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Cellular Defense against Singlet Oxygen-induced Oxidative Damage by Cytosolic $NADP^+$-dependent Isocitrate Dehydrogenase

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Singlet oxygen ($^1O_2$) is a highly reactive form of molecular oxygen that may harm living systems by oxidizing critical cellular macromolecules. Recently, we have shown that $NADP^+$-dependent isocitrate dehydrogenase is involved in the supply of NADPH needed for GSH production against cellular oxidative damage. In this study, we investigated the role of cytosolic form of $NADP^+$-dependent isocitrate dehydrogenase (IDPc) against singlet oxygen-induced cytotoxicity by comparing the relative degree of cellular responses in three different NIH3T3 cells with stable transfection with the cDNA for mouse IDPc in sense and antisense orientations, where IDPc activities were 2.3-fold higher and 39% lower, respectively, than that in the parental cells carrying the vector alone. Upon exposure to singlet oxygen generated from photoactivated dye, the cells with low levels of IDPc became more sensitive to cell killing. Lipid peroxidation, protein oxidation, oxidative DNA damage and intracellular peroxide generation were higher in the cell-line expressing the lower level of IDPc. However, the cells with the highly over-expressed IDPc exhibited enhanced resistance against singlet oxygen, compared to the control cells. The data indicate that IDPc plays an important role in cellular defense against singlet oxygen-induced oxidative injury.

Keywords: Singlet oxygen; NADPH; Isocitrate dehydrogenase; Reactive oxygen species; GSH recycling

INTRODUCTION

Singlet molecular oxygen ($^1O_2$), an electronically excited state of oxygen which results from the promotion of an electron to high energy orbitals, is produced in mammalian cells under normal and pathophysiological conditions.[1] The photodynamic action of some drugs and pigments is also mediated through singlet oxygen.[2] $^1O_2$ is a highly reactive form of molecular oxygen that may harm living systems by oxidizing critical cellular macromolecules, including lipids, nucleic acids and proteins, and it also promotes deleterious processes such as lipid peroxidation, membrane damage and cell death.[3] Biological systems have evolved to develop an effective and complicated network for defense mechanisms, to efficiently handle the harmful oxidative environments.[3–5] These defense mechanisms include non-enzymatic and enzymatic defenses. The non-enzymatic systems include reduced glutathione (GSH), ascorbic acid, α-tocopherol, uric acid and small peptide thioredoxin, while enzymatic defenses include cytosolic and mitochondrial superoxide dismutases (SODs), catalase and peroxidases.[6,7]

GSH is a well-known antioxidant which is usually present as the most abundant low-molecular-mass thiol in most organisms. It has various functions in the defense against oxidative stress and xenobiotic toxicity.[8] It can act as the electron donor for glutathione peroxidase in animal cells, and also directly reacts with reactive oxygen species (ROS). GSH is readily oxidized to glutathione disulfide (GSSG) by the glutathione peroxidase reaction, as well as the reaction with ROS.

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GSSG can be reduced to GSH by NADPH-dependent reaction catalyzed by glutathione reductase.[9] Therefore, the ultimate antioxidant capacity of a tissue is determined by the supply of reducing potentials. NADPH is an essential cofactor for the regeneration of GSH by glutathione reductase in addition to its critical role for the activity of NADPH-dependent thioredoxin system.[10–12] Both are important in the protection of cells from oxidative damage.

Glucose 6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the pentose phosphate pathway, has long been regarded as the major enzyme to generate NADPH. In fact, the role of G6PD in the cell response to oxidative stress is well established in yeast, in human erythrocytes and in the mouse embryonic stem cells.[13,14] However, cytosolic $NADP^+$-dependent isocitrate dehydrogenase (ICDH), is also responsible for the generation of cytosolic NADPH.[15] Earlier studies indicated cytosolic ICOH (IDPc) in the rat liver was 16-fold more active in producing NADPH than G6PD,[16] suggesting an important role of IDPc in the production of NADPH and eventually for the cellular defense against oxidative stress.

In the present study, the role of IDPc in cellular defense against singlet oxygen-induced oxidative injury was investigated by comparing the cellular responses after stable transfection of IDPc cDNA into NIH3T3 cells in sense and antisense orientations. Our data presented in this study showed that transformed NIH3T3 cells with high levels of transduced IDPc became more resistant to oxidative damage caused by singlet oxygen than the cells with reduced level of IDPc or control cells with the vector alone. These data provide direct evidence for the protective role of IDPc against singlet oxygen-induced cellular oxidative damage.

MATERIALS AND METHODS

Materials

β-$NADP^+$, isocitrate, 2-thiobarbituric acid, G6PD, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), rose bengal (RB), methylene blue (MB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,4-dinitophenylhydrazine (DNPH), avidin-tetramethylrhodamine isothiocyanate (TRITC), and 5-sulfosalicyclic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamide and nitroblue tetrazolium (NBT) were from Bio-Rad (Hercules, CA, USA). 2′,7′-Dichlorofluoroscin diacetate (DCFHDA) and diphenyl-1-pyrenylphosphine (DPPP) were purchased from Molecular Probes (Eugene, OR, USA).

Cell Cultures

The NIH3T3 cell lines with stable transfections with the cDNA for mouse IDPc in sense, IDPc(+), and antisense, IDPc(−), orientations[17] were a kind gift of Dr Huh (Kyungpook National University, Taegu, Korea). The NIH3T3 cell line transfected with LNCX-vector alone was used as a control. NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 10 μg/ml gentamycin at 37°C in an incubator under 5% CO_2.

IDPc Activity and Immunoblotting Analysis

Cells were collected at 10,000*g* for 10 min at 4°C and were washed once with cold PBS. Briefly, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris-Cl, pH 7.4). Cell homogenates were centrifuged at 1000*g* for 5 min, and the supernatants were further centrifuged at 15,000*g* for 30 min. The resulting supernatants were used as the cytosolic fractions. Protein concentration was determined by the method of Bradford using the reagents purchased from Bio-Rad. The supernatants were added by 1/10 volume of 10X PBS containing 1% Triton-X100, which finally made the solution 1X PBS containing 0.1% Triton-X100. The supernatants were used to measure the activities of several cytosolic enzymes. The activity of IDPc was measured by the production of NADPH at 340 nm.[18] The reaction mixture for IDPc activity contained 50 mM MOPS, pH 7.2, 5 mM threo-DS-isocitrate, 35.5 mM triethanolamine, 2 mM $NADP^+$, 1 mM ADP, 2 mM $MgCl_2$ and 1 μg/ml rotenone. One unit of IDPc activity is defined as the amount of enzyme catalyzing the production of 1 μmol of NADPH/min. A purified mouse IDPc was used to prepare polyclonal anti-IDPc antibodies in rabbits. The cytosolic homogenates from cultured cells were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected into immunoblot analysis using anti-IDPc antibodies, as described.[19]

Treatment with Singlet Oxygen

Singlet oxygen was generated by photoexcitation of the light-sensitive dye RB or MB. Cultures with various concentrations of RB or MB were irradiated with white light from a 100 W tungsten bulb at 30 cm from the petri dish.

Cell Viability

Cells (1×10^5) were grown in 35 mm plates, and cell viability after treatment with photoactivated dye was

assessed by MTT assay.[17] After 48 h of treatment with dye/light (1 h), 40 μl of MTT (5 mg/ml) was added and incubated for 4 h at 37°C. The MTT solution was discarded by aspiration, and the resulting formazan product was dissolved in 1 ml of dimethyl sulfoxide and the absorbance was measured at 540 nm. Cell viability was expressed as a percentage of untreated control cells.

Protein Carbonyl Content

The protein carbonyl content was determined spectrophotometrically using the DNPH-labeling procedure as described.[20] The crude extract (~1 mg protein) was incubated with 0.4 ml 0.2% DNPH in 2 M HCl for 1 h at 37°C. The protein hydrazone derivatives were sequentially extracted with 10% (w/v) trichloroacetic acid, treated with ethanol/ethyl acetate, 1:1 (v/v), and reextracted with 10% trichloroacetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride, and the difference spectrum of the sample treated with DNPH in HCl was examined versus a sample treated with HCl alone. Results are expressed as nmol of DNPH incorporated per mg of protein calculated from an absorbtivity of 21.0 $mM^{-1}\,cm^{-1}$ at 360 nm for aliphatic hydrazones.

Lipid Peroxidation

Thiobarbituric acid-reactive substances (TBARS) were determined as an independent measurement of lipid peroxidation. The cell extracts (500 μl) were mixed with 1 ml TBA solution (0.375% thiobarbituric acid in 0.25N HCl containing 15% (w/w) trichloroacetic acid),[21] and heated at 100°C for 15 min. Then the reaction was stopped on ice, and the absorbance was measured at 535 nm. Lipid peroxidation was also estimated by using a fluorescent probe DPPP as described by Okimoto *et al.*[22]

8-OH-dG Levels

8-OH-dG levels of U937 cells were estimated by using a fluorescent binding assay as described by Struthers *et al.*[23] After U937 cells were exposed to RB (25 μM)/light (15 min), cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 in PBS for 1 h) for fluorescent microscope with 488 nm excitation and 580 nm emission.

Measurement of Intracellular ROS

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFHDA with confocal microscopy.[24] Cells were grown at 2×10^6 cells/100 mm plate containing slide glass coated with poly-L-lysine and maintained in the growth medium for 24 h. Cells were treated with 10 μM DCFHDA for 15 min and exposed to RB (25 μM)/light (15 min). Cells on the slide glass were washed with PBS and a cover glass was put on the slide glass. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was imaged on a laser confocal scanning microscope (DM/R-TCS, Leica) coupled to a microscope (Leitz DM REB).

Cellular GSH Levels

The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4\,M^{-1}\,cm^{-1}$), and GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine.[25]

RESULTS

As mentioned in our previous paper,[17] the three kinds of recombinant IDPc were constructed. The NIH3T3 cells were transfected with the LNCX containing either an IDPc gene as a sense orientation, as an antisense orientation, or the LNCX alone. The IDPc activity of IDPc(+) cells was increased 2.3-fold compared with that of the control cells. In contrast, IDPc(−) cells exhibited 39% less IDPc activity when compared with that of the control (Fig. 1A). Immunoblot analysis using anti-IDPc antibody further confirmed the correlation between the amount of IDPc enzyme measured in cell extracts by immunoreaction and the corresponding levels of enzymatic activity (Fig. 1B). Increased expression of IDPc(+) or reduced expression of IDPc(−) did not significantly alter the activities of other antioxidant enzymes such as glutathione peroxidase, glutathione reductase, SOD, G6PD and catalase, suggesting that the transfection of IDPc cDNAs did not affect the activities of other enzymes involved in antioxidation.[17]

To study the relationship between IDPc activity and singlet oxygen-induced damage, cells were exposed to different doses of RB or MB with illumination for 1 h prior to the measurement of cell viability. As shown in Fig. 2, IDPc(+) cells were more resistant to singlet oxygen-induced cell killing than were the control and IDPc(−) cells. More than 85% of IDPc(+) cells survived, whereas about 73 and 56% of control and IDPc(−) cells survived, respectively, upon exposure to RB (25 μM)/light (1 h). Exposure of NIH3T3 cells to dye in the absence of illumination or by illumination alone did not modify the viability of cells.

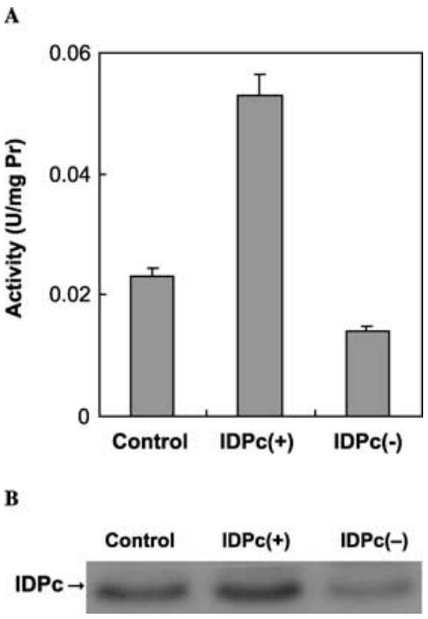

FIGURE 1 (A) Activity of IDPc in transfectant cell lines. IDPc activities are expressed as units/mg protein. Each value represents the mean ± S.D. from three independent experiments. (B) Immunoblot analysis of IDPc protein expressed in stable transformant NIH3T3 cells. The cytosolic fraction (20 μg protein) from cultured cells were separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and then subjected to immunoblot analysis using anti-IDPc antibodies.

As indicative markers of oxidative damage to cells, the occurrence of oxidative DNA damage, protein oxidation and lipid peroxidation were evaluated. Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins.[20] IDPc(−) cells elicited an approximately 4.5-fold increase of carbonyl groups, as compared to untreated cells when RB (25 μM)/light (15 min) was used (Fig. 3A). Although the carbonyl content of IDPc(+) and control cells also increased with exposure to singlet oxygen, the increase was significantly lower than that of IDPc(−) cells. The increase in lipid peroxidation is proportional to the relative degree of oxidative stress imposed to the cells. We determined whether the change in cellular IDPc level correlated with the change in MDA level as an indicator of lipid peroxidation upon exposure to singlet oxygen. The level of MDA in IDPc(−) cells was higher than in control or IDPc(+) cells either untreated or treated with singlet oxygen. Exposure of RB (25 μM)/light (15 min) increased the level of MDA 2-fold in IDPc(−) cells, however, the increase in MDA content of IDPc(+) and control cells was significantly lower than that of IDPc(−) cells (Fig. 3B). Similarly, DPPP fluorescent intensity, an indicative marker to monitor lipid peroxidation, was increased markedly in IDPc(−) cells, whereas it was increased slightly in IDPc(+) cells after exposure to singlet oxygen (Figs. 4A and 5A). The reaction of intracellular ROS with DNA resulted in numerous forms of base damage, and 8-OH-dG is one of the most abundant and most studied lesions generated. Because 8-OH-dG causes misreplication of DNA,[27] it has been implicated as a possible cause of mutation and cancer. Therefore, 8-OH-dG has been used as an indicator of oxidative DNA damage *in vivo* and *in vitro*.[28] Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC.[23] The fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in IDPc(−) cells upon exposure to singlet oxygen (Figs. 4B and 5B). In contrast, the overall DNA appeared to be intact or markedly protected in IDPc-rich cells even after exposure to the same dose

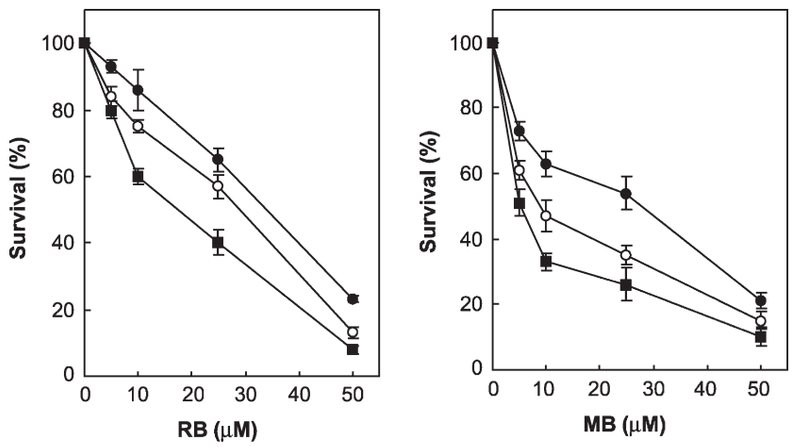

FIGURE 2 Effect of IDPc on cell viability upon exposure to singlet oxygen. Three different cells grown on 35 mm plates were exposed to different doses of RB or MB for 1 h with illumination and then incubated for 48 h prior to the measurement of cell viability. Control, IDPc(+) and IDPc(−) cells are indicated by *open circles*, *closed circles* and *closed rectangles*. Each value represents the mean ± S.E. from five independent experiments.

of photoactivated dye. These results indicate that the reduced expression of IDPc most likely leads to increased cell injury, while elevated IDPc appears to protect cells and DNA from oxidative damage.

To investigate the role of IDPc in cellular defense against singlet oxygen-induced cell damage, we determined the level of intracellular peroxide, reflected by the relative intensity of DCF,[29] in different cells before and after treatment with photoactivated dye. Deacylation by esterase to dichlorofluoroscin occurs within the cells, and the nonfluorescent dichlorofluoroscin is, subsequently, oxidized in the presence of intracellular hydroperoxides and peroxides to highly fluorescent dichlorofluorescein.[29] DCF fluorescence intensity, without treatment with singlet oxygen, increased in IDPc(−) cells but decreased in IDPc(+) cells compared with the control cells. Similarly, the fluorescence intensity increased markedly in IDPc(−) cells, whereas it only increased slightly in IDPc(+) cells after they were exposed to RB (25 μM)/light (15 min) (Figs. 4C and 5C). One important parameter of GSH metabolism is the ratio of GSSG/total GSH (GSH_t) which may reflect the efficiency of GSH turnover. When the cells were exposed to RB (25 μM)/light (15 min), the ratio for cellular [GSSG]/[GSH_t] was 2.8-fold higher in IDPc(−) cells and 37% lower in IDPc(+) cells, respectively, than that of the control cells. This data indicate that GSSG in IDPc(−) cells was not reduced as efficiently as in IDPc(+) cells. Taken together, these results strongly suggest that the decrease in the efficiency of GSH recycling may be responsible for the higher concentration of intracellular peroxides and the pronounced oxidative damage in IDPc(−) cells upon exposure to singlet oxygen.

DISCUSSION

It is well established that $^1O_2$ can be generated in cells such as under conditions of oxidative stress,[30] from decomposition of lipid peroxides or by spontaneous dismutation of superoxide.[31,32] In addition, both naturally occurring compounds such as riboflavin[33] and many xenobiotics, such as psoralene,[34] porphyrins[2] and tetracyclins[35] can generate $^1O_2$ inside cells when irradiated by visible light. Singlet oxygen is most often generated *in vitro* by photosensitization reactions. Irradiation of the sensitizer dye, such as MB or RB, mediates photoreactions through an excited triplet state which acts either by hydrogen atom or electron transfer reactions (type I) or by transferring the excited energy, forming singlet oxygen, which then reacts with the target molecules (type II).[32] Light induced a few diseases including erythropoietic protoporphyria, pellagra and cataractogenesis have been attributed in part to the toxicity of $^1O_2$.[36,37]

The ICDH family of enzymes, either the NAD^+-dependent or $NADP^+$-dependent form, exists in virtually all species and has a wide variety of functions, including major roles in the tricarboxylic acid cycle, responsible for energy (ATP) production, and other biosynthesis pathways. The presence of $NADP^+$-dependent ICDH in mitochondria, peroxisomes, and the cytoplasm has been reported,[38] however, the biological functions of this enzyme have not been clearly elucidated. We recently demonstrated that the control of cytosolic redox balance and oxidative damage is one of the primary functions of IDPc.[17] Recently, IDPc that is preferentially expressed in bovine corneal epithelium has been identified. The role of

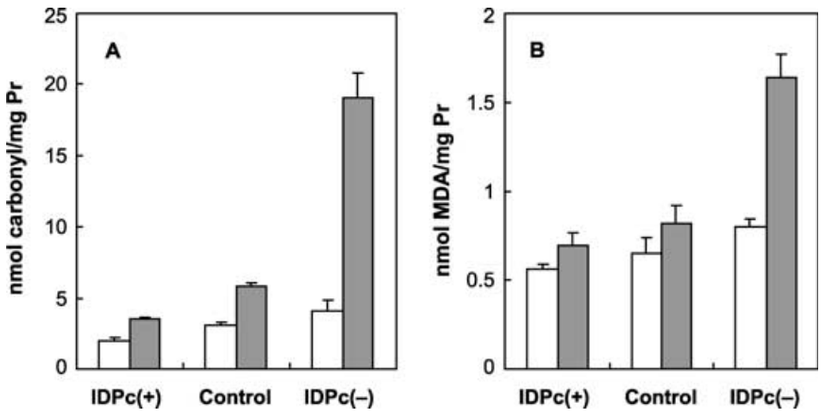

FIGURE 3 (A) Protein carbonyl content of IDPc transfectant cells exposed to RB/light. Protein carbonyls were measured in cell-free extracts by the method of Levine *et al.*[20] with the use of DNPH. *Open* and *shaded bars* represent the protein carbonyl contents in the cells unexposed and exposed to RB (25 μM)/light (15 min), respectively. Each value represents the mean ± S.D. from three independent experiments. (B) Lipid peroxidation of IDPc transfectant cells after treatment with RB/light. *Open* and *shaded bars* represent the level of MDA accumulated in the cells unexposed and exposed to RB (25 μM)/light (15 min), respectively. Each value represents the mean ± S.D. from three independent experiments.

this enzyme in contributing to corneal transparency is likely attributed to its protective effect against UV radiation.[39] We also found that *E. coli* mutant lacking $NADP^+$-dependent ICDH is sensitive to the radiation.[40] Therefore, it is reasonable to assume that IDPc performs some other functional role besides supplying NADPH for the biosynthesis pathways.

Earlier data suggest that $NADP^+$-dependent G6PD, IDPc and malic enzymes are important in production of NADPH for biosynthesis and GSH recycling.[16] McAlister-Henn and co-workers[41,42] recently reported that G6PD and IDPc are important in providing NADPH for β-oxidation of fatty acids for growth. In fact, yeast cells with disrupted genes for both G6PD and IDPc grow slowly and highly sensitive to endogenous and exogenous H_2O_2 treatment, suggesting an important role of IDPc in protection against oxidative damage.[41] To directly demonstrate the possible antioxidant role of IDPc against singlet oxygen-induced cell damage, three different NIH3T3 transformed cells with stable transfection of IDPc cDNAs in sense and antisense directions were employed. In these transformed cells, our previous data revealed that only IDPc activity was either elevated in IDPc(+) cells or

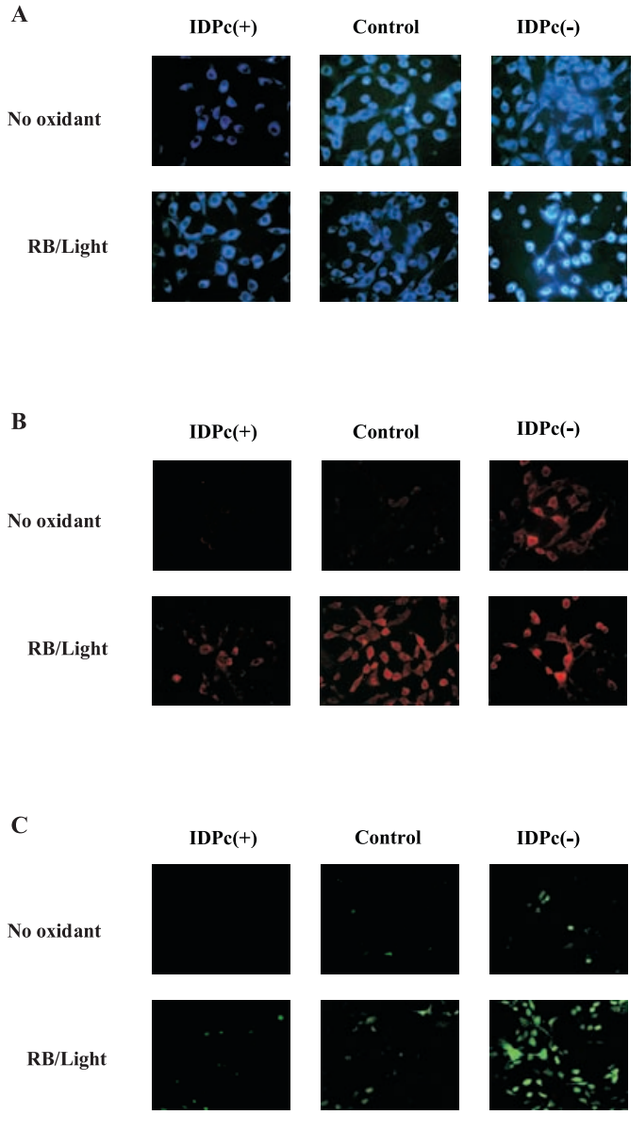

FIGURE 4 (A) Visulization of lipid peroxidation in IDPc transfectant cells. Cells (1×10^6 cells/ml) were stained with 5 μM DPPP for 15 min. Fluorescence images were obtained under microscopy from three separate experiments. (B) Effect of singlet oxygen on 8-OH-dG levels in IDPc transfected cells. Cells were fixed and permeabilized immediately after exposure to RB (25 μM)/light (15 min). 8-OH-dG levels reflected by the binding of avidin-TRITC was visualized by fluorescence microscope with 488 nm excitation and 580 nm emission. (C) DCF fluorescence in transfected cells. Typical patterns of DCF fluorescence are presented for transfected cells unexposed or exposed to RB (25 μM)/light (15 min). Fluorescence images were obtained under laser confocal microscopy from three separate experiments.

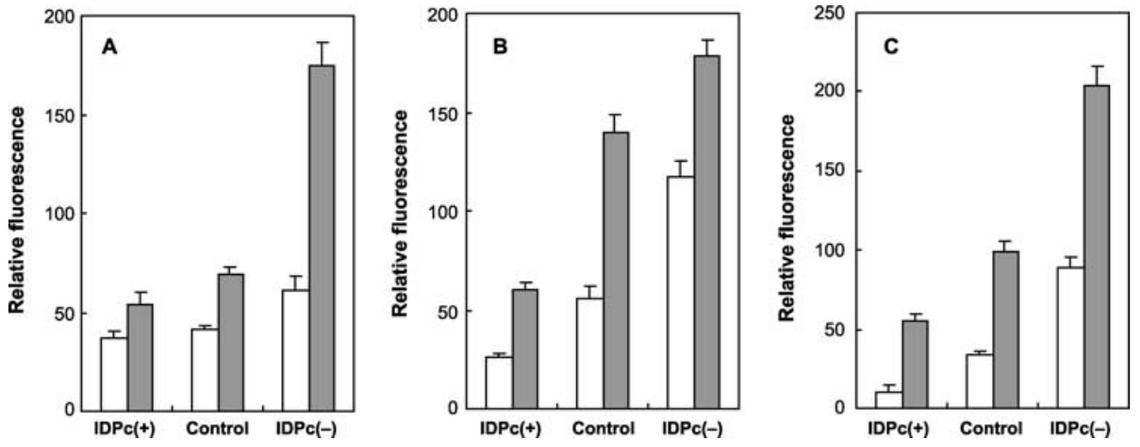

FIGURE 5 Relative intensities of (A) DPPP fluorescence, (B) avidin-FITCS fluorescence and (C) DCF fluorescence in transfected cells. The averages of fluorescence intensity were calculated as described.[26] *Open* and *shaded bars* represent the relative fluorescence in the cells unexposed and exposed to RB (25 μM)/light (15 min), respectively. Each value represents the mean ± S.D. from three independent experiments.

reduced in IDPc(−) cells compared to the control cells transformed with DNA vector alone. In contrast, the activities of other antioxidant enzymes were remained unchanged, indicating that the activities of other key enzymes involved in the cellular defense against oxidative stress is not affected by the changes in IDPc activity in IDPc(+) and IDPc(−) cells.[17] The present study revealed that the ratio of GSSG to total glutathione ($GSSG/GSH_t$) were higher in IDPc(−) cells than in the control or in IDPc(+) cells with subsequent changes in cell viability upon exposure to singlet oxygen. This result confirms the importance of IDPc in the recycling of GSH in NIH3T3 cells. Under our experimental conditions, a clear inverse relationship was observed between the amount of IDPc expressed in target cells and their cell viability. Our conclusion was further supported by the levels of intracellular peroxides generation, DNA damage reflected by an increase of 8-OH-dG level, as well as increase in lipid peroxidation and protein oxidation. Based on the results reported in this study, IDPc plays a protective role against singlet oxygen-induced oxidative injury to cells.

Acknowledgements

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