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THE ROLE OF LIPID PEROXIDATION IN THE NEPHROTOXICITY OF CISPLATIN

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Abstract—The possible role of lipid peroxidation in the nephrotoxicity of the antitumour drug cisplatin was studied *in vitro*. In contrast to Adriamycin®, cisplatin did not induce lipid peroxidation in rat kidney microsomes containing a NADPH-generating system. Pretreatment of rat kidney microsomes with cisplatin did not reduce the activity of a microsomal glutathione (GSH)-dependent protective factor against lipid peroxidation induced by Fe^{2+}–ascorbate. However, pretreatment of rat kidney microsomes with 0.1 mM *N*-ethyl maleimide (NEM) did reduce this GSH-dependent protection. Cisplatin also did not reduce the activity of a cytosolic GSH-dependent protective factor against Fe^{2+}–ascorbate-induced lipid peroxidation. The results of our experiments indicate that, in contrast to Adriamycin, cisplatin does not induce lipid peroxidation *in vitro* in various test systems. It also does not destroy microsomal and cytosolic GSH-dependent protective factors against lipid peroxidation.

Cisplatin is an important anti-neoplastic drug which is used against several types of tumour, especially of the testis and ovary [1]. The dose-limiting side-effect of cisplatin chemotherapy is nephrotoxicity [2, 3]. Pathological lesions are predominant in the S_3-segment of the proximal tubule, localized in the outer stripe of the outer medulla [2].

The molecular mechanism of the nephrotoxicity induced by cisplatin is still unknown, in contrast to the molecular mechanisms of various other nephrotoxicants [4]. The nephrotoxicity of cisplatin has been attributed to covalent binding of platinum to critical protein thiol- or thiomethyl-groups [5]. Renal brush border enzymes, such as ion-translocases, have been excluded as primary sites of toxicity [6]. Decrease in glutathione (GSH‡) levels and depression of macromolecule synthesis in the kidney may play a role in cisplatin nephrotoxicity [7].

Sugihara *et al.* [8, 9] have suggested that cisplatin exerts its nephrotoxic effects by the generation of free radicals, which may cause oxidative damage in the kidney. McGinness *et al.* [10] have shown that orgotein (superoxide dismutase) reduced to some extent the nephrotoxicity of cisplatin in rats. Orgotein plays an important role in the scavenging of superoxide anion-free radicals by catalysing their conversion to molecular oxygen and hydrogen peroxide [10]. Recently, Dobyan *et al.* [11] and Bull *et al.* [12] have demonstrated that the free radical scavenger *O*-(β-hydroxyethyl)-rutoside also affords some protection against cisplatin-induced nephrotoxicity in rats. Naganuma *et al.* [13] and Baldew *et al.* [14] have shown that sodium selenite protects rodents against the nephrotoxicity of cisplatin: in principle, this protection could be due to anti-oxidative properties of sodium selenite. The nephrotoxicity of cisplatin could be attributed to free radical-mediated lipid peroxidation similar to, for example, the nephrotoxicity of the heavy metal cadmium [15].

Xenobiotics can cause lipid peroxidation in several ways. Firstly, xenobiotics can generate free radicals, initiating the process of lipid peroxidation. For example, some metals can react with hydrogen peroxide to form hydroxyl radicals, which can cause lipid peroxidation [16]. Secondly, xenobiotics can reduce the efficiency of defense mechanisms of the body against free radical-mediated damage, making the organism more vulnerable to factors which can induce lipid peroxidation. Several defense mechanisms against lipid peroxidation have been described, including a microsomal GSH-dependent factor [17, 18] and a cytosolic GSH-dependent factor [19–21]. However, up until now it has not been known whether destruction of these defense mechanisms is involved in cisplatin-induced nephrotoxicity.

The aim of the present study was to investigate whether cisplatin can cause lipid peroxidation *in vitro*, either directly or indirectly, by reducing the microsomal or cytosolic defense mechanisms against lipid peroxidation. Adriamycin®, an antitumour drug known to induce nephrotoxicity via lipid peroxidation [22], was chosen as a model compound to test the validity of the experimental set-up.

MATERIALS AND METHODS

Chemicals. Cisplatin was synthesized as described previously [23]. GSH, adenosine 5′-diphosphate, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, thiobarbituric acid and *N*-ethyl maleimide (NEM) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Adriamycin

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‡ Abbreviations: GSH, glutathione; NEM, *N*-ethyl maleimide; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

(Adriablastina) was obtained from Farmitalia (Rotterdam, The Netherlands). All other chemicals were of analytical grade.

Preparation of kidney microsomes. Male Wistar rats, 220–250 g, were obtained from the Central Institute for the Breeding of Laboratory Animals/ Harlan Sprague–Dawley (CPB/HSD) (Zeist, The Netherlands). The rats were killed by decapitation, the kidneys were removed and homogenized (30% w/v) in ice-cold 50 mM potassium phosphate buffer pH 7.4 using a Potter homogenizer. Kidney microsomes were prepared from whole kidney homogenates as described elsewhere for rat liver microsomes [17] and stored at −80° until use.

Fe^{2+}–ascorbate system. Kidney microsomes (stored at −80°) were thawed and diluted 5-fold with ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl and, in order to remove cytosolic contamination, washed twice by centrifugation at 115,000 *g* for 40 min. Finally the pellet was resuspended in ice-cold Tris–HCl buffer. To denature proteins, microsomes were heated first for 2 min at 100° and cooled subsequently in ice. The heat-pretreated microsomes (final concentration 1.5 mg microsomal protein/mL) were incubated in Tris–HCl/KCl buffer at 37° in a shaking water bath. Cisplatin, dissolved in the Tris–HCl buffer, was added in the presence or absence of 0.2 mM ascorbic acid and 10 μM $FeSO_4$. Ascorbic acid was neutralized with KOH before addition. Reactions were started by adding a freshly prepared $FeSO_4$ solution, or by adding a freshly prepared cisplatin solution. After several time-points the reactions were stopped and lipid peroxidation was measured as described below.

NADPH-generating system. Kidney microsomes were prepared as described above and were incubated in a Tris–HCl buffer (50 mM, pH 7.4) with an NADPH-generating system, consisting of NADP (1.9 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (1.1 U/mL), magnesium chloride (4.3 mM), $FeSO_4$ (10 μM)–ascorbate (0.2 mM), and cisplatin (0.02, 0.1, 0.2. 2 or 4 mM) or Adriamycin (0.1 mM). Adriamycin is a well known lipid peroxidation-inducing agent [22].

Microsomal protecting factor. The influence of cisplatin on the GSH-dependent microsomal protecting factor was studied in two types of experiments with non-heat-pretreated kidney microsomes: in the first set of experiments microsomes were pre-incubated at 37° with cisplatin (0.1, 1, 2 or 4 mM) or 0.1 mM NEM for 30 min. NEM is known to reduce the efficacy of the microsomal protecting factor [17]. The pre-incubation was terminated by a 5-fold dilution with ice-cold Tris–HCl buffer (50 mM, pH 7.4). The diluted incubation mixture was immediately centrifuged twice with the Tris–HCl buffer (115,000 *g* at 4° for 40 min) to wash out residual cisplatin or NEM. The microsomal pellet was then resuspended in the Tris–HCl buffer. In order to stimulate lipid peroxidation, the pre-incubated microsomes were incubated with 0.2 mM ascorbate, 10 μM $FeSO_4$ and 1 mM GSH. GSH was added to activate the microsomal protecting factor. In the second set of experiments, microsomes were pre-incubated with buffer at 37° for 30 min (i.e. without cisplatin or NEM pretreatment) and subsequently incubated with cisplatin (0.1, 1, 2 or 4 mM), $FeSO_4$ (10 μM)–ascorbate (0.2 mM) and 1 mM GSH. In all experiments ascorbic acid and GSH were neutralized with KOH before use.

Cytosolic protecting factor. Kidney cytosol was prepared from male Wistar rats according to the method of McCay *et al.* [19]. Kidney microsomes were prepared from the same animals and washed twice in ice-cold potassium phosphate buffer (0.15 M, pH 7.5) by centrifugation (115,000 *g* at 4° for 40 min). The microsomal pellet was resuspended in the same buffer, heat-pretreated at 100° for 2 min (in order to inactivate the microsomal protecting factor) and subsequently cooled in ice.

The influence of cisplatin on the GSH-dependent cytosolic factor was studied in two types of experiments with these heat-pretreated kidney microsomes. In the first set of experiments the microsomes (1.5 mg protein/mL) were incubated with ascorbate (0.2 mM) and $FeSO_4$ (10 μM) to induce lipid peroxidation. The influence of the cytosolic GSH-dependent protective factor on lipid peroxidation in these microsomes was studied by adding cytosol (10 mg cytosolic protein/mL final concentration) and GSH (1 mM) to the microsomal incubation mixture. The influence of cisplatin on this system was studied by adding cisplatin (0.1, 1, 2 or 4 mM) to the incubation mixture. In the second set of experiments, it was investigated as to whether pretreatment of cytosol with cisplatin could reduce the efficacy of the cytosolic protective factor on the Fe^{2+}–ascorbate-induced lipid peroxidation in the heat-pretreated kidney microsomes. To this end, cytosol was pre-incubated with cisplatin at 37° for 40 min, dialysed to remove excess of cisplatin and then added to the heat-pretreated microsomal incubation mixture in which lipid peroxidation was induced by Fe^{2+}–ascorbate. Control experiments were also carried out with cytosolic preparations subjected to the same manipulations, but with addition of phosphate buffer instead of cisplatin.

Assays. Lipid peroxidation was measured with the standard thiobarbituric acid (TBA) assay essentially as described by Haenen and Bast [17], and was expressed as the absorbance at 535 vs 600 nm ($\Delta A_{535-600}$). Briefly: an aliquot (0.3 mL) of the incubation solution was mixed with 2.0 mL ice-cold TBA–trichloroacetic acid (TCA)–HCl-butylhydroxytoluene solution. After heating (15 min, 80°) of this incubation solution and subsequent centrifugation, the supernatant was collected and the absorbance at 535 vs 600 nm measured.

At concentrations higher than 2.5 mM in the incubation medium cisplatin interfered with the standard TBA assay: an insoluble pink precipitate was observed after centrifugation of the solution. Therefore, in these cases, the TBA assay was slightly modified: the incubations were stopped by adding 1.0 mL TCA–HCl solution (16.8% w/v in 0.125 N HCl) to 0.3 mL of the incubation mixture. After standing on ice for 15 min and subsequent centrifugation, the supernatant was collected. To 1 mL of this supernatant 1 mL of a cisplatin–TBA solution (1.20 mg cisplatin and 4.16 mg TBA in TCA–HCl-butylhydroxytoluene solution) was added. After subsequent heating (15 min at 80°) and

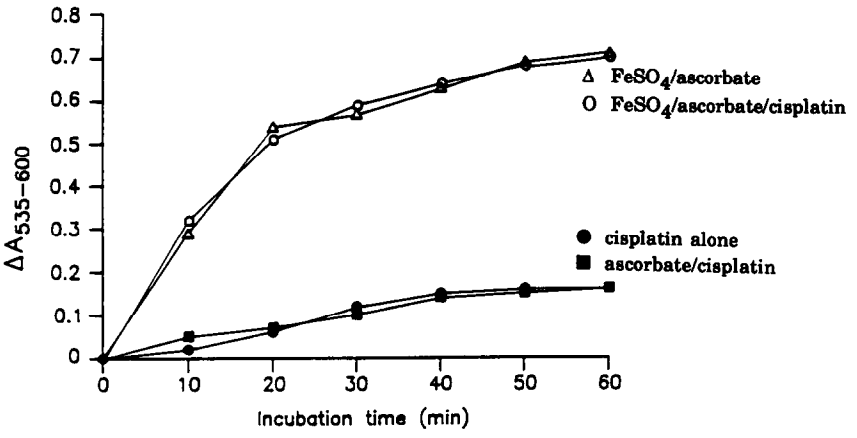

Fig. 1. Time course of lipid peroxidation in heat-pretreated rat kidney microsomes during incubation with 10 μM $FeSO_4$ plus 0.2 mM ascorbate (Δ), 10 μM $FeSO_4$, 0.2 mM ascorbate plus 2 mM cisplatin (O), 2 mM cisplatin alone (●) or 0.2 mM ascorbate plus 10 μM cisplatin (■). Similar results were obtained when other cisplatin concentrations (1, 2 or 4 mM) were used. Data represent one typical example out of three independent duplicate experiments.

centrifugation (15 min) of this solution, the pellet was dissolved in dimethylsulfoxide and the absorbance at 555 vs 600 nm ($\Delta A_{555-600}$) was measured.

Protein determinations were made according to the method of Lowry *et al.* [24] using bovine serum albumin as standard.

RESULTS

Modified TBA assay

Absorption measurements in the presence of cisplatin concentrations of 2.5 mM or higher with the modified TBA assay (see Materials and Methods) of standard malondialdehyde solutions and of microsomal test solutions in which lipid peroxidation was induced by the Fe^{2+}–ascorbate system appeared to correlate well with corresponding absorption measurements with the standard TBA assay (see Materials and Methods) in the absence of cisplatin: $(\Delta A_{555-600})_{\text{modified assay}} = 0.72\ (\pm 0.04) \times (\Delta A_{535-600})_{\text{standard assay}}$ (N = 5). At concentrations of cisplatin lower than 2.5 mM the standard TBA assay could be used accurately.

Fe^{2+}–*ascorbate system*

Optimum conditions for Fe^{2+}–ascorbate-induced lipid peroxidation in heat-pretreated kidney microsomes were established at 0.2 mM ascorbate and 10 μM Fe^{2+} (Fig. 1). Incubation of cisplatin (0.01–4 mM) did not induce appreciable lipid peroxidation in heat-pretreated kidney microsomes (Fig. 1). Co-incubation of Fe^{2+}–ascorbate with 2 mM cisplatin neither enhanced nor reduced the lipid peroxidation induced by Fe^{2+}–ascorbate (Fig. 1). To investigate whether cisplatin could replace Fe^{2+} in the microsomes with the Fe^{2+}–ascorbate system as catalyst for lipid peroxidation, cisplatin–ascorbate incubations were performed. Lipid peroxidation induced by 10 μM cisplatin in this system, however, was equal to that induced by ascorbate alone (Fig. 1). Similar results were obtained when other cisplatin concentrations (0.02–4 mM) were used (data not shown).

In order to study whether Fe^{2+} could catalyse lipid peroxidation in combination with cisplatin, kidney microsomes were incubated with both 10 μM Fe^{2+} and 0.2 mM cisplatin: in these experiments, however, no appreciable lipid peroxidation was observed. Similar results were obtained when other cisplatin concentrations (0.02–2 mM) were used (data not shown).

NADPH-generating system

Cisplatin, 0.1 mM, in contrast to both Adriamycin (0.1 mM) and Fe^{2+}–ascorbate (10 μM/0.2 mM) did not cause lipid peroxidation in heat-pretreated rat kidney microsomes containing an NADPH-generating system (Fig. 2). Moreover, 2 mM cisplatin neither enhanced nor reduced Adriamycin- or Fe^{2+}–ascorbate-induced lipid peroxidation in this test system. Similar results were obtained with other concentrations of 0.001–4 mM cisplatin in this system (data not shown).

Microsomal protecting factor

GSH (1 mM) protected non-heat-pretreated rat kidney microsomes against Fe^{2+}–ascorbate-induced lipid peroxidation as derived from the observed delay in the lipid peroxidation process (Fig. 3). Pretreatment of the microsomes with NEM (0.1 mM) reduced this microsomal GSH-dependent protection against Fe^{2+}–ascorbate-induced lipid peroxidation. However, pretreatment of the kidney microsomes with 2 mM cisplatin for 1 hr did not reduce this GSH-dependent protection factor (Fig. 3). Co-incubation of non-heat-pretreated kidney microsomes with 2 mM cisplatin also did not reduce the activity of the GSH-dependent microsomal protective factor against Fe^{2+}–ascorbate-induced lipid peroxidation (Fig. 3).

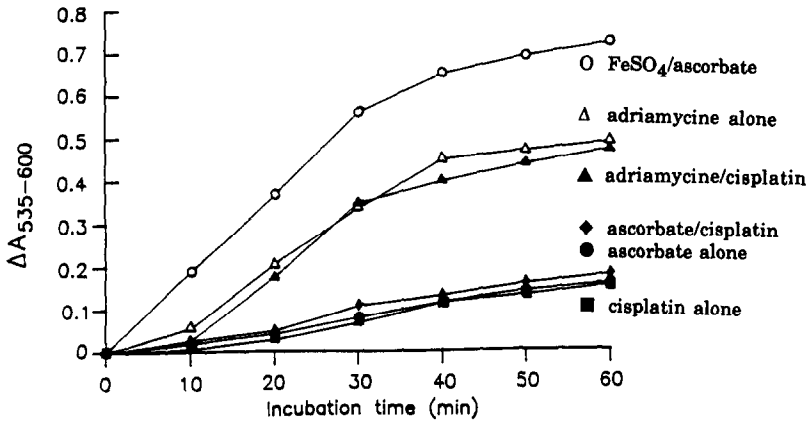

Fig. 2. Time course of lipid peroxidation in rat kidney microsomes with the NADPH-generating system and with 0.2 mM ascorbate plus 10 μM $FeSO_4$ (○), 0.1 mM Adriamycin (△), 0.1 mM Adriamycin plus 0.1 mM cisplatin (▲), 0.2 mM ascorbate (●), 0.2 mM ascorbate plus 10 μM cisplatin (◆) or 0.1 mM cisplatin (■). Data represent one typical example out of three independent duplicate experiments.

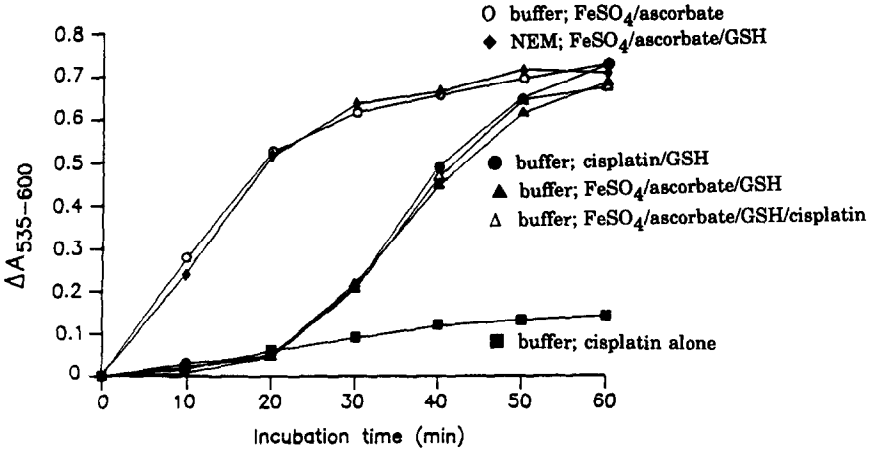

Fig. 3. The influence of NEM and cisplatin on the GSH-dependent microsomal protective factor against lipid peroxidation in (non-heat-pretreated) rat kidney microsomes. NEM (0.1 mM) pretreated microsomes were incubated with 10 μM $FeSO_4$–0.2 mM ascorbate plus 1 mM GSH (◆). Buffer-pretreated microsomes were incubated with 10 μM $FeSO_4$–0.2 mM ascorbate (○), 10 μM $FeSO_4$–0.2 mM ascorbate plus 1 mM GSH (▲), 10 μM $FeSO_4$–0.2 mM ascorbate plus 1 mM GSH and 2 mM cisplatin (△), 1 mM GSH and 2 mM cisplatin (●), or 2 mM cisplatin alone (■). Data represent one typical example out of three independent duplicate experiments.

Similar results were obtained with 0.2, 1 and 4 mM cisplatin (data not shown).

Cytosolic protective factor

Cytosolic rat kidney protein (10 mg/mL incubation mixture) provided protection against Fe^{2+}–ascorbate-induced lipid peroxidation in heat-pretreated kidney microsomes in the presence of 1 mM GSH, but not in the absence of this concentration of GSH (Fig. 4). Co-incubation of this incubation mixture with 2 mM cisplatin had no influence on the activity of the added cytosolic GSH-dependent protective factor. Pre-incubation of kidney cytosol with 2 mM cisplatin and subsequent incubation of this cytosol with heat-pretreated kidney microsomes and Fe^{2+}–ascorbate resulted in the same protection against lipid peroxidation as obtained when the cytosol was pre-incubated with buffer instead of cisplatin (data not shown). Similar results were obtained upon co-incubation and pre-incubation of kidney cytosol with 0.2, 1 and 4 mM cisplatin (data not shown).

DISCUSSION

The aim of the present study was to investigate *in vitro* in rat kidney fractions whether lipid peroxidation plays a role in the nephrotoxicity of cisplatin. Because cisplatin at concentrations higher than

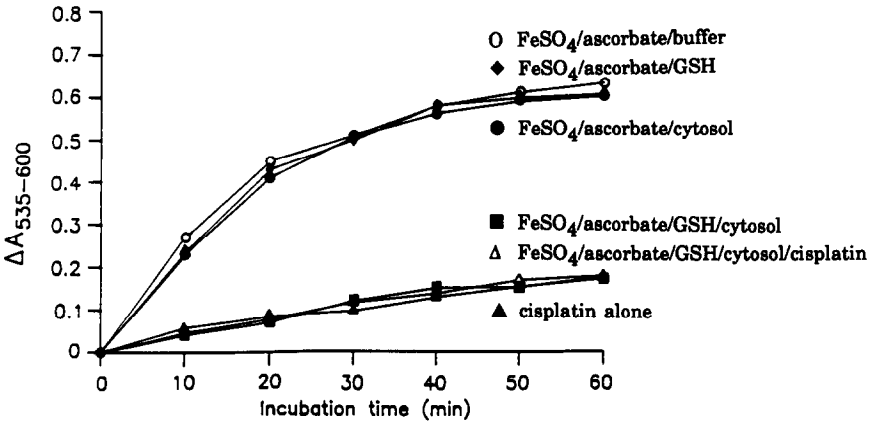

Fig. 4. The influence of cisplatin on the GSH-dependent cytosolic protective factor against lipid peroxidation in rat kidney microsomes. Heat-pretreated rat kidney microsomes were incubated with 10 μM $FeSO_4$–0.2 mM ascorbate plus phosphate buffer (○), 10 μM $FeSO_4$–0.2 mM ascorbate plus 1 mM GSH (◆), 10 μM $FeSO_4$–0.2 mM ascorbate plus 10 mg cytosolic protein/mL (●), 10 μM $FeSO_4$–0.2 mM ascorbate plus 1 mM GSH and 10 mg cytosolic protein/mL (■), 10 μM $FeSO_4$–0.2 mM ascorbate plus 1 mM GSH, 10 mg cytosolic protein/mL and 2 mM cisplatin (△), or 2 mM cisplatin only (▲).

2.5 mM interfered with the standard TBA assay, we developed first a modified TBA assay for lipid peroxidation. This modified TBA assay allowed the detection of TBA-reactive species in the presence of cisplatin at concentrations as high as 4 mM.

The results presented in this paper demonstrate that neither in the presence nor the absence of ascorbate and Fe^{2+} does cisplatin itself generate TBA-reactive species in heat-pretreated rat kidney microsomes (Fig. 1). This means that cisplatin at concentrations up to 4 mM is apparently not able to cause lipid peroxidation in heat-pretreated kidney microsomes. The kidney microsomes were briefly heat-pretreated in order to destroy GSH-dependent protective factors in the microsomes, thus making the microsomes more vulnerable to lipid peroxidation [17].

Cisplatin also did not generate TBA-reactive species in rat kidney microsomes containing an NADPH-generating system (Fig. 2). This indicates that cisplatin is not oxidized by NADP through one-electron reactions. In contrast, Fe^{2+}–ascorbate efficiently generated TBA-reactive species in this system (Fig. 2).

The anti-tumour drug Adriamycin, which was used as a positive control in this study, caused lipid peroxidation in the heat-pretreated kidney microsomes (Fig. 2). Adriamycin induces nephrotoxicity in rodents [25]. Adriamycin-induced lipid peroxidation is NADPH-dependent and requires activation of Adriamycin to a semiquinone free radical intermediate by microsomal NADPH-cytochrome P450 reductase [22] or cytochrome P450. Our experiments show that cisplatin had no influence at all on the lipid peroxidation induced by Adriamycin in kidney microsomes, indicating that cisplatin is neither reduced nor oxidized chemically or biochemically in this system.

GSH protects non-heat-pretreated kidney microsomes against Fe^{2+}–ascorbate-induced lipid peroxidation (Fig. 3). This GSH-dependent protection against lipid peroxidation is thermally labile and probably proceeds via a vitamin E-dependent microsomal protein as has been shown by Haenen and Bast [17] for liver microsomes. Apart from heat pretreatment, this GSH-dependent protection can be reduced by pre-incubation of microsomes with the thiol inactivator NEM (Fig. 3), probably as a result of inactivation of GSH and the vitamin E-dependent microsomal protein [17]. Pre-incubation of microsomes with cisplatin (instead of NEM), at concentrations as high as 4 mM, did not result in any significant change in the generation of TBA-reactive species by Fe^{2+}–ascorbate, indicating that the microsomal GSH-dependent protective factor is not inactivated by cisplatin. The lack of influence on this protective factor was found to be independent of the time of incubation of the kidney microsomes with cisplatin (Fig. 3).

In the presence of cytosol, GSH also protects heat-pretreated kidney microsomes against Fe^{2+}–ascorbate lipid peroxidation (Fig. 4). This is probably due to a GSH-dependent cytosolic protective factor as described by Gibson *et al.* [20]. Pre-incubation of heat-pretreated kidney microsomes with cisplatin did not reduce the GSH-dependent cytosolic protection against Fe^{2+}–ascorbate-induced lipid peroxidation in kidney microsomes. This lack of effect of cisplatin on the cytosolic protective factor was also found to be independent of the time of incubation with cisplatin, since both pre-incubation of the cytosol with cisplatin and co-incubation with Fe^{2+}–ascorbate yielded similar results (Fig. 4). This indicates that cisplatin is not able to inactivate the cytosolic GSH-dependent protective factor against lipid peroxidation.

Our results show that cisplatin itself does not induce lipid peroxidation in rat kidney microsomes, either directly or indirectly, by destroying microsomal or cytosolic GSH-dependent protective factors.

Sugihara and Gemba [8] have used rat renal cortical slices to study the role of lipid peroxidation in the nephrotoxicity of cisplatin. Incubation of the renal slices with 2 mM cisplatin for 2 hr increased levels of TBA-reactive species by 50%. Mannitol prevented the cisplatin-induced elevation of the levels of TBA-reactive species, an effect which was suggested to be mediated by the anti-oxidant effect of mannitol. However, it is questionable as to whether mannitol might also inactivate cisplatin by the formation of adducts with cisplatin [26, 27].

Sugihara *et al.* [28] have also observed increased levels of TBA-reactive species in rat kidneys, 72 hr after treatment with cisplatin. Cisplatin-induced nephrotoxicity is generally manifested 3–4 days after treatment with cisplatin, although early events responsible for the nephrotoxicity may have occurred already shortly after administration of cisplatin [14, 29]. In contrast, there is generally no delay of several days in the expression of lipid peroxidation after its initiation. The lipid peroxidation in kidneys of rats only 72 hr after treatment with cisplatin, observed by Sugihara *et al.* [28], may therefore be a consequence of the nephrotoxicity induced by cisplatin rather than its cause [30, 31]. This explanation is also in accordance with results of Litterst *et al.* [32] who observed that levels of TBA-reactive species in kidneys of rats treated with nephrotoxic doses of cisplatin were not elevated 24 hr after administration of the drug.

It has been shown that cisplatin nephrotoxicity can be reduced by the administration of free radical scavengers such as *O*-(β-hydroxyethyl)-rutoside [9, 11]. However, it is not known whether the protective effects of these agents and their free radical scavenging capacity are causally linked. *O*-(β-Hydroxyethyl)-rutoside, for example, is a bioflavonoid, belonging to a class of compounds with prominent anti-oxidant activities [33], probably related to metal-chelating properties [34]. This latter property or other pharmacological activities of *O*-(β-hydroxyethyl)-rutoside [34] might, however, be responsible for its protective effect against cisplatin nephrotoxicity rather than its anti-oxidant activity. The results presented in this paper suggest that the protective effect of sodium selenite against cisplatin-induced nephrotoxicity, which was observed recently [13, 14], is therefore most likely not mediated by the anti-oxidant properties of sodium selenite. A direct interaction between selenol-metabolites of selenite, formed by reactions between selenite and GSH or other thiols, and cisplatin covalently bound to essential proteins has been proposed as an alternative explanation [35].

In conclusion, the present results demonstrate that cisplatin is not able to induce lipid peroxidation *in vitro* in a variety of rat kidney test-systems, either directly or indirectly, by damaging kidney microsomal or cytosolic GSH-dependent protection mechanisms against lipid peroxidation. It is therefore unlikely that the protective effect of sodium selenite or other anti-oxidants against cisplatin-induced nephrotoxicity in rodents is due to their anti-oxidative properties.

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