function of Prx 1.[15–17] Prx 1 is involved in the control of various physiological functions such as cell growth, differentiation,

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apoptosis, and cellular homeostasis.[19–23] Based on the above, we hypothesized that Epo-C12 might target the cysteine residue(s) of Prx 1 and induce apoptosis in BALL-1 cells by inhibiting Prx 1 activity. Herein, in order to test the hypothesis, we investigated the apoptotic activity of Epo-C12, by focusing on Prx 1.

2. Results and discussion

2.1. Epo-C12 effect on intracellular ROS generation in BALL-1 cells

Reactive oxygen species (ROS) play an important role in apoptosis induction. To investigate whether ROS are involved in Epo-C12-induced apoptosis, the intracellular ROS levels in BALL-1 cells were measured by flow cytometry using the ROS-detecting fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA)[24] after treatment with Epo-C12 (1, 5, or 10 μM) (Fig. 3). Epo-C12 induced ROS in a dose-dependent manner. ROS levels after treatment with 10 μM Epo-C12 were similar to 50 μM H_2O_2 treated cells, which were used as a positive control. Epo-C12-induced apoptosis in BALL-1 cells was inhibited by 250 μM dithiothreitol (DTT), 1.5 mM glutathione (GSH), and 250 μM *N*-acetylcysteine (NAC) (Fig. 4). These results suggest that Epo-C12 induced apoptosis by increasing intracellular ROS levels in BALL-1 cells. DTT, GSH, and NAC should suppress Epo-C12-induced apoptosis by reducing ROS production. Generally, glutathione is present in millimolar concentrations (0.5–10 mM) in the cell.[25] Most of the cellular glutathione exists in the reduced form (GSH, 95%) while less than 5% is present as oxidized glutathione disulfide (GSSG).[26] Under these intracellular conditions, Epo-C12 induces apoptosis in BALL-1 cells and increases intracellular ROS levels even at concentrations below 5 μM. These results rule out the possibility that Epo-C12 directly reacts with the reducing agents to lose apoptosis-inducing activity.

2.2. Effect of Epo-C12 on peroxidase activity of peroxiredoxin 1

We first examined inhibitory activity of Epo-C12 against the peroxidase activity of recombinant Prx 1 (Fig. 5). Peroxidase activity was measured by the ferrous oxidation-xylenol orange (FOX) assay[27] by using H_2O_2 as a substrate. Epo-C12 (50 μM) decreased the peroxidase activity of Prx 1 to 56% compared to the vehicle control (DMSO). Conoidin A[28] (50 μM), an inhibitor of Prx peroxidase activity, decreased the activity to 33%. Epo-C12 showed cytotoxicity with a 50% inhibitory concentration (IC_{50}) value of 4.41 ± 2.02 μM, whereas conoidin A exhibited cytotoxicity against BALL-1 cells with an IC_{50} value of 1.05 ± 0.24 μM. Although Epo-C12 and conoidin A showed cytotoxicity at lower concentrations than those for the inhibition of peroxidase activity of Prx 1, these results suggest that Epo-C12 as well as conoidin A inactivate the peroxidase activity of Prx 1 in BALL-1 cells, while inducing cell death. This possibility is supported in a previous report by Corbett and collaborators.[29] They reported that Prx 1 plays a primary role in protecting pancreatic β-cells from H_2O_2 and peroxynitrite.

2.3. Epo-C12 effect on peroxiredoxin 1 chaperone activity

Next, the effect of Epo-C12 on Prx 1 chaperone activity was investigated by a light scattering assay of thermally induced aggregation of recombinant citrate synthase (CS) (Fig. 6).[16,30,31] Oxidized Prx 1, prepared by treating Prx 1 with H_2O_2, was used in this assay. CS aggregation causes an increase in absorbance at 450 nm (blue line). An increase in absorbance was not observed when a mixture of CS and oxidized Prx 1 was heated (pink line). These results indicate that CS aggregation was inhibited by oxidized Prx 1 due to its chaperone activity. The chaperone activity of oxidized Prx 1 was retained in the presence of Epo-C12 (light green line) or conoidin A (orange line).

2.4. Binding of Bio-Epo-C12 to Prx 1 and its mutants

To determine the cysteine(s) targeted by Epo-C12, we performed binding experiments of Bio-Epo-C12 with Prx 1 and its mutants (Fig. 7). Wild-type (WT) Prx 1, and three alanine-scanning Prx 1 mutants (C52A, C173A, and C52A/C173A) were transiently expressed with a *N*-terminal FLAG3-tag in HEK 293 T cells. Expression of the mutant proteins was confirmed by Western blot with anti-FLAG tag (**Fig. S1A** in Supplementary Material). They were then tested for the ability to bind Bio-Epo-C12. Lysates were treated with Bio-Epo-C12 and further incubated with streptavidin resin beads. Proteins were resolved by SDS-PAGE. Endogenous Prx 1 and Flag-tagged WT Prx 1, C52A, C153A, and C53A/C173A mutants that were pulled using Bio-Epo-C12 were detected by Western blot with anti-FLAG tag and anti-Prx 1 antibodies (Fig. 7**A**). Next, WT Prx 1, and five Prx 1 mutants (C71A, C83A, C52A/C71A/C173A, C52A/C83A/C173A, and C52A/C71A/C83A/C173A) were expressed and used in Bio-Epo-C12 binding experiments (Fig. 7**B**). Expression of the mutant proteins was confirmed by Western blot with anti-FLAG tag (**Fig. S1B** in Supplementary Material). Binding between Bio-Epo-C12 and C71A, C83A, and C52A/C71A/173A mutants was confirmed by Western blot. However, binding of Bio-Epo-C12 with C52A/C83A/C173A and C52A/C71A/C83A/C173A mutants was not validated. Results obtained through binding experiments suggest that Cys^{83} in Prx 1 is important in Bio-Epo-C12 binding. However, C83A mutant also binds Bio-Epo-C12. Thus, Cys^{83} and one or more cysteine residues in Prx 1 are involved in Bio-Epo-C12 and Prx 1 binding. Prx 1 peroxidase activity inhibition by Epo-C12 suggests that Bio-Epo-C12 binds to Cys^{52}, the peroxidatic cysteine of Prx 1. Previously, Chock and coworkers reported that glutathionylation of Cys^{83} alone is sufficient to induce Prx 1 decamer dissociation inhibiting its chaperone activity.[16] They also reported that glutathionylation of WT Prx 1 and its C52S/C173S mutant, greatly reduces their molecular chaperone activity in protecting CS from thermally induced aggregation. Yang and coworkers reported that triptolide, a diterpenoid triepoxide isolated from *Tripterygium wilfordii*, binds to Cys^{83} and Cys^{173} in Prx 1.[32] They showed that triptolide selectively inhibits Prx 1 chaperone activity through a direct interaction with these cysteines, thereby inducing Prx HMW oligomers dissociation. Thus, the modification of both Cys^{83} and Cys^{173} in Prx 1 by glutathione or triptolide causes a decrease in chaperone activity. Taken together, it can be speculated that Epo-C12 does not inhibit Prx 1 chaperone activity because it does not bind to Prx 1 Cys^{173}. It is worth noting that triptolide did not suppress Prx 1 peroxidase activity. The effects of Epo-C12 on Prx 1 as well as its target cysteines within Prx 1 are different from those of triptolide.

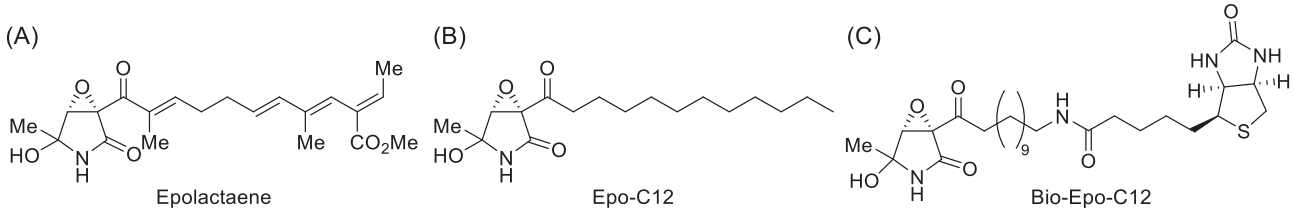


Fig. 1. Structures of epolactaene (A), Epo-C12 (B), and Bio-Epo-C12 (C).

3. Conclusion

Epo-C12 is a synthetic derivative of epolactaene, which induces apoptosis in BALL-1 cells. The present study revealed that Epo-C12-induced ROS production in BALL-1 cells. Epo-C12 induced apoptosis in BALL-1 cells was suppressed by reducing reagents such as DTT and NAC. Epo-C12 inhibited the peroxidase activity of Prx 1. Thus, we hypothesized that Epo-C12 induced apoptosis could be due to an increase in intracellular ROS concentration of BALL-1 cells. Our study demonstrated that Epo-C12 induced ROS-mediated apoptosis by inhibiting peroxiredoxin 1 peroxidase activity in BALL-1 cells. However, Epo-C12 induced apoptosis in BALL-1 cells at lower concentrations than those for the inhibition of peroxidase activity of Prx 1, suggesting the possibility that other target proteins of Epo-C12 exist. Further target validation of Epo-C12 is necessary to understand the detail mechanism for the apoptosis-inducing activity.

The binding experiments of Bio-Epo-C12 with the point-mutated Prx 1s suggest that Epo-C12 binds to Cys^{52} and Cys^{83} residues of Prx 1. The inhibition of Prx 1 peroxidase activity can be explained by Epo-C12 binding Cys^{52}. Epo-C12 did not inhibit Prx 1 chaperone activity, suggesting that it does not bind to Cys^{173} in Prx 1.

Our present study proposes a molecular mechanism underlying the effects of Epo-C12 on Prx 1. This study also provides a chemical tool for the selective inhibition of the peroxidase activity of Prx 1 without affecting its chaperone activity.

4. Experimental methods

4.1. Materials

Epo-C12 and Bio-Epo-C12, used in the present study, were prepared in our previous studies.[5,8] Roswell Park Memorial Institute (RPMI) was purchased from Nissui (Tokyo, Japan) or FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). *N*-Acetylcysteine (NAC), crystal violet, glycerol, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12), glutathione (GSH), HEPES buffer, sodium hydroxide, and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained by FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Calcium chloride, Cell Counting Reagent SF, dimethyl sulfoxide (DMSO), EDTA, ethylene glycol tetraacetic acid (EGTA), hydrogen peroxide, magnesium chloride, 2-mercaptoethanol, phosphate buffered saline (PBS), proteinase inhibitor cocktail, sodium dodecyl sulfate (SDS), Tris(hydroxymethyl)aminomethane (Tris), Triton X-100, potassium phosphate, sodium phosphate, and dithiothreitol (DTT) were purchased from Nacalai Tesque (Kyoto, Japan). TRIzol reagent, MMLV reverse transcriptase, ribonuclease inhibitor, and oligo(dT)18 were purchased from Invitrogen (Carlsbad, CA, USA). Anti-FLAG antibody, fluorescein isothiocyanate-conjugated annexin V (Annexin V-FITC) and propidium iodide (PI) were obtained from Sigma Aldrich (St Louis, MO, USA). Fluorescein isothiocyanate-conjugated annexin V (Annexin V-FITC) and propidium iodide (PI) were obtained from Sigma Aldrich (St Louis, MO, USA). Conoidin A and NADPH (sodium salt) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Funakoshi (Tokyo, Japan). Recombinant human Prx 1 and CS, which were fused to His_{20}-tag and His_{23}-tag at *N*-terminus, respectively, were obtained from Prospec-Tany Technogene (Ness Ziona, Israel). *EcoR* I and *Hind* III endonucleases and Tris-buffered saline with Tween 20 (TBST) were purchased from Takara Bio (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Biowest (Loire Valley, French). HRP-anti-mouse IgG and HRP-anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Danvers, USA). KOD-Plus polymerase was obtained from Toyobo (Osaka, Japan). PEROXsay assay kit was purchased from G-Biosciences (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from Merck Millipore (Darmstadt, Germany). The vector plasmid, pUSEamp(+), was purchased from Upstate Biotechnology (Lake Placid, NY, USA). FuGENE 6 transfection reagent was purchased from (Madison, WI, USA). Streptavidin Sepharose beads and ECL Plus Western blotting detection reagents were obtained from GE Healthcare (London, UL). Skim milk was purchased from Yukijirushi (Tokyo, Japan).

4.2. Cell lines and culturing conditions

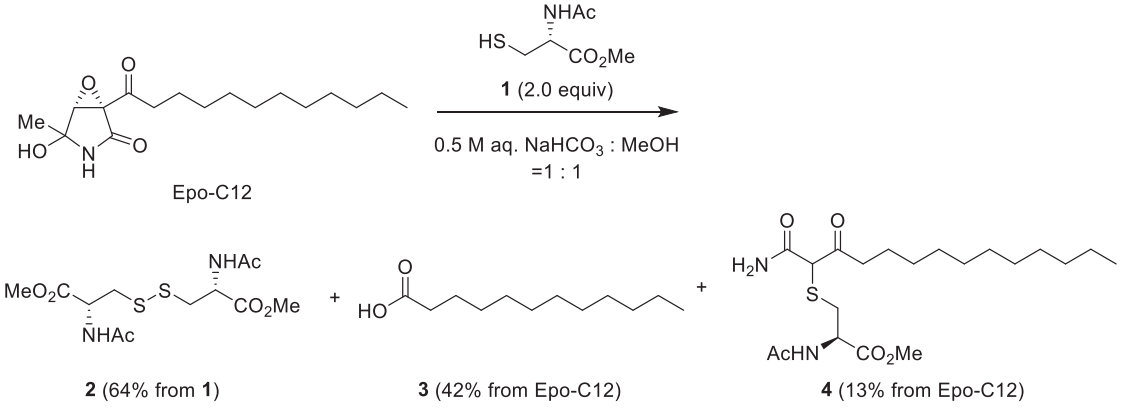
BALL-1 cells were purchased from Cell Resource Center for Biomedical Research/Cell Bank (Tohoku University, Japan). BALL-1 cells were cultured at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS.

4.3. Measurement of intracellular ROS generation in BALL-1 cells (Fig. 3)

BALL-1 cells (2×10^5 cells) were incubated with 10 μM DCFH-DA[24] for 10 min. Cells were further incubated with the vehicle control (DMSO), Epo-C12 (1, 5, or 10 μM), or H_2O_2 (50 μM) for 30 min. Cell pellets were collected by centrifugation and resuspended in PBS. Subsequently, cells were analyzed by a flow cytometer (FACS Calibur, Becton Dickinson). Fluorescence was measured at 530 nm upon excitation at 488 nm.

4.4. Effect of DTT, GSH, and NAC on Epo-C12 induced apoptosis in BALL-1 cells (Fig. 4)

Percentage of apoptosis was determined by Annexin V-FITC and PI double staining using a MEBCYTO Apoptosis kit (MBL, Nagoya, Japan). BALL-1 cells (2×10^5 cells) were incubated with DMSO or the indicated antioxidants (250 μM DTT, 1.5 mM GSH, or 250 μM NAC) in 12-well



Scheme 1. Reaction of Epo-C12 with *N*-acetylcysteine methyl ester (**1**).

plates at 37 °C for 1.5 h. Then, the cells were treated with or without Epo-C12 (5 μM) for 24 h. Cells were collected and washed with ice-cold PBS twice. The pellet was resuspended in 200 μL Annexin V binding buffer (10 mM HEPES–NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing 5 μL of Annexin V-FITC. After incubation for 10 min, cells were collected and washed with Annexin V binding buffer. The pellet was then resuspended in 360 μL Annexin V binding buffer containing 2 μg/mL PI. The cells were analyzed by flow cytometry (Coulter EPICS XL; Beckman Coulter, Miami, FL, USA).

4.5. Epo-C12 and conoidin a cytotoxic activity in BALL-1 cells

Cell growth was evaluated using a cell counting kit (Cell Counting Reagent SF) according to the manufacturer's instructions based on the WST-8 assay.[33] For the assay, the cells were cultured in a 96-well plate, with each well containing 10,000 cells in a total volume of 100 μL. The concentration of DMSO in the cell cultures was 0.1% (v/v). As a negative control, blank wells (0 cells/100 μL) and non-treated wells (10,000 cells/100 μL) were also incubated. The plates were incubated with various concentrations of each compound for 24 h. At the end of the incubation time, 10 μL of the WST-8 solution provided with the kit were added, and the resulting mixture was further incubated for 2 h at 37 °C. Absorbance values were measured at 450 nm with a 96-well plate reader. Cell growth inhibition was evaluated as the ratio of the absorbance of the sample to that of the control.

4.6. Evaluation of Prx 1 peroxidase activity (Fig. 5)

Reduction of H_2O_2 by recombinant Prx 1 was measured using the PEROXsay assay kit, which monitors the oxidation of ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions by H_2O_2.[27] Reactions were performed at 25 °C in 50 mM sodium phosphate buffer (pH 7.4) containing 0.2 mM DTT. Recombinant Prx 1 (2 μM) was preincubated with DMSO, Epo-C12 (50 μM), or conoidin A (50 μM) in PBS for 30 min, then incubated with H_2O_2 (40 μM) for 20 min. The enzymatic reaction was quenched by the addition of the assay solution (D-sorbitol, xylenol orange, ferric ammonium sulfate, and sulfuric acid), and incubated for 30 min. Absorbance was measured at 560 nm on a micro plate reader. One activity unit was defined as the amount of enzyme required to reduce 1 μmol of H_2O_2 per min.

4.7. Epo-C12 effect on recombinant Prx 1 chaperone activity (Fig. 6.)

Chaperone activity was determined by assessing the ability of oxidized Prx 1 to inhibit the thermal aggregation of recombinant CS.[16,30,31] Recombinant Prx 1 (16 μM) was preincubated with H_2O_2 (100 mM) in 40 mM potassium phosphate buffer (pH 7.0). CS (1 μM) in a buffer containing 50 mM HEPS-KOH (pH 7.0) was mixed with oxidized Prx 1 (4 μM). The mixture was incubated with DMSO, Epo-C12 (25 μM) or conoidin A (25 μM). The mixture was incubated at room temperature for 20 min. Then, the cuvette was placed into a thermocontrolled holder at 45 °C. The increase in the absorbance at 450 nm was monitored.

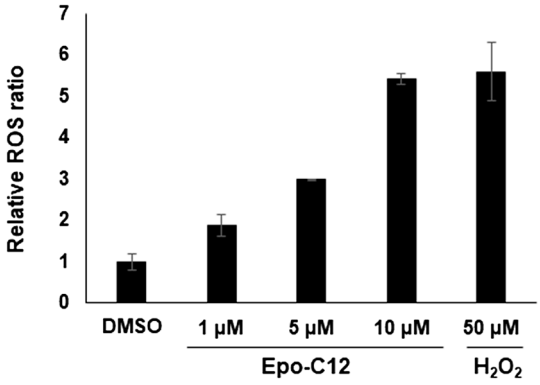


Fig. 3. Effect of Epo-C12 on intracellular ROS production in BALL-1 cells. BALL-1 cells were incubated with DCFH-DA for 10 min. The cells were then incubated with vehicle control (DMSO), Epo-C12 (1, 5, or 10 μM), or H_2O_2 (50 μM) for 30 min. After the cells were washed with PBS, the fluorescence derived from the intracellular 2′,7′-dichlorofluorescein (DCF) was measured by flow cytometry. The relative fluorescence intensity normalized to the control is shown as the mean of two independent measurements. The error bars represent the range from the measurements.

4.8. Plasmid construction

The isolation of total RNA from BALL-1 cells was performed using the TRIzol reagent method,[34] by following the manufacturer's protocol. RNAs were converted to cDNAs using MMLV reverse transcriptase, ribonuclease inhibitor, and oligo(dT)18. *PRDX1* was then amplified by polymerase chain reaction (PCR) using KOD-Plus polymerase.[35] The primer sequences were as follows: forward, 5′- GCATAAGCTTTCTTCAGGAAATGCTAAAATTGGGCACCCT-3′, reverse, 5′-ATGCGAATTCTCACTTCTGCTTGGAGAAATATTCTTTGCT-3′. After heat denaturation for 2 min at 94 °C, each PCR amplification for 30 cycles was performed as follows: 15 *sec* at 94 °C and 40 *sec* at 68 °C except that,

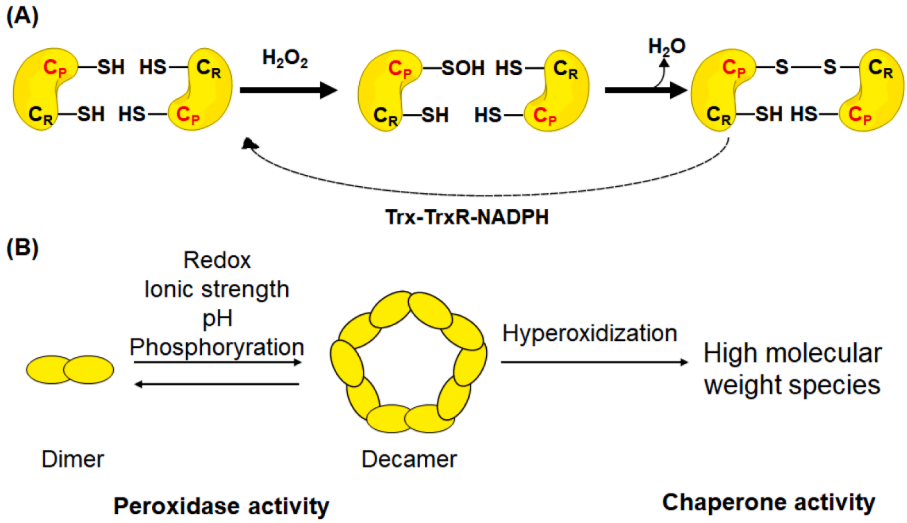


Fig. 2. Oligomerization of Prx 1. (A) Formation of a head-to-tail homodimer. Prx 1 shows peroxidase activity that removes intracellular H_2O_2 through the catalytic cycle in collaboration with the thioredoxin-thioredoxin reductase-NADPH (Trx-TrxR-NADPH) system. C_P, peroxidatic Cys^{52}; C_R, resolving Cys^{172}. (B) Formation of high molecular weight (HMW) species. Five homodimers of Prx 1 form decamers, which can then further aggregate to form HMW species. Hyperoxidation induces oligomerization from low molecular weight (LMW) to HMW species. LMW species display the peroxidase activity, whereas HMW species display chaperone activity.

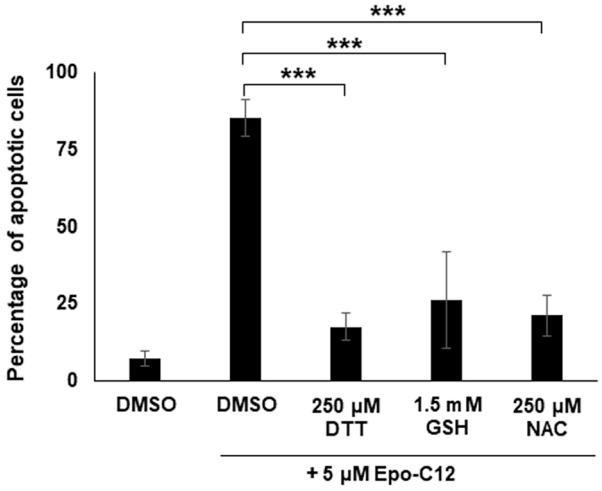


Fig. 4. Effect of DTT, GSH, and NAC on Epo-C12 induced-apoptosis in BALL-1 cells. BALL-1 cells were pretreated with DMSO or the indicated antioxidants (250 μM DTT, 1.5 mM GSH, or 250 μM NAC) for 1.5 h. The cells were treated with or without Epo-C12 (5 μM) for 24 h. Apoptotic cells were detected by flow cytometry after staining with annexin V and propidium iodide (PI). Percentages of late apoptotic cells (annexin V positive, PI positive) are indicated. The experiments were performed in octuplicate. The values represent means ± standard deviation (SD). Statistical significance was assessed by Tukey honestly significant difference (HSD) test, and the asterisks *** indicate $p < 0.001$.

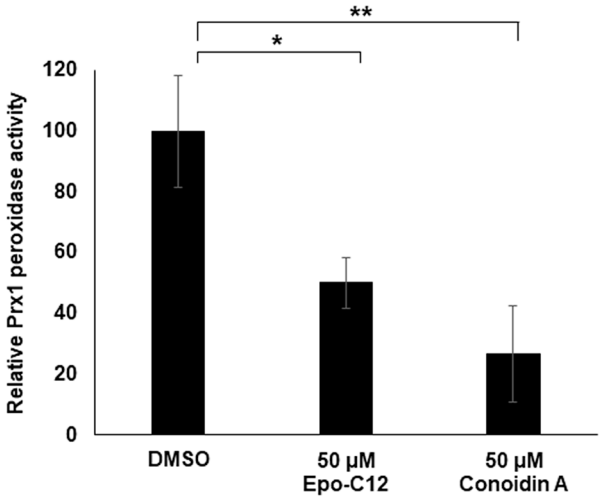


Fig. 5. Inhibition of the peroxidase activity of Prx 1 by Epo-C12 and conoidin A. Prx 1 (2 μM) was preincubated with DMSO, Epo-C12 (50 μM) or conoidin A (50 μM) for 30 min, then incubated with H_2O_2 (40 μM) for 20 min. An assay solution (D-sorbitol, xylenol orange, ferric ammonium sulfate, and sulfuric acid) was added to the media, and the resultant mixture was incubated for 30 min. Absorbance was measured at 560 nm on a micro plate reader. One activity unit was defined as the amount of enzyme required to reduce 1 μmol of H_2O_2 per min. The experiments were performed in triplicate. The values represent means ± SD. Statistical significance was assessed by Tukey HSD test, and the asterisks * and ** indicates $p < 0.05$ and 0.01, respectively, compared with the vehicle (DMSO) control.

in the last cycle, extension was carried out for 3 min, in a thermal cycler (PCR Thermal Cycler Dice, Takara, Shiga, Japan). PCR products were purified by agarose gel electrophoresis, phenol–chloroform extraction, and 70% ethanol precipitation. Purified DNAs were digested with *EcoR* I and *Hind* III restriction enzymes and concentrated by ethanol precipitation. Concentrated DNA was then ligated into pUSEamp containing a 3 × FLAG tag, digested with the same enzymes. The construct was

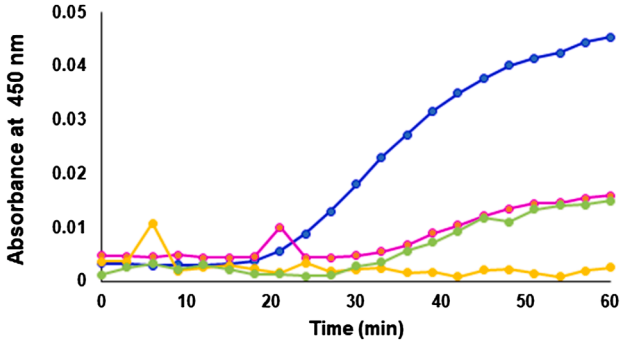


Fig. 6. Effect of Epo-C12 on the chaperone activity of Prx 1. Light scattering assay of thermally induced aggregation of 1 μM citrate synthase (CS) at 45 °C was performed. The increase in the absorbance at 450 nm was observed as CS thermally aggregated (blue line). A significant increase in the absorbance was not observed when a solution of CS and oxidized Prx 1 (4 μM) was heated (pink line). This chaperone activity of the oxidized Prx 1 was retained in the presence of 25 μM Epo-C12 (light green) or 25 μM conoidin A (orange).

transformed into competent *Escherichia coli* TOP10 and purified with the HiSpeed Plasmid Midi Kit (Qiagen, Hilden, Germany). The sequence of the construct was verified by DNA sequencing.

4.9. Construction of cysteine-to-alanine mutations

Four mutant *PRDX1* genes, each encoding an alanine residue substituting a cysteine (residues 52, 71, 83 and 173) in *PRDX1* were constructed by PCR-mediated site-directed mutagenesis.[36] The plasmid containing *PRDX1* was used as a template for sequential rounds of polymerase chain reaction (PCR). For the first round of amplification, the forward and reverse primers for *PRDX1* (5′- GCATAAGCTTTCTTCAGGAAATGCTAAAATTGGGCACCCT-3′, and 5′- ATGCGAATTCTCACTTCTGCTTGGAGAAATATTCTTTGCT-3′) were used together with mutagenic primers converting the cysteine codon (TGT or TGC) to an alanine codon (GCT or GCC; underlined below).

C52A forward primer: 5′-CCTCTTGACTTCACCTTTGTGGCCCCCACGGAGATCATT-3′
C52A reverse primer: 5′- AATGATCTCCGTGGGGGCCACAAAGGTGAAGTCAAGAGG-3′
C71A forward primer: 5′-GAATTTAAGAAACTCAACGCCCAAGTGATTGGTGCTTCT-3′
C71A reverse primer: 5′-AGAAGCACCAATCACTTGGGCGTTGAGTTTCTTAAATTC- 3′
C83A forward primer: 5′-TCTGTGGATTCTCACTTCGCCCATCTAGCATGGGTCAAT-3′
C83A reverse primer: 5′-ATTGACCCATGCTAGATGGGCGAAGTGAGAATCCACAGA-3′
C173A forward primer: 5′-ACTGACAAACATGGGGAAGTGGCCCCAGCTGGCTGGAAA-3′
C173A reverse primer: 5′-TTTCCAGCCAGCTGGGGCCACTTCCCCATGTTTGTCAGT-3′

After heat denaturation for 2 min at 94 °C, each PCR amplification for 30 cycles was performed as follows: 15 *sec* at 94 °C and 40 *sec* at 68 °C except that, in the last cycle, extension was carried out for 3 min, in a thermal cycler (PCR Thermal Cycler Dice, Takara, Shiga, Japan). Overlapping PCR products were purified by agarose gel electrophoresis, phenol–chloroform extraction and 70% ethanol precipitation. Amplified DNAs were digested with *EcoR* I and *Hind* III restriction enzymes and concentrated by ethanol precipitation. Purified DNA was then ligated into pUSEamp containing a 3 × FLAG tag and digested with the same enzymes. The constructs were transformed into competent *Escherichia coli* TOP10 and purified using the HiSpeed Plasmid Midi Kit from Qiagen. The sequence of all constructs was verified by DNA sequencing.

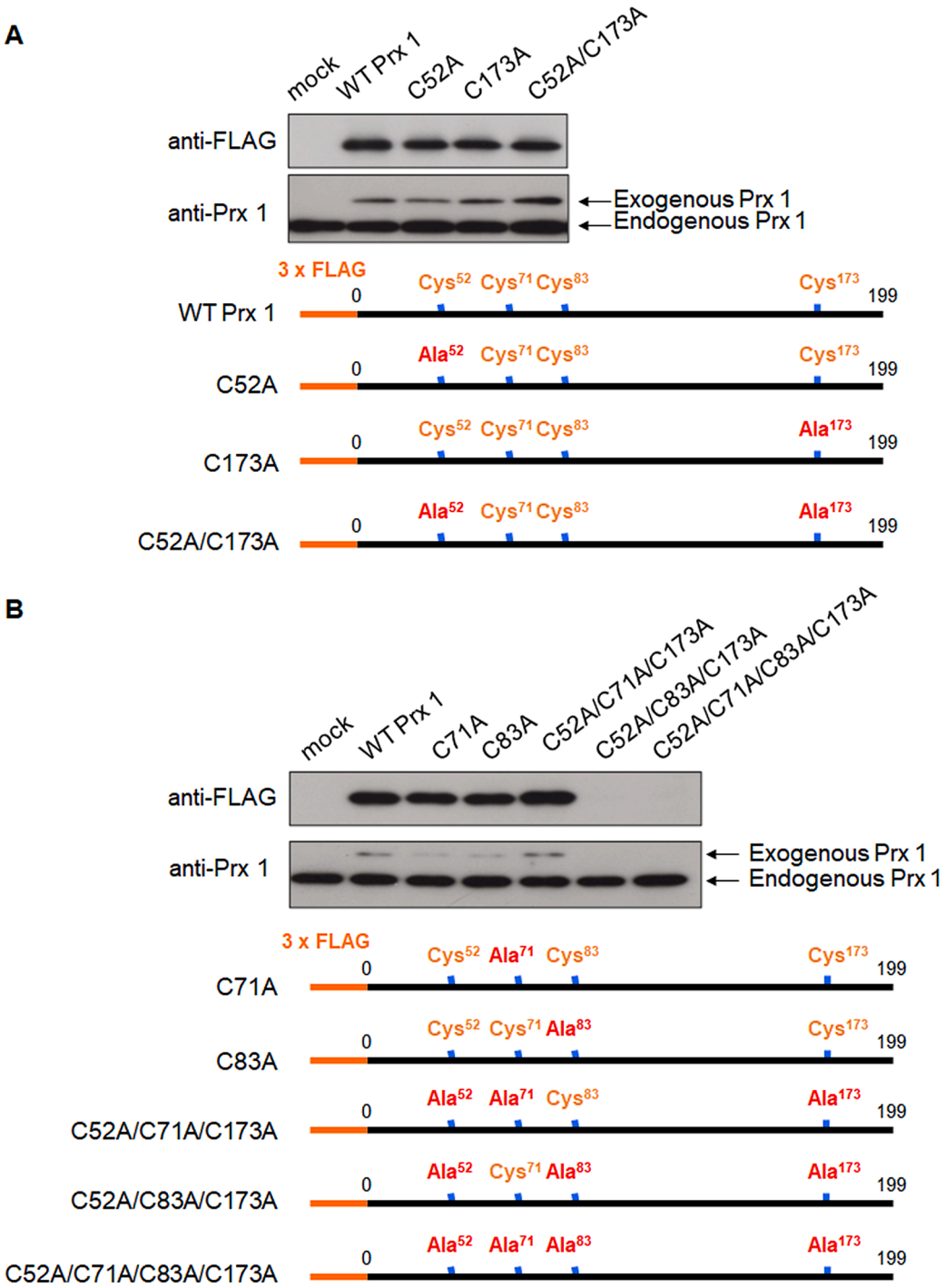


Fig. 7. Binding experiments of Bio-EpoC12 with point-mutated Prx 1s. HEK 293 T cells were transfected with the indicated expression vector. Cells were lysed after 24 h and treated with 1 μM Bio-Epo-C12 for 20 h; to precipitate the Bio-Epo-C12 complexes, samples were further incubated with streptavidin resin for 2 h. The proteins were resolved by SDS-PAGE. Endogenous and exogenous Prx 1s was detected by Western blot with anti-FLAG tag and anti-Prx 1 antibodies.

Double mutant (C52A/173A) was constructed by introducing the C173A mutant to the C52A singlet. Briefly, this mutant was constructed by employing the similar mutagenesis procedure using the following forward and reverse mutagenic primers.

C52A/C173A forward primer: 5′-ACTGACAAA-CATGGGGAAGTGGCCCCAGCTGGCTGGAAA-3′
C52A/C173A reverse primer: 5′- AATGATCTCCGTGGGGGCCA-CAAAGGTGAAGTCAAGAGG-3′

Similarly, C52A/C71A/C173A and C52A/C83A/C173A triple mutants were constructed by introducing C71A and C83A to the C52A/C173 doublet, respectively. C52A/C71A/C83A/C173A quadruple mutant was constructed by introducing C71A to the C52A/C83A/C173 triplet.

4.10. Transfection

HEK 293 T cells were cultured in DMEM/F-12 at 37 °C, 5% CO_2, in humid atmosphere. The cells were transfected in 6-well plates using

FuGENE 6[37] according to the manufacturer's protocol. After incubation at 37 °C for 24 h, the cells were washed with PBS. The cells were dissociated as clumps, and a part of the solution containing cell clumps was dissociated into single cells with 0.25% trypsin-EDTA. The cells were collected by centrifugation and washed with PBS twice.

4.11. Bio-Epo-C12 binding of point-mutated Prx 1s (Fig. 7)

Transfected HEK 293 T cells were washed three times with cold PBS and then treated with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, pH 7.5). Lysed cells were centrifuged at 20,800*g* for 5 min at 4 °C, and the supernatant was collected as the cell lysate. The protein concentration of the lysate was adjusted at 1 mg/mL after determination by bicinchoninic acid (BCA) protein assay.[38] The lysate (1 mL) was incubated with Bio-Epo-C12 (1 μM) at 4 °C for 20 h. Streptavidin agarose beads (100 μL) were added to the lysate, and incubated at 4 °C for 2 h. After the beads were washed with lysis buffer three times, the proteins were eluted by the addition of 50 μL of sample buffer (62.5 mM Tris–HCl, 1% SDS, 5% glycerol, 3% 2-mercaptoethanol, 0.05% crystal violet), followed by boiling the samples at 90 °C. The beads were separated by centrifuging at 20,800*g* for 10 min at 4 °C, and the supernatants were collected as the eluted samples. Eluates were analyzed by SDS–PAGE and blotted onto PDVF membranes. The membranes were blocked for 0.5 h at 4 °C with 5% skim milk/TBST. Proteins were detected by incubating the membranes with the primary antibody at 4 °C overnight (anti-FLAG 1:10000 and anti-Prx 1 1:1000) followed by incubation with HRP-conjugated secondary antibodies (anti-mouse IgG 1:2000 and anti-rabbit IgG 1:2000) at room temperature for 1 h. Chemiluminescence detection was performed using ECL Plus Western blotting detection reagents. Chemiluminescent signals were captured on X-ray film (Hyperfilm Amersham, Piscataway, NJ, USA).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116203.

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