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Renal protective effect of polysulfide in cisplatin-induced nephrotoxicity

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ABSTRACT

Cisplatin is a major chemotherapeutic drug for solid tumors whereas it may lead to severe nephrotoxicity. Despite decades of efforts, effective therapies remain largely lacking for this disease. In the current research, we investigated the therapeutic effect of hydrogen polysulfide, a novel hydrogen sulfide (H_2S) derived signaling molecule, in cisplatin nephrotoxicity and the mechanisms involved. Our results showed that polysulfide donor Na_2S_4 ameliorated cisplatin-caused renal toxicity *in vitro* and *in vivo* through suppressing intracellular reactive oxygen species (ROS) generation and downstream mitogen-activated protein kinases (MAPKs) activation. Additionally, polysulfide may inhibit ROS production by simultaneously lessening the activation of NADPH oxidase and inducing nucleus translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) in RPT cells. Interestingly, polysulfide possesses anti-cancer activity and is able to add on more anti-cancer effect to cisplatin in non-small cell lung cancer (NSCLC) cell lines. Moreover, we observed that the number of sulfur atoms in polysulfide well reflected the efficacy of these molecules not only in cell protection but also cancer inhibition which may serve as a guide for further development of polysulfide donors for pharmaceutical usage. Taken together, our study suggests that polysulfide may be a novel and promising therapeutic agent to prevent cisplatin-induced nephrotoxicity.

1. Introduction

Cisplatin is a widely used chemotherapeutic drug for solid tumors arising from multiple organs such as head and neck, testicular, cervical, ovaries, lung and bladder; however, clinical studies have revealed that cisplatin usage is accompanied with severe adverse effects including nephrotoxicity, ototoxicity and neurotoxicity [1]. Among these side effects, cisplatin-induced neurotoxicity is most severe and prevalent as evidence shows that over 30% of patients show symptoms of acute kidney injury (AKI) following the administration of cisplatin [2].

Cisplatin nephrotoxicity is characterized with massive renal proximal tubular (RPT) cell death, consisting of both necrosis and apoptosis [3]. As a result, renal insufficiency begins as manifested by increases of serum creatinine and blood urea nitrogen levels several days after the administration of cisplatin, along with a reduction of serum magnesium and potassium levels [4]. Oxidative stress has long been recognized as an important factor contributing to cisplatin-induced RPT cell death [5]. Numerous studies have observed the massive production of reactive oxygen species (ROS) upon cisplatin treatment in cultured renal tubular cells, kidney slices, and *in vivo* animals [6,7]. Further studies have suggested that cisplatin-induced activation of NADPH oxidase contributes to the pathophysiology as pharmacological inhibition of NADPH oxidase protects renal cells in cultured proximal tubule cells and *in vivo* animals [1,8–10]. On the other hand, whether cisplatin stimulates the production of mitochondrial ROS remains controversial in RPT cells [11,12].

Polysulfide is a category of chemical compounds comprising chains of sulfur atoms. In mammalian system, polysulfide can be generated from hydrogen sulfide (H_2S), an endogenous gasotransmitter, as described in the following equation: $2nH_2S + 1/2\ (2n\text{-}1)O_2 \rightarrow H_2S_{2n} + (2n\text{-}1)H_2S$ in the presence of oxygen [13]. Interestingly, besides directly derived from H_2S, Kimura and others [14] demonstrated that they are also generated by H_2S producing enzyme 3-mercaptopyruvate sulfurtransferase (3MST), implying its possible physiological importance. Although the biological functions of polysulfide are not fully acknowledged, existing evidence shows that polysulfide may possess various biological effects similar to H_2S. For example, Nagai *et al.* [15] found that polysulfide was able to activate TRPV channels more potently than H_2S does. Subsequently, Oosumi and others [16] determined that Cysteine 422 and Cysteine 622 in the TRPV 1 channel

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were sensitive to polysulfide. The anti-oxidant effect of polysulfide was also studied recently. Koike and colleagues [17] reported that polysulfide exhibited protective effects against cytotoxicity caused by oxidative stress in neuroblastomaSH-SY5Y cells. They also showed that polysulfide may activate the translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) into nucleus by dimerizing Kelch-like ECH-associated protein 1 (keap1) and as a result facilitate the expression of anti-oxidant genes [17]. Therefore, we hypothesized that polysulfide may prevent cisplatin nephrotoxicity by attenuating ROS generation. Besides, the effect of polysulfide on the anti-cancer activity of cisplatin was also examined in non-small cell lung cancer cell (NSCLCC) lines.

2. Materials and methods

2.1. Reagents and antibodies

N-acetyl-cysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), 2,7-dichlorofluorescein diacetate (DCFH-DA), Hoechst 33342, propidium iodide (PI) were purchased from Sigma-Aldrich (St Louis, MO. USA). Polysulfide donors including Na_2S_2, Na_2S_3 and Na_2S_4 were obtained from Dojindo Molecular Technologies Dojindo (Kumamoto, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), streptomycin/penicillin and trypsin were obtained from Hyclone Laboratories (South Logan, UT.USA). The RIPA buffer was purchased from ThermoFisher Scientific Inc (Waltham, MA. USA). The Bradford colorimetric protein assay kit (Rockford, IL. USA) was used for protein quantification. The antibody for p-p47phox was from ThermoFisher Scientific Inc (Waltham, MA. USA). All the other antibodies were obtained from Cell Signaling Technology (Beverly, MA. USA). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis,MO) unless otherwise stated.

2.2. Cell culture

The porcine RPT cell line namely LLC-PK1 was purchased from ATCC (Rockville, MA. USA) and cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% penicillin (100 U)/streptomycin (100 mg/mL) in a humidified atmosphere of 95% air and 5% CO2 at 37 ℃. The monolayer cells were deprived from FBS for about 18 h prior to experiments.

2.3. MTT assay

The cell viability was tested by MTT reduction assay as previously described [18]. For MTT reduction assay, 0.5 mg/mL MTT was incubated with treated cells for 4 h after which the formazon crystals were dissolved with DMSO. The absorbance was measured at 570 nm with a Varioskan Flash microplate reader (Waltham, MA. USA).

2.4. Hoechst 33342/propidium iodide staining

Hoechst 33342/propidium iodide (PI) staining was performed in 96 well plates. After treatment, the cells were incubated with phenol red free DMEM containing with 5 μg/mL Hoechst 33342 and 15 μg/mL PI for 15 min at 37 ˚C. The images were taken in Cytation 3 imaging reader (BioTek, VT. USA).

2.5. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) assay was performed with a commercial kit from Sigma-Aldrich (St Louis, MO. USA) according to the manufacturer's instruction. Briefly, 50 μL medium after treatment was mixed with LDH detection reagents. After incubation, the absorbance was measured at 450 nm.

2.6. Western blot assay

Renal cortical tissue and cell samples were lysed with RIPA buffer containing phosphatase and protease inhibitors. The protein content was measured using BCA colorimetric protein kit. Equal amount of protein were separated with 12% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 10% nonfat milk, the membranes were incubated with primary antibody overnight with mild shake at 4 ˚C. Then the membrane was washed for 3 times with TBST buffer followed by 1 h incubation with horseradish peroxidase-conjugated secondary antibody. The immunoblots were visualized with ECL Western blotting substrate. Protein bands were normalized with non-phosphorylated form of proteins or β-actin.

2.7. NADPH oxidase activity assay

The activity of NADPH oxidase was measured as described previously [19]. Briefly, LLC-PK1 cells were washed with ice-cold PBS and then homogenized in KH_2PO_4 buffer (20 mM, pH 7.1) containing 1 mM EGTA and protease inhibitors. After centrifugation (800*g*, 10 min, 4 ℃), 50 μL homogenates were added to 150 μL of 50 mM phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose, 50 μM lucigenin, and 100 μM NADPH in the presence or absence of 200 μM apocynin. Photon emission from lucigenin was measured every 30 s for 5 min in a luminometer. No NADPH oxidase activity was measured in the presence of 200 μM apocynin which was subtracted from the corresponding value in the absence of apocynin. The data were converted to relative light unites/min/mg of protein. NADPH oxidase activity of control cells was arbitrarily set at 100%. The protein content was measured using BCA colorimetric protein kit.

2.8. Nuclear protein extraction

The nuclear protein was extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher) according to the manufacture's instruction. Briefly, ice-cold CER I and CER II were used to extract the cytoplamsic proteins. Subsequently, NER reagent was employed to extract the nuclear proteins. The ratio of CER I: CER II: NER was maintained at 200:11:100 μL respectively during the experiment.

2.9. Intracellular ROS measurement

The intracellular ROS was measured by a fluorescence dye CM-H_2DCFDA in 96-well plate. After treatment, cells were washed with PBS and then incubated with 100 μL of DCFH-DA (10 μM, dissolved in phenol red-free DMEM) for 30 min at 37 ℃. The fluorescence intensity was detected with excitation and emission wavelengths of 485 nm and 535 nm in a Varioskan Flash microplate reader (Waltham, MA. USA).

2.10. Measurement of plasma creatinine and blood urea nitrogen

The kits used for the measurement of plasma creatinine and blood urea nitrogen (BUN) were obtained from BioAssay Systems (Hayward, CA. USA). The experiments were performed according to the manufacturer's instruction. The levels of plasma creatinine and BUN were normalized with control groups.

2.11. TUNEL staining

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling assay (TUNEL assay) was performed using an in situ cell death detection kit (Roche, Penzberg. Germany) according to the manufacturer's instructions. Briefly, tissue taken from kidneys was fixed and embedded in paraffin and 4-μm sections were prepared. After dewax and rehydrate, sections were stained with terminal