Free Radical Biology & Medicine, Vol. 32, No. 11, pp. 1185–1196, 2002

0891-5849/02/$–see front matter

PII S0891-5849(02)00815-8

Original Contribution

CYTOSOLIC NADP$^+$-DEPENDENT ISOCITRATE DEHYDROGENASE STATUS MODULATES OXIDATIVE DAMAGE TO CELLS

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(*Received* 27 *November* 2001; *Accepted* 7 *March* 2002)

Abstract—NADPH is an important cofactor in many biosynthesis pathways and the regeneration of reduced glutathione, critically important in cellular defense against oxidative damage. It is mainly produced by glucose 6-phosphate dehydrogenase (G6PD), malic enzyme, and the cytosolic form of NADP$^+$-dependent isocitrate dehydrogenase (IDPc). Little information is available about the role of IDPc in antioxidant defense. In this study we investigated the role of IDPc against cytotoxicity induced by oxidative stress 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 3–4-fold higher and 35% lower, respectively, than that in the parental cells carrying the vector alone. Although the activities of other antioxidant enzymes, such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, and G6PD, were comparable in all transformed cells, the ratio of GSSG to total glutathione was significantly higher in the cells expressing the lower level of IDPc. This finding indicates that IDPc is essential for the efficient glutathione recycling. Upon transient exposure to increasing concentrations of H_2O_2 or menadione, an intracellular source of free radicals and reactive oxygen species, the cells with low levels of IDPc became more sensitive to oxidative damage by H_2O_2 or menadione. Lipid peroxidation, 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 oxidative stress, compared to the control cells. This study provides direct evidence correlating the activities of IDPc and the maintenance of the cellular redox state, suggesting that IDPc plays an important role in cellular defense against oxidative stress.

Keywords—Isocitrate dehydrogenase, NADPH, Reactive oxygen species, Cellular redox state, Free radicals

INTRODUCTION

There is growing evidence that reactive oxygen species (ROS) are directly or indirectly involved with a variety of chronic diseases [1]. However, biological systems have evolved to develop an effective and complicated network for defense mechanisms, to efficiently handle the harmful oxidative environments [2–4]. These defense mechanisms include nonenzymatic and enzymatic defenses. The nonenzymatic 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 and peroxidases [5,6].

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 [7]. It can act as the electron donor for glutathione peroxidase in animal cells, and also directly reacts with ROS. GSH is readily oxidized to glutathione disulfide (GSSG) by the glutathione peroxidase reaction, as well as the reaction with ROS. GSSG can be reduced to GSH by NADPH-dependent reaction catalyzed by glutathione reductase [8]. 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

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system [9–11]. Both are important in the protection of cells from oxidative damage. The oxidized form of thioredoxin, with a disulfide bridge between the half-cystines, can be reduced by NADPH in the presence of a flavoprotein, thioredoxin reductase [12]. Reduced thioredoxin may provide reducing equivalents to at least two enzymes, thioredoxin peroxidases and methionine sulfoxide reductase, presumably involved in the defense against oxidative stress. NADPH is also required for the formation of active catalase tetramers, where each catalase monomer contains one NADPH binding site necessary for its enzymatic activity [13].

The isocitrate dehydrogenases (ICDHs; EC 1.1.1.41 and EC 1.1.1.42) catalyze oxidative decarboxylation of isocitrate to 2-oxoglutarate and require NAD^+ or $NADP^+$, producing NADH and NADPH, respectively [14]. It is known that mammalian tissues contain three classes of ICDH isoenzymes: mitochondrial NAD^+-dependent ICDH, mitochondrial $NADP^+$-dependent ICDH (IDPm), and cytosolic $NADP^+$-dependent ICDH (IDPc) [14]. NAD^+-dependent ICDH appears to play a major role in the tricarboxylic acid cycle, while the role of the $NADP^+$-dependent enzymes, either cytosolic or mitochondrial, remains to be established.

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 [13,15,16]. However, two other $NADP^+$-linked dehydrogenases, malic enzyme and IDPc, are also responsible for the generation of cytosolic NADPH [17]. It has also been proposed that G6PD and malic enzyme could be owing to the maintenance of the redox state of the cell [17]. Earlier study indicated IDPc in the rat liver was 16- and 18-fold more active in producing NADPH than G6PD and malic enzymes, respectively [18], suggesting an important role of IDPc in the production of NADPH and eventually for the cellular defense against oxidative stress. Despite these earlier results, the biological role of IDPc in the antioxidant defenses in mammalian cells has not been established.

In the present study, the role of IDPc in cellular defense against oxidative stress 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 stress caused by H_2O_2 or menadione treatment than the cells with reduced level of IDPc, the untransfected parental cells, or control cells with the vector alone. These data provide direct evidence for the protective role of IDPc against cellular oxidative damage.

MATERIALS AND METHODS

Materials

Hydrogen peroxide, menadione, NADPH, β-$NADP^+$, GSSG, GSH, pyrogallol, isocitrate, glucose-6-phosphate, glutathione reductase, *tert*-butyl hydroperoxide, 2-thiobarbituric acid, G6PD, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), pronase, RNase A, phenazine ethosulfate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide;thiazolyl blue (MTT), and 5-sulfosalicyclic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, ammonium persulfate, N,N,N′,N′-tetramethylenediamide, riboflavin, and nitroblue tetrazolium (NBT) were from Bio-Rad (Hercules, CA, USA). 2′,7′-Dichlorofluoroscin diacetate (DCFHDA) was purchased from Molecular Probes (Eugene, OR, USA).

Screening of the cDNA library and nucleotide sequence analysis

The amplified cDNA clone for rat IDPc [19] was labeled with [α-^{32}P]dCTP by nick translation and used to screen a Lambda ZAP II cDNA library of NIH3T3 cells (Stratagene, La Jolla, CA, USA) by plaque hybridization, as we previously described [20]. The DNA inserts of the positive cDNA clones were subcloned into plasmid pBluescript II SK(+) and their nucleotide sequences were analyzed from both ends by the deoxynucleotide-chain-termination method with Sequenase V2.0 kit (U.S. Biochemical Corp., Cleveland, OH, USA). Nucleotide sequence data were assembled and analyzed by using the computer software PC/GENE (IntelliGenetics, Mountain View, CA, USA).

Preparation of the recombinant IDPc constructs

The largest cDNA (insert size, 2.2 kb) for mouse IDPc was initially subcloned into *Xho I, Eco RI* site of pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) to generate pSK-IDPc. pSK-IDPc was digested by *Xba I* and *Kpn I* followed by ligation of the IDPc cDNA insert into pGEM7(+), then further digested by *Bam HI* and ligate the resulting insert into pGEM7(+) again to generate *Cla I* site available at both ends. To prepare various DNA constructs such as the LNCX-sense IDPc, LNCX-antisense IDPc, the LNCX retroviral vector [21] was initially digested by *Cla I*, and then ligated with *Cla I* digested the IDPc insert. The identification of sense or antisense IDPc construct was performed by the polymerase chain reaction (PCR). In the LNCX retroviral vector,

expression of sense or antisense IDPc cDNA was directed by the use of cytomegalovirus (CMV) promoter. The respective DNA construct of LNCX-sense IDPc, LNCX-antisense IDPc, or LNCX-vector alone was transfected into NIH3T3 cells, prior to selecting stable transformants in the presence of G418.

Cell culture and stable transfection

NIH3T3 cells (American Type Culture Collection) 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. At 24 h before transfection, BOSC23 retroviral packaging cells [21] were grown at a density of 1×10^6 cells in DMEM containing 10% (v/v) FBS. On the following day, BOSC23 cells were incubated with new media containing 25 μM chloroquine for 2 h, and the recombinant IDPc constructs were transfected into the BOSC23 cells by the calcium phosphate method [22]. After 10 h of incubation, the culture medium was replaced with a medium containing 10% (v/v) FBS for additional 24 h to produce retroviral particles. To separate packaging cells from the retrovirus particles, the whole culture medium was filtered through a sterile filter (0.4 μm diameter), and the filtrate used as a retrovirus source. For transfection of retrovirus particles, the retroviral solution treated with polybrene (4 mg/ml) was infected into NIH 3T3 cells (5×10^5). After 5 h of incubation, the retrovirus solution was aspirated, and a fresh DMEM containing 10% (v/v) FBS was added to the culture. Two days after transfection, cells were exposed to 400 μg/ml G-418. After 3 weeks of incubation, resistant transformant cells were expanded in a complete medium containing G-418 (100 μg/ml).

Antibody preparation and immunoblot analysis

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 (Schleicher 38 Shuell, Keene, NH, USA), and subsequently subjected into immunoblot analysis using anti-IDPc antibodies, as described [23].

Northern blot analysis

Total RNAs from cultured cells were prepared using RNAzol (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's protocol. Total RNA (20 μg/lane) from cultured cells was separated by electrophoresis on 0.66 M formamide/1% agarose gels, transferred to GeneScreen membranes, and hybridized with a ^{32}P-labeled mouse IDPc cDNA as a probe. A membrane for mouse multiple tissue Northern (MTN) blot (CLONTECH, Palo Alto, CA, USA) was hybridized with a ^{32}P-labeled DNA probe. Hybridization and subsequent procedures were the same as previously described [20].

Cytotoxicity assay

Cells were first grown on a 96 well plate at a density of 1×10^4 cells/well, overnight before treatment. After overnight culture, various concentrations of hydrogen peroxide or menadione were applied to the cells in serum free DMEM, and cells were incubated for additional 48 h at 37°C. After 48 h of oxidant treatment to cells, culture media were aspirated under vacuum, before 200 μl of MTT (1 mg/ml) was added and further incubated for 4 h at 37°C. The MTT solution was discarded by aspirating, and then the resulting formazan product converted by the viable cells was dissolved in 150 μl dimethylsulfoxide. The absorbance was read in an ELISA plate reader at 540 nm with a 620 nm reference. Cell viability is expressed as a percentage of the absorbance seen in the untreated control cells [24].

Enzyme assay

Cells were collected at 10,000 $\times$ *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 $\times$ *g* for 5 min, and the supernatants were further centrifuged at 15,000 $\times$ *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 10$\times$ PBS containing 1% Triton-X100, which finally made the solution 1$\times$ 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 [25]. The reaction mixture for IDPc activity contained 50 mM MOPS, pH 7.2, 5 mM threo-DS-isocitrate, 35.5 mM triethanolamine, 2 mM NAD^+, 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. Catalase activity was measured with the decomposition of hydrogen peroxide, which was determined by the de-

crease in absorbance at 240 nm [26]. SOD activity in cell extracts was assayed spectrophotometrically using a pyrogallol assay [27], where one unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%. Glutathione reductase activity was quantified by the GSSG-dependent loss of NADPH [28] as measured at 340 nm ($\varepsilon = 6.67\ mM^{-1}cm^{-1}$). Reaction mixture contained 0.1 mM NADPH, cell-free extract, 1 mM GSSG, 1 mM EDTA, and 0.1 M potassium phosphate, pH 7.4 in a final volume of 1.5 ml. G6PD activity was measured by following the rate of $NADP^+$ reduction at 340 nm using the procedure described [29]. Glutathione peroxidase activity in the crude extracts was measured by the standard indirect method based on NADPH oxidation by *tert*-butyl hydroperoxide in the presence of excess glutathione and glutathione reductase, as previously described [30].

Cellular NADPH and GSH levels

NADPH was measured using the enzymatic cycling method as described by Zerez et al. [31]. Briefly, the reaction mixture contained 100 mM Tris (pH 8.0), 5 mM EDTA, 2 mM phenazine ethosulfate, 0.5 mM MTT, 1.3 unit G6PD, and fixed amounts of the cell extracts was preincubated for 5 min at 37°C. The reaction was started by the addition of 1 mM glucose 6-phosphate. The absorbance at 570 nm was measured for 3 min. The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$) as described by Akerboom and Sies [32], and GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine [33]. Total GSH level was measured in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.2 mg NADPH, 30 μg DTNB, and 0.12 unit glutathione reductase. GSSG level was measured by the same method as the total GSH level but after treatment of 1 μl of 2-vinylpyridine and 3 μl of triethanolamine for 1 h.

Measurement of intracellular ROS

Intracellular peroxide concentration was measured using the oxidant-sensitive fluorescent probe DCFHDA with confocal microscopy [34]. Cells were grown at 2×10^6 cells per 100 mm plate containing slide glass coated with poly-L-lysine and maintained in the growth medium for 24 h. Cells were exposed to 10 μM DCFHDA for 15 min and treated with 1 mM hydrogen peroxide or 50 μM menadione for 5 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).

Cell morphology

Cells (2×10^5) were grown for 48 h and the morphology of IDPc cell lines in the absence and in the presence of 1 mM H_2O_2 or 50 μM menadione for 1 h were examined by phase contrast microscopy using oil immersion without staining.

Lipid peroxidation

Thiobarbituric acid-reactive substances (TBARS) were determined as an independent measurement of lipid peroxidaion. Samples were evaluated for malondialdehyde (MDA) production using a Lipid Peroxidation Assay kit (Calbiochem, La Jolla, CA, USA).

DNA isolation and single-strand DNA breaks

DNA was isolated from NIH3T3 cell pellets extracted with a buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 20 μg/ml RNase, 0.5% SDS) and the cells were digested with 100 μg/ml pronase for 3 h at 50°C, followed by phenol extraction and precipitation by 2 vol of ethanol [35]. DNA samples were applied to 0.5% agarose gels in a TAE buffer system, and electrophoresis was performed at 5 V/cm for 2 h at room temperature. Following electrophoresis, gels were stained with ethidium bromide and recorded under UV illumination.

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

RESULTS

Isolation and characterization of mouse IDPc cDNA

To isolate cDNAs for mouse IDPc, a cDNA library of NIH/3T3 cells (Stratagene, La Jolla, CA, USA) was screened with a partial cDNA for rat IDPc, which was amplified by the PCR [19] as a probe. Twelve positive cDNA clones for mouse IDPc were isolated from about two million phage plaques screened. Of these, one clone containing the largest DNA insert (2.2 kb) was purified, subcloned into plasmid pBluescript II SK(+), and its nucleotide sequence was determined. Mouse IDPc cDNA was 2177 bp long with an open reading frame

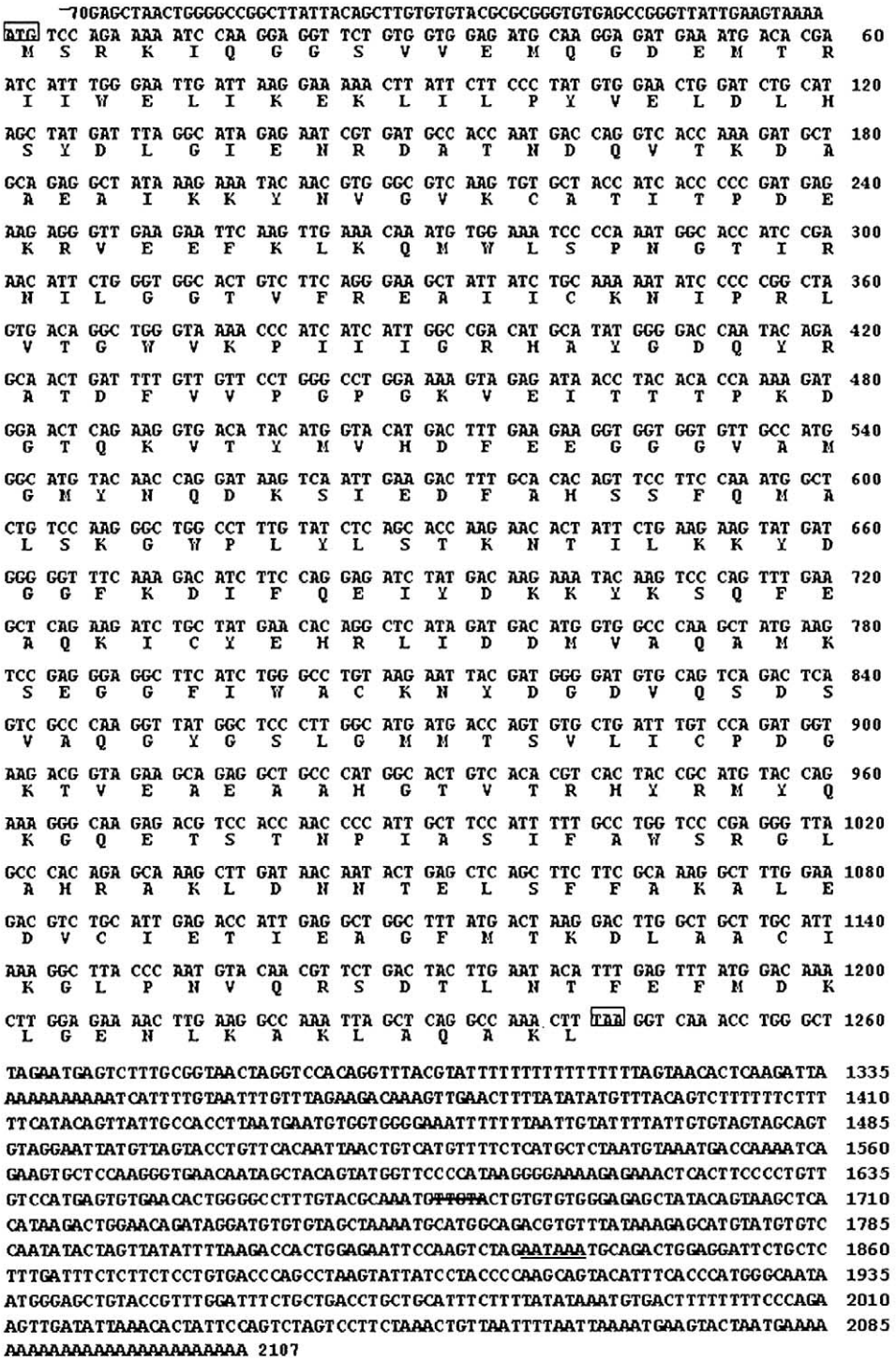

Fig. 1. Nucleotide and deduced amino acid sequences of the cDNA encoding mouse IDPc. Mouse IDPc cDNA was 2177 bp long with an open reading frame (1,245 bp) for the entire protein region of IDPc. The initiation codon, ATG and the termination codon, TAA were marked with boxes. The full-length IDPc cDNA encodes a protein of 414 amino acids with a calculated molecular mass of 46 kDa.

(1245 bp) for the entire protein coding region of IDPc (Fig. 1). The deduced amino acid sequence for the mouse IDPc protein consisted of 414 amino acid residues with the estimated molecular mass of 46,575 Da (Fig. 1). The protein homology of mouse IDPc showed 97.8% and 68.5% identity to that of rat IDPc and bovine IDPm,

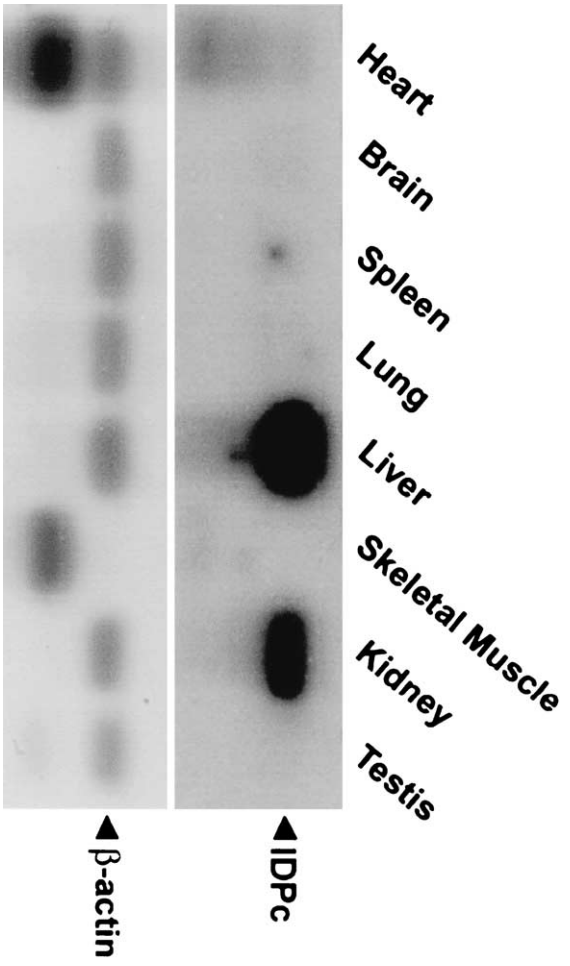

Fig. 2. Tissue-specific expression of IDPc mRNA transcript in various mouse tissues. A membrane for the mouse multiple tissue MTN blot was used to study the expression of IDPc message. The membrane was then hybridized with a [^{32}P]-labeled mouse IDPc cDNA (upper panel) or actin cDNA (bottom panel) followed by washing and autoradiography as described [20].

respectively. However, our mouse IDPc cDNA contained two different nucleotide sequences in the protein coding region and two different nucleotide sequences in the 3′-UTR region from the previously reported mouse IDPc [36].

Tissue-specific expression of IDPc

To investigate the expression pattern of IDPc in different mouse tissues, Northern analyses were performed. One major IDPc transcript (2.2 kb) was observed in several mouse tissues in a tissue-specific manner (Fig. 2). The level of IDPc expressed in mouse tissues was highest in the liver followed by kidney. In contrast, other tissues including brain and lung contained very low levels of IDPc message.

Stable transfection of IDPc constructs

To directly investigate the role of IDPc, two different transformants per recombinant IDPc constructs were isolated after stable transfection of the sense IDPc, IDPc(+)-1 and IDPc(+)-3, and antisense IDPc, IDPc(−)-1 and IDPc(−)-2, or LNCX vector alone (control) (Fig. 3A). Chromosomal integration of the transfected IDPc constructs was confirmed by the PCR (data not shown). Activity measurement revealed that IDPc(+)-1 and IDPc(+)-3 cells contained 3.5- and 2.8-fold more IDPc activity, respectively, than that of the control with the vector alone. In contrast, IDPc(−)-1 and IDPc(−)-2 cells exhibited 16% and 35% less IDPc activities, respectively, compared to that of the control (Fig. 3B). Essentially the same IDPc activity was measured in untransfected parental cells and control cells transfected with the vector alone. To demonstrate differences in response to ROS-mediated damage between cells with sense or antisense IDPc, we chose IDPc(+)-1 as sense cells, IDPc(+), and IDPc(−)-2 as antisense cells, IDPc(−). 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. 3C).

Cellular sensitivity to oxidants

Each line of transformants exhibited different sensitivities to extracellular agents such as H_2O_2 and menadione (2-methyl-1,4-naphtoquinone), known to produce oxidative stress. While the peroxides may cause oxidative injury from both outside and inside the cells, menadione may act as intracellular sources of free radicals and ROS by redox cycling. As shown in Fig. 4, when cells were exposed to hydrogen peroxide or menadione, a dose-dependent decrease in cell viability was observed. However, the rate of cell death of IDPc(−) cells was greater than that of more resistant IDPc(+) cells. More than 90% of IDPc(+) and control cells survived, whereas 44% of IDPc(−) cells died after exposure to 12.5 μM menadione. The relative sensitivity of untransfected NIH3T3 cells to oxidants was similar to that of control transfectant cells carrying the vector alone (data not shown). This finding indicates that the transfection process did not affect cellular sensitivity. These results support the view that the relative resistance of the cells to H_2O_2- or MD-mediated oxidative stress correlates to the level of intracellular IDPc content.

Cellular antioxidant system in transfectants

Because cellular antioxidants act in a concerted manner as a team, it is important to investigate whether the

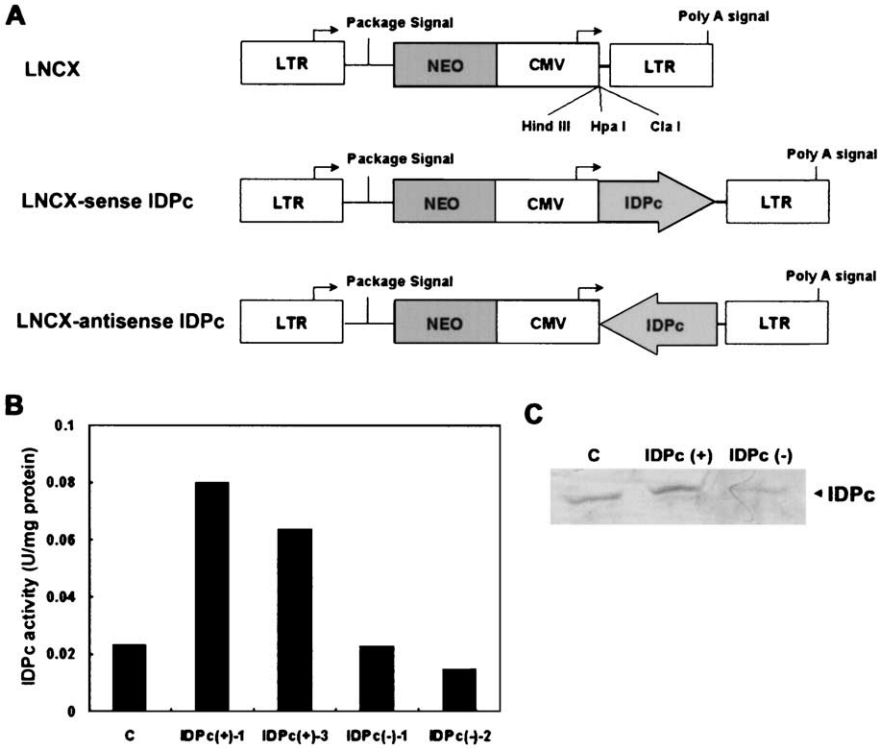

Fig. 3. Construction of different IDPc constructs. (A) Structures of the recombinant retroviral DNA constructs designed to express viral vector alone (LNCX), and IDPc cDNAs in sense (LNCX-sense IDPc) or antisense (LNCX-antisense IDPc) orientation under the CMV promoter. (B) Activity of IDPc in transfectant cell-lines. IDPc activities are expressed as units/mg protein. (C) 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 polyclonal antibodies.

modulation in cellular IDPc activity caused concomitant alterations in the activity of other major antioxidant enzymes. Therefore, we measured the activities of the following five enzymes: glutathione reductase, glutathione peroxidase, SOD, catalase, and G6PD. As shown in Table 1, the activities of these major antioxidant enzymes were similar to each other in all cells examined, suggesting that transfection of IDPc cDNA did not affect the activities for these five antioxidant enzymes. In general, the activities of antioxidant enzymes were de-

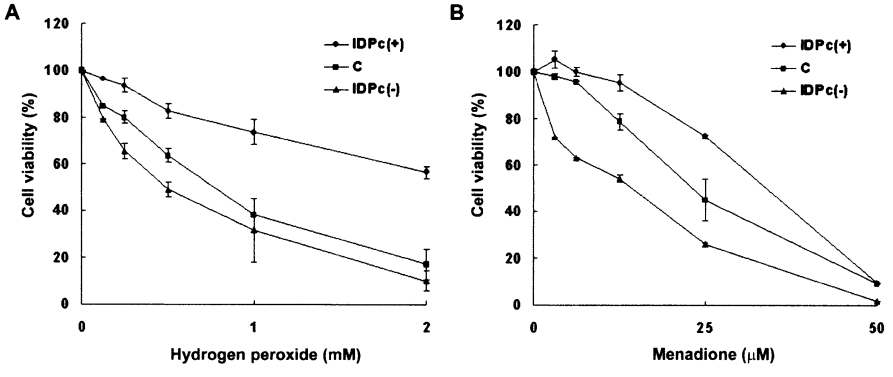

Fig. 4. Effects of transduced IDPc on cell viability upon exposure to oxidants. Three different cells (2×10^4 cells/well) in 200 μl of medium were grown in 96 well plates and then transiently exposed to different concentrations of H_2O_2 (A) or menadione (B), and cells were incubated for additional 48 h prior to the measurement of cell viability. Survival of untreated cells is expressed as 100%. Each value represents the mean $\pm$ SE from three to six independent experiments.

Table 1. Changes in Antioxidant Enzyme Activities of NIH3T3 Cells after Exposure to the Oxidants

Antioxidant enzyme	Oxidant	NIH3T3-LNCX	IDPc(+)	IDPc(−)
Catalase[a]	–	13.5 ± 2.5	10.8 ± 2.0	9.77 ± 1.2
	HP	10.9 ± 1.5	8.03 ± 0.5	7.11 ± 0.7
	MD	11.2 ± 2.2	7.02 ± 0.6	7.57 ± 1.8
G 6-P dehydrogenase[a]	–	0.156 ± 0.02	0.130 ± 0.02	0.121 ± 0.01
	HP	0.010 ± 0.01	0.114 ± 0.01	0.084 ± 0.01
	MD	0.074 ± 0.01	0.080 ± 0.01	0.079 ± 0.01
Glutathione reductase[b]	–	60 ± 5	55 ± 4	59 ± 5
	HP	42 ± 5	47 ± 4	44 ± 2
	MD	30 ± 7	40 ± 3	45 ± 3
Glutathione peroxidase[b]	–	21.3 ± 3.8	15.5 ± 2.9	13.5 ± 2.4
	HP	14.8 ± 2.9	13.5 ± 0.5	13.9 ± 1.3
	MD	16.5 ± 4.2	12.3 ± 0.1	12.3 ± 1.6
SOD[a]	–	8.46 ± 2.6	6.50 ± 0.4	6.42 ± 1.0
	HP	5.52 ± 2.6	6.20 ± 1.0	7.62 ± 0.8
	MD	6.66 ± 2.2	7.30 ± 2.0	4.84 ± 1.6

Results represent the mean ± SD calculated from three or four independent determinations. HP = 1 mM H_2O_2; MD = 50 μM menadione.
[a] Enzyme activity represents units/mg protein.
[b] Enzyme activity represents units/g protein.

creased upon exposure to both oxidants, however, the difference was not statistically significant between all different cells investigated. This result indicates that changes in the IDPc activity did not induce any compensatory increase or decrease in the activities of other key antioxidant enzymes. We also evaluated the effect of IDPc expression on the NADPH level and the GSH turnover. The total NADPH in IDPc(+) cells was 1.4-fold higher than the control cells while was 31% lower in IDPc(−). We then assessed how IDPc expression might influence the GSH turnover. One important parameter of GSH metabolism is the ratio of GSSG/total GSH (GSH_t), which may reflect the efficiency of GSH turnover. The ratio of GSSG/GSH_t in IDPc(−) cells was 3.3-fold higher than that of the control, indicating that GSSG in IDPc(−) cells was not reduced as efficiently as it was in the control cells. These results confirm that IDPc is essential for efficient glutathione recycling, through the generation of a reducing equivalent, NADPH.

Intracellular peroxide level

To investigate whether the differences in IDPc activities influenced the ROS concentrations, we measured the levels of intracellular peroxides in the different transformant cells using the oxidant-sensitive probe DCFH-DA under a laser confocal scanning microscope. 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 [37]. As shown in Fig. 5, even without treatment with an exogenous oxidant, the DCF fluorescence was higher in IDPc(−) cells when compared to the control cells and IDPc(+) cells. The level of intracellular oxidant was significantly increased in IDPc(−) cells determined by an increase in DCF fluorescence upon exposure to H_2O_2 or menadione. However, increase in DCF fluorescence was much smaller in IDPc(+) cells compared to the control cells that were still less sensitive to exogenous H_2O_2 (1 mM) or menadione (50 μM) treatment. These results further confirm that the relative rate of ROS generation is inversely proportional to the transduced IDPc level.

Cell damage

IDPc(−) cells exposed to 1 mM H_2O_2 or 50 μM menadione showed a pronounced morphological change when compared to the control cells. In contrast, the morphological change was greatly diminished in IDPc(+) cells (data not shown). The increase in lipid peroxidation was 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 lipid peroxide content upon exposure to an oxidant. After 1 h of exposure with H_2O_2 (1 mM) and menadione (50 μM), the level of lipid peroxide in IDPc(−) cells were elevated 2.1- and 1.7-fold, respectively, compare to those of the control cells (Fig. 6A). Since extensive DNA breakage had been observed in cells exposed to an oxidant, we have investigated the extent of oxidant-induced DNA breakage in relation to the intracellular level of IDPc. DNA, from different transformed NIH3T3 cells exposed to H_2O_2 or menadione for 1 h, was extracted and analyzed by electrophore-

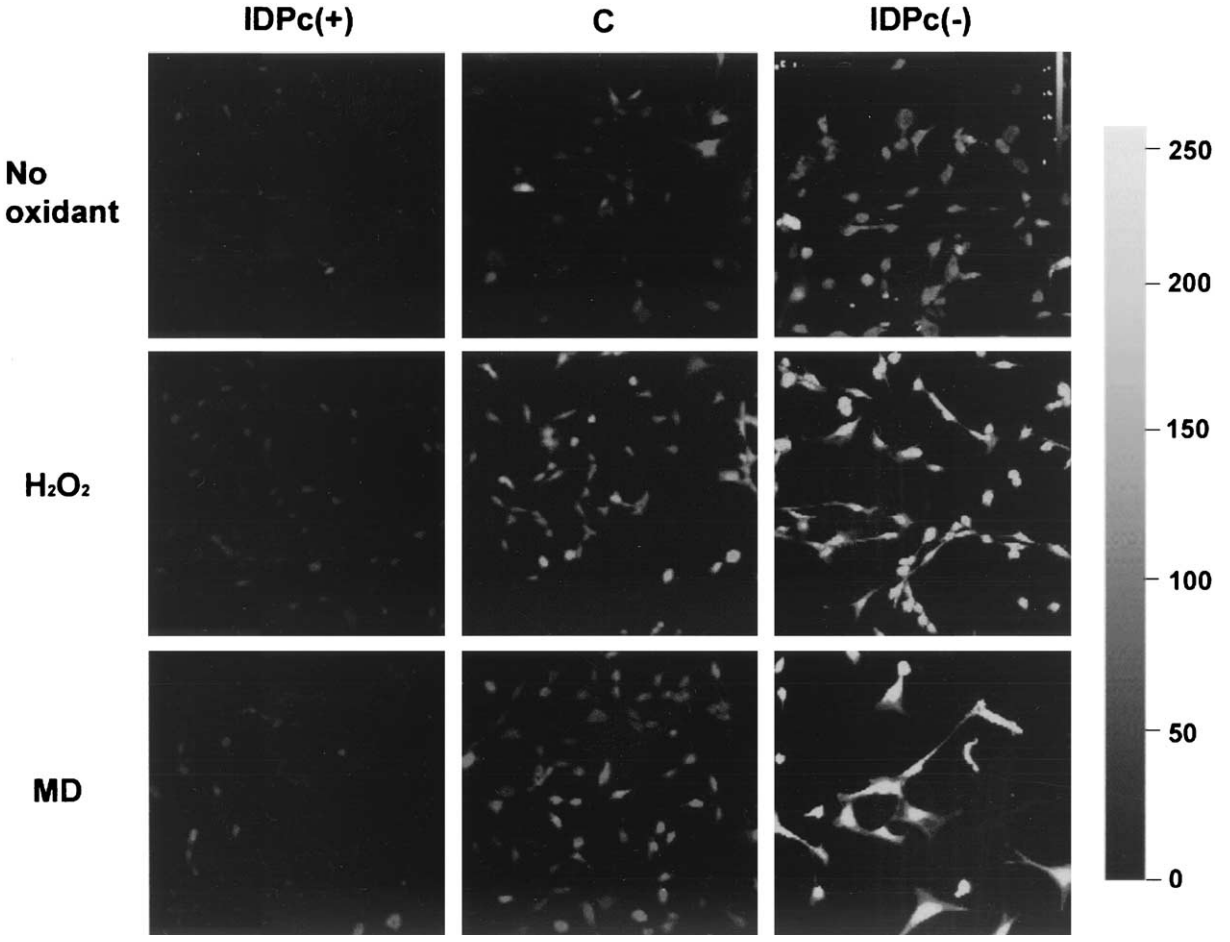

Fig. 5. Measurement of in vivo molecular oxidation by DCF fluorescence. Subcultures of NIH3T3 transfectant cells were grown on poly-L-lysine coated glasses, loaded with H_2-DCF, and then exposed to either 1 mM H_2O_2 or 50 μM menadione for 5 min. The pseudocolor images of DCF fluorescence by ROS are analyzed with the confocal microscopy.

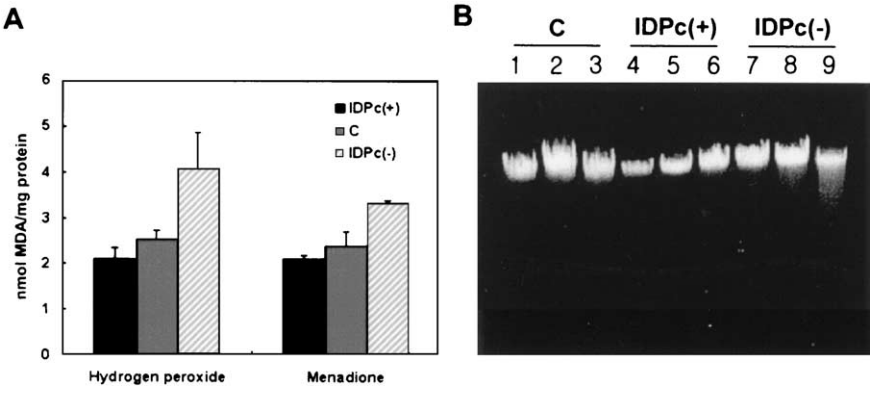

Fig. 6. Cell damage of IDPc transfectant cells. (A) Lipid peroxidation of IDPc transfectant cells after treatment with the oxidant. Cells were treated with 1 mM H_2O_2 or 50 μM menadione for 1 h. Samples were evaluated for MDA production using a Lipid Peroxidation Assay kit. Each value represents the mean $\pm$ SD from three to five independent experiments. (B) Effect of oxidant on DNA strand breaks in IDPc transfectant cells. DNA from cells were analyzed by 0.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Lanes 1, 4, and 7 from untreated cells; lanes 2, 5, and 8 from 1 mM H_2O_2-treated cells; lanes 3, 6, and 9 from 50 μM menadione-treated cells.

sis on 0.5% agarose gels. As shown in Fig. 6B, more fragmented DNA in IDPc(−) cells were observed after exposure to increasing concentrations of oxidants. For instance, DNA was more extensively damaged in IDPc(−) cells treated with 50 μM menadione. In contrast, the overall DNA appeared to be intact or markedly protected in IDPc-rich cells even after exposure to the same oxidant. These results indicate that reduced expression of IDPc most likely leads to increased cell injury, while elevated IDPc appears to protect cells and DNA from oxidative damage.

DISCUSSION

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 ICDHs in mitochondria, peroxisomes, and the cytoplasm has been reported [38], however, the biological functions of this enzyme have not been clearly elucidated. One of the functions of the mitochondrial enzyme has been known either to catalyze the decarboxylation of isocitrate into α-ketoglutarate in the tricarboxylic acid cycle or to mediate the reversal reaction for the generation of isocitrate from glutamic acid for gluconeogenesis [38]. We recently demonstrated that the control of mitochondrial redox balance and oxidative damage is one of the primary functions of IDPm [39]. In the ovary, the cytosolic form is thought to supply NADPH for fatty acid synthesis and for fatty acid chain elongation and desaturation reactions [40]. Although the relationship between the mitochondrial ICDH and its cytosolic counterpart is unknown, it is likely that these two $NADP^+$-dependent isoenzymes are derived from two different genes, based on the lack of cross-immunoreactivity [40], different chromosomal loci and gene sequences [41,42], different tissue-specific expression [43,44], and different subcellular localization [16,43]. Recently, IDPc that is preferentially expressed in bovine corneal epithelium has been identified. The role of this enzyme in contributing to corneal transparency is likely attributed to its protective effect against UV radiation [45]. We also found that *E. coli* mutant lacking $NADP^+$- dependent ICDH is sensitive to the radiation [46]. Therefore, it is reasonable to assume that IDPc performs some other functional role besides supplying NADPH for the biosynthesis pathways.

Plaut and co-workers [44] reported that high levels of IDPc activity are observed in liver and kidney while IDPm activity is mainly detected in heart and skeletal muscles. Consistent with the biochemical determinations, our earlier data of Northern analyses for different isoforms of IDH demonstrated that NAD^+-dependent IDH is localized in all tissues examined [47], while IDPm is mainly expressed in the heart and muscles [39]. Our current Northern analysis revealed that IDPc transcript is also expressed in a tissue-specific manner. Liver and kidney contained highest levels of IDPc transcript, while other organs contain much lower levels of IDPc message. In fact, brain contained very little, if any, detectable level of IDPc transcript. Our current Northern data are in agreement with the biochemical data [44]. Furthermore, the tissue-specific expression of IDPc may explain the reason why different tissues such as brain and liver may have different susceptibility to oxidative organ damage. It has been reported that brain is one of the most vulnerable organs to oxidative stress and ischemic injury [48]. NADPH produced at different rates in different tissues may indicate a possibility of differential protection against oxidative damage [49,50]. However, the importance of an IDPc-dependent antioxidant mechanism against oxidative injury in different tissues remains to be evaluated.

It is well established that oxidative modifications of proteins and DNA are related to pathogenesis of many chronic disease states including aging, alcoholism, and various neurodegenerative diseases. Individual cells or target proteins isolated from patients and appropriate animal models were shown to be more sensitive to endogenous and exogenous oxidative challenges than the normal counterparts. In this regard, the aim of present work was to evaluate the role of IDPc in the protection of NIH3T3 cells, used as a model, from oxidative stress caused by exposure to hydrogen peroxide or menadione during aerobic growth. A large part of the cytotoxic effects of these oxidants could be mediated by the production of hydroxyl radicals ($^{\bullet}OH$) via Fenton-type reactions in the presence of iron [51]. The reduced toxicity of such oxidants on cells can be achieved by removal of potential precursors of highly toxic hydroxyl radicals, namely O_2^- and H_2O_2.

Earlier data suggest that $NADP^+$-dependent G6PD, IDPc, and malic enzymes are important in production of NADPH for biosynthesis and GSH recycling [18]. McAlister-Henn and co-workers [52,53] recently reported that mammalian 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. To directly demonstrate the possible antioxidant role of IDPc, we prepared three different NIH3T3 transformed cells with stable transfection of IDPc cDNAs in sense and antisense directions. In these transformed

cells, our data revealed that only IDPc activity was either elevated in IDPc(+) cells or reduced in IDPc(−) cells compared to the control cells transformed with DNA vector alone. In contrast, the activities of other antioxidant enzymes 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. The ratio of GSSG to total glutathione ($GSSG/GSH_t$) were higher in IDPc(−) cells than in the wild-type or in IDPc(+) with subsequent changes in cell viability upon exposure to oxidants. These results confirm the importance of IDPc in the recycling of glutathione 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, DNA damage reflected by an increase of single strand breaks, lipid peroxidation, and changes in cellular morphology studied with a light microscope.

In summary, we prepared three different transformed cells with different levels of transduced IDPc to investigate the biological role of IDPc against oxidative damage. Our results demonstrate a clear inverse relationship between the level of IDPc activity and the relative degree of oxidative damage. Our biochemical data demonstrate that IDPc is responsible for the production of NADPH, which is then used for GSH recycling and the removal of H_2O_2 via thioredoxin/glutathione-dependent peroxidases. Based on the results reported in this study, IDPc has a protective role against oxidative damage.

Acknowledgement — This work was supported by a grant (No. 991473) from the Basic Research Program of the Korea Science and Engineering Foundation.

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ABBREVIATIONS

DCFHDA—2′,7′-dichlorofluoroscin diacetate
DTNB—5,5′-dithio-bis(2-nitrobenzoic acid)
G6PD—glucose 6-phosphate dehydrogenase
ICDH-$NADP^+$—dependent isocitrate dehydrogenase
IDPc—cytosolic ICDH
IDPm—mitochondrial ICDH
MDA—malondialdehyde
MTT—3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide;thiazolyl blue
NBT—nitroblue tetrazolium
ROS—reactive oxygen species
TBARS—thiobarbituric acid-reactive substances