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Management of Oxidative Stress by Heme Oxygenase-1 in Cisplatin-induced Toxicity in Renal Tubular Cells

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Induction of heme oxygenase-1 (HO-1) may serve as an immediate protective response during treatment with the cytostatic drug cisplatin (CDDP). Oxidative pathways participate in the characteristic nephrotoxicity of CDDP. In the present study, cultured tubular cells (LLC-PK1) were used to investigate whether induction of HO provided protection against CDDP by maintaining the cellular redox balance. The antioxidants, α-tocopherol (TOCO) and *N*-acetylcysteine (NAC), were used to demonstrate that elevation of ROS levels contribute to the development of CDDP-induced cytotoxicity. Chemical modulators of HO activity were used to investigate the role of HO herein. Hemin was used to specifically induce HO-1, while exposure of the cells to tin-protoporphyrin (SnPP) was shown to inhibit HO activity. Hemin treatment prior to CDDP-exposure significantly decreased the generation of ROS to control levels, while inhibition of HO increased the ROS levels beyond the levels measured in cells treated with CDDP alone. Furthermore, HO induction protected significantly against the cytotoxicity of CDDP, although this protection was limited. Similar results were obtained when the cells were preincubated with TOCO, suggesting that mechanisms other than impairment of the redox ratio are important in CDDP-induced loss of cell viability *in vitro*. In addition, SnPP treatment exacerbated the oxidative response and cytotoxicity of CDDP, especially at low CDDP concentrations. We therefore conclude that HO is able to directly limit the CDDP-induced oxidative stress response and thus serves as safeguard of the cellular redox balance.

Keywords: Heme oxygenase; CDDP; Renal tubular cells; ROS; Antioxidants; Cytotoxicity

INTRODUCTION

Induction of heme oxygenase-1 (HO-1) is thought to serve as an immediate protective response against the nephrotoxicity of cisplatin (CDDP), a common chemotherapeutic agent. Heme oxygenase (HO) catalyses the first and rate limiting step in the degradation of heme to biliverdin and bilirubin, respectively. Carbon monoxide (CO) and iron are released by the HO-catalyzed reaction. CO is thought to serve as a second messenger, whereas iron induces the synthesis of ferritin, which in turn efficiently chelates its inducer. The second step in this heme degrading pathway is catalyzed by biliverdin reductase, a cytosolic enzyme, and leads to the formation of bilirubin (for a review see[1]).

Three HO isoenzymes, products of distinct genes, have been characterized to date.[2,3] HO-1 is a widely distributed heat-shock protein and is induced by a variety of stimuli, including UV(A) irradiation, heavy metals, inflammation and prooxidant states.[1,4] HO-2 is expressed constitutively, predominantly in brain and testicular tissue. Recently, HO-3 has been described having properties similar to HO-2.[5]

Although the mechanisms underlying induction of HO are complex, HO-1 induction is proposed to be a general response to oxidant stress in mammalian cells. Intracellular redox levels thus appear to play an important role in this response.[6–8] The general assumption is that as a result of HO activity the concentration of the prooxidant heme is decreased

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and the levels of biliverdin and bilirubin are increased resulting in a favorable redox balance, as the latter compounds have effective antioxidative activity.[9]

Cis-diamminedichloroplatinum(II) [cisplatin, CDDP] is a widely used chemotherapeutic agent for the treatment of human malignancies, including ovarian and testicular cancer.[10] However, CDDP produces severe side effects, the most prominent being its nephrotoxicity, which limits its clinical use considerably.[11] It is generally accepted that selective accumulation in the proximal tubular cells, resulting from active secretory transport, provides the basis for CDDP-mediated nephrotoxicity.[11] However, the detailed mechanism of CDDP-induced renal damage has not been elucidated as of yet.[4,11]

The kidney is a major site for oxidative processes and is therefore extremely vulnerable to oxidant-damage.[4] Oxidative stress, such as reduction in cellular glutathione levels, enhancement of lipid peroxidation and generation of superoxide anion in a cell-free system,[4] has been implicated in CDDP-mediated toxicity. Correspondingly, oxidant-scavenging enzymes and several antioxidants were shown to protect against CDDP-induced renal damage.[12–15] In addition, a critical role for iron in CDDP-mediated enhancement of lipid peroxidation in rat kidney cortical slices has been suggested.[16,17] Moreover, studies using mouse proximal tubular cells provided evidence that the induction of apoptosis following exposure to low concentrations of CDDP is caused by the generation of oxygen free radicals.[18]

Previous studies have suggested a protective role for HO in the nephrotoxicity of CDDP.[6] The observed induction of HO-1 following CDDP exposure might be linked to the CDDP-related depletion of cellular glutathione, as HO induction often follows glutathione depletion.[19,20] Interestingly, exogenous induction of HO was shown to protect against CDDP-induced nephrotoxicity, while inhibition deteriorated its toxic effects.[6] The protection provided by HO-1 induction against the renotoxicity of CDDP has been studied *in vitro*[4] and *in vivo*.[6,11,21] Despite the increasing evidence for its antioxidative function, the direct influence of HO induction on the CDDP-induced oxidative response has not been reported as of yet.

In the present study cultured tubular cells (LLC-PK1) were used to evaluate the oxidative mechanisms involved in CDDP-mediated toxicity. To this end, the effect of widely used antioxidants (α-tocopherol (TOCO) and *N*-acetylcysteine (NAC)) on ROS production and cytotoxicity following CDDP exposure was determined. Furthermore, chemical modulators of HO activity, such as hemin and tin-protoporphyrin were used to investigate the role of altered HO activity on the oxidative response and toxicity of CDDP in LLC-PK1.

MATERIALS AND METHODS

Chemicals

Cisplatin (CDDP) was obtained from Sigma (St. Louis. MO, USA). H_2DCF-DA was obtained from Molecular Probes (Leiden, The Netherlands). Hemin and SnPP (tin-protoporphyrin) were from Porphyrin Products Laboratories (Logan, UT, USA). Phenolred-free DMEM/F12 was obtained from Life Technologies (Breda, The Netherlands). All other chemicals were of analytical grade and purchased from Sigma (St. Louis. MO, USA).

Cell Culture

LLC-PK1 cells (ATCC number CL-101), originally derived from the pig proximal tubule, were routinely seeded at 1.5×10^5 cells/75 cm^2 flask and subcultured every 6–7 days. Two days prior to an experiment, LLC-PK1 cells were plated at a density of 3×10^6 cells/75 cm^2 culture area in Medium 199 supplemented with 5% FCS, 2 mM *L*-glutamine, 100 u/mL penicillin, 100 μg/mL streptomycin. This combination of supplements covers a broad antibiotic spectrum and is preferential over other broad-spectrum antibiotics such as gentamicin, which exert renal toxicity at low concentrations. Cells were maintained in a 5% CO_2 humidified atmosphere at 37°C.

Determination of ROS Production

Cells were plated in 96-well culture plates. Following defined preincubation periods (see below) cells were rinsed with PBS. To each well serum-free DMEM/F12 without phenolred containing 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) with or without treatment was added. H_2DCF-DA is generally used to detect a broad range of intracellular oxidizing reactions.[22] After 60 min. incubation at 37°C, cells were rinsed once and treatments were started as described. ROS production was measured between 4 and 24 h after addition of CDDP (always prepared freshly) on a microplate reader equipped with a spectrofluorometer (cytofluor 2300 Fluorescence Measurement System, Millipore corp. Bedford, MA, USA) at an emission wavelength of 538 nm and extinction wavelength of 485 nm. Relative ROS production was expressed as an increase in fluorescence compared to fluorescence of the appropriate controls (100%).

Determination of Cell Viability

Cell viability was assessed by the MTT assay as well as by the neutral red uptake cytotoxicity test (NR

assay). The MTT assay, which measures the reduction of dimethylthiazol diphenyl tetrazolium bromide (MTT) to formazan by the mitochondrial enzyme succinate dehydrogenase was based on Denizot and Lang.[23] Cells were grown on 96-well plates, 60–80% confluent cells were treated as described in the text. Following the given incubation period, the medium was discarded and 0.6 mg/ml MTT solution (final concentration; 3 mg/ml stock dissolved in PBS) was added to the wells. After 2–4 h at 37°C in a humidified atmosphere the MTT solution was discarded and the formazan product in each well was solubilized in 100 µl acidic isopropanol (containing 0.1 M HCl). Absorbance at 595 nm was determined on a Biorad 3550 microplate reader equipped with a spectrophotometer (Biorad, Veenendaal, The Netherlands) Cell viability was expressed as (A_{595} treated cells/A_{595} of appropriate control) × 100%, after correction for background absorbance.

The NR test was performed according to Riddell *et al.*[24] by adding 50 µg/ml neutral red (stock 0.2 mg/ml in distilled H_2O) to the medium of the cells. After 2 h incubation at 37°C cells were rinsed twice with PBS. Lysosomal uptake of neutral red was determined by adding destain buffer (1% (v/v) acetic acid, 50% (v/v) ethanol and 49% (v/v) distilled water) to the cells and placing the culture plates for 15 min on an orbital shaker. The plates were read spectrophotometrically at 540 nm to quantify NR uptake. Viability was expressed as (A_{540} − treated cells/A_{540} − control cells) × 100% after correction for background absorbance.

Determination of HO Activity

The activity of microsomal HO was assayed as described by Kutty and Maines with minor modifications.[25] The reaction mixture (1.5 ml) contained 3–4 mg protein of the cell homogenate, 25 µM hemin, 2 mM NADP, 5 mM glucose-6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 1.5 mM $MgCl_2$ and 3 mg 100,000*g* pig liver supernatant (as source for biliverdin reductase and was determined to have a biliverdin reductase activity of approximately 600 nmol/min mg protein), in 0.05 M phosphate buffer (pH 7.4). SnPP, being a competitive HO inhibitor, was added to the mixture at 10 µM during measurement of HO activity in homogenates of SnPP-treated (HO inhibited) cells. The incubation was carried out at 37°C for 60 min. The reaction was terminated by the addition of 7.5 ml chloroform. After centrifugation (4300*g*, 5°C, 10 min) the organic phase was transferred to a clean tube and concentrated to 1 ml by evaporation under a stream of N_2. The production of bilirubin was calculated by measuring the difference in absorption of the chloroform extract between 465 and 530 nm using an extinction coefficient of 40 $mM^{-1} cm^{-1}$. Absorbance was measured on a split beam spectrophotometer (UV 2101PC, UV VIS scanning, Shimadzu Benelux, 's Hertogenbosch, The Netherlands). All steps were performed under subdued light conditions.

Preparation of Cell Homogenates

Cells were grown on 100 mm culture dishes and treated as described. To determine HO activity cell homogenates were prepared by scraping the cells in cold phosphate buffer sodium (PBS). The cells were pelleted at 166*g* for 5 min at 4°C. The pellet was resuspended in 1.25 ml HO-assay-buffer (0.05 M phosphate buffer (pH 7.4) with 1.5 mM $MgCl_2$). Cells were snap-frozen in liquid N_2 and then allowed to thaw at room temperature. This procedure was repeated three times to completely lyse all cells. After homogenation of the lysate 1 ml was used to determine HO activity, the remaining fraction was used for protein determination.

Western Blot Analysis

Cells were seeded on 100 mm culture dishes at 2×10^6 cells/dish and cultured for 48 h prior to the start of the experiments. Cells were treated with hemin or Sn-protoporhyrin as described above. The incubations were terminated by discarding the medium. The monolayers were rinsed with cold PBS and the cells were scraped in RIPA buffer (1% Igepal CA-360 (Sigma, St. Louis, MO), 0.5% sodium deoxycholate, 0.1% sodiumdodecylsulfate in PBS supplemented with 100 µg/ml phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate). Samples were homogenized and centrifuged shortly (12,000*g*, 5 min 4°C). 30 µg of protein from the supernatant fraction from the respective samples were separated on SDS-polyacrylamide gels in a Mini-Protean 3 Cell (Biorad, Veenendaal, The Netherlands) as described by Laemmli *et al.*[26] and electroblotted onto a polyvinyliden difluoride membrane according to the method of Towbin *et al.*[27] Polyclonal antibodies raised against rat HO-1 (from Sanbio bv, Uden, The Netherlands, Stressgen catalog no. SPA 895) were used for immunological staining as described by Wortelboer *et al.*[28]

Protein Determination

Protein content of the samples was determined using the method described by Lowry.[29]

Statistical Analysis

Significance of differences between different experiments was first determined using a one-way

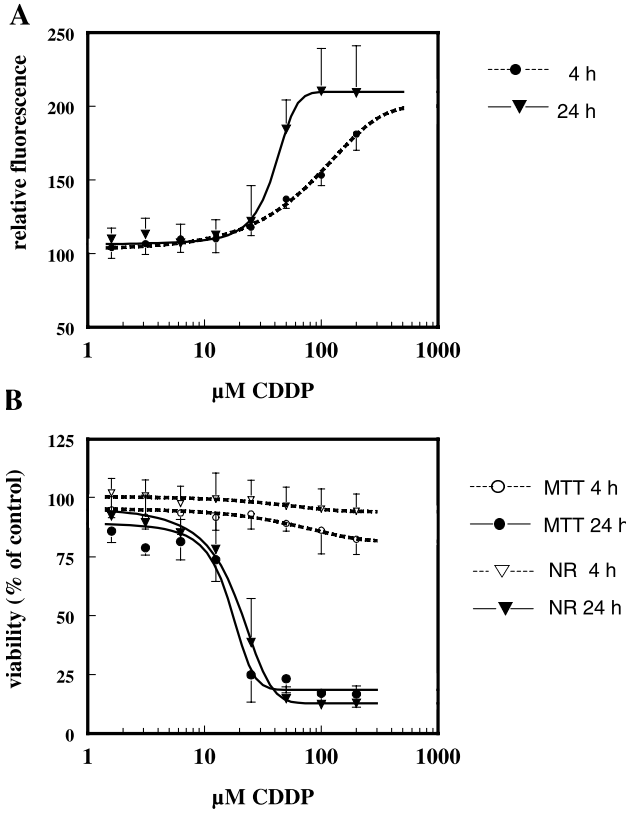

FIGURE 1 CDDP-induced generation of ROS (A) and decrease of cell viability (B) in LLC-PK1 cells. (A) ROS generation was determined after incubation of the cells for 4 and 24 h to increasing concentrations of CDDP (0–200 µM). (B) Impairment of cell viability by increasing concentrations of CDDP (0–200 µM) as determined by the MTT and NR assay. The data represent means ± SD from quadruplicate measurements from at least two independent experiments. Results of statistical analyses are delineated in the text.

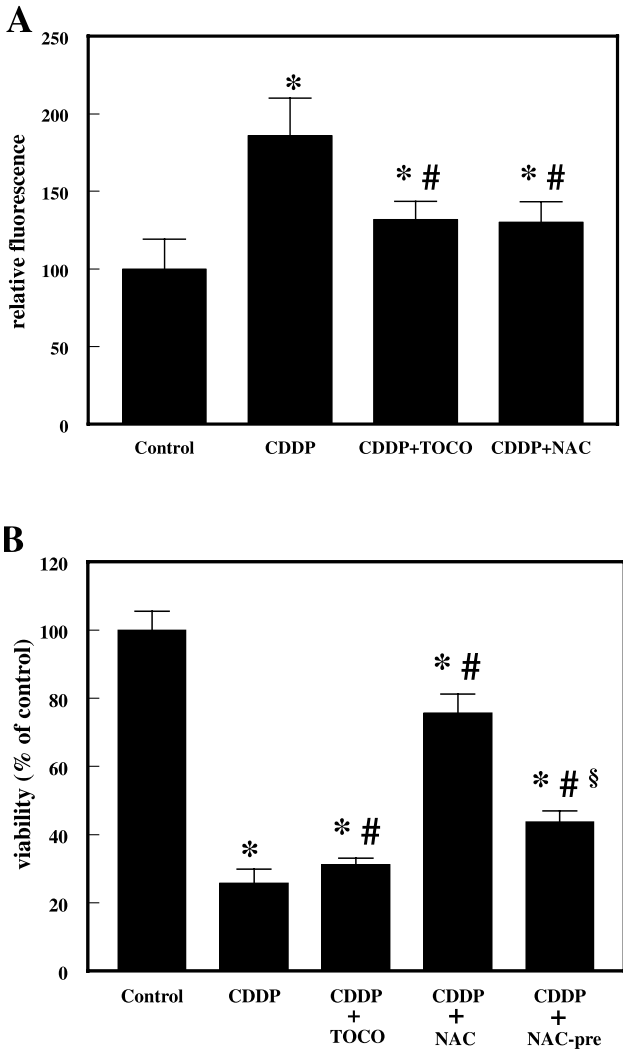

FIGURE 2 The effect of α-tocopherol (TOCO) and *N*-acetylcysteine (NAC) on the CDDP-induced ROS generation (A) and loss of viability (B). (A) The cells were preincubated (24 h) with 5 µM TOCO or 1 mM NAC, respectively, prior to exposure to 100 µM CDDP. After 24 h exposure to CDDP the ROS response was determined. (B) The cells were preincubated (24 h) with 5 µM TOCO or 1 mM NAC, respectively, prior to exposure to 25 µM CDDP. The antioxidant, TOCO or NAC remained present during CDDP exposure or was omitted prior to incubation with CDDP (NAC-pre). After 24 h exposure to CDDP cell viability was determined using the NR assay. The data represent means ± SD from quadruplicate measurement from at least two independent experiments. *Significantly different from cell viability of control treated cells ($P < 0.05$). #Significantly different from cell viability of CDDP-treated LLC-PK1 cells ($P < 0.05$). §Significantly different from cell viability of CDDP + TOCO- and CDDP + NAC-treated LLC-PK1 cells ($P < 0.05$).

ANOVA within a study and then by Bonferroni comparison of the groups of data (treatments) using SYSTAT 8.0 software. Differences were considered significant if $P < 0.05$.

RESULTS

CDDP-induced Oxidative Stress and Toxicity

CDDP, at concentrations exceeding 25 µM, significantly elevated the levels of ROS in renal tubular cells already after a 4 h exposure to CDDP (4 h, $P < 0.05$) (Fig. 1A). The ROS production increased concentration-dependently, reaching an apparent maximum in ROS production after 24 h exposure to 100 µM CDDP. Therefore, CDDP concentrations between 25 and 100 µM were used in all subsequent experiments. The CDDP-induced elevation of ROS production was also time-dependent, as the ROS levels after 24 h exposure to CDDP concentrations between 25 and 100 µM were significantly higher as compared to a 4 h exposure to corresponding concentrations ($P < 0.05$). In addition to the induction of oxidative stress, the effect of increasing concentrations of CDDP on cell viability was determined. Cell viability of the LLC-PK1 cells also decreased concentration- and time-dependently (Fig. 1B). After 4 h of incubation, cell viability was significantly decreased for high concentrations of CDDP (100 and 200 µM, MTT assay, $P < 0.05$). After 24 h cell viability was already significantly decreased at a CDDP concentration of 1.6 µM as compared to

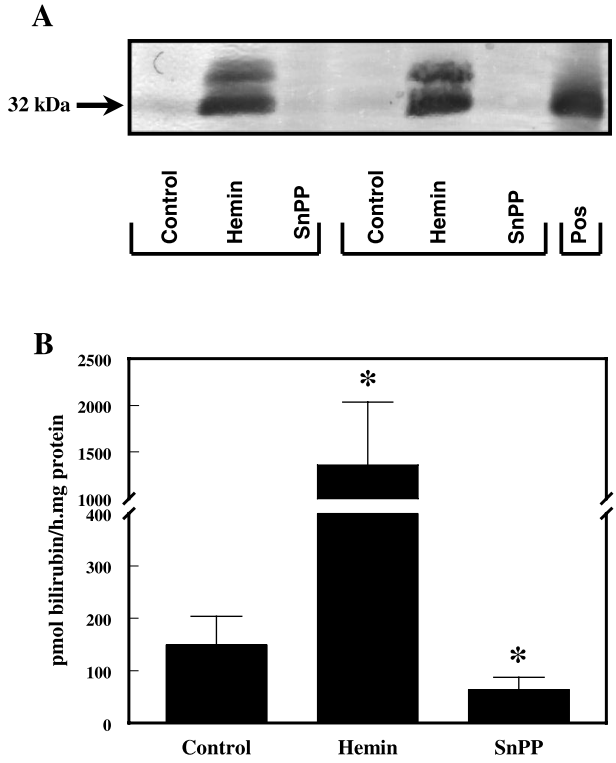

FIGURE 3 The effect of hemin and SnPP on HO-1 protein expression (A) and HO activity (B) in LLC-PK1 cells. (A) Cells were incubated for 1 h with 100 μM hemin and then incubated in hemin-free medium for 6 h before determination of HO-1 protein expression. The SnPP-treated cells were preincubated for 7 h with 10 μM SnPP before determination of HO-1 protein expression. The control cells were treated with solvent (0.1 % DMSO). Proteins were separated on a SDS-polyacrylamide gel, and HO-1 apoprotein was detected by immunoblotting with anti-rat-HO-1 antibodies. The Western blot shows duplicate samples of one typical experiment. The right lane (POS) shows the hepatocyte homogenate, which is used as a reference. (B) The cells were treated as described in A. HO activity in the cell homogenates was determined as described in the material and methods. The results of the determination of HO activity are presented as means ± SD from triplicate measurements from at least two independent experiments. *Significantly different from solvent-treated cells ($P < 0.05$).

solvent-treated cells (Fig. 1B, 24 h, MTT assay, $P < 0.05$). In addition, exposure to 25 μM CDDP and higher resulted in a significant higher loss of cell viability after a 24 h incubation as compared to exposure to corresponding CDDP concentrations for 4 h incubation (Fig. 1B, 24 vs. 4 h exposure, $P < 0.05$).

Figure 1B shows that the results on cell viability as determined by either the MTT or NR assay were not statistically different. This is further illustrated by the estimated IC_{50} values, which were 18.5 ± 4.9 and 16.5 ± 6.5 μM for the NR and MTT assay, respectively. As one of the targets of CDDP toxicity are the mitochondria and the MTT assay reflects mitochondrial activity, cell viability was assessed in subsequent experiments using the NR assay.

Effect of Antioxidants

Preincubation with α-tocopherol (TOCO) as well as by *N*-acetyl-cysteine (NAC) decreased CDDP-induced ROS generation significantly to almost control levels (Fig. 2A, $P < 0.05$ as compared to solvent- and CDDP-treated cells). Preincubation with 5 μM TOCO could almost completely prevent elevation of ROS for CDDP concentrations up to 100 μM. Similar effects were observed with pretreatment and concomitant incubation with 1 mM NAC.

Cells preincubated with TOCO showed a significantly higher viability as compared to cells incubated with CDDP (25 μM) alone, although this protective effect of TOCO on cell viability was not pronounced. In contrast, preincubation with 1 mM NAC significantly protected against the cytotoxic effect of CDDP, as judged by the NR-assay (Fig. 2B). NAC provided the greatest protection, when NAC remained present during the CDDP incubation. Omission of NAC just prior to CDDP exposure diminished the protective effect, although this protection was still greater than observed after preincubation with TOCO (NAC-pre; Fig. 2B). Altogether, this suggests that antioxidant agents, especially NAC, can protect cells against the cytotoxicity of CDDP (Fig. 2B).

Modulation of HO-1

To investigate the putative protective role of increased HO activity prototypical modulators of HO activity, hemin and tin-protoporphyrin (SnPP) were used. Before the role of altered HO activity in CDDP-mediated oxidative damage could be monitored, the effects of these chemicals on HO-1 protein expression and HO activity in LLC-PK1 cells were determined. Figure 3A shows that HO-1 expression is clearly induced by hemin pretreatment, while HO-1 protein is just above the detection limit in the samples from the control and the SnPP treated cells. No obvious difference in HO-1 protein expression between control and SnPP treated cells was observed. In line with these observations, Fig. 3B shows that HO activity is strongly induced (about 8–10 fold) after pretreatment with hemin ($P < 0.05$ as compared to the solvent-treated cells). In contrast, SnPP treatment resulted in a significant decrease of HO activity ($P < 0.05$ as compared to solvent-treated cells). These treatments did not affect the viability of the cells as determined by the MTT-assay (data not shown).

Effect of HO Modulation on CDDP-mediated Oxidative Response and Cytotoxicity

Following treatment of the cells with hemin or SnPP, respectively, as described above, the cells were

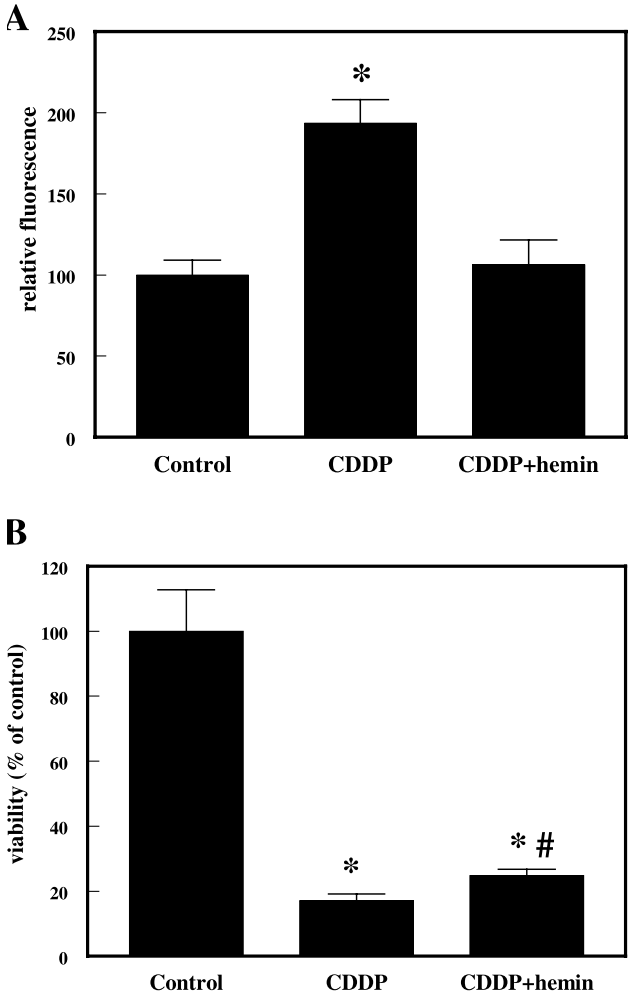

FIGURE 4 The effect of hemin preincubation on CDDP-induced ROS generation (A) and loss of viability (B) in LLC-PK1 cells. (A) Cells were incubated for 1 h with 100 μM hemin, followed by 6 h incubation in hemin-free medium. Thereafter cells were exposed to 100 μM of CDDP for 24 h and ROS levels were determined as described in the material and methods. (B) LLC-PK1 cells were treated with hemin as described in A. Following the 6 h incubation with hemin-free, cells were exposed to 100 μM CDDP. Cell viability was determined 24 h later using the NR assay. The control cells (A and B) were treated with solvent (0,1 % DMSO). Results are presented as means ± SD from quadruplicate measurements from at least two independent experiments. *Significantly different from solvent-treated cells ($P < 0.05$). #Significantly different from ROS production in LLC-PK1 cells treated with CDDP ($P < 0.05$).

exposed to CDDP and the oxidative response was measured. Figure 4A shows that hemin-treatment significantly prevented the typical CDDP-mediated elevation of ROS levels up to 100 μM CDDP ($P < 0.05$ as compared to solvent-treated cells). Pretreatment with hemin significantly protected the cells against the decrease in viability following exposure to CDDP (Fig. 4B; $P < 0.05$ as compared to CDDP-treated cells). This protective effect was only partial and was more profound at lower CDDP concentrations, while at higher concentrations of CDDP hemin treatment provided no prevention of loss of viability (Table I).

TABLE I Effect of hemin treatment on the cytotoxicity of CDDP

	Cell viability (% of control)[a]	
CDDP (μM)	Control	Hemin treated
0	100 ± 4.4	101.2 ± 5.5
12.5	62.4 ± 7.6*	84.2 ± 13.7*,**
100	17.2 ± 2.0*	24.7 ± 2.0*,**
200	12.5 ± 6.0*	15.3 ± 8.2*

[a] Control-treated cells exposed to 0 μM CDDP. Cells were incubated with solvent or 100 μM hemin for 1.5 h followed by 6 h incubation in hemin-free medium. Cells were then exposed to 0, 12.5, 100 and 200 μM hemin. Cell viability was determined 24 h later using the NR uptake assay. The data represent mean ± SD from at least two independent experiments. *Significantly different from cells treated with 0 μM CDDP ($P < 0.05$). **Significantly different from cells treated with corresponding concentration of CDDP alone (Control) ($P < 0.05$).

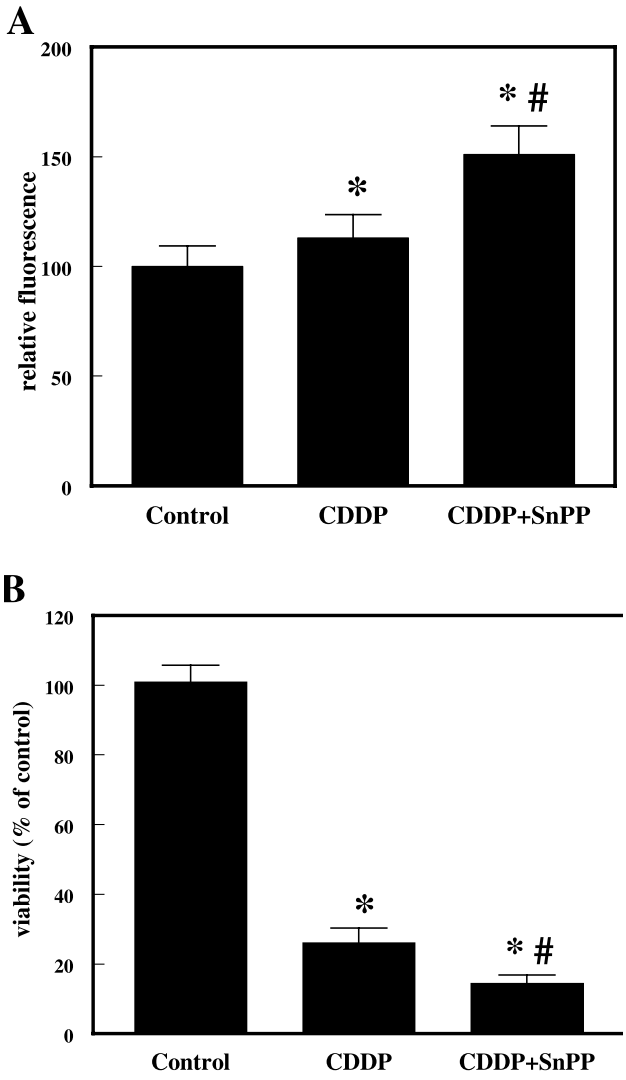

FIGURE 5 The effect of SnPP on CDDP-mediated ROS response (A) and decrease in viability (B) of LLC-PK1 cells. (A) Cells were preincubated for 7 h with 10 μM SnPP prior to exposure for 24 h to 25 μM CDDP. ROS levels were determined as described in the material and methods. (B) Cells were treated as described in A. Cell viability was determined using the NR assay. The control cells (A and B) were treated with solvent (0,1 % DMSO). The results are presented as means ± SD from quadruplicate measurements. *Significantly different from solvent-treated cells ($P < 0.05$). #Significantly different from ROS production in LLC-PK1 cells treated with CDDP ($P < 0.05$).

In contrast, the CDDP-induced (25 μM) ROS response after inhibition of HO by SnPP exceeded that of solvent- and CDDP-treated cells (Fig. 5A, $P < 0.05$). In these experiments the lowest concentration of CDDP (25 μM) that induced a detectable ROS response was chosen to illustrate the effects of HO-inhibition. In addition, HO inhibition by SnPP prior to CDDP exposure (25 μM) exacerbated its cytotoxic effects (Fig. 5B). These data again suggest the contribution of ROS to the cytotoxicity of CDDP.

DISCUSSION

Using renal tubular cells (LLC-PK1 cells) as a model, the role of HO in the development of oxidative cell damage following exposure to different CDDP concentrations was studied. CDDP is known to react with cellular macromolecules including DNA, RNA, proteins and cellular thiols finally resulting in disruption of many cellular processes including maintenance of the redox balance (reviewed by Trimmer and Essigman[11]). In the present study, CDDP induced a time- and concentration dependent increase in cellular ROS levels. The profound decrease in cell viability after exposure to CDDP concentrations exceeding 25 μM correlated with the significant increase in ROS levels observed. This suggests the involvement of oxidative stress in CDDP-induced cytotoxicity. Furthermore, the observation that 4 h incubation with 25 μM CDDP induced a significant increase of ROS levels, while a significant decrease of cell viability by 25 μM CDDP was observed only after 24 h exposure, suggests that oxidative stress preceded and thus contributed to the observed cytotoxicity. The CDDP-induced oxidative damage in renal tubular epithelial cells is in line with observations of others.[4,30]

α-tocopherol (TOCO) is a well-known antioxidant, particularly effective in scavenging lipid peroxides.[9] As anticipated, the CDDP-mediated oxidative response was effectively reduced by preincubation with TOCO. However, the CDDP-induced loss of viability was only slightly affected by TOCO pretreatment. This lack of profound protection against high CDDP concentrations, suggests that ROS-independent pathways are primarily responsible for the CDDP-mediated toxicity *in vitro*, as is also described by others.[16,18] For example, Lieberthal *et al.*, using human primary tubular cells, showed that high CDDP concentrations caused necrosis, and that this process was not affected by antioxidants.[18]

N-acetylcysteine (NAC) is an effective radical scavenger, but is also known to increase the levels of both GSH and cysteine.[31] NAC-treatment not only effectively prevented the CDDP-induced increase in ROS levels, but almost completely protected the cells against the cytotoxicity of CDDP. Thus, the observed protective effect of NAC may be, at least partially, attributed to effects secondary to ROS scavenging. Cysteine is a potent ligand for CDDP and binds CDDP both extra- and intracellularly, eliminating its immediate reactivity. In addition, it reduces the uptake of CDDP in epithelial cells.[32] The observation that the protective effect of NAC was more profound when it was continuously present is in agreement with the above-mentioned studies.[18] HO activity in the renal tubular cells was clearly induced by hemin pretreatment. Hemin treatment prior to CDDP exposure prevented the CDDP-induced elevation of ROS. In light of the presented effects of TOCO and NAC, the observed attenuation of CDDP oxidative toxicity can be attributed to an increased HO activity. Several studies have ascribed the protective effect of increased HO activity against oxidative-stress related toxicity to increased levels of bilirubin.[21] Bilirubin, the endproduct of HO-catalyzed heme-degradation, is like TOCO a potent lipid peroxide scavenger.[9] The protective effect of HO induction was comparable to the effect of TOCO treatment on CDDP-induced oxidative stress and cytotoxicity. Taken together, this suggests that lipid peroxidation does not contribute significantly to the toxicity of CDDP. The modest protection of increased HO activity may be due to the limitation of intracellular heme levels to provide sufficient concentrations of bilirubin as has been proposed by others.[33]

Treatment of LLC-PK1 cells with tin-protoporphyrin (SnPP) significantly reduced basal HO activity. Subsequently, the oxidative stress response and cytotoxicity induced by CDDP observed after treatment of LLC-PK1 cells with SnPP, are in agreement with other studies, which showed that inhibition of basal HO activity deteriorates the toxic effects of CDDP *in vitro*.[4]

The present data suggest that basal HO levels may serve to maintain the redox-balance during exposure to low concentrations (< 25 μM) of the drug, while at higher CDDP concentrations the oxidative response clearly exceeds the antioxidant defense of the cells expressing physiological levels of HO. The induction of HO as observed *in vivo* in response to CDDP insult can thus be regarded as a clear protective response in toxic nephropathy.[6,21] The assumption that HO may serve as a safeguard of cellular redox levels concurs with the reports describing the first case of human hereditary HO-1 deficiency showing the important role of HO-1 in protecting the kidney against chronic low-level oxidative insult.[34] Moreover, studies using transgenic mice deficient in HO-1 (−/−) showed that absence of HO-1 resulted in more severe renal failure and greater renal injury as compared to wild type mice treated with CDDP.

Although the contribution of ROS to the toxicity of high CDDP concentrations *in vitro* appears to be limited, previous studies show the significant involvement of ROS-related mechanisms in acute renal injury *in vivo* following CDDP-intoxication.[35] This discrepancy may be explained by the influences of secondary effects *in vivo* such as altered renal hemodynamics.[35] Furthermore, increased HO activity was shown to protect against the nephrotoxicity of CDDP in rats,[6,21] while inhibition of HO exacerbated its toxic effects.[4,6] Our results suggest that the protection provided by HO *in vivo* to CDDP toxicity may proceed through oxidative pathways. In addition, Liang and colleagues showed the involvement of a redox-based mechanism in the induction of HO-1 in LLC-PK1 cells.[36] Taken together, these observations point to a protective feedback mechanism in HO induction.

CDDP is directly accumulated in the proximal tubule,[11] leading to high local concentrations. Together with the observation that CDDP induced ROS formation at relatively high concentrations ($>25\,\mu M$), this may indicate that CDDP-related oxidative stress plays a significant role in its site-specific toxicity. Furthermore, the proximal tubule contains relatively the highest intrarenal levels of CYP450.[37] The presence of high heme levels may further contribute to the oxidative stress and tubular toxicity of CDDP, as the heme-moiety from CYP450 provides catalytic iron. Iron, capable of catalyzing free radical reactions, has been implicated in the toxicity of CDDP.[38] Therefore, the protection provided by increased HO activity in renal tubular cells *in vitro* as well as *in vivo* may not only be attributed to increased levels of antioxidants, but also to the consequent reduction of free heme (prooxidant) levels. This mechanism has also been suggested by Baliga and colleagues using the same renal tubular cell line (LLC-PK1).[38]

Exogenously induced HO may thus provide additional antioxidative defense ammunition, limiting the toxic side effects of CDDP. It will be important to assess if such an increased antioxidant defense interferes with the DNA reactivity of CDDP and thus its antitumor efficacy. Furthermore, if iron, released during HO-catabolized degradation of heme, is not properly sequestered, increased HO activity may have adverse effects. In conclusion, our results show that HO is able to limit the CDDP-induced oxidative response and partially limit the CDDP toxicity. Whether HO is able to directly restore the redox balance *in vivo* remains to be assessed.

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References

[1] Dennery, P.A. (2000) "Regulation and role of heme oxygenase in oxidative injury", *Curr. Top. Cell. Regul.* **36**, 181–199.
[2] Maines, M.D. (1997) "The heme oxygenase system: a regulator of second messenger gases", *Annu. Rev. Pharmacol. Toxicol.* **37**, 517–554.
[3] Kutty, R.K., Kutty, G., Rodriguez, I.R., Chader, G.J. and Wiggert, B. (1994) "Chromosomal localization of the human heme oxygenase genes: heme oxygenase-1 (HMOX1) maps to chromosome 22q12 and heme oxygenase-2 (HMOX2) maps to chromosome 16p13.3", *Genomics* **20**, 513–516.
[4] Shiraishi, F., Curtis, L.M., Truong, L., et al., (2000) "Heme oxygenase-1 gene ablation or expression modulates cisplatin-induced renal tubular apoptosis", *Am. J. Physiol. Renal Physiol.* **278**, F726–F736.
[5] McCoubrey, W.K., Huang, T.J. and Maines, M.D. (1997) "Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3", *Eur. J. Biochem.* **247**, 725–732.
[6] Agarwal, A., Balla, J., Alam, J., Croatt, A.J. and Nath, K.A. (1995) "Induction of heme oxygenase in toxic renal injury: a protective role in cisplatin nephrotoxicity in the rat", *Kidney Int.* **48**, 1298–1307.
[7] Applegate, L.A., Luscher, P. and Tyrrell, R.M. (1991) "Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells", *Cancer Res.* **51**, 974–978.
[8] Applegate, L.A., Noel, A., Vile, G., Frenk, E. and Tyrrell, R.M. (1995) "Two genes contribute to different extents to the heme oxygenase enzyme activity measured in cultured human skin fibroblasts and keratinocytes: implications for protection against oxidant stress", *Photochem. Photobiol.* **61**, 285–291.
[9] Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N. and Ames, B.N. (1987) "Bilirubin is an antioxidant of possible physiological importance", *Science* **235**, 1043–1046.
[10] Lebwohl, D. and Canetta, R. (1998) "Clinical development of platinum complexes in cancer therapy: an historical perspective and an update", *Eur. J. Cancer* **34**, 1522–1534.
[11] Trimmer, E.E. and Essigmann, J.M. (1999) "Cisplatin", *Essays Biochem.* **34**, 191–211.
[12] Appenroth, D. and Winnefeld, K. (1993) "Role of glutathione for cisplatin nephrotoxicity in young and adult rats", *Renal Fail.* **15**, 135–139.
[13] Dobyan, D.C., Bull, J.M., Strebel, F.R., Sunderland, B.A. and Bulger, R.E. (1986) "Protective effects of *O*-(beta-hydroxyethyl)-rutoside on *cis*-platinum-induced acute renal failure in the rat", *Lab Investig.* **55**, 557–563.
[14] McGinness, J.E., Proctor, P.H., Demopoulos, H.B., Hokanson, J.A. and Kirkpatrick, D.S. (1978) "Amelioration of *cis*-platinum nephrotoxicity by orgotein (superoxide dismutase)", *Physiol. Chem. Phys.* **10**, 267–277.
[15] Brady, H.R., Zeidel, M.L., Kone, B.C., Giebisch, G. and Gullans, S.R. (1993) "Differential actions of cisplatin on renal proximal tubule and inner medullary collecting duct cells", *J. Pharmacol. Exp. Ther.* **265**, 1421–1428.
[16] Kim, Y.K., Jung, J.S., Lee, S.H. and Kim, Y.W. (1997) "Effects of antioxidants and Ca2 + in cisplatin-induced cell injury in rabbit renal cortical slices", *Toxicol. Appl. Pharmacol.* **146**, 261–269.
[17] Kameyama, Y. and Gemba, M. (1991) "The iron chelator deferoxamine prevents cisplatin-induced lipid peroxidation in rat kidney cortical slices", *Jpn J. Pharmacol.* **57**, 259–262.
[18] Lieberthal, W., Triaca, V. and Levine, J. (1996) "Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: apoptosis vs. necrosis", *Am. J. Physiol.* **270**, F700–F708.
[19] Ewing, J.F. and Maines, M.D. (1993) "Glutathione depletion induces heme oxygenase-1 (HSP32) mRNA and protein in rat brain", *J. Neurochem.* **60**, 1512–1519.
[20] Fulton, B. and Jeffery, E.H. (1994) "The temporal relationship between hepatic GSH loss, heme oxygenase induction, and cytochrome P450 loss following intraperitoneal aluminum

administration to mice", *Toxicol. Appl. Pharmacol.* **127**, 291–297.
[21] Agarwal, A. and Nick, H.S. (2000) "Renal response to tissue injury: lessons from heme oxygenase-1 GeneAblation and expression", *J. Am. Soc. Nephrol.* **11**, 965–973.
[22] Hempel, S.L., Buettner, G.R., O'Malley, Y.Q., Wessels, D.A. and Flaherty, D.M. (1999) "Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123", *Free Radic. Biol. Med.* **27**, 146–159.
[23] Denizot, F. and Lang, R. (1986) "Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability", *J. Immunol. Methods* **89**, 271–277.
[24] Riddell, R.J., Clothier, R.H. and Balls, M. (1986) "An evaluation of three *in vitro* cytotoxicity assays", *Food Chem. Toxicol.* **24**, 469–471.
[25] Kutty, R.K. and Maines, M.D. (1982) "Oxidation of heme c derivatives by purified heme oxygenase. Evidence for the presence of one molecular species of heme oxygenase in the rat liver", *J. Biol. Chem.* **257**, 9944–9952.
[26] Laemmli, U.K. (1970) "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature* **227**, 680–685.
[27] Towbin, H., Staehelin, T. and Gordon, J. (1979) "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications", *Proc. Natl Acad. Sci. USA* **76**, 4350–4354.
[28] Wortelboer, H.M., de Kruif, C.A., van Iersel, A.A., Falke, H.E., Noordhoek, J. and Blaauboer, B.J. (1991) "Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures. The effects of model inducers on apoproteins and biotransformation activities", *Biochem. Pharmacol.* **42**, 381–390.
[29] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with Folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.
[30] Masuda, H., Tanaka, T. and Takahama, U. (1994) "Cisplatin generates superoxide anion by interaction with DNA in a cell-free system", *Biochem. Biophys. Res. Commun.* **203**, 1175–1180.
[31] Borud, O., Mortensen, B., Mikkelsen, I.M., Leroy, P., Wellman, M. and Huseby, N.E. (2000) "Regulation of gamma-glutamyltransferase in cisplatin-resistant and sensitive colon carcinoma cells after acute cisplatin and oxidative stress exposures", *Int. J. Cancer* **88**, 464–468.
[32] Kroning, R., Lichtenstein, A.K. and Nagami, G.T. (2000) "Sulfur-containing amino acids decrease cisplatin cytotoxicity and uptake in renal tubule epithelial cell lines", *Cancer Chemother. Pharmacol.* **45**, 43–49.
[33] Nutter, L.M., Sierra, E.E. and Ngo, E.O. (1994) "Heme oxygenase does not protect human cells against oxidant stress", *J. Lab. Clin. Med.* **123**, 506–514.
[34] Ohta, K., Yachie, A., Fujimoto, K., et al., (2000) "Tubular injury as a cardinal pathologic feature in human heme oxygenase- 1 deficiency", *Am. J. Kidney Dis.* **35**, 863–870.
[35] Matsushima, H., Yonemura, K., Ohishi, K. and Hishida, A. (1998) "The role of oxygen free radicals in cisplatin-induced acute renal failure in rats", *J. Lab. Clin. Med.* **131**, 518–526.
[36] Liang, M., Croatt, A.J. and Nath, K.A. (2000) "Mechanisms underlying induction of heme oxygenase-1 by nitric oxide in renal tubular epithelial cells", *Am. J. Physiol. Renal Physiol.* **279**, F728–F735.
[37] Schaaf, G.J., de Groene, E.M., Maas, R.M., Commandeur, J.N.M. and Fink-Gremmels, J. (2001) "Characterization of primary rat proximal tubular cells", *Chem.-Biol. Interact.* **134**, 167–190.
[38] Baliga, R., Zhang, Z., Baliga, M., Ueda, N. and Shah, S.V. (1998) "Role of cytochrome P-450 as a source of catalytic iron in cisplatin- induced nephrotoxicity", *Kidney Int.* **54**, 1562–1569.