Expression of Ascorbate Peroxidase Derived from *Cyanidioschyzon merolae* in Mammalian Cells

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Abstract. *Background/Aim: Ascorbate peroxidase (APX) derived from Cyanidioschyzon merolae, a primitive red alga living in high temperature and acidic environments, has greater anti-oxidative capacity than similar peroxidases occurring in other plants. In the present study, we examined whether expression of Cyanidioschyzon merolae-derived APX (cAPX) in mammalian cells increases cellular anti-oxidative capacity. Materials and Methods: The cAPX gene was introduced into the mouse fibroblast-like cell line C3H10T1/2. Production of reactive oxygen species (ROS) and/or cell viability was assessed after heat, H_2O_2 and acid stimulation. Results: Heat and H_2O_2 stimulation resulted in ROS production. cAPX-expressing cells were more tolerant to oxidative stress induced by heat, H_2O_2 and acid stimulations than control cells lacking cAPX. Conclusion: Introduction of cAPX increases the anti-oxidative capacity in mammalian cells.*

High levels of reactive oxygen species (ROS) are produced under excessive stress and pathological conditions (1, 2). For example, heat stress increases mitochondrial superoxide levels (3, 4) whilst acid stress induces intracellular acidification and impairment of mitochondrial function. Together, this leads to ROS production (5, 6). ROS promotes autophagy, apoptosis and necrosis (7). Previous studies

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Key Words: Ascorbate peroxidase, Cyanidioschyzon merolae, fibroblast, anti-oxidative stress, reactive oxygen species.

report that excess ROS causes widespread damage to biological macromolecules. ROS induced damage promotes cell death and apoptosis related processes such as increased DNA double-strand breaks, increased histone H2AX phosphorylation, increased caspase-3 and an elevation in the BAX/Bcl-2 ratio (6, 8-10).

In order to protect against excess ROS, various antioxidant systems are physiologically present in cells and tissues. The general endogenous antioxidant system consists of antioxidant enzymes, hydrophilic antioxidants and lipophilic radical antioxidants (7). The antioxidant enzyme superoxide dismutase catalyzes the conversion of superoxide into H_2O_2. Catalase and glutathione peroxidase catalyze H_2O_2 removal (7). In addition, green plants and other photosynthetic protists have ascorbate peroxidase (APX) which catalyzes the reduction of H_2O_2 to water using ascorbic acid as the specific electron donor (3, 11). However, ROS frequently exceeds the capacity of the cellular antioxidant systems and leads to tissue damage during inflammation, injury and cancer (1, 2).

The primitive red alga *Cyanidioschyzon merolae* (*C. merolae*) is a small unicellular organism which inhabits extreme high temperature and acidic environments (42°C, pH 2.5) (12, 13). In an earlier study, *C. merolae* was shown to be more tolerant to methylviologen, a ROS generator, than *Arabidopsis thaliana* (*A. thaliana*), a popular model organism in plant biology and genetics (10, 12). Furthermore, *C. merolae*-derived APX (cAPX) has been shown to have higher ROS-scavenging capacity than *A. thaliana*-derived APX under high-temperature stress (12). These reports indicate that cAPX exerts higher anti-oxidative ability than APXs of other organisms. Therefore, we hypothesized that ectopic expression of cAPX in mammalian cells, in addition to the general endogenous antioxidants, could protect against oxidative stress-induced cell damage.

The purpose of this study was to generate a mammalian cell line with high anti-oxidative capacity by expressing the
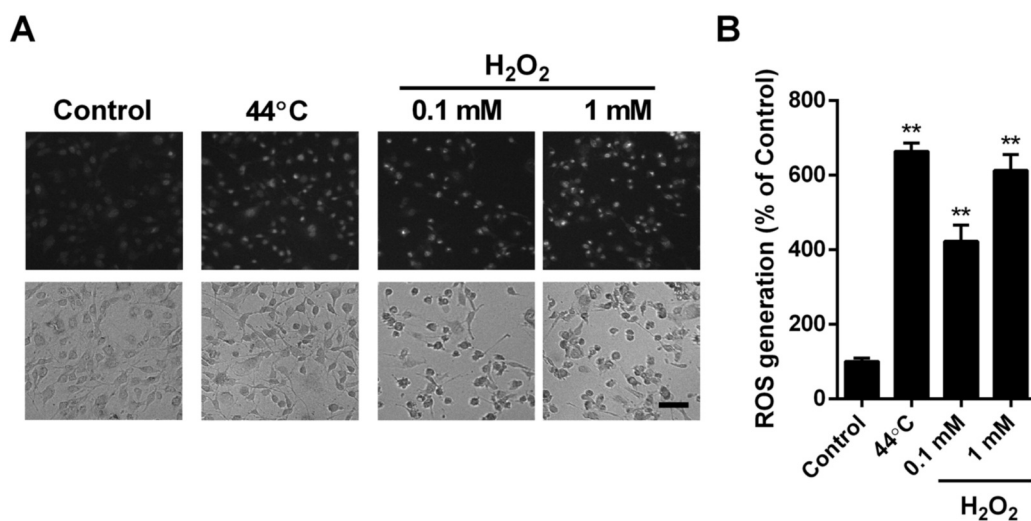
Figure 1. *Production of reactive oxygen species (ROS) in C3H10T1/2 cells. (A) Representative images of ROS generation after heat stimulation (44°C) and 0.1 or 1 mM H_2O_2 application for 2 h. Upper: Dark field; Lower: Bright field. Scale bar is 50 μm. (B) Percentage change in ROS generation in C3H10T1/2 cells after heat stimulation (44°C) and 0.1 or 1 mM H_2O_2 treatment for 2 h. **p<0.01 compared with Control; Dunnett's multiple comparisons test following one-way ANOVA.*

cAPX gene and to further explore the function of cAPX activity. The cAPX gene was introduced into the mouse fibroblast-like cell line C3H10T1/2 and the protective functions of the cAPX against ROS induced by heat, H_2O_2 or acid stimulations were evaluated.

Materials and Methods

Construction of the expression vector. The coding region of cAPX (NM_005536994) was obtained by PCR amplification of *C. merolae* genomic DNA using PrimeSTAR HS DNA polymerase (TaKaRa, Ohtsu, Japan) and cloned into a pcDNA 3.1 D/V5-His-TOPO (cAPX-V5-His) or pcDNA3.1(+) vector [cAPX-pcDNA3.1(+)]. All constructs were confirmed by sequencing. Genomic DNA of *C. merolae* was kindly provided by Dr. Osamu Misumi (Yamaguchi University). Myc-cAPX-IRES-EGFP vector was obtained by inserting the sequences of Myc, IRES and EGFP into cAPX-pcDNA3.1(+). Myc was subcloned from Myc-TLE3 (9). IRES and EGFP were subcloned from pCMV-IRES-GFP (Addgene; Watertown, MA, USA).

Cell culture, transfection and selection of G418 resistant clones. C3H10T1/2 murine embryonic fibroblasts and COS-7 African green monkey fibroblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured with Dulbecco's Modified Eagle's Medium (DMEM; FUJIFILM Wako Pure Chemical, Osaka, Japan) containing 10% fetal bovine serum (FBS; Nichirei, Tokyo, Japan) (14). C3H10T1/2 and COS-7 cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instruction. C3H10T1/2 cells transfected with pcDNA-cAPX-V5-His or pcDNA-V5-His empty vector were treated with G418 (Roche, Basel, Switzerland) for 2 weeks until G418-resistant clones emerged.

ROS measurement. C3H10T1/2 cells were seeded in 96-well plates at a density of 10,000 cells per well. ROS measurement was performed with a ROS Detection Cell-Based Assay Kit (Cayman Chemical, An Arbor, MI, USA). Briefly, cells were incubated for 2 h in a humidified 5% CO_2 chamber with ROS staining buffer at 37°C or 44°C and with ROS staining buffer containing 0.1 mM or 1 mM H_2O_2 at 37°C. Next, the ROS staining buffer was aspirated and replaced with cell-based assay buffer. Cells were observed with a fluorescence microscope (IX71, Olympus, Tokyo, Japan) with filter sets capable of measuring at an excitation wavelength between 480-520 nm and an emission wavelength between 570-600 nm. Fluorescence intensities were determined per well by subtracting the mean fluorescence of the background from that of the selected 20-30 cells. The assay was performed in triplicate.

Cell viability assay. Cytotoxicity of H_2O_2, low pH and heat were examined using a cell counting kit-8 (Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 5,000 cells per well and incubated with DMEM at 37°C. For heat stimulation, the cells were incubated at 44°C or 37°C (control) in a humidified 5% CO_2 chamber for 2 h. For acid stimulation, the cells were incubated at HCl-adjusted pH 3, 5 and 7 (control) at 37°C for 5 min. According to manufacturer recommendations, cell viability was calculated as the percentage of absorbance (450 nm) against the control treatment. The assay was performed in triplicate.

Western blot analysis. The following antibodies were used for western blot analysis: anti-V5-tag mouse monoclonal antibody (M167-3) (MBL, Aichi, Japan), anti-Myc-tag rabbit polyclonal antibody (562) (MBL), and anti-β-Actin mouse monoclonal (A1978) (Sigma-Aldlich, St. Louis, MO, USA). The target proteins were detected using an anti-mouse or anti-rabbit IgG antibody conjugated with a horseradish peroxidase (Cell Signaling, Beverly, MA, USA) and visualized using ImmunoStar LD (FUJIFILM Wako Pure Chemical).

Cell staining. To visualize C3H10T1/2 cell nuclei, cells were mounted in Hard Set Mounting Medium with DAPI (Vector laboratories, Burlingame, CA, USA). To visualize the cytoskeleton, cells were stained with Rhodamine Phalloidin (Thermo Fisher Scientific). Fluorescence was observed with a fluorescence microscope (BZ-9000, Keyence, Tokyo, Japan) equipped with the appropriate filters.

Statistical analysis. Data are presented as the mean±SEM. An unpaired Student's *t*-test was used to compare differences between two different groups. Dunnett's *post hoc* test following one-way AVOVA was used to analyze three or more groups. Significance was determined at $p<0.05$.

Results

Compared to control cells incubated at 37˚C, application of heat stress (44˚C) or H_2O_2 increased ROS generation in C3H10T1/2 cells (Figure 1A). The ROS levels after heat and H_2O_2 application were significantly higher than that of control cells (Figure 1B).

C. merolae is a small unicellular organism (Figure 2A). cAPX an intronless gene, was amplified from genomic DNA by PCR and transfected into C3H10T1/2 mouse fibroblast cells. The size of the amplified PCR product matched the predicted size (1315 bp) of the cAPX coding sequence (Figure 2B). The cAPX coding sequence was then inserted into a mammalian expression plasmid (Figure 2C). After transfection of cAPX gene into C3H10T1/2 cells, the cAPX protein was detected by western blotting analysis using the anti-V5 antibody (Figure 2D). No apparent differences in cell morphology were observed between cAPX-expressing cells and control cells (Figure 2E). Cell proliferation of cAPX-expressing cells and control cells were similar at all observed time points (Figure 2F).

To determine the anti-oxidative capacity of cAPX-expressing C3H10T1/2 cells, we measured cell viability following oxidative stress induced by heat, H_2O_2 and acid. Compared with the control 37˚C condition, viability of control non-transfected cells was decreased at 44˚C (Figure 3A). However, cAPX-expressing cells remained viable at 44˚C with significantly higher cell viability compared to control cells (Figure 3A). The endogenous antioxidant glutathione has been reported to exert anti-oxidative action at high temperatures (15). However, addition of glutathione into culture medium did not change cell viability of either the control or cAPX-expressing cells at 44˚C (Figure 3A). H_2O_2 treatment for 2 h decreased viability of both, control and cAPX-expressing cells in a dose-dependent manner. Compared with the control cells, cAPX-expressing cells had higher cell viability when treated with 0.1 mM H_2O_2 (Figure 3B). Extracellular acidification at pH 3 for 5 min decreased cell viability of both, control cells and cAPX-expressing cells (pH 7) (Figure 3C). The cAPX-expressing cells showed higher viability compared to control cells at pH 3 (Figure 3C).

To utilize cAPX-expressing cells for future research work with human cells, we created a cell labeling and tracing system. An IRES-GFP was placed downstream of Myc-
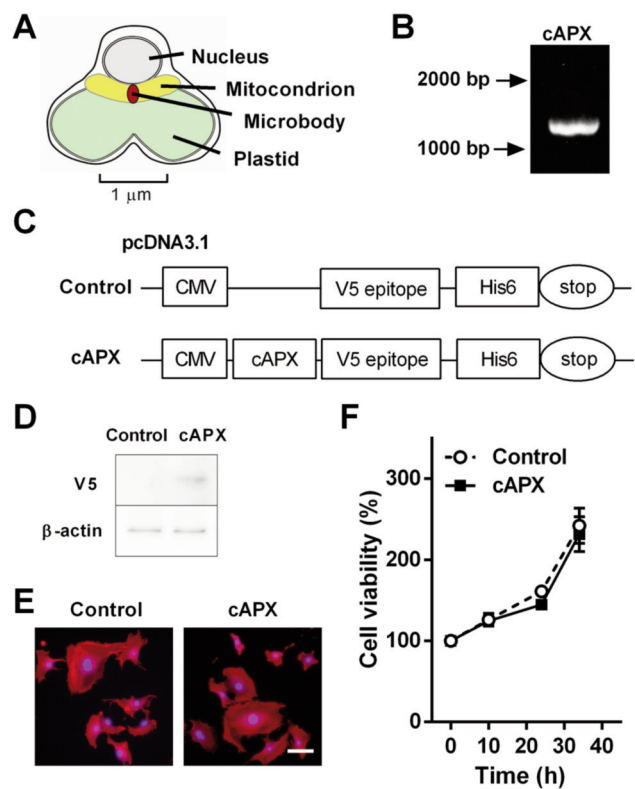

Figure 2. *Cyanidioschyzon merolae (C. merolae)-derived ascorbate peroxidase (cAPX) was introduced into C3H10T1/2 cells. (A) Schematic of the unicellular red alga C. merolae is shown. (B) The coding sequence of cAPX was amplified by PCR from C. merolae genomic DNA. (C) Schematics of the structure of CMV-V5-His plasmid (Control) and cAPX-V5-His plasmid (cAPX). (D) Protein levels of cAPX-V5-His and β-actin in C3H10T1/2 cells stably expressing cAPX-V5-His (cAPX) or empty vector (Control) were determined by western blotting analysis. (E) Cell morphology of empty vector (Control) or C3H10T1/2 cells stably expressing cAPX-V5-His (cAPX) are shown. Scale bar is 10 μm. (F) Cell viability of empty (Control) or C3H10T1/2 cells stably-expressing cAPX-V5-His (cAPX) was assessed.*

cAPX in the pcDNA3.1 expression vector (Figure 4A). Confirmation of cell transfection with this plasmid was performed by western blotting using anti-Myc antibody (Figure 4B) and by fluorescence microscopy (Figure 4C).

Discussion

The present study demonstrates that introduction of the APX gene of *C. merolae* into C3H10T1/2 cells led to a higher resistance against cell death induced by oxidative stress. Heat stimulation at 44˚C and H_2O_2 treatment caused intracellular ROS production and promoted cell death. Under oxidative stress with heat at 44˚C, acidic conditions (pH 3) and 1 mM H_2O_2 treatment, cell viabilities of the cAPX-expressing cells were higher than that of the control cells. The results suggest that cAPX enhances anti-oxidative capacity of mammalian
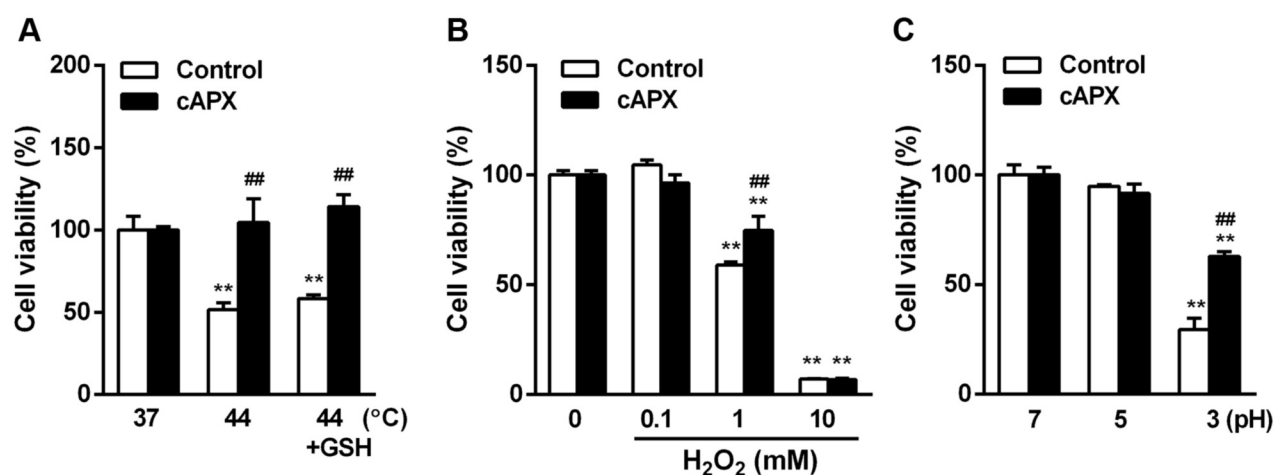
Figure 3. *Anti-oxidative effect of Cyanidioschyzon merolae (C. merolae)-derived ascorbate peroxidase (cAPX) in C3H10T1/2 cells. (A) Cell viabilities of cAPX-expressing and control cells cultured at 37°C, 44°C and 44°C+glutathione for 2 h. (B) Cell viabilities of cAPX-expressing and control cells after treatment with H_2O_2 for 2 h. (C) Cell viabilities of cAPX-expressing and control cells cultured at pH 3, 5 or 7 for 5 min. $^{##}p<0.01$ compared with Control; unpaired t-test; $^{**}p<0.01$ compared with 37°C, 0 mM or pH 7, respectively; Dunnett's multiple comparisons test following one-way ANOVA.*

cells. Although there was no difference in cell viability after 10 mM H_2O_2 treatment between control and cAPX-expressing cells, the oxidative stress at this concentration was perhaps too high for the anti-oxidative capacity of cAPX. In the future, introduction of cAPX could be a tool for the treatment of various medical conditions involving oxidative stress such as radiation burns, heat burns, ischemia, reperfusion, cancer or Alzheimer's disease (2).

APX is an antioxidant enzyme, which catalyzes the removal of H_2O_2. APX activity has been reported in green plants and photosynthetic protists but has not been reported in animals (3). H_2O_2 is generally neutralized by catalase and glutathione peroxidase using glutathione as a reductant in animals. In the present study, cAPX was successfully introduced into mammalian cells. Furthermore, the expression of cAPX reduced oxidative stress-induced cell death. This suggests that the introduction of exogenous cAPX removed ROS synergistically with endogenous catalase and glutathione in the mammalian cells.

Since APX-mediated anti-oxidative action requires ascorbate as an electron donor (11), APX is inactivated when ascorbate concentration is below 20 μM in plants (2). Hence, ascorbate is essential for the anti-oxidative ability of cAPX. In the present study, enhancement of anti-oxidative capacity of cells was observed by cAPX expression in the absence of any additional ascorbate supplementation to murine C3H10T1/2 cells. This might be because murine cells can synthesize ascorbate (14). Although most animals and plants can synthesize ascorbate from glucose (16), primates including human cannot produce ascorbate endogenously because of the absence of the gene encoding *l*-gulonolactone oxidase, an enzyme required for the last step in ascorbate
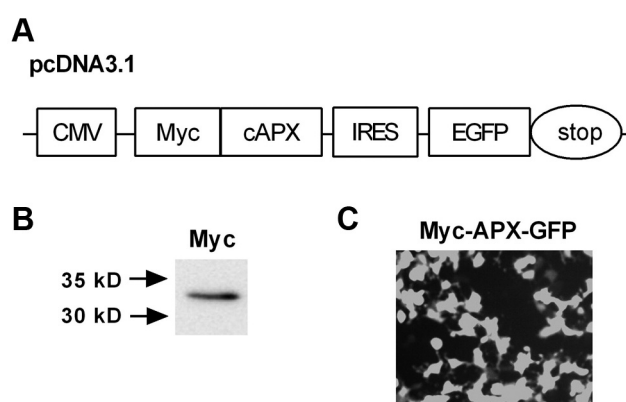

Figure 4. *Construction of plasmid to trace Cyanidioschyzon merolae-derived ascorbate peroxidase (cAPX)-expressing cells. (A) Schematic of the structure of Myc-cAPX-IRES-EGFP plasmid. (B) COS-7 cells were transiently transfected with Myc-cAPX-IRES-EGFP plasmid. Myc-cAPX proteins were detected by western blot analysis. (C) GFP was detected by fluorescence microscopy.*

synthesis. Ascorbate is essential for life in humans. Therefore, ascorbate is obtained as dietary vitamin C through cellular uptake *via* Na-dependent transporters (11, 17). Thus, consideration of ascorbate metabolism will be required if cAPX is expressed in human cells for clinical purposes.

Recently, as a cytotherapeutic approach for tissue injury in humans, cell sheet transplantation has been applied to esophagus, periodontal tissue, cornea, heart and lung injuries (7, 18). The cell sheets consist of autologous cells, allogenic cells or iPS-derived cells from various biological tissues and organs (7). Cell sheets can be used as tissue transplantation into regions of injury caused by disease or trauma. In the

present study, we labelled cAPX cells with GFP which could potentially allow cell tracing and identification in a future clinical setting. Addition of cAPX-expressing cells to cell sheets would enable greater resistance against oxidative stress.

Although further studies examining the anti-oxidative effects of cAPX in human cells and animal models are needed, in this study, we demonstrated that APX derived from *C. merolae* could increase heat and acid tolerance in mouse fibroblasts.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

Authors' Contributions

SH, SK and KO designed the project and experiments. SH, SK, KM, YS and IU performed the experiments. SH, KM and KO performed data analysis and statistical analysis. SH, SK, KM and KO wrote/revised the article. All Authors read and approved the final article.

Acknowledgements

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