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Glutathionylation regulates cytosolic NADP$^+$-dependent isocitrate dehydrogenase activity

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Abstract
Cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDPc) is susceptible to inactivation by numerous thiol-modifying reagents. This study now reports that Cys^{269} of IDPc is a target for S-glutathionylation and that this modification is reversed by dithiothreitol as well as enzymatically by cytosolic glutaredoxin in the presence of GSH. Glutathionylated IDPc was significantly less susceptible than native protein to peptide fragmentation by reactive oxygen species and proteolytic digestion. Glutathionylation may play a protective role in the degradation of protein through the structural alterations of IDPc. HEK293 cells treated with diamide displayed decreased IDPc activity and accumulated glutathionylated enzyme. Using immunoprecipitation with an anti-IDPc IgG and immunoblotting with an anti-GSH IgG, we purified and positively identified glutathionylated IDPc from the kidneys of mice subjected to ischemia/reperfusion injury and from the livers of ethanol-administered rats. These results suggest that IDPc activity is modulated through enzymatic glutathionylation and deglutathionylation during oxidative stress.

Keywords: *Glutathionylation, IDPc, structural alterations, oxidative stress*

Introduction

Tripeptide glutathione (GSH) is present in cells at millimolar concentrations and the ratio of GSH to glutathione disulphide (GSSG) is critical for the cellular redox balance [1]. Under conditions of moderate oxidative stress, the reversible formation of mixed disulphides between protein sulphydryl groups and GSH, a process known as S-glutathionylation, can occur [2]. In addition, S-glutathionylation can also occur in physiologically-relevant situations, where it modulates discrete effects on protein function [1]. Glutathionylation is reversed by the actions of the enzyme glutaredoxin (thioltransferase) [3,4] and may prevent the irreversible oxidation of cysteine to cysteine sulphinic (Cys-SO_2H) and sulphonic acid (Cys-SO_3H). Therefore, the regulated formation of mixed disulphides between protein thiols and glutathione redox changes may act as a reversible switch similar to phosphorylation [5]. Numerous enzymes, including carbonic anhydrase III [6], tyrosine hydroxylase [7], creatine kinase [8], cAMP-dependent protein kinase [9] and HIV-1 protease [10] are potentially influenced by the formation of protein adducts with glutathione. Transcription factors such as c-Jun [5] and NFκB [11–13] also appear to be redox-regulated by mechanisms that include protein S-thiolation and Ras proteins become glutathionylated, with the concomitant activation of p21ras, when cells are exposed to oxidants [14].

The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyse oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD$^+$ or NADP$^+$, producing reducing equivalents in the form of NADH and NADPH, respectively [15]. NADPH is an essential electron donor for the

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regeneration of GSH by glutathione reductase and for the activity of NADPH-dependent thioredoxin system [16,17], both of which are important in protecting 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 (IDH), mitochondrial $NADP^+$-dependent ICDH (IDPm) and cytosolic $NADP^+$-dependent ICDH (IDPc) [15]. We previously reported that $NADP^+$-dependent ICDHs help supply the NADPH needed for GSH production against cytosolic and mitochondrial oxidative damage [18,19]. Although, the relationship between IDPc and its mitochondrial counterpart is unknown, it is likely that these two $NADP^+$-dependent isoenzymes are derived from different genes, based on the lack of cross-immunoreactivity [20], different chromosomal loci and gene sequences [21,22], different tissue-specific expression and different subcellular localizations [23]. Recently, we reported that the activity of IDPm is regulated by glutathionylation [24].

In this study, we demonstrate that IDPc is inactivated by glutathionylation. This inactivation was reversed not only by dithiothreitol (DTT) but also by cytosolic thioltransferase (glutaredoxin 1, Grx1), a thiol-disulphide oxidoreductase that is specific for glutathionyl mixed disulphide substrates and specifically utilizes GSH as a co-substrate. This mechanism suggests an alternative modification to the redox regulation of cysteine in IDPc and suggests a possible *in vivo* mechanism in the regulation of IDPc activity.

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Materials and methods

Materials

Isocitrate, β-$NADP^+$, NADPH, sodium selenite, N-acetyl-DL-penicillamine (SNAP), GSH, GSSG, cysteine, DTT, trypsin, N-ethylmaleimide (NEM), diamide, rose bengal and 8-anilino-1-naphthalene sulphonic acid (ANSA) were purchased from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxynonenal (HNE) was obtained from Calbiochem (La Jolla, CA). The anti-GSH antibody was purchased from VitroGen (Watertown, MA). Streptavidin-HRP was purchased from Cell Signaling (Beverly, MA). A purified mouse IDPc was used to prepare polyclonal anti-IDPc antibodies in rabbits. The human Grx1 was purchased from Abfrontier (Seoul, Korea).

IDPc expression and purification

To prepare recombinant IDPc, *Escherichia coli* was transformed with the plasmid pET-22b(+) containing an insert of the human IDPc gene fused with a C-terminal His × 6 tag were grown and lysed and then the human IDPc was purified by affinity chromatography using a nickel-nitrilotriacetic acid-agarose column (Qiagen).

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′-GCTTCATCTGGGCCAGTAAAAACTATG-3′ for C269S and 5′-GGACTTGGCTGCTAGCATTAAAGGTTTAC-3′ for C379S, in which the substituted serine codon is underlined. To prepare recombinant proteins, *E. coli* transformed with pET14b containing the cDNA insert for human IDPc or mutant IDPc (C269S and C379S) constructs was grown and lysed and His-tagged proteins were purified on nickel-nitrilotriacetic acid agarose.

In vitro *glutathionylation*

Recombinant IDPc was incubated with various concentrations of GSSG at 37°C for the indicated times. Glutathionylated samples were subjected to SDS-PAGE for immunoblotting with an anti-GSH IgG. *In vitro* experiments with purified IDPc were also done with biotin-labelled GSSG. Biotin-GSSG was synthesized by coupling biotin to the primary amino acids of GSSG using EZ-Link^{TM} Sulfo-NHS-Biotin (Thermo) as the biotinylating reagent, as described previously [25]. Biotin labelling was revealed with streptavidin-HRP.

Cell culture

The human embryonic kidney cell line HEK293 was purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin sulphate. Cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Measurement of IDPc activity

IDPc (6.5 μg) or the cytosolic fraction was added to 1 ml of 40 mM Tris buffer (pH 7.4) containing $NADP^+$ (2 mM), $MgCl_2$ (2 mM) and isocitrate (5 mM). The activity of IDPc was measured by the production of NADPH at 340 nm and 25°C [18]. One unit of IDPc activity is defined as the amount of enzyme catalysing the production of 1 μmol of NADPH/min. To determine the IDPc activity in HEK293 cells, cells were collected at 1000 × g for 10 min at 4°C and were washed once with cold PBS. Cells were then homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris-Cl, pH 7.4), the cell homogenate was centrifuged

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at 1000 × g for 5 min, then the supernatant was further centrifuged at 15 000 × g for 30 min. The supernatant was used to measure the activity of IDPc. Protein levels were determined by the Bradford method using reagents purchased from Bio-Rad.

Immunoblot analysis

Proteins were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and subsequently subjected to immunoblot analyses using the appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labelled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). For glutathionylation detection by immunoblot, IDPc was mixed with 5X SDS sample buffer, without reducing reagents and supplement with 5 mM NEM to block unreacted thiol groups and then subjected to SDS-PAGE followed by electroblotting onto nitrocellulose membranes.

Immunoprecipitation

Cytosolic fractions were pre-cleared with protein-A Sepharose (Amersham Pharmacia Biotech) and preserum for 1 h at 4°C. Supernatants were then incubated with rabbit polyclonal anti-IDPc (5 μg) for 12 h at 4°C followed by a protein-A Sepharose incubation for 1 h at 4°C. Immunoprecipitated proteins were washed, separated by SDS-PAGE and visualized by Western blotting with an anti-GSH antibody.

Structural analysis

Steady-state intrinsic fluorescence measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer with the sample compartment maintained at 22°C. A 150 W xenon source was used with the slit-width fixed at 5 nm for excitation and emission. Unless otherwise stated, samples were excited at 278 nm and the emission was monitored between 300–400 nm. ANSA (100 μM) was incubated with the various forms of proteins in 25 mM potassium phosphate buffer, pH 7.0/50 mM KCl. The fluorescence emission spectra (excitation, 370 nm) of the different mixtures were monitored on the spectrofluorometer. ANSA binding to the protein was determined by subtracting the emission spectrum of ANSA alone from that of ANSA in the presence of enzyme.

Preparation of cytosolic fractions from tissues

Kidneys from mice subjected to 30 min of bilateral renal ischemia and reperfusion for 24 h were the kind gift of Dr K.M. Park (Kyungpook National University, Korea). Livers from rats injected intraperitoneally with ethanol (2.5 g/kg/day, 7 days) were the kind gift of Dr E. S. Yang (Kyungpook National University, Korea). Tissues in sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4) on ice were homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000 × g for 5 min and the supernatant was centrifuged at 15 000 × g for 30 min. The supernatant was used to purify IDPc by immunoprecipitation.

Replicates

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

Results

GSSG inhibited the activity of IDPc in a time- and concentration-dependent manner (Figure 1A and B). Incubation of IDPc with 5 mM GSSG for 30 min completely eliminated activity, while 5 mM GSH did

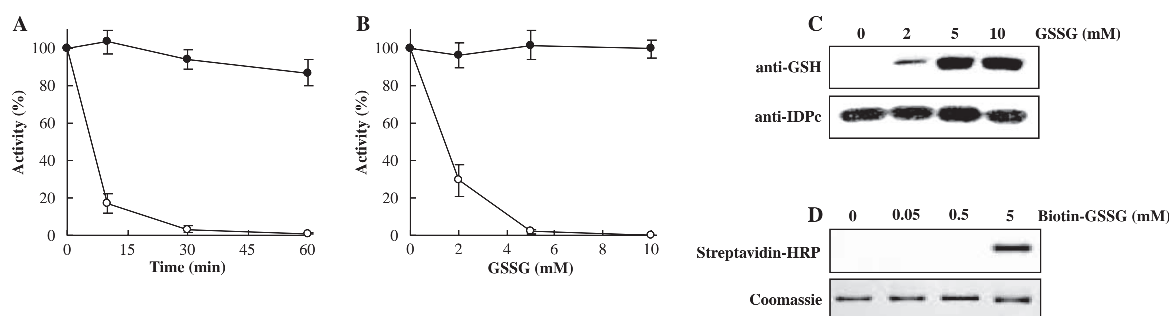

Figure 1. Inactivation of IDPc by GSSG and detection of GSS-IDPc. (A) Time-dependent inactivation of IDPc by GSSG. IDPc was incubated with 5 mM GSSG (open circles) or 5 mM GSH (closed circles) at 37°C. An aliquot of the incubation mixture was taken at the indicated times and the remaining activity was determined. Activities are given as a percentage of the control value. Data are presented as means ± SD of five separate experiments. (B) Concentration-dependent inactivation of IDPc by GSSG. IDPc was incubated with various concentrations of GSH or GSSG for 30 min at 37°C and the remaining activity was determined. Activities are given as a percentage of the control value. Data are presented as means ± SD of five separate experiments. (C) Immunochemical analysis of GSS-IDPc. After incubation with various concentrations of GSSG for 30 min at 37°C samples were subjected to SDS-PAGE for immunoblotting with an anti-GSH IgG. (D) Recombinant IDPc was incubated with various concentrations of biotin-GSSG. Western blot probed with streptavidin-HRP and developed with the ECL reagent.

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not noticeably inhibit IDPc activity. Compared to a previous report on IDPm [24], IDPc was significantly more susceptible to inactivation by glutathionylation. When IDPc was incubated with various concentrations of GSSG and subjected to non-reducing Western blot analysis with a polyclonal anti-GSH antibody, the intensity of the immunoreactive 45 kDa band was increased in a concentration-dependent manner (Figure 1C). As a complementary approach, the recombinant IDPc was treated with biotin-GSSG. Figure 1D shows non-reducing Western blots probed with streptavidin-HRP to detect IDPc undergoing S-glutathionylation following this treatment.

It has been proposed that the cysteine residue(s) in IDPc could be potential targets of sulphydryl modifying agents [26]. To determine whether glutathionylated cysteine(s) in IDPc are susceptible to sulphydryl modifying agents, IDPc was reacted simultaneously with GSSG and various concentrations of diamide, SNAP, selenite and HNE, a lipid peroxidation product. As shown in Figure 2A, a dose-dependent decrease of glutathionylated IDPc was observed. The addition of 5 mM DTT or GSH completely reversed inhibition (Figure 2B), suggesting that GSSG modifies susceptible cysteine(s) on the protein through the formation of a mixed disulphide. Grx1 is known to specifically reverse protein-glutathione mixed disulphides by utilizing GSH as an electron donor. More than 90% of the original IDPc activity was recovered by the enzyme-catalysed disulphide exchange with Grx1 (5 μg) in the presence of 0.5 mM GSH (Figure 2B). However, the recovery of IDPc activity was not pronounced with 0.5 mM GSH alone. Western blotting with an anti-GSH antibody revealed a correlation between the recovery of IDPc activity and the reduction in glutathionylated IDPc level (Figure 2B). The remaining activity of the C269S mutant was not affected by GSSG and no glutathionylated IDPc was observed with 5 mM GSSG (Figure 2C), confirming that Cys^{269} is a target of IDPc glutathionylation. Glutathionylation of the C379S mutant IDPc was similar to that of wild-type IDPc.

Under the conditions of oxidative stress, reactive oxygen species (ROS) can cause oxidative damage to cellular proteins, including fragmenting the peptides. The fragmentation of IDPc by oxidative damage was measured by the disappearance of the native IDPc band at 45 kDa in denaturing electrophoresis gels. Glutathionylated IDPc was protected from the peptide fragmentation caused by RB/light (Figure 3A), which generates singlet oxygen. Furthermore, glutathionylated IDPc was significantly less susceptible than the native protein to proteolytic digestion by trypsin (Figure 3B). Glutathionylation may play an important protective role in the degradation of IDPc by ROS or proteases, presumably through structural changes that render IDPc less susceptible to attack. To reveal increases in protein flexibility during the partial unfolding of glutathionylated IDPc, binding of the fluorescent probe ANSA was used to detect the accessibility of the hydrophobic regions on the protein. When IDPc was exposed to GSSG for 30 min, it bound the hydrophobic probe ANSA more efficiently than the native protein did (Figure 3C). To determine the effects of glutathionylation on the IDPc conformation, the intrinsic fluorescence of the aromatic amino acids in each of the various enzyme forms was determined. Native IDPc exhibits a fluorescence emission spectrum typical for

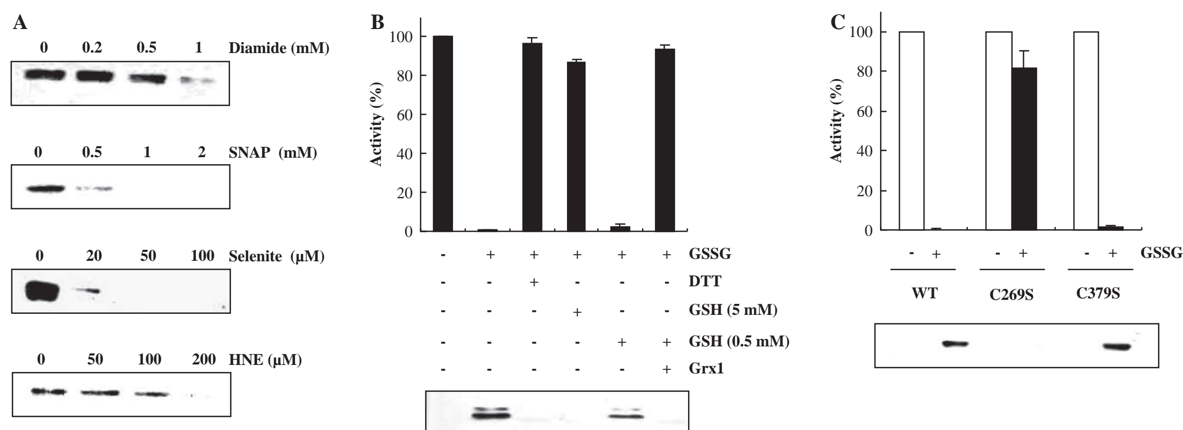

Figure 2. Effect of sulphydryl modifying agents on IDPc glutathionylation and the reversibility of glutathionylation. (A) After incubating IDPc with 5 mM GSSG in the presence or absence of diamide, selenite, SNAP and HNE for 30 min at 37°C, samples were characterized by SDS-PAGE followed by immunoblotting with an anti-GSH IgG. (B) IDPc was incubated with 5 mM GSSG for 30 min and subsequently treated with 5 mM DTT, 5 mM GSH or Grx1 (5 μg) in the presence of 0.5 mM GSH for 30 min at 37°C. Activities are given as a percentage of the control value. Data are presented as means ± SD of three separate experiments. Samples were characterized by SDS-PAGE followed by immunoblotting with an anti-GSH IgG. (C) Wild-type and mutant IDPc were treated with 5 mM GSSG for 30 min at 37°C and the remaining activity was determined. Activities are given as a percentage of the control value. Data are presented as means ± SD of three separate experiments. After incubation with 5 mM GSSG for 30 min at 37°C, wild-type and mutant IDPc were characterized by SDS-PAGE followed by immunoblotting with an anti-GSH IgG.

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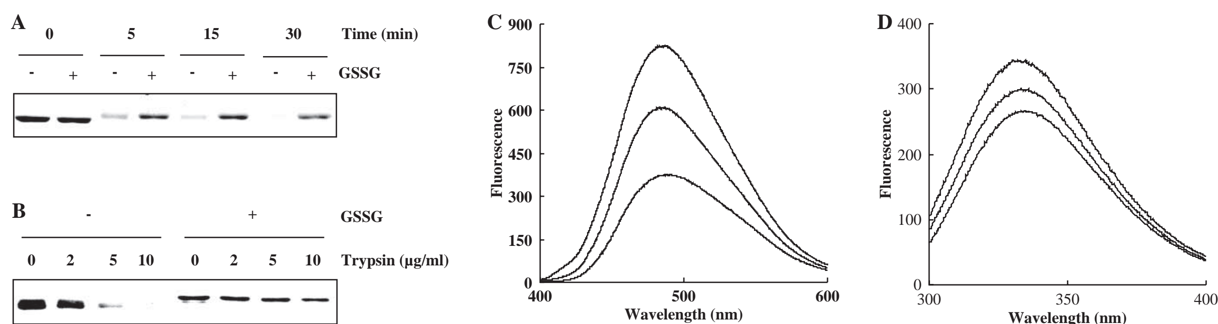

Figure 3. Effect of glutathionylation on the oxidative fragmentation and proteolytic digestion of IDPc and structural changes in glutathionylated IDPc. IDPc was treated with or without 5 mM GSSG for 30 min at 37°C. Then the reaction mixtures passed through microspin G25 columns. Glutathionylated and untreated control IDPc were incubated with 10 μM rose bengal/light for the indicated times (A) or with various concentrations of trypsin for 15 min at 4°C (B). Samples were separated by SDS-PAGE and protein bands were visualized by Coomassie staining. (C) Spectrofluorometric analysis of ANSA binding to glutathionylated IDPc. Emission spectra from 400–600 nm (excitation, 370 nm) of ANSA (100 μM) bound to native IDPc (lower trace) or IDPc treated with 2 or 5 mM GSSG for 30 min at 37°C (middle and upper traces, respectively). The increase in fluorescence intensity at 490 nm resulting from ANSA binding to the enzyme was determined by subtracting the emission spectrum of ANSA alone from that of ANSA in the presence of the different forms of the enzyme. (D) Steady-state emission spectra of the intrinsic fluorescence of native IDPc (upper trace) or IDPc treated with 2 or 5 mM GSSG for 30 min at 37°C (middle and lower traces, respectively) were analysed in a spectrofluorometer. Spectra were obtained using an excitation wavelength of 278 nm and excitation and emission slits of 5 nm.

tryptophan residues in proteins. Upon excitation of native IDPc at 278 nm, an emission spectrum with a maximum at 333 nm was observed. The fluorescence spectra of native and GSSG-treated IDPc, normalized to the protein content, showed that modified IDPc displays a dose-dependent decrease in the quantum yield of the emission spectra (Figure 3D).

Because GSSG readily glutathionylates IDPc *in vitro*, we examined IDPc activity and glutathionylation in the embryonic kidney cell line HEK293 after diamide treatment. Chemical oxidants such as diamide can reportedly serve as catalysts in promoting the formation of protein-mixed disulphides with glutathione [27]. We observed a concentration-dependent decrease of IDPc activity in diamide-treated cells (Figure 4A). Cytosolic fractions from both control and diamide-treated cells were subjected to immunoprecipitation with anti-IDPc antibody followed by separation on SDS-PAGE. A Western blot analysis of purified IDPc with an anti-GSH IgG revealed a concentration-dependent increase of immunoreactive bands in diamide-treated cells, while no increase was found in the

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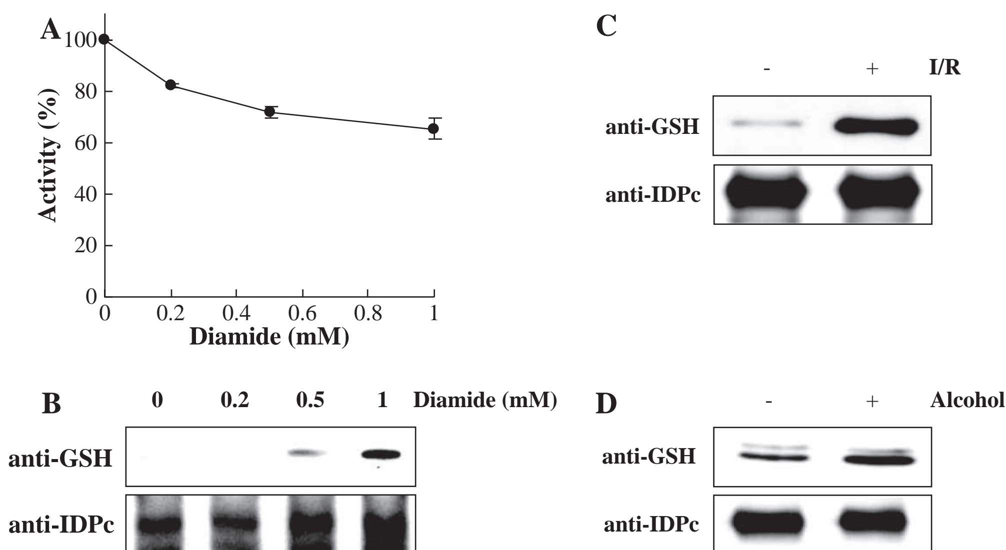

Figure 4. IDPc glutathionylation in HEK293 cells treated with diamide and tissues from animals under oxidative stress. (A) HEK293 cells were incubated with various concentrations of diamide for 30 min at 37°C and disrupted by sonication. The cytosolic fraction was prepared and the activity of IDPc was determined. Activities are given as a percentage of the control value. Data are presented as means ± SD of five separate experiments. (B) IDPc was purified from control and oxidant-treated HEK293 cells using immunoprecipitation with an anti-IDPc antibody as described, then characterized by SDS-PAGE followed by immunoblotting. Purified IDPc was probed with an anti-GSH IgG. Kidney (C) and liver (D) IDPc were purified from control and ischemia/reperfusion-induced mice and alcohol-administered rats, respectively, using immunoprecipitation with an anti-IDPc as described. Purified IDPc was probed with an anti-GSH IgG.

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control cells (Figure 4B). To verify the physiological significance of our finding, direct evidence for the enhanced glutathionylation of IDPc in tissues under oxidative stress was evaluated by immunoprecipitation with an anti-IDPc antibody followed by immunoblotting with an anti-GSH antibody. Ischemia/reperfusion [28] and alcohol administration [29] are reported to markedly increase ROS production and to subsequently induce a pro-oxidant state of tissues. IDPc from the kidneys of ischemia/repefusion-induced mice and the livers of ethanol-administered rats was purified by immunoprecipitation with an anti-IDPc antibody. Western blot analysis of these IDPc proteins with an anti-GSH IgG showed a significant increase of glutathionylated IDPc (Figure 4C and 4D).

Discussion

NADPH is used by the cell to maintain a level of GSH and reduced thioredoxin to combat oxidative damage. Glutathione reductase converts GSSG to GSH in the cell using NADPH as a reducing equivalent [16]. The oxidized form of thioredoxin, with a disulphide bridge between the half-cystines, is reduced by NADPH in the presence of the flavoprotein, thioredoxin reductase [30]. Reduced thioredoxin may provide reducing equivalents to several enzymes including thioredoxin peroxidases and methionine sulphoxide reductase, presumably aiding in the defense against oxidative stress. Although the pentose phosphate pathway is considered to be a major source of cellular reducing power, with G6PD catalysing the key NADPH-producing step [31], it is possible that other enzymes generate NADPH. IDPc of rat liver has been proven to have an $\sim$20-times higher specific activity than G6PD [32]. Recently, an IDPc species that is preferentially expressed in the bovine corneal epithelium was identified. The role of this enzyme in corneal transparency is likely due to its protective effect against UV radiation [33]. We also previously reported that the control of the cellular redox balance and oxidative damage are primary functions of IDPc in NIH3T3 cells [18].

The purpose of this study was to examine the mechanism of IDPc regulation, inactivation by glutathionylation. We hypothesized that the IDPc is a likely target of glutathionylation based on the fact that it contains a highly reactive cysteine at physiological pH [26] and that modification by sulphydryl-specific reagents inhibit enzyme activity. The sulphydryl groups of IDPc reportedly are susceptible to modification by ROS, reactive nitrogen species and lipid peroxidation products [29,34–36]. Experiments performed *in vitro* demonstrated that IDPc is inactivated by reversible glutathionylation. Sulphydryl modifying agents such as diamide, selenite, SNAP and HNE inhibited the glutathionylation of IDPc. The main feature that makes S-glutathionylation a possible regulatory mechanism is its reversibility [1]. Treatment of glutathionylated IDPc with DTT or Grx1 in the presence of GSH recovered IDPc activity, indicating the formation of protein-SSG species. The mutant protein C269S is resistant to GSSG-induced inactivation, suggesting that Cys^{269} is a target for glutathionylation.

If a cysteine is glutathionylated, it is not available for other oxidative reactions. In this respect, glutahionylation is often considered a way to protect sensitive cysteines from other, possibly irreversible, forms of oxidation, thus allowing the cells to restore the cognate function of the protein when oxidative stress conditions are overcome [37]. Supportive evidence for this arises, for example, from the S-glutahionylation of the γ-glutamyl transpeptidase, which seems to protect this protein from the irreversible oxidative damage by hydrogen peroxide during γ-glutamyl transpeptidase-mediated metabolism of GSH [38]. Glutathionylated IDPc was less susceptible to ROS-mediated fragmentation and protease attack. We also demonstrated that structural changes in IDPc protein were imposed by glutathionylation, thus indicating a potential for a regulatory function of S-glutathionylation. Thus, it is tempting to speculate that IDPc glutathionylation, at least partially responsible for maintaining cellular redox status against oxidative stress by avoiding irreversible inactivation of important antioxidant enzymes.

Under basal conditions the concentration of GSH dominates GSSG by more than an order of magnitude. However, in many diseases the redox equilibrium shifts with a loss of GSH and an accumulation of GSSG When GSSG accumulates in tissues, it results in the formation of S-glutathionylated proteins [39]. In this respect, the ready formation of glutathione mixed disulphide on IDPc has pathological significance. Immunoprecipitation and Western blot analysis revealed that the GSSG-protein adduct of IDPc is present in the kidneys from ischemia/reperfusion-induced mice and the livers from ethanol-administered rats. Thus, the possibility that IDPc is regulated by glutathionylation in various oxidative stress-related diseases merits further consideration.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A. S-glutathionylation in protein redox regulation. Free Radic Biol Med 2007;43:883–898.

[2] Chai YC, Ashraf SS, Rokutan K, Johnston RB, Thomas JA. S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. Arch Biochem Biophys 1994;310:273–281.

[3] Barrett WC, DeGnore JP, Konig S, Fales HM, Keng YF, Zhang ZY, Yim MB, Chock PB. Regulation of PTP1B via glutathionylation of the active site cysteine 215. Biochemistry 1999;38:6699–6705.

[4] Chrestensen CA, Starke DW, Mieyal JJ. Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. J Biol Chem 2000;275:26556–26565.

[5] Klatt P, Molina EP, Lamas S. Nitric oxide inhibits c-Jun DNA binding by specifically targeted S-glutathionylation. J Biol Chem 1999;274:15857–15864.

[6] Mallis RJ, Hamann MJ, Zhao W, Zhang T, Hendrich S, Thomas JA. Irreversible thiol oxidation in carbonic anhydrase III: protection by S-glutathiolation and detection in aging rats. Biol Chem 2002;383:649–662.

[7] Borges CR, Geddes T, Watson JT, Kuhn KM. Dopamine biosynthesis is regulated by S-glutathionylation: potential mechanism of tyrosine hydroxylase inhibition during oxidative stress. J Biol Chem 2002;277:48295–48302.

[8] Konorev EA, Kalyanaraman B, Hogg N. Modification of creatine kinase by S-nitrosothiols: S-nitrosation vs. S-thiolation. Free Radic Biol Med 2000;28:1671–1678.

[9] Humphries KM, Juliano C, Taylor SS. Regulation of camp-dependent protein kinase activity by glutathionylation. J Biol Chem 2002;277:43505–43511.

[10] Davis DA, Brown CA, Newcomb FM, Boja ES, Fales HM, Kaufman J, Stahl SJ, Weld PW, Yarchoan R. Reversible oxidative modification as a mechanism for regulating retroviral protease dimerization and activation. J Virol 2003;77:3319–3325.

[11] Reynaert NL, van der Vliet A, Guala AS, McGovern T, Hristova M, Pantano C, Heintz NH, Heim J, Ho YS, Matthews DE, Wouters EFM, Janssen-Heininger YMW. Dynamic redox control of NF-κB through glutaredoxin-regulated S-glutathionylation of inhibitory κB kinase β. Proc Natl Acad Sci USA 2006;103:13086–13091.

[12] Qanungo S, Starke DW, Pai HV, Mieyal JJ, Nieminen AL. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFκB. J Biol Chem 2007;282:18427–18436.

[13] Kil IS, Kim SY, Park J-W. Glutathionyaltion regulates IκB. Biochem Biophys Res Commun 2008;373:169–173.

[14] Adachi T, Pimentel DR, Heibeck T, Hou X, Lee YJ, Jiang B, Ido Y, Cohen RA. S-glutathiolation of Ras mediates redox-sensitive signaling of angiotensin II in vascular smooth muscle cells. J Biol Chem 2004;279:29857–29862.

[15] Koshland DE, Walsh K, LaPorte DC. Sensitivity of metabolic fluxes to covalent control. Curr Top Cell Regul 1985;27:13–22.

[16] Kirsch M, de Groot H. NAD(P)H, a directly operating antioxidant? FASEB J 2001;15:1569–1574.

[17] Nakamura H. Thioredoxin and its related molecules: update 2005. Antioxid Redox Signal 2005;7:823–828.

[18] Lee SM, Koh HJ, Park DC, Song BJ, Huh TL, Park J-W. Cytosolic $NADP^+$-dependent isocitrate dehydrogenase status modulates oxidative damage to cells. Free Radic Biol Med 2002;32:1185–1196.

[19] Jo SH, Son MK, Koh HJ, Lee SM, Song IH, Kim YO, Lee YS, Jeong KS, Kim YB, Park J-W, Song BJ, Huh TL. Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial $NADP^+$-dependent isocitrate dehydrogenase. J Biol Chem 2001;276:16168–16176.

[20] Jennings GT, Sadleir JW, Stevenson PM. Purification and properties of NADP(+)-dependent isocitrate dehydrogenase from the corpus luteum. Biochim Biophys Acta 1990;1034:219–227.

[21] Huh TL, Ryu JH, Huh JW, Sung HC, Oh IU, Song BJ, Veech RL. Cloning of a cDNA encoding bovine mitochondrial NADP(+)-specific isocitrate dehydrogenase and structural comparison with its isoenzymes from different species. Biochem J 1993;292:705–710.

[22] Oh IU, Inazawa J, Kim YO, Song BJ, Huh TL. Assignment of the human mitochondrial NADP(+)-specific isocitrate dehydrogenase (IDH2) gene to 15q26.1 by *in situ* hybridization. Genomics 1996;38:104–106.

[23] Plaut GWE, Cook M, Aogaichi T. The subcellular location of isozymes of NADP-isocitrate dehydrogenase in tissues from pig, ox and rat. Biochim Biophys Acta 1983;760:300–308.

[24] Kil IS, Park J-W. Regulation of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase activity by glutathionylation. J Biol Chem 2005;280:10846–10854.

[25] Eaton P, Wright N, Hearse DJ, Shattock MJ. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. J Mol Cell Cardiol 2002;34:1549–1560.

[26] Fatania HR, Al-Nassar KE, Thomas N. Chemical modification of rat liver cytosolic NADP(+)-linked isocitrate dehydrogenase by N-ethylmaleimide. Evidence for essential sulphydryl groups. FEBS Lett 1993;322:245–248.

[27] Kosower NS, Kosower EM. Diamide: an oxidant probe for thiols. Methods Enzymol 1995;251:123–133.

[28] Kim J, Kil IS, Seok YM, Yang ES, Kim DK, Lim DG, Park J-W, Bonventre JV, Park KM. Orchiectomy attenuates post-ischemic oxidative stress and ischemia/reperfusion injury in mice. J Biol Chem 2006;281:20349–20356.

[29] Lee JH, Yang ES, Park J-W. Inactivation of $NADP^+$-dependent isocitrate dehydrogenase by peroxynitrite: Implications for cytotoxicity and ethanol-induced liver injury. J Biol Chem 2003;278:51360–51371.

[30] Holmgren A. Antioxidant function of thioredoxin and glutaredoxin systems. Antioxid Redox Signal 2000;2:811–820.

[31] Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L. Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. EMBO J 1995;14:5209–5215.

[32] Veech RL, Eggleston LV, Krebs HA. The redox state of free nicotinamide-adenine dinucleotide phosphate in the cytoplasm of rat liver. Biochem J 1969;115:609–619.

[33] Sun L, Sun TT, Lavker RM. Identification of a cytosolic $NADP^+$-dependent isocitrate dehydrogenase that is preferentially expressed in bovine corneal epithelium. A corneal epithelial crystalline. J Biol Chem 1999;274:17334–17341.

[34] Lee SM, Huh TL, Park J-W. Inactivation of $NADP^+$-dependent isocitrate dehydrogenase by reactive oxygen species. Biochimie 2001;83:1057–1065.

[35] Yang ES, Richter C, Chun JS, Huh TL, Kang SS, Park J-W. Inactivation of $NADP^+$-dependent isocitrate dehydrogenase by nitric oxide. Free Radic Biol Med 2002;33:927–937.

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[36] Yang JH, Yang ES, Park J-W. Inactivation of $NADP^+$-dependent isocitrate dehydrogenase by lipid peroxidation products. Free Radic Res 2004;38:241–249.
[37] Ghezzi P. Regulation of protein function by glutathionylation. Free Radic Res 2005;39:573–580.
[38] Dominici S, Valentini M, Maellaro E, Del Bello B, Paolicchi A, Lorenzini E, Tongiani R, Comporti M, Pompella A. Redox modulation of cell sulface protein thiols in U937 lymphoma cells: the role of g-glutamyl transpeptidase-dependent H2O2 production and S-thiolation. Free Radic Biol Med 1999;27:623–635.
[39] Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 2001;30:1191–1212.

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