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Protective effect of sulforaphane against cisplatin-induced mitochondrial alterations and impairment in the activity of NAD(P)H: Quinone oxidoreductase 1 and γ glutamyl cysteine ligase: Studies in mitochondria isolated from rat kidney and in LLC-PK1 cells

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ABSTRACT

This work was designed to further study the mechanism by which sulforaphane (SFN) exerts a renoprotective effect against cisplatin (CIS)-induced damage. It was evaluated whether SFN attenuates the CIS-induced mitochondrial alterations and the impairment in the activity of the cytoprotective enzymes NAD(P)H: quinone oxidoreductase 1 (NQO1) and γ glutamyl cysteine ligase (γGCL). Studies were performed in renal epithelial LLC-PK1 cells and in isolated renal mitochondria from CIS, SFN or CIS + SFN treated rats. SFN effectively prevented the CIS-induced increase in reactive oxygen species (ROS) production and the decrease in NQO1 and γGCL activities and in glutathione (GSH) content. The protective effect of SFN on ROS production and cell viability was prevented by buthionine sulfoximine (BSO), an inhibitor of γGCL, and by dicoumarol, an inhibitor of NQO1. SFN was also able to prevent the CIS-induced mitochondrial alterations both in LLC-PK1 cells (loss of membrane potential) and in isolated mitochondria (inhibition of mitochondrial calcium uptake, release of cytochrome *c*, and decrease in GSH content, aconitase activity, adenosine triphosphate (ATP) content and oxygen consumption). It is concluded that the protection exerted by SFN on mitochondrial alterations and NQO1 and γGCL enzymes may be involved in the renoprotection of SFN against CIS.

1. Introduction

Cisplatin (cis-diamminedichloroplatinum II, CIS) is an important chemotherapeutic agent in the clinical treatment of several human cancers including those of the ovary, breast, head, neck, testis, bladder, esophageal and cervical (Lebwohl and Canetta, 1998). However, the usefulness of this antineoplastic is limited because it causes nephrotoxicity (Pabla and Dong, 2008), ototoxicity, neurotoxicity and hepatotoxicity (Tsang et al., 2009).

Nephrotoxicity is the major collateral effect in the CIS antitumor therapy. Both *in vitro* and *in vivo* studies suggest that reactive oxygen species (ROS) generation, depletion of antioxidant enzymes and enhanced oxidative/nitrosative are involved in CIS-induced nephrotoxicity (Chirino and Pedraza-Chaverri, 2009). In addition, CIS inhibits mitochondrial function and antioxidant activity, induces deoxyribonucleic acid (DNA) damage and glutathione (GSH) depletion (Chirino and Pedraza-Chaverri, 2009; Santos et al., 2007). Mitochondrial dysfunction has been shown to be an early event in CIS-induced renal failure (Gordon and Gattone 2nd, 1986). Exposure of freshly isolated porcine proximal tubular cells in suspension to CIS resulted in loss of mitochondrial membrane potential (MMP) and mitochondrial inhibition of complexes I to IV, preceding cellular death (Kruidering et al., 1997). Also, CIS-induced nephrotoxicity is associated with an important diminution in mitochondrial adenosine triphosphate (ATP) synthesis by inhibition of oxidative phosphorylation (Santos et al., 2007), as well as the mitochondrial permeability transition (mPT), a Ca^{2+} dependent unspecific permeabilization process, often associated with cytochrome *c* release – a hallmark of mitochondrial apoptotic pathway activation – leading to cellular death (Correa et al., 2008b).

Oxidative/nitrosative stress plays an important role in CIS-induced nephrotoxicity and mitochondrial dysfunction (Jung et

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al., 2009). In this context, it has been shown that free radical scavengers and synthetic antioxidants ameliorate or prevent CIS-induced mitochondrial dysfunction (Rodrigues et al., 2010; Santos et al., 2008). In addition CIS-induced damage is ameliorated or prevented by natural antioxidants such as lycopene, naringenin, vitamin E and C, quercetin, resveratrol, etc. (Chirino and Pedraza-Chaverri, 2009).

Sulforaphane (SFN) is a naturally occurring isothiocyanate produced by the enzymatic action of myrosinase on glucoraphanin, a glucosinolate contained in cruciferous vegetables such as broccoli, cabbage, brussel sprouts, etc. (Dinkova-Kostova, 2008). It has been shown that SFN is an indirect antioxidant that dissociates the Nrf2/Keap1 complex, thereby permitting the translocation of Nrf2 into the nucleus where it forms a heterodimer with other transcription factors such as small Maf, which in turn binds to the 5-upstream cis-acting regulatory sequence, termed antioxidant response element or electrophile response elements located in the promoter region of genes encoding various antioxidant and phase 2 detoxifying enzymes including glutathione reductase, glutathione peroxidase, glutathione-S-transferase, catalase, heme oxygenase-1, γ glutamyl cysteine ligase (γGCL, previously known as gamma-glutamyl cysteine synthetase) and NAD(P)H: quinone oxidoreductase 1 (NQO1) (Yoon et al., 2008). Phase 2 enzymes play a major role in the detoxification of ROS produced by xenobiotics (Dinkova-Kostova and Talalay, 2008). In fact, it has been found that SFN exhibits cardioprotective (Angeloni et al., 2009), renoprotective (Yoon et al., 2008) and neuroprotective effects (Innamorato et al., 2008). We have previously shown that SFN indeed induces nuclear Nrf2 translocation in LLC-PK1 cells and protects these cells from CIS-induced toxicity (Guerrero-Beltrán et al., 2010). It was also found in rats that SFN attenuates CIS-induced renal dysfunction, structural damage, oxidative/nitrosative stress, GSH depletion, enhanced urinary hydrogen peroxide excretion and the decrease in antioxidant enzymes (catalase, glutathione peroxidase and glutathione-S-transferase). The renoprotective effect of SFN on CIS-induced nephrotoxicity was associated with the attenuation in oxidative/nitrosative stress and the preservation of antioxidant enzymes (Guerrero-Beltrán et al., 2010). To further study how SFN confers renoprotection against CIS-induced oxidative stress damage, we investigated its effect in mitochondria isolated from rat kidney evaluating the respiratory chain function, calcium retention capacity, mitochondrial permeability transition pore (mPTP) triggering and cytochrome *c* release. In addition the potential role of two cytoprotective enzymes (NQO1 and γGCL) was evaluated in LLC-PK1 cells in culture. NQO1 and γGCL are two Nrf2-dependent enzymes which are expected to be increased by SFN pretreatment. NQO1 is a flavoenzyme that catalyses the two-electron reduction of reactive quinones to non-toxic, radical scavenging hydroquinones (Lind et al., 1990). It has a role in antioxidant defense via the generation of antioxidant forms of ubiquinone and vitamin E (Ross et al., 2000). γGCL catalyses the gamma carboxy linkage from glutamate to cysteine, the first and rate-limiting step of de novo GSH synthesis (Franklin et al., 2009). GSH is a tripeptide composed of glutamate, cysteine and glycine and is the most abundant intracellular antioxidant. This points to γGCL as a crucial enzyme in the regulation of the intracellular redox homeostasis.

2. Materials and methods

2.1. Reagents

Porcine renal epithelial cells Lily Laboratory Culture Porcine Kidney (LLC-PK1) were obtained from American Type Culture Collection (Rockville, MD, USA). SFN (Cat. no. S8044, batch 26815401) was purchased from LKT laboratories (St. Paul, MN, USA). CIS (Cat. no. P4394-1Gm batch 087K1349), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), 2,3-naphthalenedicarboxyaldehyde (NDA), ATP, nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$), flavin adenine dinucleotide (FAD), L-glutamic acid, L-cysteine, 5-sulfosalicylic acid (SSA), menadione, dicoumarol (DIC), and L-buthionine-(S,R)-sulfoximine (BSO) were from Sigma–Aldrich (St. Louis, MO, USA). Monochlorobimane was purchased from Fluka (Schnelldorf, Germany). Dulbecco's Modified Eagle Medium (DMEM), DMEM without phenol red, fetal bovine serum (FBS), trypsin, antibiotic (10,000 U/mL penicillin and 10,000 μg/mL streptomycin) and other tissue culture reagents were obtained from Gibco (México, DF). Cell culture plates were obtained by Nunc (Roskilde, Denmark). Anti-(cytochrome *c*) mAb (Clone 7H8.2C12) and biotin-conjugated secondary antibodies were from Zymed Laboratories (San Francisco, CA, USA). Polyclonal antibody against voltage-dependent anion channel (rat) (VDAC) was purchased from Alexis Biochemicals (Axxora, LLC, CA, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide (JC-1), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-DHFDA), and dihydroethidium (DHE) were obtained from Molecular Probes (Invitrogen). All other reagent chemicals were of reagent grade from Sigma–Aldrich unless otherwise specified.

2.2. Studies in LLC-PK1 cells

2.2.1. Cell culture and viability

LLC-PK1 cells were maintained in DMEM supplemented with 10% FBS and 1% of antibiotic. Cells were cultured under permissive conditions: 37 °C and 5% CO_2. Cells were seeded at a density of 4×10^4 cells/cm^2 onto 12 well plates and were used for experiments on the following day. Cells were exposed to different concentrations of CIS (0–50 μM) or SFN (0–100 μM) for 24 h. In some experiments, DIC (100 μM) and BSO (50 μM), inhibitors of NQO1 (Asher et al., 2006) and γGCL (Griffith and Meister, 1979), respectively, were added 1 h before the addition of SFN to the incubation medium. The cell viability was assessed by reduction of MTT to formazan as previously described (Orozco-Ibarra et al., 2009). After treatment, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), which was followed by the addition of MTT (0.125 mg/mL of DMEM). Next, the cells were incubated at 37 °C for 1 h in humidified air supplemented with 5% CO_2. Medium was then discarded and the formazan crystals, formed in cells, were dissolved in isopropanol acid (500 μL/well). The absorbance was measured at 570 nm. In additional experiments bright field images were obtained by phase contrast microscopy Nikon Eclipse TS100F (Nikon Co, Tokyo, Japan) to compare cell morphology in all experimental conditions.

2.2.2. Determination of superoxide anion ($O_2^{\bullet -}$) and ROS production

The fluorescent markers DHE and carboxy-DHFDA were used to assess $O_2^{\bullet -}$ and ROS production, according to Hernández-Fonseca et al. (2008). DHE enters the cells and is oxidized mainly by $O_2^{\bullet -}$ to ethidium (Et) in the cytosol, then it is retained within the cell nucleus because of its interaction with DNA, staining the nucleus with bright red fluorescence. The compound carboxy-DHFDA is deacetylated, oxidized by ROS and reactive nitrogen species, and converted to the fluorescent compound 5-(and 6-)carboxy-2,7-dichlorofluorescein (carboxy-DCF), staining the cell cytoplasm with bright green fluorescence (Hernández-Fonseca et al., 2008). After treatment, both fluorescent probes were loaded in DMEM without phenol red containing 20 μM of each fluorescent during 30 min at 37 °C. Cells were visualized under epifluorescence microscope (using the fluorescent cubes B-2AC filter (excitation 450–490 nm, emission 515 nm) and G-2A (excitation 510–560 nm, emission of 515 nm) from Nikon Co. for the carboxy-DCF and Et detection, respectively). The intensity of Et or carboxy-DCF fluorescence was measured in 5 different fields per well per condition in three independent experiments using the AxioVision AC 4.4 image analyzer (Carl Zeiss Imaging Systems).

2.2.3. γGCL enzymatic activity assay

γGCL enzymatic activity was measured in LLC-PK1 cells using the method previously described by White et al. (2003). LLC-PK1 cells were cultured onto 6 well plates at a density of 1×10^6 cells/cm^2. After the experimental protocol, the cells were washed twice with PBS, trypsinized and collected by centrifugation ($200 \times g$ for 3 min) and then resuspended in 50 μL of ice-cold TES-SB buffer [20 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, 2 mM L-serine, 20 mM boric acid]. The cell pellet was sonicated and centrifuged at $5000 \times g$ for 10 min at 4 °C to remove cellular debris. Twenty microlitres of sample (in duplicate) or GSH standards in TES-SB buffer were added in 20-s intervals to prewarmed microcentrifuge tubes containing 50 μL of reaction cocktail (400 mM Tris base, 40 mM ATP, 20 mM L-glutamic acid, 2 mM EDTA, 20 mM boric acid, 2 mM L-serine and 40 mM magnesium chloride hexahydrate). The γGCL reaction was initiated by adding 50 μL of 2 mM L-cysteine to one of each of the sample tubes (γGCL tube). After incubating for 20 min at 37 °C, the γGCL reaction was stopped by adding 50 μL 200 mM SSA to all tubes. Immediately after the addition of SSA, 50 μL of 2 mM L-cysteine (dissolved in TES-SB buffer) were added to the second tube of each sample (the baseline GSH tube) and to the GSH standard tubes. After the 20 min incubation on ice, all tubes were centrifuged for 5 min at $2000 \times g$ at 4 °C. Twenty microlitres of supernatant were aliquoted in triplicate into the wells of a 96-well flat-bottomed, black-sided microplate. One hundred and eighty microlitres of NDA solution (50 mM Tris, pH 10, 0.5 N NaOH, and 10 mM NDA in dimethylsulfoxide (DMSO), v/v/v 1.4/0.2/0.2) was added to all wells of this plate. The plate was covered to protect the wells from room light and incubated at