Gene Ther Mol Biol Vol 11, 143-150, 2007

Transfection with glutathione-dependent dehydroascorbate reductase genes exerts cytoprotective effects against hydroperoxide-induced cell injury through vitamin C regeneration and oxidative-stress diminishment

Research Article

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Key words: glutathione-dependent dehydroascorbate reductase, dehydroascorbic acid, ascorbic acid, reactive oxygen species, oxidative stress, cell death

Abbreviations: 6-carboxy-2', 7'- dichlorodihydrofluorescein diacetate, di (acetoxymethyl ester), (CDCFH-DA); Chinese hamster ovary, (CHO); dehydroascorbic acid, (DehAsc); Dithiothreitol, (DTT); fluorescein isothiocyanate, (FITC); Glutathionee, (GSH); L-ascorbic acid, (Asc); MDA reductase, (MDAR); Monodehydroascorbate, (MDA); tert-butyl hydroperoxide, (t-BuOOH)

Received: 7 May 2007; Revised: 25 May 2007
Accepted: 29 May 2007; electronically published: July 2007

Summary

To evaluate the potential for utilization of overexpression of glutathione (GSH)-dependent dehydroascorbate (DehAsc) reductase (DHAR) as a tool to alleviate deleterious effects induced by an oxidative stress, the effect of overexpressed DHAR on tert-butylhydroperoxide (t-BuOOH)-induced cell injury was examined using DHAR gene (dhar)-transfected Chinese hamster ovary (CHO) cells. The transfected dhar products-derived DHAR was shown to be expressed all over the cytoplasm rather than in the nucleus, and repressed t-BuOOH-induced cell death, DNA strand cleavages, and intracellular reactive oxygen species (ROS)-generation in cells pretreated with DehAsc plus GSH isopropylester, but not without such pretreatment in contrast to no repression in their vector-transfected counterparts with such pretreatment. Upon the similar pretreatment, the intracellular levels of both ascorbic acid (Asc) and total vitamin C (Asc plus DehAsc) were 1.4- to 1.7-fold higher in dhar-transfected cells than in the vector-transfectants. Thus, our results suggest that overexpressed DHAR exerts cytoprotective effects against hydroperoxide-induced cell injury, and that more abundant intracellular Asc accumulation induced by expression of exogenous dhar may be involved in the mechanism.

I. Introduction

L-ascorbic acid (Asc) plays an important role in cellular defense mechanisms against oxidative stress. Asc not only scavenges a variety of aqueous reactive oxygen species (ROS) including superoxide anion radical, singlet oxygen, hydroxyl radical, and water-soluble peroxyl radicals (Halliwell and Gutteridge, 1990), but also takes part in the prevention of lipid peroxidation by reductive regeneration of alpha-tocopheroxyl radicals to alpha-tocopherol at the membrane interface (Niki, 1991). These antioxidant properties of Asc are based on its univalent or divalent oxidation **(Figure 1)**. The univalent oxidation of Asc produces the short-lived radical to Asc and dehydroascorbic acid (DehAsc). The divalent oxidation of Asc leads to the formation of DehAsc, which readily undergoes irreversible hydrolysis to 2, 3-

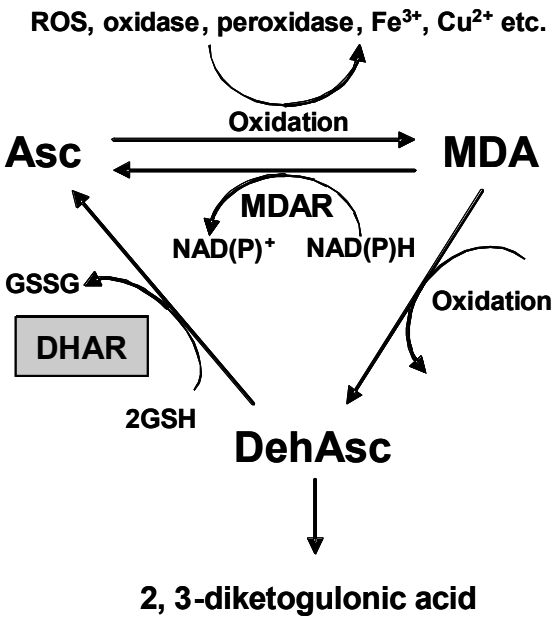

Figure 1. Metabolism of ascorbic acid in mammalian cells. Ascorbic acid (Asc) is oxidized to monodehydroascorbic acid (MDA) by various types of oxidative stress, such as ROS. MDA is reversibly converted to Asc by MDA reductase (MDAR), localized mainly in the outer mitochondrial membrane, microsomes, and the Golgi apparatus. MDA is further oxidized to dehydroascorbic acid (DehAsc), or, alternatively, nonenzymatically converted to Asc and DehAsc through a disproportionation reaction. DehAsc is easily and irreversibly hydrolyzed to 2, 3-diketogulonic acid unless reduced to Asc by DehAsc reductase (DHAR) using glutathione as the reductant.

diketogulonic acid. Although Asc and its oxidized forms are generally unstable in physiological environments, it is known that plants and animals possess an Asc regeneration system. Humans and other primates cannot synthesize Asc due to lack of L-gulono-1, 4-lactone oxidoreductase, which is required for the final step in Asc synthesis. Therefore, for humans and primates who can get Asc only from dietary sources, systems for regeneration of Asc from its oxidized forms would be quite important to protect cells from harmful ROS-induced oxidation.

The regeneration system for Asc mainly consists of two pathways associated with MDA or DehAsc reduction. MDA is converted to Asc through spontaneous disproportionation or enzymatic reduction by MDA reductase (MDAR), which is an NAD(P)H-dependent enzyme localized at subcellular membranes of mitochondria (Ito et al, 1981), microsomes, and the Golgi apparatus (Hara and Minakami, 1971; Green and O'Brien, 1973). On the other hand, DehAsc is converted to Asc through nonenzymatic reduction by glutathione (GSH) or diverse enzymes such as with glutaredoxin (Wells et al, 1990; Park and Levine, 1996), protein disulphide disulfide isomerase (Wells et al, 1990), 3α-hydroxysteroid dehydrogenase (Del et al, 1994) and thioredoxin reductase (May et al, 1997). Because of the slowness in the nonenzymatic reducing reaction, much attention has been directed to enzymatic reduction of DehAsc. Recently, a novel GSH-dependent dehydroascorbate reductase (DHAR) was purified from rat liver cytosol (Maellaro et al, 1994) and human red cells (Xu et al, 1996). Ishikawa et al. cloned the corresponding gene (dhar) from a rat liver cDNA library and achieved the functional expression of DHAR in Chinese hamster ovary (CHO) cells (Ishikawa et al, 1998). They demonstrated that the DHAR-expressing cells accumulated 1.7-fold more total vitamin C than nontransfected cells.

Based on these results, we speculated that overexpressed DHAR can suppress ROS generation and oxidative stress-induced cellular damages by increasing of intracellular Asc. In the present study, we used dhar-transfected CHO cells to investigate the repressive effects of DHAR on oxidative stress-induced cellular injuries resulting from the chemical oxidant tert-butyl hydroperoxide (t-BuOOH), an analogue of short-chain lipid hydroperoxide.

II. Materials and Methods

A. Cell culture

Rat liver glutathione-dependent dehydroascorbate reductase gene (dhar) in a pRC/CMV vector-transfected CHO cells (DHAR (+)) and the corresponding nontransfectant (transfected with pRc/CMV only) CHO cells (DHAR (-)) were obtained by electroporation and the subsequent colony selection (Ishikawa et al, 1998). DHAR (+) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FBS, GIBCO BRL), 50 units/mL penicillin, 50 μg/mL streptomycin, and 400 μg/mL geneticin disulfate (Wako Pure Chemical Industries, Osaka, Japan) at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. DHAR (-) cells were cultured in the same medium without geneticin disulfate.

B. Evaluation of cell-growth ratio

Cell-growth ratio was assessed based on mitochondrial enzymatic conversion of WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2.4-disulfophenyl)-2H-tetrazolium, sodium salt] (Dojindo, Kumamoto, Japan) to yellowish formazan, which is indicative of the number of viable cells. After t-BuOOH treatment, cells were rinsed with phenol red (PR)-free DMEM and then incubated for 3 hr in PR-free DMEM containing 10% WST-1 at 37 ºC. The absorbance at 450 nm was measured with a microplate reader (model 3550, Bio-Rad, Hercules, CA, USA).

C. Immunocytochemical staining of DHAR

Cells were grown on poly-L-lysine-coated chamber slides (ASAHI TECHNO GLASS, Chiba, Japan) and washed with phosphate-buffered saline (PBS) and fixed in a freshly prepared solution of 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were permeabilized in PBS containing 0.5% Triton X-100 for 5 min on ice, and then incubated with primary antibody against rat DHAR diluted 1 : 100 in PBS containing 1% bovine serum albumin for 1 hr at 37 ºC. The cells were then washed three times with PBS and incubated with a secondary antibody, fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG, for 30 min at 37 ºC. The slides were washed with PBS, mounted with anti-fading solution, and observed with a laser scanning confocal fluorescence microscope MRC-600 (Bio-Rad).

D. TUNEL staining

Apoptotic nuclei were detected in situ by the TUNEL (Terminal deoxyribonucleotidyl transferase-mediated dUTP nick

end labeling) assay with an In situ Apoptosis Detection Kit (Takara, Kyoto, Japan). Cells were grown on poly-L-lysine-coated chamber slides (ASAHI TECHNO GLASS). After t-BuOOH treatment, cells were washed three times with PBS and fixed in a freshly prepared solution of 4% paraformaldehyde in PBS for 30 min at room temperature. For blocking endogenous peroxide, cells were treated with methanol containing 0.3% hydrogen peroxide for 15 min at room temperature. Then, the cells were permeabilized with permeabilization buffer for 5 min on ice. To label DNA strand cleavage termini, cells were incubated with TUNEL reaction mixture containing TdT and FITC-labeled dUTP in the binding buffer and incubated for 90 min at 37 ºC in a humidified atmosphere. Thereafter, the slides were washed three times with PBS and mounted with anti-fading solution. The slides were observed with a laser scanning confocal fluorescence microscope ECLIPSE E600 (Nikon, Tokyo, Japan), and pseudo-color images were produced using AQUACOSMOS software (HAMAMATSU Photonics, Shizuoka, Japan). All of the TUNEL stains were done at the same time and photographed under the same conditions.

E. Measurement of intracellular ROS

Intracellular ROS production was determined based on oxidative conversion of 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate, di (acetoxymethyl ester) (CDCFH-DA) (Molecular Probes, Eugene, OR, USA) to CDCF, which is indicative of the amount of intracellular peroxide production. Cells were rinsed with PR-free DMEM and incubated for 45 min in PR-free DMEM containing 100 μM CDCFH-DA at 37ºC. After they were rinsed with PR-free DMEM again, the cells were treated with t-BuOOH. Then, the cells were rinsed three times with PR-free DMEM and the fluorescence intensity was measured with a fluorescence microplate reader (CytoFluor 2350, Millipore, Bedford, MA, USA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively. Finally, the cells were observed with a fluorescence microscope ECLIPSE E600 (Nikon).

F. Determination of intracellular Asc and total Asc

Cells were washed with PBS and collected by trypsinization. The cell number was measured using a particle analyzer CDA-500 (Sysmex, Hyogo, Japan). Then, the cells were centrifuged at 500×g for 5 min at 4 ºC and resuspended in HPLC mobile phase (99% 0.1 M KH_2PO_4-H_3PO_4-1% methanol buffer (pH 2.5) containing 50 mg/L octanesulfonic acid and 5 mg/L EDTA-2Na) containing 50 μM dithiothreitol (DTT). The cells were lysed by sonication and the freeze-thaw method, the lysate was centrifuged at 500×g for 5 min at 4 ºC and the supernatant was collected. After removal of proteins with an ULTRA FILTER UNIT USY-1 (ADVANTEC, Tokyo, Japan), a 20 μL aliquot was injected on an Eicompak SC-5DS column of 3.0×150 mm (Eicom, Kyoto, Japan) connected to an HPLC pump (Shimazu LC-10AT ; Shimazu; Kyoto, Japan), and developed with a mobile phase at a flow rate of 0.5 mL/min at 25 ºC. Asc was detected with an amperometric electrochemical detector (ECD) Eicom ECD-300 (Eicom) operated at 600 mV. For the measurement of total Asc, DTT was added to an aliquot of the same sample for Asc measurement (final 12.5 mM) for reduction of DehAsc to Asc. Standard Asc solutions of 0-500 nM were prepared in the HPLC buffer and freshly diluted just before use.

G. Statistical analysis

The unpaired Student's t-test was used to evaluate the significance of differences between groups, and the criterion of statistical significance was taken as $P < 0.05$.

III. Results

A. Expression and distribution of DHAR in dhar-transfected DHAR (+) cells

To confirm the expression of DHAR proteins, we conducted immunocytochemical staining and observed the distribution of DHAR. In DHAR (+) cells, DHAR was abundantly expressed and widely scattered in the cytoplasm, but not present in the nucleus **(Figure 2)**. In contrast, DHAR was scarcely detected in DHAR (-) cells.

B. Preventive effect of DHAR on t-BuOOH-induced cell death

The effect of DHAR overexpression and administration with DehAsc and/or GSH-iPr on cells exposed to t-BuOOH was examined by the WST-1 assay. DehAsc is transported into cells via glucose transporters (Vera et al, 1993; Welch et al, 1995; Wilson, 2005), and GSH-iPr readily diffuses into cells and is hydrolyzed by intracellular esterases to GSH. The cell-growth ratio of both DHAR (+) and DHAR (-) cells was notably decreased in a dose-dependent manner by treatment with t-BuOOH (0, 150, or 200 μM) for 27 hr **(Figure 3)**. Thus, the decrease of cell-growth ratio was not alleviated only by overexpressin of DHAR without supplied enzymatic substrates. And the recovery of cell-growth ratio was not observed by previous administration with either DehAsc or GSH-iPr alone in DHAR (-) cells, either. However, previous administration with DehAsc and GSH-iPr significantly attenuated the decrease of cell-growth ratio in DHAR (+) cells as compared with DHAR (-) cells.

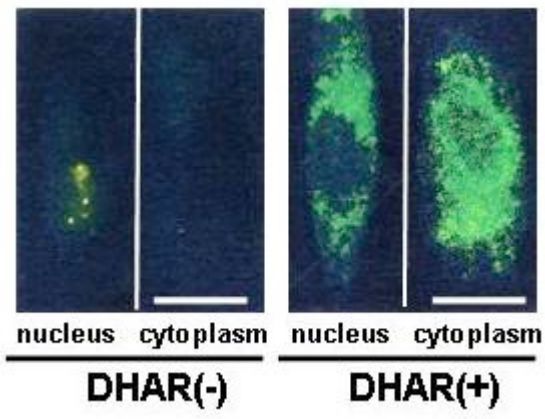

Figure 2. Immunocytochemical detection of DHAR in dhar-transfected (DHAR (+)) or non-transfected (DHAR (-)) Chinese hamster ovary (CHO) cells. Cells were plated at a density of 1.0 x 10^4 cell/cm^2 on a chamber slide. After preincubation for 18-21 hr, the cells were subjected to immunocytochemical staining, and the FITC-derived fluorescence was detected by fluorescence microscopy. The "nucleus"- and "cytoplasm"- photographs were obtained by focusing on the nucleus and cytoplasmic areas, respectively. The scale bar indicates 10 μm.

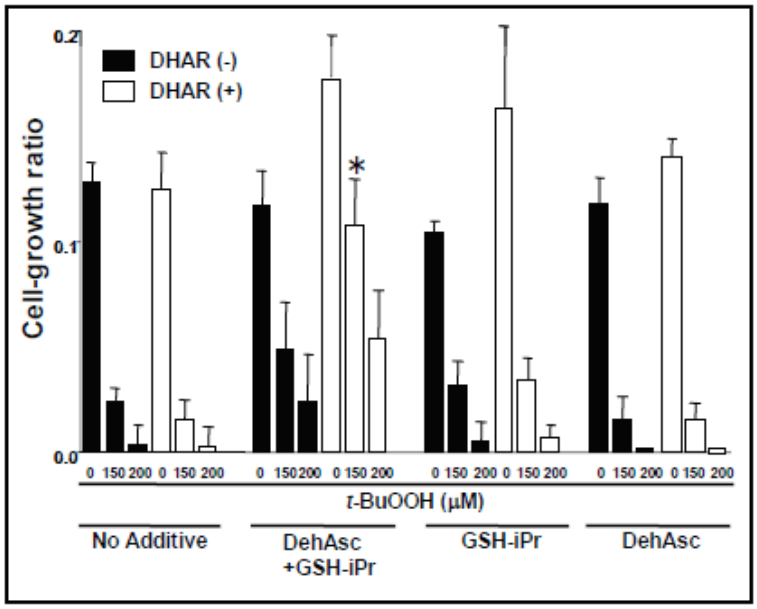

Figure 3. Preventive effect of dhar transfection and administration with DehAsc and/or GSH-iPr as DHAR substrates on t-BuOOH-induced cell death. DHAR (+) or DHAR (-) cells were seeded at a density of 1.0×10^4 cells/cm^2 in 24-well plates, and preincubated for 21 hr. The cells were treated with 100 μM DehAsc and/or 50 μM GSH-iPr for 2 hr, and then treated with various concentrations of t-BuOOH (0, 150, or 200 μM) for 27 hr. Cell-growth ratio was assessed by the WST-1 assay. The bar represents the S.D. of triplicate wells. Significantly different from DHAR (-) cells: *$P < 0.05$.

Therefore, administration with DehAsc plus GSH-iPr induced a cytoprotective effect against the cytotoxic response to t-BuOOH, and this effect was more notable in DHAR (+) cells than in DHAR (-) cells.

C. Inhibitory effect of administration with DehAsc plus GSH-iPr on t-BuOOH-induced nuclear DNA strand cleavages in DHAR (+) cells

The effect of administration with DehAsc plus GSH-iPr on t-BuOOH-induced nuclear DNA strand cleavages was investigated by the TUNEL staining assay. Cells that were treated with t-BuOOH showed an intensely red pseudo-color corresponding to brightly fluorescent staining in the nuclei **(Figure 4A, center)** and an increase in the frequency of DNA cleavages **(Figure 4B, center)**, indicating the incorporation of fluorescein dUTP onto nicked DNA strand terminals. The nuclear DNA strand cleavages were strongly suppressed by administration with DehAsc plus GSH-iPr, but not in their absence **(Figure 4A, B)**. These results suggest that administration with DehAsc plus GSH-iPr exerted protective effects against t-BuOOH-induced nuclear DNA strand cleavages in DHAR (+) cells.

D. Preventive effect of DHAR and administration with DehAsc plus GSH-iPr on t-BuOOH-induced intracellular oxidative stress

To examine whether DHAR and administration with DehAsc plus GSH-iPr could prevent t-BuOOH-induced intracellular ROS production, we quantified the intracellular ROS levels using fluorometry and the fluorescein derivative CDCFH-DA as a redox indicator.

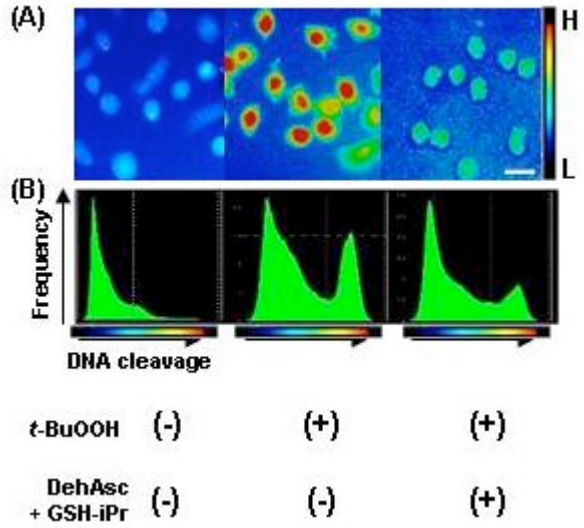

Figure 4. Preventive effect of dhar transfection and administration with DehAsc plus GSH-iPr as DHAR substrates on t-BuOOH-induced nuclear DNA strand cleavages in DHAR (+) cells. (**A**) Cells were seeded as in Figure 2 and preincubated for 21 hr. The cells were treated with 100 μM DehAsc plus 50 μM GSH-iPr for 2 hr before exposure to t-BuOOH at 400 μM for 1 hr, the maximum period for DNA cleavages, followed by TUNEL stain and the subsequent fluorography. (**B**) The histograms represent the fluorescence distribution, which reflects the degree of DNA cleavages and its frequency. The scale bar indicates 15 μm.

After permeation into cells, CDCFH-DA is esterolyzed to

CDCFH, being made membrane-impermeable, and oxidized to highly fluorescent CDCF primarily by H_2O_2, hydroperoxides, and diverse peroxides (Sejda et al, 1984). Treatment with t-BuOOH significantly increased the intracellular ROS levels in both DHAR (+) and DHAR (-) cells **(Figure 5(A))**. However, previous administration with DehAsc plus GSH-iPr markedly suppressed the increase in intracellular ROS in DHAR (+) cells, but not in DHAR (-) cells. Similar results were also observed using fluorescence microscopy **(Figure 5(B))**. Previous administration with DehAsc plus GSH-iPr strongly inhibited the irradiance of intracellular fluorescence from the redox indicator CDCFH.

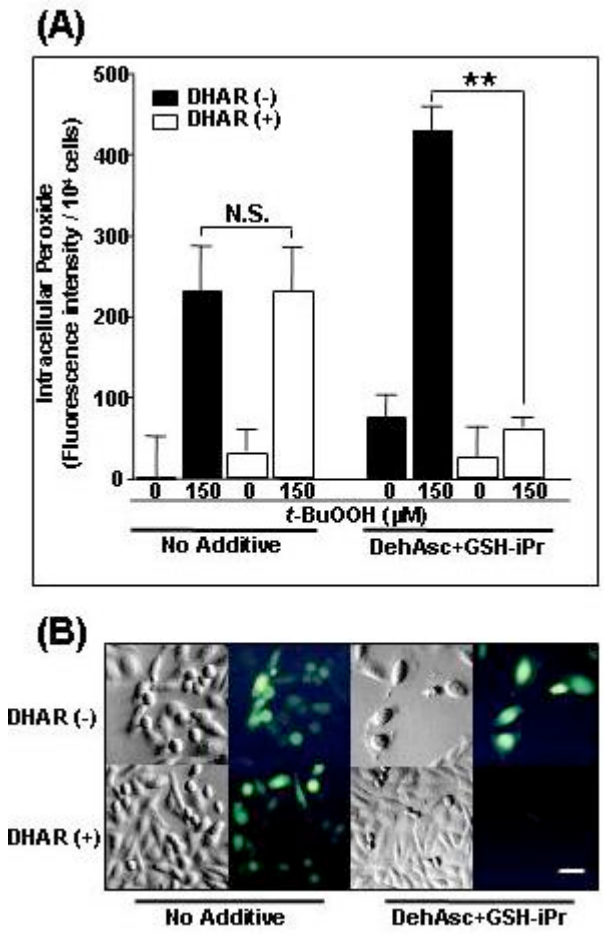

Figure 5. Preventive effect of dhar-transfection and administration with DehAsc plus GSH-iPr as DHAR substrates on t-BuOOH-induced intracellular reactive oxygen species (ROS) production.(**A**) Cells were seeded as in Figure 3 and preincubated for 18 hr. The cells were treated with 100 μM DehAsc plus 50 μM GSH-iPr for 2 hr, and then incubated with CDCFH-DA for 45 min. Thereafter, the cells were incubated with or without 150 μM t-BuOOH for 0.5 hr, and intracellular fluorescence was measured with a fluorescence microplate reader (ex: 485 nm; em: 530 nm). The bar represents the S.D. of triplicate wells. Significantly different from 0-μM t-BuOOH group with respective treatment: *P < 0.01; **P < 0.01; N.S.: not significant. (**B**) Cells were treated in the same manner as in (**A**). After t-BuOOH exposure, the fluorescence intensity was detected by fluorescence microscopy. The scale bar indicates 15 μm.

E. Accumulation of intracellular vitamin C in DHAR (+) cells

To examine the changes of intracellular total vitamin C (Asc plus DehAsc) and Asc levels after administration with DehAsc plus GSH-iPr, we quantified the intracellular amounts of both total vitamin C and Asc by HPLC and amperometric ECD detection. Intracellular levels of both total vitamin C and Asc increased for 2 hr after administration with DehAsc plus GSH-iPr in either DHAR (+) or DHAR (-) cells **(Figures 6(A), (B))**. The intracellular amounts of both total vitamin C and Asc in DHAR (+) cells were approximately 1.5- to 1.7-fold higher than those in DHAR (-) cells, respectively. These results suggested that the capacities to accumulate total vitamin C and Asc in DHAR (+) cells were superior to those in DHAR (-) cells.

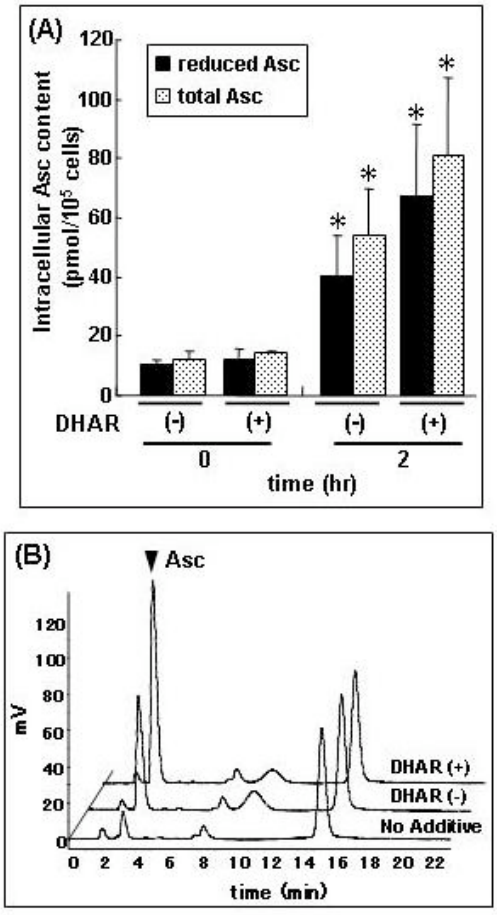

Figure 6. Intracellular total vitamin C (Asc + DehAsc) and Asc levels after administration with DehAsc plus GSH-iPr in DHAR (+) and DHAR (-) cells. (**A**) Cells were seeded at a density of $1.0x10^4$ cells/cm^2 on a 60-mm dish and preincubated for 18 hr. Cells were treated with 100 μM DehAsc plus 50 μM GSH-iPr for 0 or 2 hr, and then intracellular total vitamin C and Asc were measured by HPLC and amperometric ECD detection. Error bars are S.D. of 4 dishes. Significantly different from 0 hr: *P < 0.05. (**B**) Typical chromatograms of no additive (0 hr in DHAR (+) cells), DHAR(-) (2hr in DHAR(-) cells), and DHAR(+) (2hr in DHAR(+) cells) are shown. The first and second peaks at approximately 3 and 15 min are assigned to Asc and DTT, respectively.

IV. Discussion

In the present study, we paid attention to DHAR, which enables recycling of Asc by reduction of DehAsc to Asc, and tried to determine the possibility of utilization of overexpressed DHAR as a tool to alleviate deleterious effects induced by an oxidative stress. Our results showed that t-BuOOH-induced cell death was suppressed only by administration with DehAsc plus GSH-iPr in DHAR (+) cells **(Figure 3)**, and not by administration with either DehAsc or GSH-iPr alone. Thus, coexistence of both DehAsc and the GSH derivative is necessary to exert protective effects against oxidative stress in DHAR (+) cells. These results suggest that the cytoprotective effects are due to the reductive reaction by DHAR, which reduces DehAsc to Asc by using GSH as the reductive cofactor. Our results also showed that t-BuOOH-induced DNA strand cleavages, and ROS generation were suppressed by administration with DehAsc plus GSH-iPr in DHAR (+) cells **(Figures 4, 5)**. Because it is known that excessive ROS induces DNA strand cleavages and subsequently cell death (Saitoh et al, 2003; Saitoh and Miwa, 2004), the suppression of ROS generation in DHAR (+) cells seems to be a most critical point for the prevention of cell death.

Our results also demonstrated that the intracellular amounts of both Asc and total vitamin C (Asc plus DehAsc) were 1.4- to 1.7-fold higher in DHAR (+) cells than DHAR (-) cells upon administration with DehAsc plus GSH-iPr **(Figures 6)**. It was previously demonstrated that DHAR-transfected CHO cells could accumulate a 1.7-fold higher amount of total vitamin C compared with that of nontransfected cells (Ishikawa et al, 1998), but it was not clear about intracellular amounts of Asc. Asc, one of the major intracellular water-soluble antioxidant substances (Halliwell and Gutteridge, 1990), promptly scavenges ROS at an initial stage of ROS generation. Plasma lipoproteins exposed to aqueous peroxy radicals undergo no hydroperoxidation until depletion of endogenous Asc, which is consumed more rapidly and earlier than other plasma ROS-scavengers such as SH groups, alpha-tocopherol, bilirubin and urate, suggesting that Asc efficiently protects biomolecules, including lipids, from oxidative damages (Frei et al, 1984). Moreover, it is also well-known that Asc is transported into cells and accumulated to high concentrations (Washko et al, 1990; Welch et al, 1995; Saitoh et al, 1997). Therefore, Asc plays an important role for the protection of cellular components from oxidative stress-induced injury, and the enhancement of intracellular amounts of Asc was quite important for cytoprotection against oxidative stress. Since the decrease of cell-growth ratio in DHAR (+) cells was significantly attenuated as compared with that in DHAR (-) cells **(Figure 3)**, we supposed that the enhancement of intracellular Asc accumulation by overexpressed DHAR was correlated with the intracellular antioxidant potential and suppressed the ROS generation, resulting in decreased DNA strand cleavages and cell death. On the other hand, our result implied the presence of endogenous DHAR in CHO cells, because Asc was accumulated also in DHAR (-) cells. However, our data indicated that exogenous DHAR exerts the inherent enzymatic function similar to that of endogeous DHAR, because overexpressed DHAR was more effective against t-BuOOH-induced cell injury.

In summary, our results suggest that overexpressed DHAR exerts cytoprotective effects against hydroperoxide-induced cell injury, and that overexpressed DHAR-induced more abundant intracellular Asc accumulation may contribute its cytoprotective mechanism. Thus, DHAR is suggested to be one of the possible candidates for controlling of oxidative stress-induced cell injury.

Acknowledgments

The authors thank Dr. Norio Nagao, Mr. Liao Feng and Ms. Kikuko Yoshimitsu for their technical assistance. The present study was supported in part by Grant-in-Aid for Scientific Research, Basis Research (C), 17590064, in 2005-2007 to N.M from the Ministry of Education, Science, Sports and Culture, Japan.

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