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Cellular defense against heat shock-induced oxidative damage by mitochondrial $NADP^+$-dependent isocitrate dehydrogenase

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Abstract

Heat shock may increase oxidative stress due to increased production of reactive oxygen species and/or the promotion of cellular oxidation events. Mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) produces NADPH, an essential reducing equivalent for the antioxidant system. The protective role of IDPm against heat shock in HEK293 cells, an embryonic kidney cell line, was investigated in control and cells transfected with the cDNA for IDPm, where IDPm activity was 6–7 fold higher than that in the control cells carrying the vector alone. Upon exposure to heat shock, the viability was lower and the protein oxidation, lipid peroxidation and oxidative DNA damage were higher in control cells as compared to HEK293 cells in which IDPm was over-expressed. We also observed the significant difference in the cellular redox status reflected by the endogenous production of reactive oxygen species, NADPH pool and GSH recycling between two cells. The results suggest that IDPm plays an important role as an antioxidant defense enzyme in cellular defense against heat shock through the removal of reactive oxygen species.

Keywords: *Heat shock, NADPH, isocitrate dehydrogenase, reactive oxygen species, antioxidant enzyme*

Introduction

Most living cells have an essential, highly conserved, and exquisitely regulated cellular response to sudden heat exposure. Moderately elevated temperature induces the selective synthesis of a small number of highly conserved proteins [1]. These are referred to as heat shock proteins (HSPs), and they appear to impart resistance towards elevated temperatures [2]. HSPs also can be induced by a variety of oxidizing agents including hydrogen peroxide and menadione, as well as by radiation [3–6], whose cytotoxicity is thought to be primarily due to the generated reactive oxygen species (ROS) [7]. Exposure to oxygen induces HSPs in *Drosophila* [8], Chinese hamster ovary cells [9], and liver [10]. In contrast, heat shock induced superoxide dismutase (SOD) in mammalian cells [11] and manganese SOD (MnSOD) in *Escherichia coli* [12]. These lines of evidence support the proposal that heat shock is a form of oxidative stress.

ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) are generated *in vivo* from the incomplete reaction of oxygen during aerobic metabolism or from exposure to environmental agents such as radiation, redox cycling agents, or stimulated host phagocytes [3,13,14]. These oxygen species can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, and DNA base modifications and strand breaks [15]. Biological systems have evolved an effective and complicated network of defense mechanisms, which enable cells to cope with

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lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as SOD, which catalyze the dismutation of O_2^- to H_2O_2 and O_2 [16], catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides [17].

The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyze oxidative decarboxylation of isocitrate to a-ketoglutarate and require either NAD^+ or $NADP^+$, producing NADH and NADPH, respectively. NADPH is an essential reducing equivalent for the regeneration of reduced glutathione (GSH) by glutathione reductase and for the activity of NADPH-dependent thioredoxin system [18], both are important in the protection of cells from oxidative damage. Therefore, $NADP^+$-dependent isocitrate dehydrogenase (ICDH) may play an antioxidant role during oxidative stress. We recently proposed that mitochondrial ICDH (IDPm) is involved in the supply of NADPH needed for GSH production against mitochondrial oxidative damage [19].

In the present study the role of IDPm in cellular defense against heat shock was investigated using the HEK293 cells, an embryonic kidney cell line, transfected with the cDNA from IDPm. The results suggest that IDPm has an important protective role in heat shock-induced cellular damage, presumably, through acting as an antioxidant enzyme.

Materials and methods

Materials

RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin were obtained from GIBCO-BRL (Rockville, MD, USA). Pyrogallol, -$NADP^+$, GSSG, glucose-6-phosphate, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and avidin-tetramethylrhodamine isothiocyanate (TRITC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2′,7′-Dichlorofluoroscin diacetate (DCFH-DA) and diphenyl-1-pyrenylphosphine (DPPP) were purchased from Molecular Probes (Eugene, OR, USA). A peptide representing the N-terminal 16 amino acids of mouse IDPm (ADKRIKVAKPVVEMPG) was used to prepare polyclonal anti-IDPm antibodies [19].

Cell culture and cytotoxicity assay

The HEK 293 cell line, an embryonic kidney cell line, with stable transfection with the cDNA for mouse IDPm in a sense orientation was prepared as described [19]. The HEK293 cell line transfected with LNCX-vector alone was used as a control. HEK293 cells were grown in DMEM containing 10% fetal bovine serum and 50 U/ml penicillin/50 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Cytotoxicity was determined using the trypan blue exclusion assay [20]. Briefly, an aliquot of the cell suspension was diluted 1:1 (*v*/*v*) with 0.4% trypan blue. After 5–10 min of incubation, the number of cells excluding or staining positively for uptake of trypan blue was counted under a light microscope. Cell viability was expressed as a percentage of the counts relative to the untreated controls.

Enzyme assay

Cells were collected at 1,000 × *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 homogenate was centrifuged at 1,000 × *g* for 5 min and the supernatants further centrifuged at 15,000 × *g* for 30 min. 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 precipitates were washed twice with sucrose buffer to collect mitochondria pellet. The mitochondrial pellets were resuspended in 1X PBS containing 0.1% Triton-X100, disrupted by ultrasonication (4710 Series, Cole-Palmer, Chicago, IL) twice at 40% of maximum setting for 10 s, and centrifuged at 15,000 × *g* for 30 min. The supernatants were used to measure the activity of IDPm. The protein levels were determined by the method of Bradford using reagents purchased from Bio-Rad. Catalase activity was measured with the decomposition of hydrogen peroxide, which was determined by the decrease in absorbance at 240 nm. SOD activity in cell extracts was assayed spectrophotometrically using a pyrogallol assay, where one unit of activity is defined as the quantity of enzyme which reduces the superoxide-dependent colour change by 50%. Glutathione reductase activity was quantified by the GSSG-dependent loss of NADPH 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. Glucose 6-phosphate dehydrogenase (G6PD) activity was measured by following the rate of $NADP^+$ reduction at 340 nm using the procedure described [19]. For the measurement of IDPm activity, mitochondrial extract was added to 1 ml Tris buffer, pH 7.4, containing 2 mM $NADP^+$, 2 mM $MgCl_2$, and 5 mM isocitrate. Activity of IDPm was measured by the production of NADPH at 340 nm at 25°C [19]. One unit of IDPm activity is defined as the amount of enzyme catalyzing the production of 1 mmol of NADPH/min.

Cellular redox status

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFH-DA

with confocal microscopy [21]. 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 treated with 10 μM DCFH-DA for 15 min and exposed to heat at 42°C for 1 h. 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). NADPH was measured using the enzymatic cycling method as described by Zerez et al. [22] Briefly, the reaction mixture, which combined 100 mM Tris (pH 8.0), 5 mM EDTA, 2 mM phenazine ethosulfate, 0.5 mM MTT, 1.3 unit G6PD, and appropriate 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 the method described by Akerboom and Sies [23], and GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine [24].

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 0.25 N HCl containing 15% (*w*/*w*) trichloroacetic acid [25], 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. [26] After U937 cells were incubated with 5 μM DPPP for 15 min in the dark, cells were exposed to heat at 42°C for 1 h. The images of DPPP fluorescence by reactive species were analyzed by the Zeiss Axiovert 200 inverted microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm).

Protein oxidation

The protein carbonyl content was determined spectrophotometrically using the DNPH-labeling procedure as described [27]. 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.

DNA damage

8-OH-dG levels of HEK293 cells were estimated by using a fluorescent binding assay as described by Struthers et al. [28] After HEK293 cells were exposed to heat at 42°C for 1 h, 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.

Quantitaion of relative fluorescence

The averages of fluorescence intensity from fluorescence images were calculated as described [29].

Replicates

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

Results

To study the relationship between IDPm activity and heat shock-induced cell damage, the two kinds of HEK293 transfectant cells were constructed. The HEK293 cells were transfected with the LNCX containing a IDPm gene as a sense orientation, IDPm(+), or the LNCX alone, control. Chromosomal integration of the transfected IDPm constructs was confirmed by polymerase chain reaction (PCR) (data not shown). The IDPm activity of IDPm(+) cells was increased 6–7-fold compared with that of the control cells (Figure 1A). Immunoblot analysis using anti-IDPm antibody further confirmed the correlation between the amount of IDPm enzyme measured in cell extracts by immunoreaction and the corresponding levels of enzyme activity (Figure 1B). Because cellular antioxidants act in a concerted manner as a team, it is important to investigate whether the modulation in cellular ICDH activity caused concomitant alterations in the activity of other major antioxidant defense enzymes. Increased expression of IDPm did not significantly alter the activities of other antioxidant enzymes such as SOD, catalase, G6PD and glutathione reductase (data not shown), suggesting that the transfection of IDPm cDNA did not affect the activities of other enzymes involved in antioxidation.

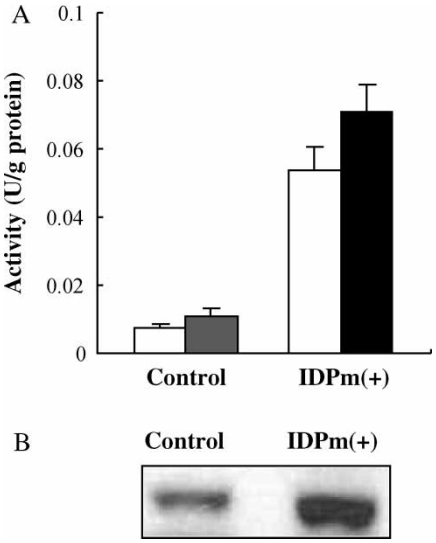

Figure 1. (A) Activity of IDPm in transfectant cell lines. Control (*open bars*) and IDPm(+) (*shaded bars*) HEK293 cells were untreated and treated with heat at 42°C for 1 h and then activity of IDPm was determined. IDPm activities are expressed as units/mg protein. The results shown are the mean ± S.D. of five independent experiments. (B) Immunoblot analysis of IDPm protein expressed in stable transformant HEK293 cells. The mitochondrial fraction (20 μg protein) from cultured cells was separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and subjected to immunoblot analysis using anti-IDPm IgG.

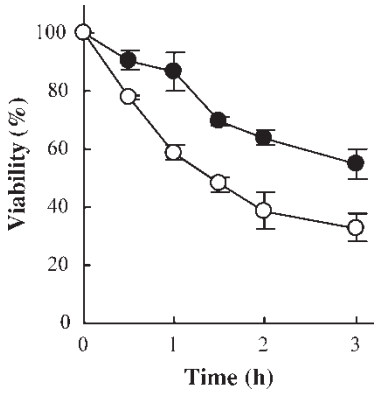

Figure 2. Viability of control (*open circles*) and IDPm(+) (*closed circles*) HEK293 cells upon exposure to heat shock. After HEK293 cells were exposed to heat stress for various lengths of time at 42°C, viability of cells was determined by trypan blue exclusion assay. Survival of untreated cells was expressed as 100%. The results shown are the mean ± S.D. of five independent experiments.

It has been known that the expression of some antioxidant defense enzymes is enhanced in response to many types of stresses. Although ICDH activity in HEK293 cells was increased by heat shock at 42°C (Figure 1A), no significant difference in the activities of other antioxidant defense enzymes occurred with exposure to heat (data not shown). Despite their role in the cellular defense mechanisms, the antioxidant defense enzymes are susceptible to inactivation by ROS. However, it is also possible that prokaryotes and eukaryotes compensate for inactivation of antioxidant defenses enzymes by an enhance expression of enzymes [30]. The results shown in this study indicate that the activity of IDPm is elevated under heat shock, possibly in a compensatory mechanism against elevated ROS.

To measure thermo-tolerance, control and IDPm(+) cells were incubated at 42°C for various lengths of time and cell viability was observed. As shown in Figure 2, control cells were significantly more resistant to heat stress than IDPm(+) cells. Control cells showed a survival of 39% after exposure at 42°C for 2 h whereas IDPm(+) cells showed a survival of 64%. The cells exhibiting the lower IDPm activity was killed to a greater extent upon exposure to heat shock, indicating that IDPm may be involved in protecting cells from damage induced by ROS generated from heat stress.

Reduction of cell viability by hyperthermia might be caused by several mechanisms such as oxidative stress, a reduction of bcr/abl tyrosine kinase activity, and TNF-α-induced autocrine suicidal loops [15,31,32]. To investigate whether or not the difference in viability of the HEK293 transfectant cells upon exposure to heat is associated with ROS formation, the levels of intracellular peroxides in HEK293 cells were measured by confocal microscopy with the oxidant-sensitive probe DCFH-DA. As shown in Figure 3A, DCF fluorescence intensity was significantly decreased in IDPm(+) cells as compared to that of the control upon exposure to heat shock. The data strengthen the conclusion that increased activity of IDPm provides the protective action to cells by decreasing the steady-state level of intracellular oxidants during heat stress. 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 heat, the ratio for cellular [GSSG]/[GSH_t] was significantly higher in control cells compared to that in IDPm(+) cells (Figure 3B). This data indicate that GSSG in control cells was not reduced as efficiently as in IDPm(+) 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 control cells upon exposure to heat. NADPH, required for GSH generation by glutathione reductase, is an essential factor for the cellular defense against oxidative damage. The ratio for [NADPH]/[$NADP^+$ + NADPH] was significantly decreased in control cells treated with heat shock, however, the decrease in this ratio was much less pronounced in IDPm(+) cells (Figure 3C).

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

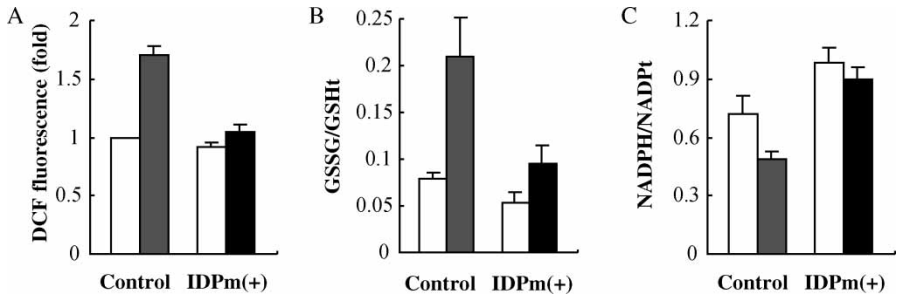

Figure 3. Heat shock-mediated modulation of cellular redox status. (A) Relative intensity of DCF fluorescence in IDPm transfectant cells. Open and shaded bars represent the DCF fluorescence in the cells unexposed and exposed to heat at 42°C for 1 h, respectively. The results shown are the mean ± S.D. of three independent experiments. (B) Ratios of GSSG versus total GSH pool and (C) NADPH versus total NADP pool in HEK293 transfectant cells. Open and shaded bars represent the ratios in the cells unexposed and exposed to heat at 42°C for 1 h, respectively. The results shown are the mean ± S.D. of three independent experiments.

[27]. Although the carbonyl content of IDPm(+) and control cells increased with exposure to heat shock, the increase was significantly lower in IDPm(+) cells compared to that of control cells (Figure 4A). 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 IDPm level correlated with the change in MDA levels as an indicator of lipid peroxidation upon exposure to heat shock. As shown in Figure 4B, exposure of heat shock increased the level of MDA 2.5-fold in control cells, however, the increase in MDA content of IDPm(+) cells was significantly lower than that of control cells. Similarly, DPPP fluorescence intensity, an indicative

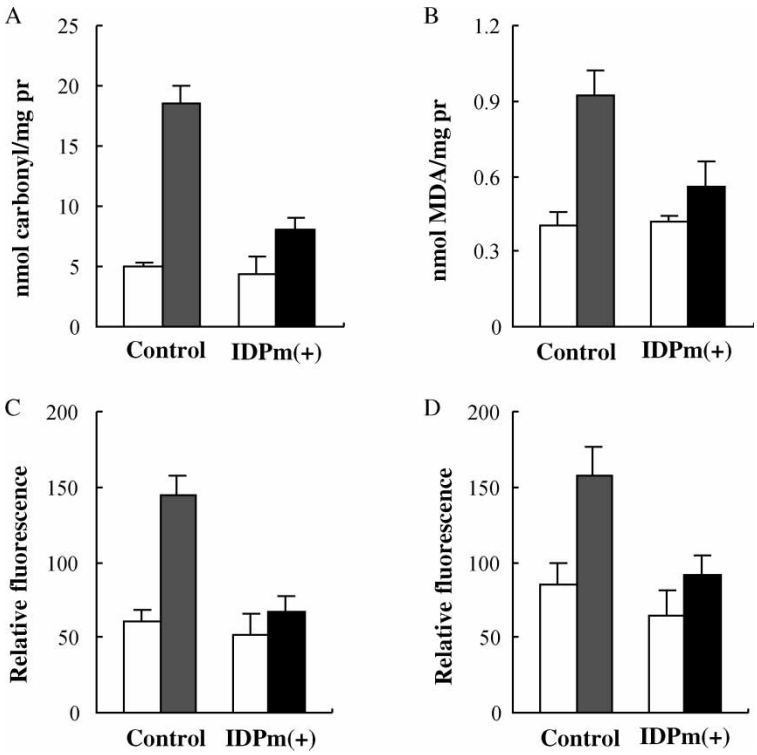

Figure 4. Heat shock-mediated oxidative damage of HEK293 cells. Control and IDPm(+) HEK293 cells were exposed to heat shock at 42°C for 1 h. (A) Protein carbonyl content of HEK293 cells exposed to heat. Protein carbonyls were measured in cell-free extracts using the method of Levine et al., [27] with the use of DNPH. The results shown are the mean ± S.D. of five independent experiments. (B) Lipid peroxidation of HEK293 cells exposed to heat. The level of MDA accumulated in the cells was determined using a TBARS assay. The results shown are the mean ± S.D. of five independent experiments. (C) To visualize lipid peroxidation in HEK293 cells exposed to heat, cells (1×10^6 cells/ml) were stained with 5 μM DPPP for 15 min. Fluorescence images were obtained under microscopy. The average of fluorescence intensity was calculated as described [29]. The results shown are the mean ± S.D. of five independent experiments. (D) Effect of heat shock on 8-OH-dG levels in HEK293 cells. Cells were fixed and permeabilized immediately after exposure to heat. 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized by a fluorescence microscope with 488 nm excitation and 580 nm emission. The average of fluorescence intensity was calculated as described [29]. The results shown are the mean ± S.D. of five independent experiments. Open and shaded bars represent HEK293 cells unexposed and exposed to heat, respectively.

marker to monitor lipid peroxidation, was increased markedly in control cells, whereas it was increased slightly in IDPm(+) cells after exposure to heat shock (Figure 4C). 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 [33], 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* [34]. Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC [28]. The fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased when control cells were exposed to heat (Figure 4D). In contrast, the overall DNA appeared to be markedly protected in IDPm-rich cells even after exposure to heat shock. These results indicate that elevated IDPm appears to protect cells and DNA from oxidative damage induced by heat shock.

Discussion

The exact role of oxidative stress in heat shock is unknown at present. However, it was suggested that heat shock may induce oxidative stress due to increased production of ROS and/or the promotion of cellular oxidation events [35]. Although limited work has indicated that cross-resistance to killing by oxidative stress or heat [36], the fact that the deletion of the genes which encode the antioxidant enzymes results in sensitization of cells to heat shock stress and the over-expression of catalase and SOD genes caused an increase in thermotolerance [37] suggests that heat and oxidative stress have common cellular effects.

IDPm is a key enzyme in cellular defense against oxidative damage by supplying NADPH in the mitochondria. NADPH is an essential cofactor for the regeneration of GSH, the most abundant low-molecular-mass thiol in most organisms, by glutathione reductase in addition to its critical role for the activity of NADPH-dependent thioredoxin system [38,39]. 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 [40]. Reduced thioredoxin may provide reducing equivalents to several enzymes including thioredoxin peroxidases, which remove hydrogen peroxide using hydrogen provided by the NADPH-dependent thioredoxin system [38,39], and methionine sulfoxide reductase, which can reactivate damaged proteins at their methionine residues [40], presumably, involved in the defense against oxidative stress. Elevation of mitochondrial NADPH and GSH by IDPm in turn suppressed the oxidative stress and concomitant ROS-mediated damage. It is well established that mitochondrial dysfunction is directly and indirectly involved in a variety of pathological states caused by genetic mutations as well as exogenous compounds or agents [41]. Mitochondrial GSH becomes critically important against ROS-mediated damage because it not only functions as a potent antioxidant but is also required for the activities of mitochondrial glutathione peroxidase and mitochondrial phospholipid hydroperoxide glutathione peroxidase [42], which removes mitochondrial peroxides. NADPH is a major source of reducing equivalents and cofactor for mitochondrial thioredoxin peroxidase family/peroxiredoxin family including peroxiredoxin III/protein SP-22 [43–45] and peroxiredoxin V/AOEB166 [46]. Therefore, any mitochondrial NADPH producer, if present, becomes critically important for cellular defense against ROS-mediated damage.

To demonstrate the heat-dependent intracellular oxidation we evaluated intracellular oxidants after heat exposure. We noted that heat stress caused an increase in intracellular oxidants such as hydroperoxides in HEK293 cells. However, the increase in hydroperoxides was significantly higher in control cells compared to IDPm(+) cells. These data suggest that the enzymatic action of IDPm protects cells from the cytotoxic actions of heat exposure by decreasing the steady-state level of intracellular oxidants. Intracellular oxidants may induce the damage to protein, DNA, and lipid, which results in cellular damage. Cells have the higher activity of IDPm show a significant decrease in oxidative damage to protein, lipid and DNA, emphasizing the essential protective role of IDPm in mitigating such damage.

The results of this study demonstrate distinct differences between cells exhibit the higher activity of IDPm and control cells in regard to the number of surviving cells and the accumulation of peroxides, oxidized proteins, lipid peroxidation products and 8-OH- dG in DNA upon exposure to heat shock. These results provide support for the role of IDPm as an important antioxidant defense enzyme to protect c ells against heat shock-mediated oxidative stress.

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