Mitochondrial NADP⁺-Dependent Isocitrate Dehydrogenase Protects Cadmium-Induced Apoptosis

In Sup Kil, Seoung Woo Shin, Hyun Seok Yeo, Young Sup Lee, and Jeen-Woo Park
School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Taegu, Korea
Received February 14, 2006; accepted June 19, 2006

ABSTRACT
Cadmium is known to exhibit high affinity for thiol groups and may therefore severely disturb many cellular functions. We have demonstrated that the control of mitochondrial redox balance and oxidative damage is one of the primary functions of mitochondrial NADP⁺-dependent isocitrate dehydrogenase (IDPm). When exposed to cadmium, IDPm was susceptible to loss of enzyme activity and structural alterations. Site-directed mutagenesis confirms that binding of cadmium occurs to a Cys⁸⁷⁹ of IDPm. We examined the antioxidant mechanism-mediated protective role of IDPm against cadmium-induced apoptosis with human embryonic kidney 293 cells transfected with the IDPm cDNA in sense and antisense orientations. As a result, we observed a clear inverse relationship between the amount of IDPm expressed in target cells and their susceptibility to cadmium-induced modulation of cellular redox status and apoptosis. In addition, loss of glutaredoxin (Grx, thioltransferase) activity by cadmium was more pronounced in antisense cells compared with the sense cells. When oxalomalate, a competitive inhibitor of IDPm, was administered to mice, inhibition of IDPm and Grx and enhanced susceptibility to apoptosis were observed upon their exposure to cadmium. These results suggest that IDPm plays an important protective role in cadmium-induced apoptosis by maintaining cellular redox status and by protection of Grx activity.

ABBREVIATIONS: ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione; ICDH, isocitrate dehydrogenase; IDPm, mitochondrial NADP⁺-dependent isocitrate dehydrogenase; HEK, human embryonic kidney; DTT, dithiothreitol; NEM, N-ethylmaleimide; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); ANSA, 8-anilino-1-naphthalene sulfonic acid; PI, propidium iodide; HA, hemagglutinin; DCFH-DA, 2',7'-dichlorofluorescin diacetate; PARP, poly(ADP-ribose) polymerase; Ac-DEVD-\textit{p}-NA, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; G6PDH, glucose 6-phosphate dehydrogenase.
catalyzes the reduction of protein-S-S-glutathione mixed disulfide (Gravina and Mieyal, 1993). Oxidized Grx is selectively recycled to the reduced form by GSH with the formation of oxidized glutathione (GSSG) and regeneration of GSH by coupling with NADPH and GSSG reductase, termed the GSH-regenerating system (Gan and Wells, 1986). These characteristic interactions of Grx with GSH distinguish it from Trx, which favors intramolecular disulfide substrates and is turned over by NADPH and thioredoxin reductase independently of GSH. Functional overlap or cross-talk between the two systems, however, has been indicated (Casagrande et al., 2002). Grx also partially shares its function as a redox sensor with Trx (Song and Lee, 2003), and overexpression of Grx inhibited oxidation of Akt and protected cells from apoptosis (Urata et al., 2003).

The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyze oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD⁺ or NADP⁺, producing NADH and NADPH, respectively (Koshland et al., 1985). NADPH is an essential reducing equivalent for the regeneration of GSH by glutathione reductase and for the activity of NADPH-dependent thioredoxin system (Kwon et al., 1994); both are important in the protection of cells from oxidative damage. Therefore, ICDH may play an antioxidant role during oxidative stress. In mammals, three classes of ICDH isoenzymes exist: mitochondrial NAD⁺-dependent ICDH, mitochondrial NADP⁺-dependent ICDH (IDPm), and cytosolic NADP⁺-dependent ICDH (Koshland et al., 1985). We recently reported that IDPm is involved in the supply of NADPH needed for GSH production against mitochondrial oxidative damage (Jo et al., 2001). Hence, the damage of IDPm may result in the perturbation of the balance between oxidants and antioxidants and subsequently lead to a prooxidant condition. Because cysteine residues play an essential role in the catalytic function of IDPm (Smyth and Colman, 1991), the highly reactive sulhydryl group in IDPm could be potential targets of nitric oxide, peroxynitrite, 4-hydroxynonenal, H₂O₂, diamide, and heavy metals. Based on the reactive nature of cysteine residue(s) in IDPm at physiological pH and that sulhydryl modification results in inactivation of enzyme, we hypothesized that IDPm may play a regulatory role in cadmium-induced apoptosis.

In this study, we report that IDPm is inactivated by the formation of complex between Cys³⁷⁸ and cadmium and that this inactivation is protected by thiols. In addition, overexpression of IDPm protected HEK293 from cadmium-induced apoptosis by protection of Grx inhibition and by reduction of ROS presumably to supply NADPH for antioxidant systems.

Materials and Methods

Materials. Isocitrate, β-NADP⁺, NADPH, CdCl₂, IDPm from pig heart, glutathione reductase from yeast, GSH, GSSG, dithiothreitol (DTT), N-ethylmaleimide (NEM), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 8-anilino-1-naphthalene sulfonic acid (ANS), propidium iodide (PI), anti-hemagglutinin (HA) IgG, and cadmium chloride were purchased from Sigma-Aldrich (St. Louis, MO). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Invitrogen (Eugene, OR). Antibodies against Bcl-2, lamin B, caspase-3, phospho-JNK, JNK, and poly(ADP-ribose) polymerase (PARP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). N-Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) was obtained from Calbiochem (San Diego, CA). Electrophoreses reagents and Bio-Rad protein assay kit were purchased from Bio-Rad (Hercules, CA). Antibody against IDPm was prepared from IDPm-immunized rabbits, and the antibody was purified by protein A affinity chromatography.

Cell Culture. HEK293, a human embryonic kidney cell line, with stable transfections with the cDNA (a gift from Dr. T. L. Huh, Kyungpook National University, Taegu, Korea) for IDPm in sense and antisense orientations was prepared as described previously (Jo et al., 2001). HEK293 with pcDNA-HA-Grx1 (a gift from Dr. Y. J. Lee, University of Pittsburgh, Pittsburgh, PA) was also prepared. The HEK293 cell line transfected with LNCX-vector or pcDNA alone was used as a control. HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Enzyme Assays. IDPm (6.5 μg) was added to 1 ml of 40 mM Tris buffer, pH 7.4, containing 2 mM NADP⁺, 2 mM MgCl₂, and 5 mM isocitrate. Activity of IDPm was measured by the production of NADPH at 340 nm at 25°C. One unit of IDPm activity is defined as the amount of enzyme catalyzing the production of 1 μmol of NADPH/min. For the determination of IDPm activities in mammalian cells, cells were collected at 1000g for 10 min at 4°C and were washed once with ice-cold PBS. In brief, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose and 10 mM Tris-Cl, pH 7.4). Cell homogenates were centrifuged at 1000g for 5 min, and the supernatants were further centrifuged at 15,000g for 30 min. The precipitates were washed twice with sucrose buffer to collect the mitochondrial pellets. The mitochondrial pellets were resuspended in 1× PBS containing 0.1% Triton X-100, disrupted by ultrasonication (Ultrasonic Homogenizer 4710 Series; Cole-Palmer Instrument Co., Chicago, IL) twice at 40% of maximum setting for 10 s, and centrifuged at 15,000g for 30 min. The supernatants were used to measure the activity of IDPm. The protein levels were determined by the method of Bradford (1976) using reagents purchased from Bio-Rad. Grx activity was determined as described previously (Lushman and Holmgren, 1982) by measuring the reduction of 0.7 mM hydroxyethyl disulfide by 1 mM GSH in the presence of NADPH and yeast glutathione reductase at 25°C. The decrease in NADPH was monitored at 340 nm. Any contribution of NADPH oxidation in the absence of GSH was subtracted out.

Immunoblot Analysis. Proteins were separated on 10 to 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). For glutathionylation detection by immunoblot, IDPm was mixed with 5× SDS sample buffer, without reducing reagents, and supplemented with 5 mM NEM to block unreacted thiol groups, and then subjected to SDS-PAGE followed by electroblotting onto nitrocellulose membranes. The relative quantity of protein in a band on a Western blot was estimated by using a densitometer (Biomed Instruments, Fullerton, CA).

Structural Analysis. For CD spectroscopy, samples of IDPm were desalted on Econo-Pac 10 DG column (Bio-Rad) equilibrated in 20 mM Tris buffer, pH 7.4, and fractions containing the protein were pooled. CD spectra were recorded on a temperature-controlled spectropolarimeter (J-810; Jasco, Tokyo, Japan). Spectra were recorded at 25°C in 0.05-cm quartz cells from 190 to 250 nm with protein concentrations of 0.05 mg/ml at a digital resolution of 0.5 nm, with scan speed of 5 nm/min, and the supernatants were further centrifuged at 15,000g for 30 min. The precipitates were washed twice with sucrose buffer to collect the mitochondrial pellets. The mitochondrial pellets were resuspended in 1× PBS containing 0.1% Triton X-100, disrupted by ultrasonication (Ultrasonic Homogenizer 4710 Series; Cole-Palmer Instrument Co., Chicago, IL) twice at 40% of maximum setting for 10 s, and centrifuged at 15,000g for 30 min. The supernatants were used to measure the activity of IDPm. The protein levels were determined by the method of Bradford (1976) using reagents purchased from Bio-Rad. Grx activity was determined as described previously (Lushman and Holmgren, 1982) by measuring the reduction of 0.7 mM hydroxyethyl disulfide by 1 mM GSH in the presence of NADPH and yeast glutathione reductase at 25°C. The decrease in NADPH was monitored at 340 nm. Any contribution of NADPH oxidation in the absence of GSH was subtracted out.

Immunoblot Analysis. Proteins were separated on 10 to 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). For glutathionylation detection by immunoblot, IDPm was mixed with 5× SDS sample buffer, without reducing reagents, and supplemented with 5 mM NEM to block unreacted thiol groups, and then subjected to SDS-PAGE followed by electroblotting onto nitrocellulose membranes. The relative quantity of protein in a band on a Western blot was estimated by using a densitometer (Biomed Instruments, Fullerton, CA).

Structural Analysis. For CD spectroscopy, samples of IDPm were desalted on Econo-Pac 10 DG column (Bio-Rad) equilibrated in 20 mM Tris buffer, pH 7.4, and fractions containing the protein were pooled. CD spectra were recorded on a temperature-controlled spectropolarimeter (J-810; Jasco, Tokyo, Japan). Spectra were recorded at 25°C in 0.05-cm quartz cells from 190 to 250 nm with protein concentrations of 0.05 mg/ml at a digital resolution of 0.5 nm, with scan speed of 5 nm/min for wavelengths above and below 190 nm, respectively. Multiple spectra were recorded for duplicated samples. These spectra were averaged and corrected for baseline contribution from the buffer. Intrinsic fluorescence steady-state fluorescence measurements were performed on an RF-5301 PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) with the sample compartment maintained at 22°C. A 150-W xenon source was used. The slit-width was fixed at 5 nm for excitation and emission. Unless otherwise
stated, samples were excited at 278 nm, and the emission was monitored between 300 and 400 nm. ANSA (100 μM) was incubated with the various forms of IDPm in 25 mM potassium phosphate buffer, pH 7.0, and 50 mM KCl. The fluorescence emission spectra (excitation, 370 nm) of the different mixtures were monitored on a spectrofluorometer. Binding of ANSA to the protein was evidenced by subtracting the emission spectrum of ANSA from that of ANSA in the presence of enzyme.

**Vector Construction.** For the construction of the His-tagged IDPm purification vector pET14b-IDPm, a 1.3-kilobase DNA encoding the IDPm gene was amplified from LNCX containing the cDNA insert for IDPm by polymerase chain reaction. In brief, the 5′-primer oligonucleotide (5′-GGAATTCATAGCTGAGAAGAGGA-3′), which annealed to the 5′ end of the IDPm gene (IDH2) and introduced an NdeI site, and 3′-primer oligonucleotide (5′-CAGGATCCATTCTGCTTGCCCA-3′), complementary to the 3′ terminus of the IDPm gene but inserted a BamHI site, were used as primers.

**Site-Directed Mutagenesis and Preparation of Recombinant Proteins.** Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following mutagenic primers were used: 5′-GCTTTGTGGCGCTTTCAAGAACTATGATG-3′ for C269S and 5′-GACCTGGCTGTTTCTATTCCATGGGCTCAG-3′ for C379S, respectively, in which the substituted serine codon is underlined. To prepare recombinant proteins, E. coli transformed with pET14b containing the cDNA insert for mouse IDPm or mutant IDPm (C269S, C379S, and C269S/C379S double mutant) constructs was grown and lysed, and Histagged proteins were purified on nickel-nitrilotriacetic acid agarose as described previously (Hoffmann and Roeder, 1991).

**Cellular Redox Status.** NADPH was measured using the enzymatic cycling method as described previously (Zerez et al., 1987). The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm (ε = 1.36 × 10^4 M⁻¹ cm⁻¹) according to the method described by Akerboom and Sies (1981), and GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine (Anderson, 1985). Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy (Jo et al., 2001).

**DNA Fragmentation Assay.** To determine the degradation of chromosomal DNA into nucleosome-sized fragments, a 500-μl aliquot of the lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to the cell pellet (2 × 10⁶ cells) and incubated at 37°C overnight. DNA was obtained by ethanol precipitation, separated in a 0.8% agarose gel, and visualized under UV light.

**Fluorescence-Activated Cell Sorting.** To determine the portion of apoptotic cells, cells were analyzed with PI staining (Darzynkiewicz et al., 1980). Untreated or cadmium-treated HEK293 cells were collected at 2000g for 5 min and washed once with ice-cold PBS and fixed in 70% ethanol. Ethanol was decanted by centrifugation, and cells were stained with 1 ml of solution containing 50 mg/ml PI, 1 mg/ml RNase A, and 1.5% Triton X-100 for at least 1 h in the dark at 4°C. Labeled nuclei were subjected to flow cytometric analysis and then gated on light scatter to remove debris. Cells (percentage) with nuclei with a sub-G₁ content were considered apoptotic.

**Caspase Activity Assay.** Cells were washed three times with chilled PBS and then incubated with 75 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, and 0.5 mM phenylmethylsulfonyl fluoride) for 30 min at 37°C. Theretofore, the contents from three wells were pooled and centrifuged at 20,000g for 20 min at 4°C. The supernatant was mixed (1:1) with reaction buffer (100 mM HEPES, 1 mM EDTA, 10 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol). Reaction began with addition of 5 μl (5 mg/ml) of the colorimetric agent Ac-DEVD-pNA (caspase-3 substrate), and caspase activity was measured at the

![Fig. 1](https://example.com/image1.jpg)

**IDPm Protects Cd²⁺-Induced Apoptosis**

---

**Fig. 1.** Inactivation of IDPm by cadmium and protection by thiols. A, time- and concentration-dependent inactivation of IDPm by cadmium. IDPm was incubated without (open circles) or with 20 (rectangles), 50 (triangles), and 100 μM cadmium (closed circles) at 37°C and an aliquot of the incubation mixture was taken at indicated times and the remaining activity was determined (top). Activities are given as a percentage of the control value. Means of triplicate assays are shown. Immunochemical analysis of cadmium-treated IDPm (bottom). After incubation with various concentrations of cadmium for 1 h at 37°C, samples were subjected to SDS-PAGE for immunoblotting with anti-IDPm IgG. B, IDPm was incubated with 50 μM cadmium in the absence or in the presence of 1 mM GSH or 1 mM DTT. Activities are given as a percentage of the control value. Data are presented as means ± S.D. of three separate experiments. C, inactivation of IDPm in cadmium-treated HEK293 cells. HEK293 cells were incubated with various concentrations of cadmium for 12 h at 37°C and disrupted by sonication. The mitochondrial fraction was prepared, and activity of IDPm was determined. Activities are given as a percentage of the control value. Data are presented as means ± S.D. of five separate experiments.
absorbance at 405 nm 1 h after incubation of the mixture at 37°C. Caspase activity was calculated as (absorbance per milligram of protein in treated sample)/(absorbance per milligram of protein in control sample).

Mice and Administration of Chemicals. ICR mice obtained from Hyochang Science (Taegu, Korea) were allowed free access to standard rodent chow (Harlan Teklad 7001; Harlan Teklad, Madison, WI) and tap water. Male mice aged 6 to 8 weeks were used in this study. Mice were injected with either saline or oxalomalate (25 mg/kg i.p.) dissolved in saline every day for 10 days. Control animals received the same volume of vehicle alone. After injection with oxalomalate for 10 days, the mice were injected with either saline or cadmium (2 mg/kg i.p. two times for 2 days). At 3 h after the last injection (n = 6/group), kidneys were rapidly removed.

Statistical Analysis. The difference between two mean values was analyzed by Student’s t test and was considered to be statistically significant when p < 0.05.

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

Results

Inactivation of IDPm by Cadmium. Incubation of IDPm with cadmium at pH 7.4 at 37°C resulted in a time- and concentration-dependent loss of enzyme activity as shown in Fig. 1A (top). At 50 μM, enzyme activity was inhibited approximately 80% after 1-h incubation, and this inhibition was not induced by fragmentation or degradation of the enzyme (Fig. 1A, bottom). To gain insight into the mechanism of IDPm damage by cadmium, IDPm was allowed to react simultaneously with cadmium and thiols such as DTT and GSH. As shown in Fig. 1B, IDPm was protected from cadmium by cotreatment of DTT or GSH, suggesting that cadmium binds to cysteine residue(s) of IDPm, which presumably induce inactivation of the enzyme. To test whether cellular IDPm was inactivated, HEK293 cells were treated with various concentrations of cadmium for 12 h. As shown in Fig. 1C, intracellular IDPm activity was inhibited approximately 45% in HEK293 cells treated with 100 μM cadmium for 12 h. IDPm inactivation in HEK293 cells requires higher concentrations of Cd2+ with longer incubation compared with the purified enzyme. This apparent difference in the inhibitory effects in situ and in vitro can reasonably be attributed to differences in the conditions of the two experiments. In particular, cellular reducing agents may protect Cd2+-induced inactivation of IDPm in situ. Thus, the in situ experiment probably reflects an underestimation of the actual sensitivity of IDPm to the inactivation by cadmium. In addition, it is possible that there may be more than one form of the enzyme, although which other forms are not known, and only

Fig. 2. Identification of target cysteine residue on IDPm. A, activities of wild type, C269S, C379S, and C269S/C379S mutant IDPm. Wild-type and mutant IDPm proteins were untreated (open bars) or treated with 50 (dotted bars) and 100 μM (shaded bars) cadmium for 1 h at 37°C, and the remaining activity was determined. Activities are given as a percentage of the control value. Data are presented as means ± S.D. of three separate experiments. B, effect of NADP+ on the inactivation of IDPm by cadmium. After incubation of IDPm with various concentrations of cadmium in the absence (closed circles) or in the presence of 1 mM NADP+ (open circles) for 1 h at 37°C, activities of IDPm were determined. Activities are given as a percentage of the control value. Means of triplicate assays are shown.

Fig. 3. Structural changes in cadmium-bound IDPm. A, analysis of cadmium-bound IDPm by CD. Untreated (thick line) and IDPm treated with 50 μM cadmium for 1 h at 37°C (thin line) were analyzed in a spectropolarimeter. Spectra were recorded from 190 to 250 nm, and mean residue ellipticity is plotted as a function of wavelength. B, spectrofluorometric analysis of ANSA binding to the cadmium-bound IDPm. Emission spectra from 400 to 600 nm (excitation, 370 nm) of 100 μM ANSA bound to native IDPm (bottom) and IDPm treated with 50 and 100 μM cadmium for 1 h at 37°C (middle and top traces, respectively). The increase in fluorescence intensity at 490 nm resulting from the binding of ANSA to the enzyme was determined by subtracting the emission spectrum of ANSA from that of ANSA in the presence of the different forms of the enzyme. C, steady-state emission spectra of intrinsic fluorescence of native (top trace) and IDPm treated with 50 and 100 μM cadmium for 1 h at 37°C (middle and bottom traces, respectively) were analyzed in a spectrofluorimeter. Spectra were obtained using an excitation wavelength of 278 nm and excitation and emission slits of 5 nm.
one form of which is susceptible to inactivation by cadmium. It has been proposed that cysteine residue(s) in IDPm could be a potential target of sulfhydryl-modifying agents (Smyth and Colman, 1991). The total number of cysteine residues in the mammalian IDPm has been reported to be 8.2 to 8.8 mol/mol subunit (Johanson and Colman, 1981). In these cysteines, Cys269 and Cys379 were regulated by NEM, and in the main site of modified cysteines (Smyth and Colman, 1991). Cys269 has a catalytic role, most likely in the strengthened binding of Mn2+ in the presence of isocitrate, whereas Cys379 is not essential for catalysis, but it is the binding site for NADP+. To determine which cysteine residues are targets for cadmium, IDPm wild type and cysteine mutants were treated with 50 μM cadmium for 1 h at 37°C. As shown in Fig. 2A, the remaining activity of C379S/C269S double mutant and C379S mutant was not affected by cadmium, but C269S was inhibited similar to IDPm wild type. These results indicate that Cys379 is a target for the inhibition of IDPm by cadmium. To evaluate the protective effect of NADP+ on the inhibition of IDPm by cadmium, IDPm was incubated with cadmium in the presence of NADP+ for 1 h at 37°C. As shown in Fig. 2B, although the incubation of IDPm with 50 μM cadmium resulted in 80% inhibition of IDPm activity, the addition of 1 mM NADP+ completely protected IDPm from inactivation (Fig. 2B). However, other substrates did not exhibit a protective effect against Cd2+-induced inhibition of IDPm. The protective effect of NADP+ supports the suggestion that cadmium reacts with cysteine residue residing in the NADP+ binding site.

Structural Changes in Modified IDPm. To examine the secondary structure of the IDPm species after modification, far UV-CD spectra of native and cadmium-treated IDPm were recorded and analyzed for specific elements of secondary structure. The CD spectrum of IDPm is very similar to that of the protein after modification with 50 μM cadmium, suggesting that cadmium-treated IDPm does not appreciably change the secondary structure of the protein (Fig. 3A). To reveal increases in flexibility of a partial unfolding of cadmium-treated IDPm, the binding of the fluorescent probe ANSA was used to detect the accessibility of the hydrophobic regions on the protein. When IDPm was exposed to 50 μM cadmium for 1 h, it binds the hydrophobic probe ANSA more efficiently than does the native protein. The representative result with cadmium is shown in Fig. 3B. In an attempt to determine the effects of cadmium on the conformation of IDPm, intrinsic fluorescence of the aromatic amino acids in each of the various forms of the enzyme were determined. Native IDPm exhibits a fluorescence emission spectrum typical for tryptophan residues in proteins. Upon excitation of native IDPm at 278 nm, an emission spectrum with a maximum at 333 nm was observed. The fluorescence spectra of native and cadmium-treated IDPm, normalized to the protein content, show that modified IDPm displays a dose-dependent decrease in quantum yield of the emission spectra and a blue shift of the maximum emission wavelength (Fig. 3C).

Redox Status of Cadmium-Treated IDPm Transfectant HEK293 Cells. To study the relationship between IDPm activity and cadmium-induced apoptotic cell death, three kinds of HEK293 transfectant cells were constructed. The HEK293 cells were transfected with LNCX containing either an IDPm gene in a sense orientation [IDPm(+)], in an antisense orientation [IDPm(−)], or LNCX alone (control). Chromosomal integration of the transfected IDPm constructs was confirmed by polymerase chain reaction (data not shown). The IDPm activity of IDPm(+) cells was increased 6-fold compared with that of the control cells. In contrast, IDPm(−) cells exhibited 30% less IDPm activity compared with that of the control (Fig. 4A, top). 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 (Fig. 4A, bottom). To investigate the role of IDPm in cellular defense against cadmium-induced oxidative stress, we determined the cellular redox status in IDPm transfectants unexposed or exposed to cadmium. The levels

![Fig. 4. Effect of IDPm on the redox status of cadmium-treated HEK293 transfectant cells. A, activity of IDPm in HEK293 transfectant cells (top). Control, IDPm(+), and IDPm(−) denote the cells expressing LNCX-vector alone, LNCX-sense IDPm, and LNCX-antisense IDPm, respectively. Data are presented as means ± S.D. of three separate experiments. *p < 0.05 compared with the control cells. Immunoblot analysis of IDPm protein expressed in HEK293 transfectant cells (bottom). The mitochondrial fraction (20 μg of protein) from cultured cells were separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and then subjected to immunoblot analysis using anti-IDPm IgG. B, ROS generation in IDPm transfectant cells treated with cadmium. Typical patterns of DCF fluorescence are presented for IDPm transfectant cells untreated or treated with 20 μM cadmium for 12 h. Fluorescence images were obtained under laser confocal microscopy. The averages of fluorescence intensity were calculated as described previously (Sundaresan et al., 1995). Ratios of NADPH versus total NADP pool (C) and GSSG versus total GSH pool (D) in IDPm transfectant cells. Data are presented as means ± S.D. of five separate experiments. Open and shaded columns represent the cells unexposed and exposed to cadmium, respectively.]

Downloaded from neopharm.aspetjournals.org at ASPET Journals on August 27, 2017
of intracellular peroxides in HEK293 cells were evaluated with an oxidant-sensitive probe DCFH-DA. Increase in DCF fluorescence was observed in IDPm(−) cells when they were exposed to 20 μM cadmium for 12 h. The increase in fluorescence was significantly reduced in IDPm(+) cells (Fig. 4B). 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 IDPm(−) cells treated with cadmium; however, the decrease in this ratio was much less pronounced in IDPm(+) cells (Fig. 4C). One important parameter of GSH metabolism is the ratio of GSSG/total GSH, which may reflect the efficiency of GSH turnover. When the cells were exposed to 20 μM cadmium for 12 h, the ratio for cellular [GSSG]/[total GSH] was significantly higher in IDPm(−) cells than in control and IDPm(+) cells (Fig. 4D). These data indicate that GSSG in IDPm(−) cells was not reduced as efficiently as in IDPm(+) cells. Taken together, these results suggest that the decrease in the efficiency of GSH recycling may be responsible for the higher concentration of intracellular peroxides in IDPm(−) cells treated with cadmium.

**Protective Effect of IDPm against Cadmium-Induced Apoptosis.** Exposure of HEK293 cells to 20 μM cadmium for 12 h caused shrinkage of the cell and plasma membrane blebbing that were apparent by light microscopy (data not shown). To assess whether these changes were attributable to apoptotic changes, DNA fragmentation and flow cytometry using PI were used. As shown in Fig. 5A, DNA fragmentation was more apparent in IDPm(−) cells treated with cadmium compared with IDPm(+) cells. Figure 5B shows a typical cell cycle plot of IDPm transfectant cells that were untreated or treated with 20 μM cadmium for 12 h. Apoptotic cells were estimated by calculating the number of subdiploid cells in the cell cycle histogram. When cells were exposed to cadmium, apoptotic cells were increased markedly in IDPm(−) cells compared with IDPm(+) cells. We evaluated changes in the apoptotic marker proteins as a result of treatment with cadmium and the activity of IDPm on these proteins. Caspase-3 activation in HEK293 cells was assessed by caspase colorimetric assay and by immunoblot analysis of lysates from cells that had been exposed to cadmium. Caspase-3 activity increased significantly higher in IDPm(−) cells compared with control and IDPm(+) cells upon exposure to cadmium (Fig. 6A). As shown in Fig. 6B, cadmium-induced cleavage of caspase-3 cleavage was more pronounced in IDPm(−) cells compared with that in IDPm(+) cells. Induction of the formation of fragments, which represents proteolytic cleavage of PARP and lamin B, indicates an ongoing apoptotic process. The cleaved products of PARP and lamin B increased markedly in IDPm(−) cells compared with IDPm(+) cells upon exposure to cadmium. Taken together, cadmium induced cleavage of procaspase-3 into the active form of caspase-3, and caspase-3 induced degradation of PARP or lamin B. The results also indicate that IDPm exhibits a protective effect on the cadmium-induced apoptosis. The role of mitochondrial pathway of apoptosis in the cadmium-induced death of HEK293 cells was examined by immunoblot analysis of the abundance of Bcl-2, an antiapoptotic protein. As shown in Fig. 6B, the abundance of Bcl-2 in HEK293 cells was significantly decreased in IDPm(−) cells compared with that of IDPm(+) cells when exposed to cadmium. The transmission of signals from external stresses during apoptosis is accompanied by the activation of JNK (Kryriakis and Avruch, 1996). Activation of JNK by cadmium was more pronounced in IDPm(−) cells compared with that of IDPm(+) cells.

**Protection of Grx1 Inhibition.** It has been reported that cytosolic Grx (Grx1) has a vital role in sulfhydryl homeostasis and cell survival (Chrestensen et al., 2000). To evaluate the cellular function of Grx1 in cadmium-induced apoptosis, we set out to elevate the level of Grx1 by transfecting cells with Grx1 cDNA, Grx1(+) (Fig. 7A). The Grx1 activity of Grx1(+) cells was increased 2-fold compared with that of the control.
cells transfected with vector alone. As shown in Fig. 7B, the cleaved products of PARP and lamin B decreased markedly in Grx1(+) cells compared with the control cells upon exposure to cadmium. When we tested Grx1 activity in IDPm transfectant cells exposed to cadmium, inhibition of Grx1 was significantly more pronounced in IDPm(−) cells compared with that in IDPm(+) cells (Fig. 8A). As shown in Fig. 8B, purified Grx1 was inactivated by cadmium, and the inactivation was protected by GSH. However, NADPH did not exhibit the protective effect. These results suggest that IDPm plays a protective role for Grx1 indirectly through the supply of NADPH for GSH recycling.

Effect of IDPm against Cadmium-Induced Apoptosis in Vivo. The kidneys as well as the testis and liver have traditionally been considered to be the main targets of cadmium toxicity. Oxalomalate, a tricarboxylic acid (α-hydroxy-β-oxaloxy succinic acid) formed in vitro and in vivo by condensation of oxaloacetate and glyoxylate, has been known to be a potent competitive inhibitor of IDPm (Ingebretsen, 1976). The enzymatic activity of IDPm in the kidneys of oxalomalate-treated mice was decreased approximately 20% (Fig. 9A). As shown in Fig. 9B, the activity of Grx1 from the kidneys of oxalomalate-treated mice was significantly lower than that of the control mice exposed to cadmium. In contrast, the mitochondrial Grx (Grx2) activity was affected by neither oxalomalate nor cadmium (Fig. 9C). It has been reported that unlike other Grx isoforms, Grx2 is relatively insensitive to oxidative inactivation (Ludberg et al., 2001). We evaluated changes in the apoptotic marker proteins as a result of cadmium exposure and the influence of IDPm inhibition by oxalomalate on these proteins. As shown in Fig. 9D, the cadmium-induced cleavage of caspase-3, PARP, and lamin B was significantly increased in the kidneys from oxalomalate-treated mice. In the meantime, the abundance of Bcl-2, an antiapoptotic protein, was significantly decreased in oxalomalate-treated mice compared with that in the control mice. These results suggest that IDPm inhibition by oxalomalate enhanced susceptibility to cadmium-induced apoptosis in vivo.

Discussion

It has been reported that IDPm contains reduced cysteinyl residues that play an essential role in enzyme activity (Smyth and Colman, 1991). The sulphydryl groups of IDPm are susceptible to modification by ROS, reactive nitrogen species, various oxidants, and lipid peroxidation products (Benderdour et al., 2003; Lee et al., 2003). In this study, we present evidence indicating that IDPm can be inhibited by cadmium. The inactivation of IDPm by cadmium was competed with sulphydryl modifying agents. Because IDPm inactivation was prevented by adding its substrate, NADP⁺, we conclude that cadmium binding sites are likely to include a cysteine residue near the NADP⁺ binding site. Site-directed mutagenesis results revealed that Cys³⁷⁹, a residue that

![Fig. 6. Analysis of apoptotic marker proteins. A, activation of caspase-3 in HEK293 transfectant cells exposed to 20 μM cadmium for 12 h. HEK293 cells were lysed and centrifuged. The supernatant was then added to Ac-DEVD-pNA and subjected to caspase colorimetric activity. Protease activity of caspase-3 was calculated by monitoring the absorbance at 405 nm. Data are presented as means ± S.D. of three separate experiments. * p < 0.05 and ** p < 0.01 compared with the control cells. B, immunoblot analysis of various apoptosis-related proteins in HEK293 transfectant cells untreated or treated with 20 μM cadmium for 12 h. Cell extracts were subjected to 10–12.5% SDS-PAGE and immunoblotted with antibodies against cleaved caspase-3, cleaved PARP, lamin B, Bcl-2, and phospho-JNK.](image)

![Fig. 7. Cadmium-induced apoptosis in Grx1 transfectant cells. A, immunoblot analysis of HA-Grx1 protein expressed in Grx1 transfectant HEK293 cells. The cell-free extracts (20 μg of protein) were subjected to 10 to 12.5% SDS-PAGE and immunoblotted with anti-HA IgG. B, immunoblot analysis of apoptotic marker proteins in Grx1 transfectant cells untreated or treated with 20 μM cadmium for 12 h. Cell extracts were subjected to 10 to 12.5% SDS-PAGE and immunoblotted with antibodies against cleaved caspase-3 and cleaved lamin B. β-Tubulin was run as an internal control.](image)

![Fig. 8. Protective effect of IDPm on cadmium-induced Grx1 inactivation. A, activities of Grx1 in IDPm transfectant HEK293 cells exposed to various concentrations of cadmium for 12 h. B, Grx1 was incubated with 100 μM cadmium for 1 h in the absence or in the presence of 1 mM GSH or 1 mM NADPH. Any contribution of NADPH oxidation in the absence of GSH was subtracted. Activities are given as a percentage of the control value. Data are presented as means ± S.D. of three separate experiments.](image)
presumably resides in the NADP⁺ binding site, is a target for cadmium. There are several lines of evidence obtained from the present study indicating that binding of cadmium to IDPm results in structural alterations. These findings are reflected in the changes in intrinsic tryptophan fluorescence and binding of ANSA. However, the CD spectrum and therefore the secondary structure content of IDPm were not altered by cadmium, which suggests that only subtle, not drastic, conformational changes may occur in modified protein. The lower fluorescence quantum yield documents the alteration of the conformational integrity in cadmium-bound IDPm. Modification of IDPm by cadmium may lead to a slight disruption of protein structure, which is presumably responsible for the inactivation of enzymes, at least in part. Among the techniques aimed at following conformational changes of proteins, binding of the fluorescent probe ANSA has been used to detect the accessibility of the hydrophobic regions on a protein upon increases in flexibility or partial unfolding. Binding can be easily monitored, because it is accompanied by an increase in fluorescence associated with the transfer of the ANSA from a hydrophilic to a hydrophobic environment (Szweda and Stadtman, 1993). A change in ANSA fluorescence at 490 nm in IDPm modified by cadmium indicates conformational changes of the protein.

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 (Kwon et al., 1994). IDPm is a key enzyme in cellular defense against oxidative damage by supplying NADPH in the mitochondria, which is needed for the regeneration of mitochondrial GSH or thioredoxin. 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 (Jaeschke et al., 2002). Mitochondrial GSH becomes critically important against ROS-mediated damage because it not only functions as a potent antioxidant but also is required for the activities of mitochondrial glutathione peroxidase and mitochondrial phospholipid hydroperoxide glutathione peroxidase (Arai et al., 1999), which removes mitochondrial peroxides. NADPH is a major source of reducing equivalents and a cofactor for mitochondrial thioredoxin peroxidase family/peroxiredoxin family, including peroxiredoxin III/protein SP-22 (Kang et al., 1998; Araki et al., 1999) and peroxiredoxin VA/OEB166 (Knoops et al., 1999). Therefore, any mitochondrial NADPH producer, if present, becomes critically important for cellular defense against ROS-mediated damage. In this regard, the inactivation of IDPm by cadmium may result in the disruption in regulating the mitochondrial redox balance by providing NADPH. Furthermore, it has been shown that the disruption of the mitochondrial redox balance by inactivation of IDPm influences the cytosolic redox status such as the NADPH pool and GSH recycling (Lee et al., 2003).

Glucose 6-phosphate dehydrogenase (G6PDH) is important in the production of NADPH for GSH recycling in cytosol. It has been shown that a lower concentration of Cd²⁺ treatment increases the expression of G6PDH as measured by mRNA levels and enzyme activity in rat hepatocytes (Xu et al., 2003). Furthermore, it has been demonstrated that G6PDH activity in the ovary extract was not diminished with 1 mM Cd²⁺ (Carattino et al., 2004).

Cadmium is toxic to number of tissues and induces apoptosis in vitro and in vivo. Cellular apoptosis could be initiated by addition of sensitive sulfhydryl groups on specific proteins, and when repair mechanisms for these proteins, such as Gpx and thioredoxin systems, are overwhelmed, an apoptotic response occurs (Chrestensen et al., 2000). Gpx specifically catalyzes the reduction of glutathione-containing mixed disulfides, in particular protein-SSG. Cellular Gpx activity can be affected both directly (inactivation/inhibition) and indirectly (depletion of the second substrate, GSH) (Chrestensen et al., 2000). Cadmium treatment of cells inhibited intracellular Gpx. Our results indicate that the Gpx inhibition by cadmium is protected by IDPm. Therefore, cadmium inactivation of IDPm exacerbates oxidative stress via the perturbation of the antioxidant defense system as well as disruption of the protective role of IDPm on Gpx activity.
Therefore, this results in an increased susceptibility to cadmium-induced apoptosis.

In conclusion, IDPm plays an important protective role in cadmium-induced apoptosis by maintaining the cellular redox status and by the protection of Grx activity.

References


