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Isothiocyanate–cysteine conjugates protect renal tissue against cisplatin-induced apoptosis via induction of heme oxygenase-1

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ABSTRACT

Heme oxygenase-1 (HO-1) is a redox sensitive inducible enzyme endowed with important antioxidant and cytoprotective activities. Here we report that two water-soluble isothiocyanate–cysteine conjugates, S-[N-benzyl(thiocarbamoyl)]-L-cysteine (BTTC) and S-[N-(3-phenylpropyl)(thiocarbamoyl)]-L-cysteine (PTTC), potently increase HO-1 protein expression and heme oxygenase activity in renal tubular epithelial cells at 5 and 10 μM, while higher concentrations are themselves cytotoxic and pro-apoptotic. Inhibitors of the pro-survival pathways ERK, MAPK and PI3K almost completely abolished the increase in HO-1 induction and heme oxygenase activity, while the JNK pathway appeared to be mainly involved in the apoptosis triggered by the isothiocyanates. We also found that renal cells exposed to 50 μM cisplatin (CDDP), a chemotherapeutic agent known for its nephrotoxic actions, displayed a marked increase in caspase-3 activity and the number of apoptotic cells. These effects were abolished by pre-incubation of cells with concentrations of BTCC or PTCC that maximize HO-1 induction and were reversed by the inhibitor of heme oxygenase activity tin protoporphyrin IX (SnPPIX). Moreover, in a model of CDDP-induced nephrotoxicity *in vivo*, pre-treatment of rats with a daily dose of BTCC or PTCC (25 mg/kg, i.p.) completely abolished the increase in serum creatinine and urea levels and markedly reduced the severity of renal tissue apoptosis caused by CDDP. The renoprotective effects of BTCC and PTCC *in vivo* were markedly attenuated by administration of rats with SnPPIX. These findings indicate that water-soluble isothiocyanates counteract renal dysfunction and apoptosis by up-regulating the HO-1 system and could be used as a supplementary treatment to mitigate CDDP-induced nephrotoxic effects.

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II), CDDP) is a potent anti-neoplastic agent that is particularly effective for the treatment of solid tumours [1]. However, its clinical use is limited by its nephrotoxic effects which occur even after a single dose injection [2,3]. CDDP accumulates preferentially in the S3 segment of the proximal tubules of the kidney where it forms adducts with DNA, inhibits protein synthesis, damages mitochondria and decreases glutathione, thus implicating several cellular targets in its renal damaging effects [1,3,4]. CDDP also causes apoptosis to renal proximal and tubular cells as well as cancer cell death [3,5], suggesting that strategies aimed at reducing CDDP renal injury should not compromise its anti-cancer effects.

Heme oxygenase-1 (HO-1), which converts heme into carbon monoxide (CO), bilirubin and iron, is a redox sensitive inducible enzyme endowed with important antioxidant and cytoprotective activities. In the last two decades substantial scientific evidence has been collected on the function of HO-1 in cell homeostasis emphasizing that HO-1 is a fundamental 'sensor' of cellular stress and directly contributes to limit or prevent tissue damage in different tissues and organs, including the kidney [6–10]. The induction of HO-1 has been consistently associated with protection of kidneys against ischaemia–reperfusion and acute renal injury as well as renal toxicity caused by compounds containing heavy metals including cisplatin (CDDP) [10–12]. In this context, Nath et al. have demonstrated that HO-1 knockout mice treated with CDDP exhibited a more severe kidney dysfunction and renal tubular apoptosis which were accompanied by 100% mortality when compared to wild-type mice [13]. The crucial role of HO-1 as a reno-protective enzyme is also supported by findings confirming that CDDP administration results in HO-1 induction in a time- and dose-dependent manner and inhibition of heme oxygenase

Abbreviations: BTCC, S-[N-benzyl(thiocarbamoyl)]-L-cysteine; CDDP, cisplatin; HO-1, heme oxygenase-1; PTCC, S-[N-(3-phenylpropyl)(thiocarbamoyl)]-L-cysteine; SnPPIX, tin protoporphyrin IX.

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activity aggravates CDDP-induced renal injury [12]. HO-1-derived CO may be one of the products directly involved in protection since our group has previously reported that CORM-3, a compound that delivers controlled amounts of CO to biological tissues, significantly protects against CDDP-induced nephrotoxicity in renal cells *in vitro* and in kidneys *in vivo* [14].

Several natural substances containing electrophilic moieties, including curcumin, chalcones and carnosol, have been reported to enhance the expression of HO-1 protein resulting in increased heme oxygenase activity [15–19]. The HO-1 gene is regulated by the Nrf2/Keap1 system, a transcriptional machinery that is highly responsive to electrophilic natural compounds and plays a crucial role in mounting an antioxidant/cytoprotective in response to different stressful stimuli [20]. Isothiocyanates are among the first substances originally identified for their chemoprotective properties and as regulators of the Nrf2 transcription factor [21–23]. They are biologically active products generated by myrosinases enzymes from the precursor glucosinolates, present in high amounts in cruciferous vegetables, with sulforaphane being a classical example but including also benzyl isothiocyanate, phenyl isothiocyanate and others [21,24,25]. Apart from interacting with Nrf2, their beneficial effects are also due to modulation of inflammation and apoptosis [24]. However, in their natural form, these substances are limited by their scarce solubility in water and toxic effects and attempts have been undertaken to synthesize chemical derivatives with increased biological activity and reduced toxicity.

The aim of the present study was to investigate whether S-[N-benzyl(thiocarbamoyl)]-L-cysteine (BTTC) and S-[N-(3-phenylpropyl)(thiocarbamoyl)]-L-cysteine (PTTC), two water-soluble isothiocyanate–cysteine conjugates developed as analogues of phenylalkyl isothiocyanate and exhibiting higher potency and lower toxicity compared to the parent compound [26], stimulate HO-1 expression and exert renal protection in models of CDDP-induced damage *in vitro* and *in vivo*. The direct effect of these derivatives on cellular apoptosis was also examined to differentiate between their beneficial and deleterious properties.

2. Materials and methods

2.1. Chemicals and reagents

S-[N-(benzylthiocarbamoyl)]-L-cysteine (BTCC) and S-[N-(3-phenylpropyl)(thiocarbamoyl)]-L-cysteine (PTCC) were purchased from Axxora Corporation (Bingham, Nottingham, UK). Stock solutions of BTCC and PTCC (1.25 mM) for the *in vitro* experiments on cells were freshly prepared in distilled water. However, since the *in vivo* treatments required in average 7.5 mg of compound to be injected, stock solutions of PTCC and BTCC in this case were prepared in aqueous solutions containing 0.1 mM NaOH/10% ethanol. Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma–Aldrich (Poole, Dorset, UK) and foetal bovine serum (FBS) was from Gibco (Paisley, UK). Hemin (ferriprotoporphyrin IX chloride) was obtained from Porphyrin Products Inc. (Logan, UT, USA) and the heme oxygenase inhibitor tin protoporphyrin IX (SnPPIX) was from Frontier Scientific (Carnforth, Lancashire, UK). Stock solutions of these two porphyrin compounds were prepared in 10 mM NaOH/phosphate buffer. HO-1 monoclonal antibodies were obtained from Stressgen (Victoria, Canada). All other reagents were purchased from Sigma–Aldrich (Poole, Dorset, UK) unless otherwise specified.

2.2. Cell culture

Porcine renal tubular epithelial cells (LLC-PK1) were obtained from the European Collection of Animal Cell Culture (Salisbury, Wiltshire, UK). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) as previously described [17]. Cells were grown in 75 cm^2 tissue culture flasks and maintained at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. Confluent cells were exposed to the various agents using DMEM supplemented with 10% FBS except in experiments performed to assess lactate dehydrogenase release (LDH) and cytotoxicity by the Alamar Blue assay in which cells were incubated in medium supplemented with 1% FBS.

2.3. Cell viability and cytotoxicity assays

LLC-PK1 cells were grown in 24-well plates (Sarstedt Ltd., Nümbrecht, Germany) and incubated for 24 h with various agents. Cell viability was determined using an Alamar Blue assay kit (Serotec, Kidlington, UK). Briefly, the assay is based on the detection of metabolic activity of living cells using a redox indicator, which changes from an oxidized (blue) form to a reduced (red) form. The intensity of the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 and 600 nm and expressed as a percentage of control. Cell injury was determined using a lactate dehydrogenase (LDH) assay kit and carried out according to the manufacturer's instructions (Roche Diagnostics, UK). Samples were read on a plate reader (Molecular Devices VERSAmax) at 490 nm with a reference wavelength of 690 nm and blanked against cell culture medium. Samples were run in triplicate, and treatment of cells with 1% triton was used as a positive control (100% LDH release).

2.4. Heme oxygenase activity assay

Heme oxygenase activity was determined in renal epithelial cells at 6, 18 and 36 h after various treatments using a method previously described by our group [27–29]. Briefly, harvested cells were subjected to three cycles of freeze thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 μM). The reaction was conducted at 37 °C in the dark for 1 h and terminated by the addition of 1 ml of chloroform; the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$).

2.5. Western blot analysis

Samples of renal epithelial cells and renal tissues were analyzed for HO-1 expression by Western immunoblot technique as already reported by our group [18,30,31]. Briefly, an equal amount of proteins (30 μg) for each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1000 dilution in Tris-buffered saline, pH 7.4). After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A). To assess nuclear translocation of Nrf2, nuclear enrichment was performed using a commercial nuclear isolation kit from Active Motif (La Hulpe, Belgium) according to the manufacturer's instructions. Nrf2 antibody (clone C-20 rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to determine the nuclear presence of Nrf2 in response to treatments. Lamin A/C (clone N-18, goat polyclonal, Santa Cruz Biotechnology) was used as a nuclear control.