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Excretion of multiple urinary biomarkers for radical-induced damage in rats treated with three different nephrotoxic compounds

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In the present study the urinary excretion of seven aldehydes, acetone and coproporphyrin III as non-invasive *in vivo* biomarkers of free radical damage was measured in rats after treatment with three nephrotoxic compounds: cisplatin, mercuric chloride ($HgCl_2$) and *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-Nac). A clear difference between the different nephrotoxic compounds was found in the time interval between dosage and maximal toxicity, as measured by clinical chemical parameters in urine. In rats treated with TFE-Nac and $HgCl_2$ this was fast: 12 h and 24 h after treatment, respectively. In the rats treated with cisplatin, however, nephrotoxicity occurred later: 96 h–108 h after treatment. Urinary creatinine excretion was decreased in all treatments. Therefore, the excretion of the proposed biomarkers was expressed as amount excreted per 12 h urine fraction as well as amount excreted per mol creatinine in each 12 h urine fraction. Urinary excretion of coproporphyrin III was decreased in almost all 12 h urine fractions with all treatments, however, when expressed per mol creatinine, increases were found in urine of rats treated with cisplatin and $HgCl_2$. In cisplatin-treated rats an increase was found in the excretion of formaldehyde per 12 h, but acetaldehyde, propanal and MDA levels were decreased. Expressed per mol creatinine, MDA levels were decreased, but other aldehydes were increased. In $HgCl_2$-treated rats urinary aldehyde excretion expressed per mol creatinine was increased. In TFE-Nac treated animals the urinary levels of acetaldehyde per 12 h were increased and per mol creatinine the levels of some aldehydes were only slightly increased. With none of the treatments did the increase in the biomarkers expressed per mol creatinine exceed the decrease in creatinine excretion. Similar time intervals were found between dosage and maximal excretion of biomarkers as for the time intervals between dosage and maximal toxicity. With all treatments significant increases in the excretion of acetone were found both per 12 h and per mol creatinine, probably related to the increased glucose excretion. It was concluded that no convincing evidence for free radical damage was found in the present study with the employed biomarkers.

Keywords: radical damage, biomarkers, cisplatin, $HgCl_2$, kidney damage, non-invasive.

Abbreviations: ACN, acetonitril; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUTA, butanal; CCl4, carbon tetrachloride; cisplatin, *cis*-platinum(II)-diamine dichloride; EC, electrochemical detection; Fe-NTA, Fe(II)nitrilotriacetate; GC/ECD, gas chromatography with electron capture detection; GGT, γ-glutamyltransferase; GLDH, glutamate dehydrogenase; $HgCl_2$, mercuric chloride; HPLC, high performance liquid chromatography; MDA, malondialdehyde; MeOH, methanol, MTBA, 4-methylthiobenzoic acid; NAG, *N*-acetyl-ß-D-glucosaminidase; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; PFB, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydro-chloride; TEP, 1,1,3,3-tetraethoxy-propane; TFE-Nac, *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

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Introduction

Free radicals are continuously formed in the body as a consequence of aerobic and anaerobic metabolism (Halliwell and Gutteridge 1984, Van der Vliet and Bast 1992, Loft and Poulsen 1996). In addition, many xenobiotics, such as environmental pollutants and cytostatic agents, induce higher production of reactive oxygen species and other radicals which are capable of reacting with cellular macromolecules such as DNA, lipids and proteins (Jamieson 1989, Starke-Reed and Oliver 1989). Currently, there is an increasing interest in the development of non-invasive and non-destructive biomarkers to investigate early (patho)physiological effects of radicals *in vivo* (Hageman *et al.* 1992). Ideally, these biomarkers should be selective, measurable at low levels in non-invasively obtainable biological samples, detectable before irreversible damage has occurred and, finally, be related to toxic effects.

Potential biomarkers for radical damage are degradation products of hydroxylated DNA nucleotides such as 8-hydroxy-2′-deoxyguanosine (8-OH-dG). This product is formed upon reaction of reactive oxygen species with DNA and may be excreted in urine as the result of nucleotide excision repair (Cundy *et al.* 1988, Shigenaga and Ames 1991). Other biomarkers for radical damage that have been proposed are porphyrins, e.g. coproporphyrin III (Miller and Woods 1993). Porphyrins may be derived from oxidized porphyrinogens and are excreted in urine without further metabolization (Miller and Woods 1993, Hermanns *et al.*, accepted).

One of the major processes resulting from radical damage is lipid peroxidation. Degradation of lipid peroxides generates a wide variety of compounds, such as alkanes, alkanals, alkenals, and hydroxyalkenals (Benedetti *et al.* 1979, Esterbauer 1982). Some of these degradation products may be distributed from the organ or tissue of origin into the bloodstream and can subsequently be excreted in urine (Comporti 1989, Esterbauer *et al.* 1991, Hageman *et al.* 1992).

In several *in vivo* studies the increased excretion of formaldehyde, acetaldehyde, MDA and acetone has been suggested to be caused by free radical damage. These include studies in which CCl_4, menadione, paraquat, adriamycin were used as potential radical inducing agents (Bagchi *et al.* 1993, Bagchi *et al.* 1995). Recently, we have developed an automated method for the simultaneous determination of low concentrations of seven different aldehydes and acetone in urine using gas chromatography with electron-capture detection (GC/ECD) (de Zwart *et al.* 1997).

In rats treated with either carbon tetrachloride (CCl_4; causing hepatotoxicity) or Fe(II)-nitrilotriacetate (Fe-NTA; causing nephrotoxicity) the urinary excretion of these aldehydes, acetone, 8-OH-dG and coproporphyrin III in urine was measured. A significant increase in the excretion of most aldehydes and acetone was found in both studies. 8-OH-dG and coproporphyrin III excretion, however, was increased only after treatment with Fe-NTA (De Zwart *et al.*, in press; Hermanns *et al.*, accepted). In the present study, urinary excretion of seven aldehydes, acetone and coproporphyrin III was studied upon administration of three different nephrotoxins to rats, viz. cisplatin, mercuric chloride ($HgCl_2$) and *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-Nac).

Cisplatin is a drug with antineoplastic activity towards several human tumours. A major side effect limiting its clinical use is dose-related nephrotoxicity (Goldstein and Mayor 1983, Safirstein *et al.* 1986). The exact biochemical mechanism of cisplatin nephrotoxicity has not yet been defined. Cisplatin has been

shown to generate oxygen free radicals *in vitro* (Masuda *et al.* 1994); however, in other studies this has not been found (Vermeulen and Baldew 1992). Glutathione-depletion has been shown to precede cisplatin-induced lipid peroxidation *in vitro* (Zhang and Lindup 1993) and *in vivo* (Somani *et al.* 1995). Cisplatin is also known to react with nucleophilic groups in DNA and proteins (such as methylthio-group) probably leading to toxicity. Other nucleophiles, e.g. 4-methylthiobenzoic acid (MTBA), were shown to prevent cisplatin toxicity *in vitro* (Boogaard *et al.* 1991).

HgCl2 is a potent nephrotoxic agent that may cause acute renal failure and irreversible cell injury and is often used as model compound for nephrotoxicity (Weinberg 1993). It has been demonstrated that HgCl2 potently stimulates generation of hydrogen peroxide *in vitro* and *in vivo* in the kidney (Nath *et al.* 1996).

TFE-Nac was shown to cause proximal tubular toxicity in the rat kidney, without any effect on the liver (Commandeur *et al.* 1988). The mechanism of toxicity of this compound is dependent on active uptake and activation by renal ß-lyase. *In vivo*, TFE-Nac is first deacetylated in the liver or kidney. The L-cysteine conjugate formed after uptake in the proximal tubules is substrate for the enzyme ß-lyase, and an instable thiol-compound is formed which can rearrange to form a difluorothionoacyl fluoride (Commandeur *et al.* 1989). This product is highly reactive and may bind covalently to nucleophilic groups in biomacromolecules (Commandeur *et al.* 1988, 1995).

The aim of the present study was to further evaluate the utility of the above proposed non-invasive urinary biomarkers for radical damage (short chain *n*-alkanals, acetone and coproporphyrin III) in rats treated with three nephrotoxic compounds, viz. cisplatin, $HgCl_2$ and TFE-Nac. All three compounds may eventually cause free radical damage but most likely not as a primary cause of toxicity and the exact role of free radical damage in the toxicity mechanism of these compounds is not clear as yet.

Experimental and animal treatments

Chemicals

O-(2,3,4,5,6-pentafluorbenzyl)hydroxylamine hydrochloride (PFB), 3-bromofluoro-benzene, acetaldehyde, propanal, butanal, pentanal, hexanal were purchased from Fluka Chemie AG (Buchs, Switzerland). Acetone and sulphuric acid (H_2SO4) were obtained from Riedel-de Haën AG (Seelze, Germany). 1,1,3,3-Tetraethoxypropane (TEP), $HgCl_2$, acetic acid, formic acid and sodium wolframate were purchased form Merck (Darmstadt, Germany). Formaldehyde, sodium acetate, *n*-heptane, EDTA disodium salt and ammonium hydroxide (25) % were purchased form J. T. Baker BV (Deventer, The Netherlands). Sodium sulphate was obtained from Janssen Chimica (Beerse, Belgium). Coproporphyrin III dihydrochloride was obtained from Porphyrin Products Inc. (Utah, USA). Nanopure water was from a Milli-Q system (Millipore, Bedford, MA, USA). Methanol (MeOH) and acetonitril (ACN) were of HPLC grade and purchased from Rathburn Chemical Ltd (Walkerburn, UK). Lithium-heparin was obtained from Organon NV (Oss, The Netherlands). TFE-Nac was synthesized as described by Commandeur *et al.* (1988). Hypnorm®, consisting of fentanylcitrate (0·315 mg ml^{-1}) and fluanisone (10 mg ml^{-1}), was obtained from Janssen Pharmaceuticals (Oxford, U.K.) and Dormicum®, containing midazolamhydrochloride (5 mg ml^{-1}), was obtained from Roche BV (Mijdrecht, The Netherlands). *Cis*-platinum(II)-diamine dichloride (cisplatin) was purchased from Sigma (St Louis, MO, USA).

Apparatus

GC system: A Hewlett Packard 5890 series II gas chromatograph equipped with a 15 mCi ^{63}Ni electron-capture detector (GC-ECD) was used. The GC was equipped with a 30 m HP 5 (CP Sil 8 CB) column (0·32 i.d., 0·25 μm film thickness, Hewlett Packard, Amstelveen, The Netherlands). For data acquisition and processing the HP Chemstation software package implemented on a HP Vectra

486/33VL was used. The temperature of the GC oven was programmed from 50 (1 min initial time) to 150 °C, at 7 °C min^{-1}, and then to 270 °C, at 20 °C min^{-1}, and kept at 270 °C for 5 min. The temperatures of the injection port and detector were 200 °C and 300 °C respectively. The column flow rate was 2·8 ml min^{-1}.

HPLC system: The HPLC system consisted of a Gyncotech 480 pump equipped with a vacuum degasser (ISI Labo Systems, Tokyo, Japan), a Triathlon auto-sampler, a Nucleosil C18 100 *4 mm column (Macherey-Nagel GmbH, Duren, Germany), a Marathon column oven set at 25 °C and a fluorescence detector LS-5B (Perkin Elmer Norwalk, CT USA) equipped with a 50 µl flowcell. The data were processed with Gyncosoft 5·3 chromatographic software.

Animal treatments

Male Wistar rats (WU, outbred), weighing about 225 g, were obtained from Charles River Wiga (Sulzfeld, Germany). Water and a commercial laboratory chow (Hope Farms, Woerden, The Netherlands) were available *ad libitum*. During the experiment, the rats were housed individually in metabolism cages for the separate collection of urine and faeces. Animal facilities were maintained at 22 °C and on a 12 h light/dark cycle. Urine fractions of 12 h were collected 24 h prior to and during 6 days following treatments. Eighteen rats were divided into four groups. One group served as a control and received a single i.p. dose of saline ($n = 4$). The other three groups were treated with a single i.p. dose of either 7·5 mg kg^{-1} cisplatin ($n = 5$), 2·5 mg kg^{-1} $HgCl_2$ ($n = 5$) or 13·2 mg kg^{-1} TFE-Nac ($n = 4$). All compounds were dissolved in saline and all animals received a volume of 5 ml kg^{-1}.

Urine-collecting vessels were positioned in containers filled with dry ice which permitted the instant freezing of the urine over 12 h periods. Urine volumes were determined and fractions of it stored at –20 °C until further analysis.

After 6 days rats were anaesthesized by s.c. injection of 0·75 ml kg^{-1} Hypnorm® and 1 ml kg^{-1} Dormicum®, blood was taken and treated with lithium-heparin (25 U ml^{-1} blood). Blood plasma was obtained after centrifugation for 10 min at 4000 *g*, and stored at –20 °C until further analysis (within 20 days).

Clinical chemistry

The clinical chemical analyses in blood plasma and urine were performed on a Hitachi 911 Automatic Analyzer of Boehringer Mannheim (Almere, The Netherlands), consisting of a photometrical unit and a central processing unit. Analyses in blood were carried out at 37 °C in lithium-heparin plasma. All analyses were done according to the instructions of the manufacturer of the automatic analyser (Accreditation Compliance sheets of the BM/Hitachi 911 analyzer, Boehringer Mannheim, Almere, The Netherlands, 1996) (Tietz 1986).

The enzymes aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) were determined according to the International Federation of Clinical Chemistry (IFCC) kinetic method. Alkaline phosphatase (ALP) according to the Dutch Foundation of Clinical Chemistry (NVKC) recommendations, γ-glutamyltransferase (GGT) according to the kinetic method of Szasz, glutamate dehydrogenase (GLDH) according to the optimized Deutsche Gesellschaft für Klinische Chemie (DGKC) method, *N*-acetyl-ß-D-glucosaminidase (NAG) according to the cresolsulphonphtalein method. Determinations were done according to the 2,5-dichlorophenyl diazonium salt (DPD) method for bilirubin, the hexokinase method for glucose, the urease/NADH method for urea-N, the copper biuret method for total plasma protein, the benzethonium chloride method for urinary protein, the phenol 4-aminophenazon peroxidase (PAP) method for creatinine and the bromocresolgreen method for albumin.

Analysis of aldehydes and acetone in urine

The concentrations of the lipid peroxides degradation products, formaldehyde, acetaldehyde, acetone, propanal, butanal, pentanal, hexanal and MDA, in rat urine were determined using a GC method with ECD detection. This is a very selective and sensitive method with detection limits ranging from 39 ± 5·3 to 500 ± 23 fmol per µl injected dependent on the aldehyde studied (De Zwart *et al.* 1997).

Briefly, to 0·5 ml of urine in capped 2 ml glass tubes, 15 µl of 3·3 M H_2SO_4 was added in order to hydrolyse protein-bound aldehydes. The mixture was vortexed and allowed to stand at room temperature for 10 min. The proteins were then precipitated with 75 µl of 0·3 M sodium wolframate. After shaking and centrifuging for 10 min at 4000 *g*, 0·5 ml of the supernatant was used for derivatization with 0·5 ml PFB-reagent. PFB-reagent consisted of a solution of 1 mm PFB in 1·5 M sodium acetate buffer (pH 5·0). To derivatize the sample, it was vortexed and maintained at room temperature for 1 h. The oximes formed were extracted from the aqueous samples by adding 0·5 ml *n*-heptane containing 10 µM 3-bromofluoro-benzene (internal standard) and shaking for 30 s. The heptane layer was removed and washed once with 0·5 ml 0·1 N HCl to remov[illegible]

The heptane layer was dried over anhydrous sodium sulphate and 1 µl was injected onto the GC column. Quantification of all seven aldehydes and acetone was done using calibration curves of mixtures of aldehydes and acetone in urine. A standard response curve was constructed by plotting the ratios of peak areas and the internal standard (3-bromofluorobenzene) individually against the concentrations in the range of 0·05 to 4 µM.

Analysis of coproporphyrin III in urine

Urinary coproporhyrin III was analysed using fluorescence detection at 400 nm excitation and 600 nm emission wavelength according to Richard *et al.* (1986). Separation was achieved using isocratic elution with a mobile phase consisting of 40 % ACN in 50 mM ammonium formate buffer (pH 3·6) at a flow of 1 ml min^{-1}. Prior to injection, urine samples were acidified with HCl (final concentration 0·4 M). Quantification was carried out using a standard curve of coproporphyrin III in 0·4 M HCl. Peak areas were linearly related to the amount injected onto the column over a concentration range of 0·5–16 µM for coproporphyrin III.

Statistical methods

Experimental results were evaluated statistically using the Student's *t*-test and were considered significant at $p < 0·05$.

Results

Clinical chemical parameters

Several clinical chemical parameters were measured in plasma collected 1 week after treatment and in 12 h urine fractions collected throughout the experiment. Both urea and creatinine levels in plasma were increased in cisplatin and TFE-Nac-treated rats (figure 1), indicative for reduced glomerular filtration. No such increases were found in $HgCl_2$-treated rats. Furthermore, an increase in plasma bilirubin and a decrease in ALP activity was found in cisplatin-treated animals. Plasma ALT, AST, GLDH, sodium, potassium, total protein and albumin remained unchanged when compared with control levels with all treatments (data not shown). Several parameters (urine volume, creatinine excretion) showed a circadian rhythm.

Cisplatin: The urinary excretion of creatinine per 12 h was decreased upon cisplatin treatment (figure 2(A)). Excretion of creatinine remained low during the whole experiment with minimal excretion levels 96 h after administration of cisplatin. The urine production per 12 h was significantly decreased from 48 h till

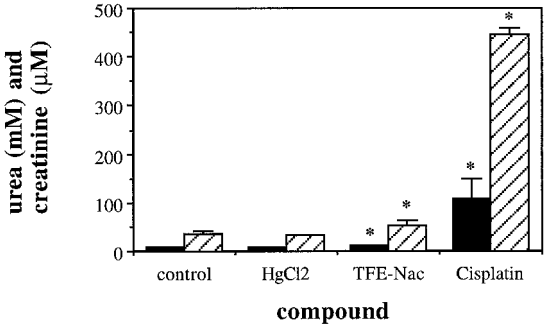

Figure 1. Urea (■) and creatinine (◪) levels in plasma of rats treated with a single i.p. dose of $HgCl_2$ (2·5 mg kg^{-1}), TFE-Nac (13·2 mg kg^{-1}) or cisplatin (7·5 mg kg^{-1}). The values are given as mean values ± sem of four or five animals. *: Significantly different with $p < 0·05$ in the Student's *t*-test with the corresponding control group.

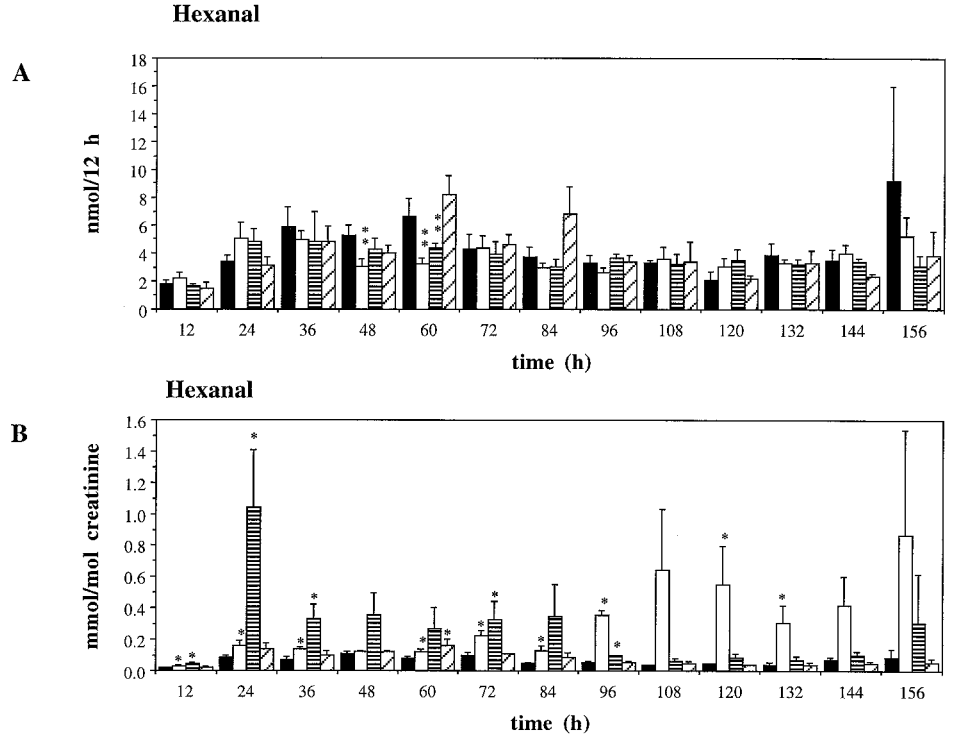

Figure 2. (A) Urinary excretion of creatinine per 12 h. (B) Excretion of urine per 12 h. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with $HgCl_2$ (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *:Significantly higher with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group. **:Significantly lower with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group.

96 h after treatment (figure 2(B)). Therefore, all urinary parameters were expressed as amount excreted per 12 h as well as amount per mol creatinine.

Excretion of glucose/12 h was maximal 84 h after treatment with cisplatin (figure 3(A)). Protein/12 h was statistically significantly decreased 24, 36 and 48 h after treatment (figure 4(A)). Both urinary sodium and potassium excretion per 12 h decreased significantly 12 h after treatment, and remained low during the experiment with a minimum 96 h after treatment (data not shown). Urinary GLDH/12 h was maximal 108 h after treatment and decreased rapidly thereafter; NAG/12 h was significantly increased 72 h after treatment (0·18 ± 0·02 vs 0·09 ± 0·01) and remained increased; ALP/12 h was significantly increased 96 h after treatment (3900 ± 1500 vs 8 ± 5) and remained high and GGT/12 h was increased 96 h, 108 h and 120 h after treatment, but interindividual variation was high (data not shown).

Urinary glucose/creatinine was maximal 108 h after treatment and protein/creatinine was statistically significantly increased 60 h after treatment and was maximal 108 h after treatment (figures 3(B) and 4(B)). Urinary sodium/creatinine was decreased from 12 h after treatment until the end of the experiment, but potassium/creatinine was unchanged when compared with the control values (data not shown). Urinary GLDH/creatinine was maximal 108 h after treatment and decreased rapidly thereafter; NAG/creatinine was m[illegible]

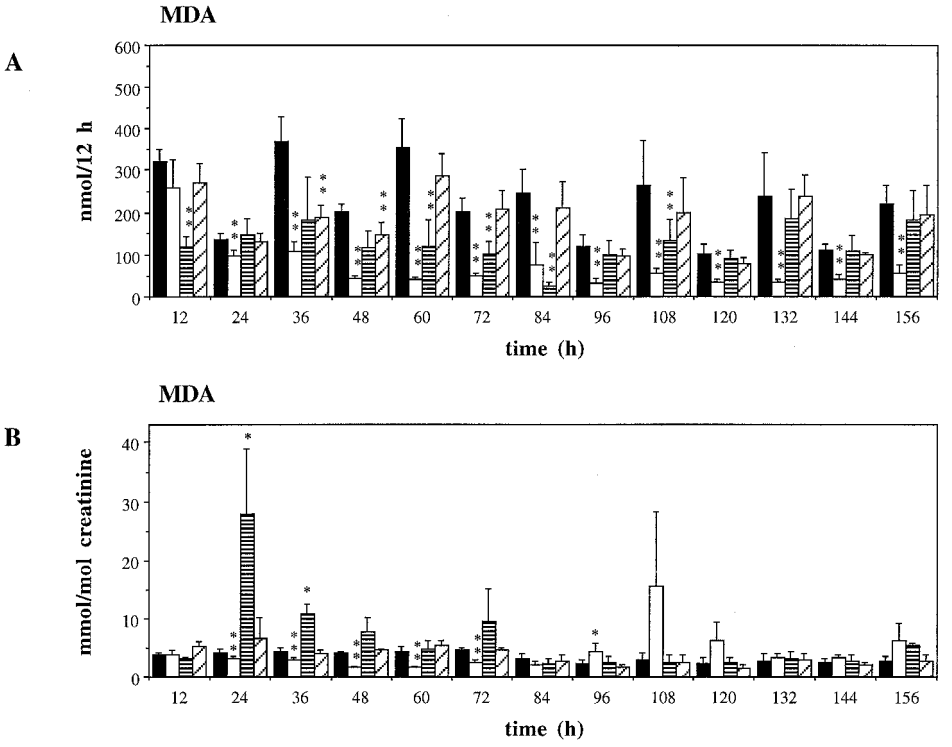

Figure 3. Urinary excretion of glucose in every 12 h fraction of four groups of animals. (A) Glucose excretion per 12 h. (B) Glucose excretion per mol creatinine. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with $HgCl_2$ (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *:Significantly higher with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group.

treatment (40 ± 14 vs 1·3 ± 0·2); ALP/creatinine was significantly increased 96 h after treatment and remained high (500 ± 150 vs 0·16 ± 0·09) and GGT/creatinine was increased 96 h, 108 h and 120 h after treatment, but interindividual differences were high (data not shown).

$HgCl_2$: Urinary creatinine per 12 h was decreased. Minimal excretion was observed already 24 h after treatment, thereafter excretion increased again, but did not completely reach control level (figure 2(A)). No significant change in urine production per 12 h was found (figure 2(B)).

Urinary glucose/12 h was maximally increased 36 h after treatment, and it remained higher than control levels until the end of the experiment (figure 3(A)). Urinary protein/12 h was slightly increased, although interindividual differences were high (figure 4(A)). Sodium and potassium per 12 h were statistically significantly decreased throughout the whole experiment. GLDH, NAG, ALP and GGT per 12 h (data not shown) started to increase 12 h or 24 h after treatment, but variation between animals was high. ALP/12 h remained high throughout the whole experiment.

Urinary glucose and protein per mol creatinine were statistically significantly increased, with a maximum 36 h after treatment (figures 3(B) and 4(B)). Sodium/creatinine was only decreased 12 h and 24 h after treatment and at the end of the experiment (i.e. 96 h, 108 h and 132 h), but potassium/creati[illegible]

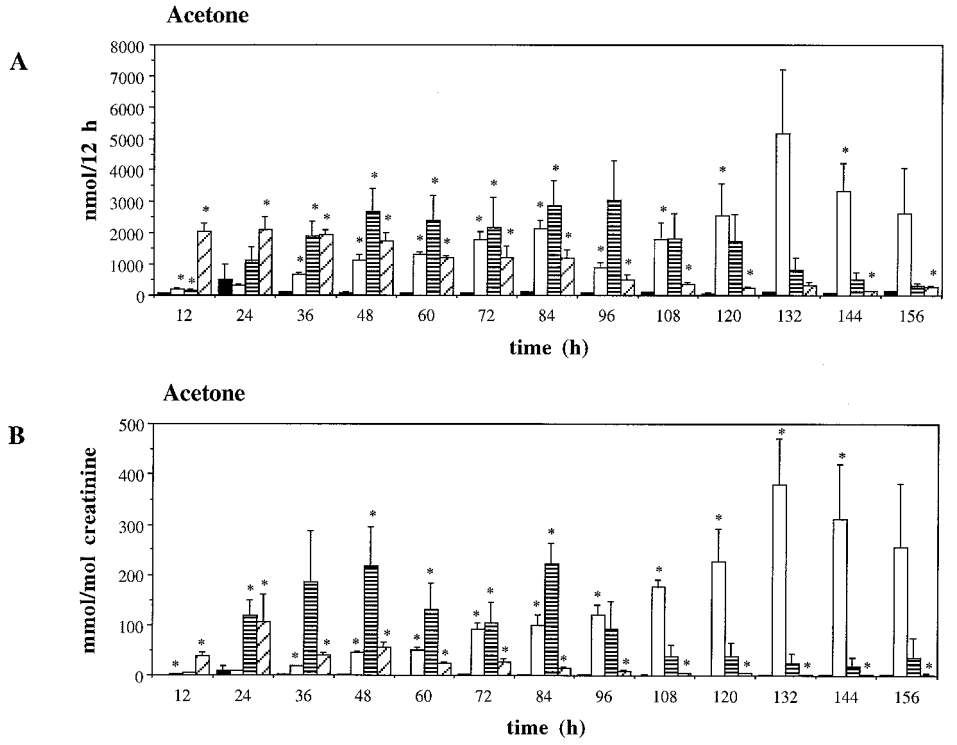

Figure 4. Urinary excretion of protein in every 12 h fraction of four groups of animals. (A) Protein excretion per 12 h. (B) Protein excretion per mol creatinine. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with $HgCl_2$ (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *:Significantly higher with $p < 0{\cdot}05$ in the Student's *t*-test with the corresponding control group. **:Significantly lower with $p < 0{\cdot}05$ in the Student's *t*-test with the corresponding control group.

12 h, 24 h and 36 h after treatment (data not shown). NAG/creatinine was maximally increased 36 h after treatment (65 ± 7 vs 1·8 ± 0·1); GLDH/creatinine was maximal 24 h after treatment (60 ± 60 vs 0·16 ± 0·08), with high variation between animals. GGT/creatinine in urine was only increased 12 h (0·7 ± 0·6 vs 0·14 ± 0·06) and 24 h after treatment (1800 ± 600 vs 0·12 ± 0·1). ALP/creatinine in urine was maximal 84 h after treatment (500 ± 250 vs 0·4 ± 0·2).

TFE-Nac: Urinary creatinine excretion was decreased in the first urine fraction until 60 h after treatment and thereafter returned to control level (figure 2(A)). Urine production was strongly increased throughout the whole experiment (figure 2(B)).

Urinary glucose/12 h was strongly increased 12 h after treatment, thereafter levels declined and 120 h after treatment levels were back to control value (figure 3). Urinary protein/12 h was statistically significantly increased 12 h, 24 h and 36 h after treatment (figure 4). Sodium and potassium levels per 12 h were decreased, but less than with the other two treatments. Levels of sodium and potassium also returned to control level at the end of the experiment. All four other urinary enzyme activities (i.e. GLDH, GGT, NAG and ALP) per 12 h and per creatinine were significantly increased in the first 12 h after treatment (data not shown). Urinary glucose/creatinine and protein/creatinine showed large[illegible]

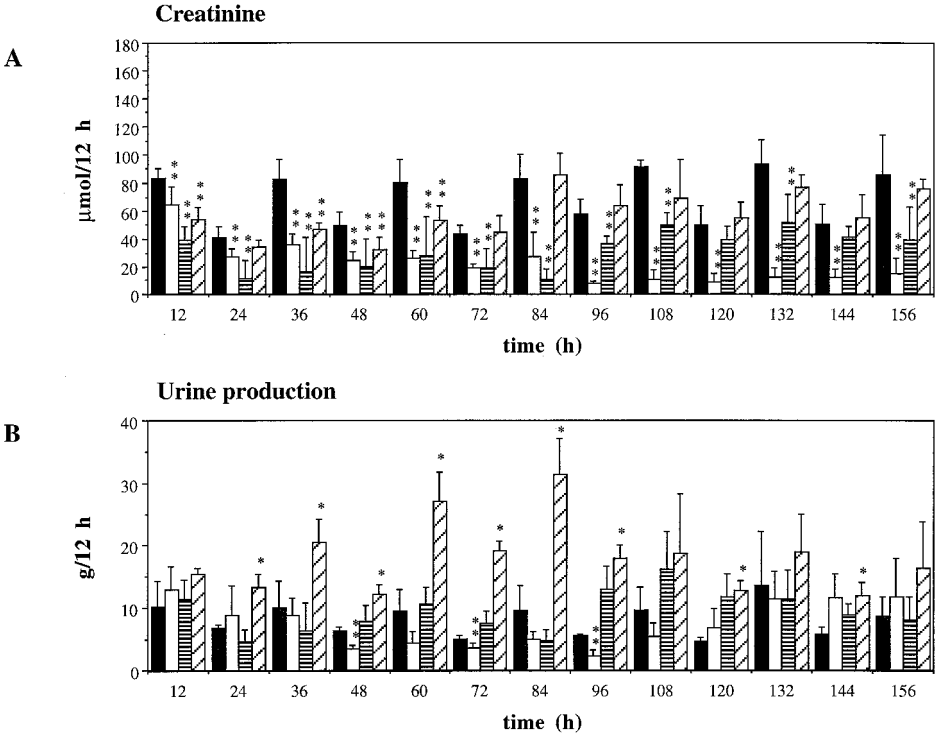

Figure 5. Urinary excretion of coproporphyrin III in every 12 h fraction of four groups of animals. (A) Coproporphyrin III excretion per 12 h. (B) Coproporphyrin III excretion per mol creatinine. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with $HgCl_2$ (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *:Significantly higher with $p < 0{\cdot}05$ in the Student's *t*-test with the corresponding control group. **:Significantly lower with $p < 0{\cdot}05$ in the Student's *t*-test with the corresponding control group.

as expressed per 12 h. Urinary sodium and potassium per mol creatinine, however, were largely unchanged throughout the experiment (data not shown).

Urinary excretion of coproporphyrin III

Cisplatin: Urinary excretion of coproporphyrin III per 12 h was already statistically significantly decreased 12 h after treatment (figure 5(A)). Minimal coproporphyrin III excretion was observed 48 h after treatment, and the levels remained low until the end of the experiment. Coproporphyrin III excretion expressed per mol creatinine was decreased in the first 84 h after treatment (figure 5(B)).

$HgCl_2$: Urinary excretion of coproporphyrin III per 12 h was slightly decreased 36 h after treatment, then above control levels 60 h after treatment (figure 5(A)), although with high variation between animals, and finally remained significantly decreased from 84 h after treatment until the end of the experiment. The excretion per mol creatinine was significantly increased 12 h after treatment and remained slightly increased (figure 5(B)), although with a high variation, until 72 h after treatment; from 96 h after treatment levels in urine were decreased again.

TFE-Nac: Coproporphyrin III levels per 12 h and per m

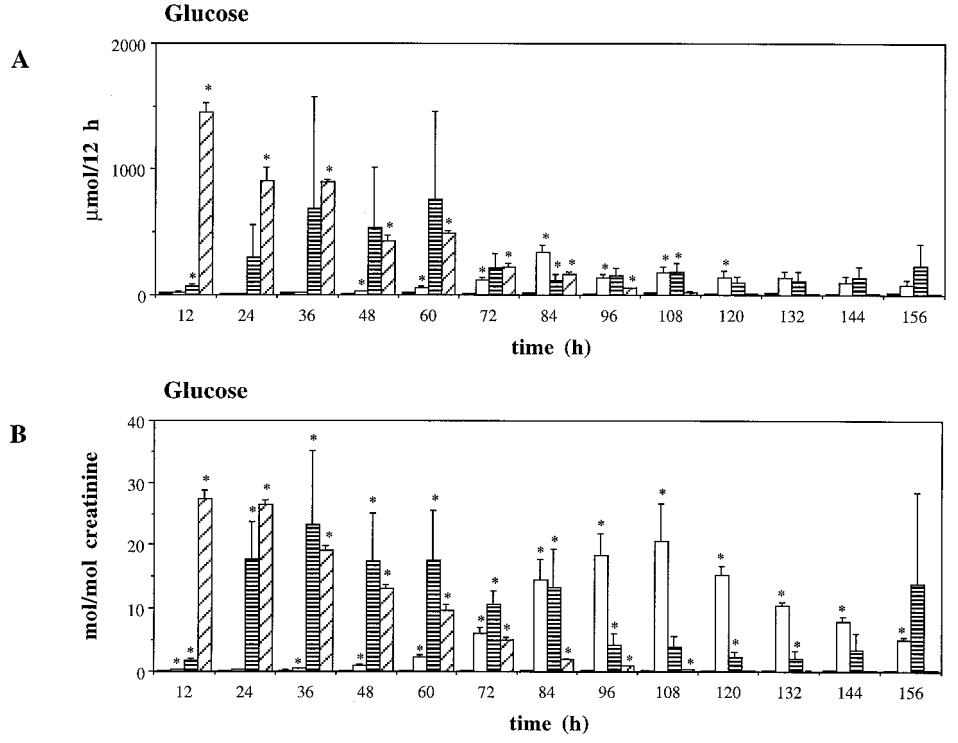

Figure 6. Urinary excretion of propanal in every 12 h fraction of four groups of animals. (A) Propanal excretion per 12 h. (B) Propanal excretion per mol creatinine. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with HgCl2 (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *: Significantly higher with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group. **: Significantly lower with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group.

already significantly decreased 12 h after treatment and remained low throughout the experiment, with a minimum 36 h and 48 h after treatment (figure 5).

Urinary excretion of lipid peroxide degradation products

Cisplatin: In rats treated with cisplatin a slight but statistically significant increase in excretion of formaldehyde per 12 h was found compared with the corresponding control group 24 h, 48 h, 72 h and 144 h after treatment. Urinary excretion of acetaldehyde (data not shown) and propanal (figure 6(A)) per 12 h was decreased until 36 h and 60 96, 108 h after treatment. Butanal excretion was decreased in urine fractions as well, be it only from 96 h until 132 h after treatment (data not shown). Pentanal (data not shown) and hexanal (figure 7(A)) excretion per 12 h remained largely unchanged. MDA excretion per 12 h, however, was decreased from 24 h onwards throughout the whole experiment (figure 8(A)). Urinary excretion of acetone was increased to the greatest extent; it was already significantly increased in the first 12 h after treatment, reaching a maximum 132 h after treatment and remaining high until the end of the experiment (figure 9).

Urinary formaldehyde, acetaldehyde, pentanal (data not shown), propanal (figure 6(B)) and hexanal (figure 7(B)) excretion per mol crea

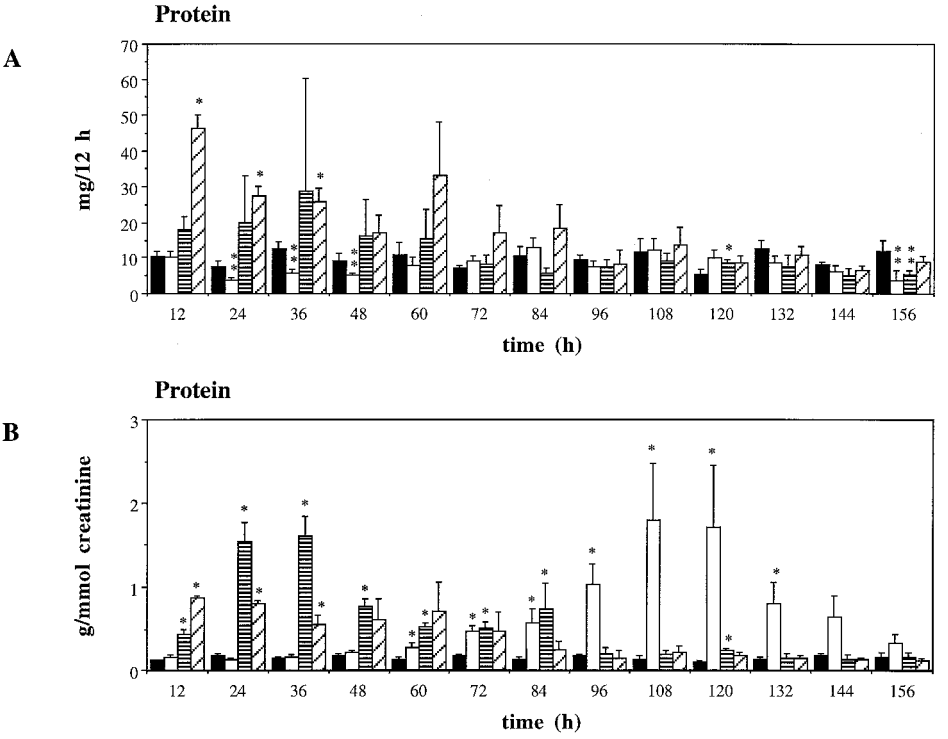

Figure 7. Urinary excretion of hexanal in every 12 h fraction of four groups of animals. (A) Hexanal excretion per 12 h. (B) Hexanal excretion per mol creatinine. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with $HgCl_2$ (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *: Significantly higher with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group. **: Significantly lower with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group.

from 24 h after treatment until the end of the experiment with a maximum 108 h after treatment. MDA excretion per mol creatinine, however, was decreased from 24 h after treatment until 72 h after treatment. 96 h and 108 h after treatment an increase was found, be it with very high variation between animals 108 h after treatment (figure 8(B)).

$HgCl_2$: Urinary excretion of formaldehyde, acetaldehyde, pentanal (data not shown) and hexanal (figure 6(A)) per 12 h remained unchanged. Statistically significant decreases were found in the excretion of propanal (figure 6(A)), butanal (data not shown) and MDA (figure 8(A)) per 12 h throughout the rest of the experiment. Urinary acetone excretion per 12 h showed statistically significant increases during the experiment (figure 9(A)). This increase was already significant in the first 12 h urine samples after exposure, reached a maximum 96 h after treatment and declined thereafter, though it did not return completely to the control level before the end of the experiment. Acetone excretion per mol creatinine was significantly increased 24 h after treatment and remained high throughout the experiment although with high interindividual variations (figure 9(B)). Maximal excretion of acetone per mol creatinine was reached 48 h after treatment.

When expressed per mol creatinine, all seven aldehydes

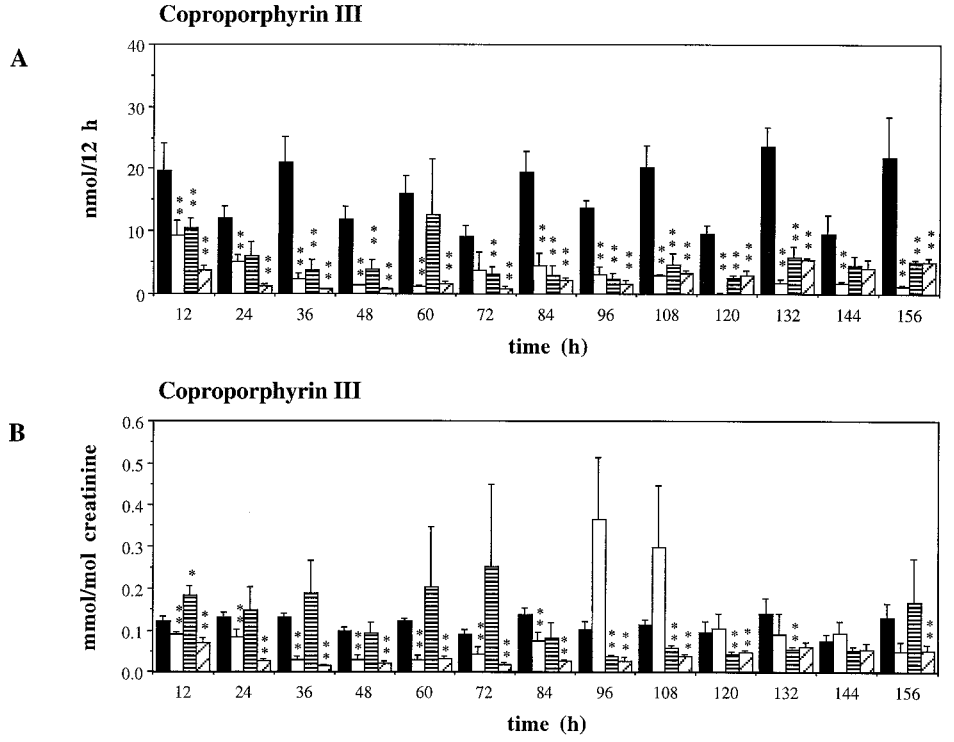

Figure 8. Urinary excretion of MDA in every 12 h fraction of four groups of animals. (A) MDA excretion per 12 h; (B) MDA excretion per mol creatinine. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with $HgCl_2$ (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *:Significantly higher with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group. **:Significantly lower with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group.

increased 24 h after treatment. Formaldehyde, acetaldehyde, pentanal, (data not shown), propanal and hexanal levels (figures 6(B) and 7(B)) remained higher than control level until 84 h after treatment, with sometimes high variation between animals.

TFE-Nac: Pentanal (data not shown) and hexanal (figure 7(A)) per 12 h excretion remained at control level during the whole experiment. A slight, statistically significant decrease was found in the urinary excretion of butanal (data not shown), propanal and MDA (figures 6(A) and 8(A)) per 12 h. Urinary formaldehyde per 12 h (data not shown) was slightly (statistically significant) increased 72 and 84 h after treatment. An increase in the excretion of acetaldehyde was found with a maximum 60 h after treatment (data not shown). The increase in the excretion of acetone per 12 h was already maximal 12 h after exposure and diminished thereafter, but remained significantly higher than the control group until the end of the experiment (figure 9(A)).

Urinary excretion of butanal (data not shown) and MDA (figure 8(B)) per mol creatinine remained at control level throughout the experiment. Urinary excretion of formaldehyde, acetaldehyde, pentanal (data not shown) and hexanal (figure 7(B)) per mol creatinine was slightly increased 60 h after treatment. Acetone excretion per mol creatinine was significantly increased throughout the

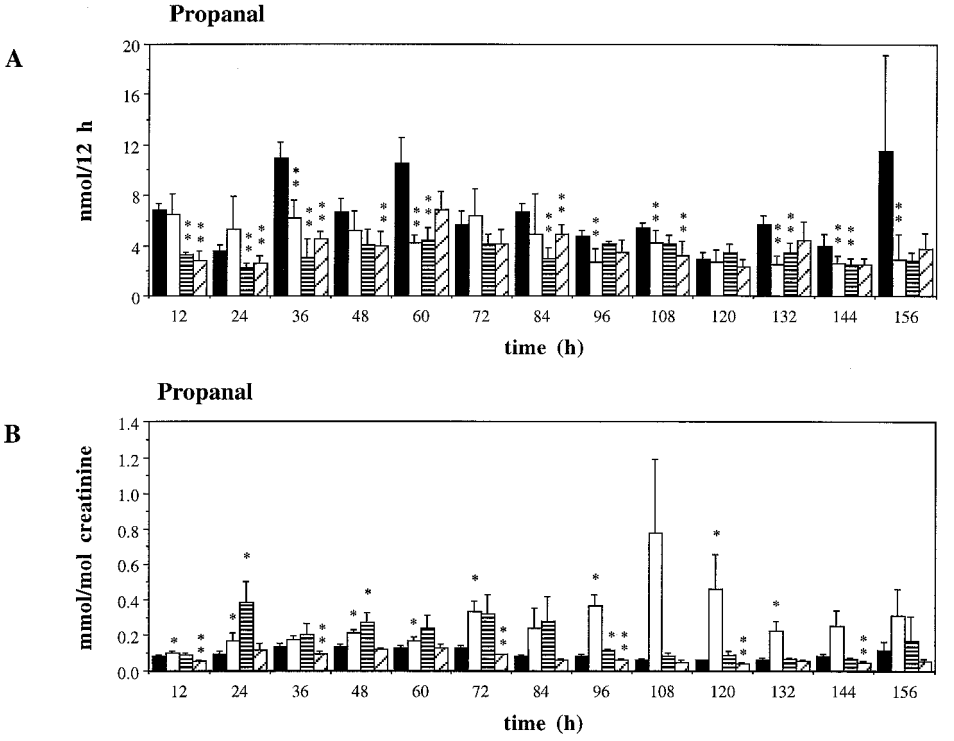

Figure 9. Urinary excretion of acetone in every 12 h fraction of four groups of animals. (A) Acetone excretion per 12 h. (B) Acetone excretion per mol creatinine. Bars represent mean values ± sem of four or five animals. ■ Indicates the control group, □ the group treated with cisplatin (7·5 mg kg^{-1}), ▤ the group treated with $HgCl_2$ (2·5 mg kg^{-1}) and ▨ the group treated with TFE-Nac (13·2 mg kg^{-1}). *: Significantly higher with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group. **: Significantly lower with $p < 0{\cdot}05$ in the Student's t-test with the corresponding control group.

maximum 24 h after treatment (figure 9(B)). Propanal excretion per mol creatinine was slightly decreased throughout the experiment (figure 6(B)).

Discussion

The primary aim of this study was to investigate whether a number of recently proposed non-invasive *in vivo* biomarkers of free radical damage could be used to study the mechanism of induction of renal damage by three different nephrotoxic compounds. The nephrotoxic compounds used for this purpose were cisplatin, $HgCl_2$ and TFE-Nac. These are well known, selective nephrotoxins with different toxicity mechanisms. In cisplatin-treated rats impaired glomerular and tubular functions were observed, resulting in reduced reabsorption of water (Miura *et al.* 1987). Cisplatin causes extensive necrosis of the S_3 segment of the proximal tubule located on the outer stripe of the outer medulla in the kidney (Nagano *et al.* 1995). Free radical damage is thought to play a role in the cisplatin-mediated toxicity, but whether this is a causal mechanism or a result of toxicity is unclear up till now (Vermeulen and Baldew 1992). $HgCl_2$ damages the glomerulus both directly or as the result of formation of auto-antibodies that are deposited in the glomerulus and affect glomerular filtration (Aten 1995), and it causes severe necrosis in the S

segment of the proximal tubule (Gartland *et al.* 1989). Furthermore, it has been shown that $HgCl_2$ stimulates renal generation of hydrogen peroxide *in vitro* and *in vivo*, causing oxidative stress (Nath *et al.* 1996). TFE-Nac is also known to cause toxicity in the S_3 segment of the proximal tubule. The mechanism of toxicity involved, deacetylation to *S*-(1,2,2-tetrafluoroethyl)-L-cysteine (TFE-cys) and subsequent formation of reactive intermediates by ß-lyase resulting in covalent binding to nucleophilic centra in bio-macromolecules (Commandeur *et al.* 1989). Exposure of cells to TFE-cys has been shown to lead to oxidative stress *in vitro*. This oxidative stress most probably is the consequence of covalent binding to mitochondria, which results in production of oxygen radicals (Van de Water *et al.* 1996).

Nature and extent of renal toxicity

At the dose levels selected, severe nephrotoxicity was induced by all three nephrotoxins. This was indicated by the reduced glomerular filtration as was derived from the decreased excretion of creatinine in urine (figure 2(A)) and the increases in plasma urea and creatinine (Koren 1989). In TFE-Nac-treated rats plasma urea and creatinine levels were only slightly increased when compared to cisplatin treated rats (figure 1). No increase in plasma creatinine and urea levels was found in rats treated with $HgCl_2$, which might be due to a significant recovery of the renal damage after 1 week (the time that the plasma samples were taken). The reduced glomerular filtration rate may be the result of (partial) blockage of the tubules by shedding of damaged proximal tubular cells. However, cisplatin in also known to cause damage to the glomeruli directly (Miura *et al.* 1987). The decrease found in the urinary excretion of sodium upon all treatments is in agreement with a reduced glomerular filtration rate (Winter 1981, Koren 1989). The increases found in the urinary excretion of glucose indicate that also tubular reabsorption had decreased (figure 3).

An increase in urine production was found in rats treated with TFE-Nac, in which also large increases in glucose levels were found. This is probably caused by decreased reabsorption of solutes by the proximal tubules, which results in a higher osmotic value of the primary urine (Stanton and Koeppen 1993). In $HgCl_2$-treated animals no increase was found in the urine production, while in the cisplatin-treated animals even a decrease in urinary volume was found, probably because glomerular filtration was decreased due to direct toxic effect of cisplatin on the glomeruli (figure 2(B)). The latter agrees with previously reported data that both glomerular and tubular funtions were impaired and that the fractional reabsorption of water and potassium were reduced (Miura *et al.* 1987). Furthermore, a decrease in renal plasma flow was found in cisplatin-treated rats (Fillastre and Raguenez-Viotte 1989).

Another clear difference between the different nephrotoxic compounds chosen in the present study is the time interval between dosage and maximal toxicity, as measured by clinical chemical parameters in urine. In rats treated with TFE-Nac, maximal toxicity was found almost immediately after treatment (12 h). In $HgCl_2$-treated rats a rapid response was found as well, i.e. maximal toxicity was observed 24 h to 36 h after treatment. Cisplatin treatment, however, produced maximal toxicity only 96 h or 108 h after treatment. Possible explanations for this difference in time interval between dosage and toxicity could be the doses of the drug, the

disposition of the drug, a slow uptake in the kidney or glutathione depletion preceding the toxicity (Somani *et al.* 1995) but remain speculative.

Influence of renal damage on urinary biomarker excretion

Urinary parameters are usually corrected for differences in urine volume by expressing them as amount excreted per mol creatinine. However, the excretion of creatinine in the present study is severely reduced by all three nephrotoxins. Therefore, if data were corrected for creatinine levels this would result in an overestimation of the excretion of the urinary parameters. For this reason, only if the increase in excretion of a urinary biomarker expressed per mol creatinine exceeds the decrease in creatinine excretion for that period could this increase be considered meaningful. This was found for the urinary excretion of, for example, glucose, protein and acetone.

Urinary excretion of coproporphyrin III

It has been reported that porphyrinogens are readily oxidized to porphyrins by free radicals (Woods and Calas 1989). Oxidized porphyrins are not further metabolized in mammals *in vivo*, but are excreted unchanged in urine (De Matteis 1988). Recently, Miller and Woods suggested that porphyrins might be potential urinary biomarkers for radical damage in the kidney based on the increased porphyrin excretion in rats treated with cephaloridine and methyl mercury hydroxide. Cephaloridine is thought to cause oxidative stress through redox cycling (Miller and Woods 1993). It has also been shown previously that Hg^{2+}-ions induce porphyrinuria (Woods *et al.* 1990a, b). In the present study, however, no increase in the excretion of coproporphyrin III per 12 h was found in any of the treatments (figure 5(A)). Remarkably, a decrease was found instead. This decrease in coproporphyrin III excretion per 12 h was most pronounced in cisplatin and TFE-Na- treated rats, but was found after treatment with $HgCl_2$ as well. The decrease in coproporhpyrin III was also observed when the excreted amounts were corrected for the urinary creatinine in TFE-Na-treated animals. However, in $HgCl_2$- and cisplatin-treated rats a slight increase was found, although not exceeding the decrease found in creatinine excretion in these periods (figure 5(B)). In another study, in which rats were treated with increasing doses of Fe-NTA, an oxygen radical-inducing nephrotoxic agent, a clear increase in the urinary excretion of coproporphyrin III was found, already before toxicity occurred (Hermanns *et al.*, accepted). The relation of excretion of coproporphyrin III and free radical damage needs to be further established, for example by investigating the effect of antioxidants on both the nephrotoxicity of these compounds and the excretion of coproporphyrin III.

Urinary excretion of lipid peroxides degradation products

The urinary excretion of eight different lipid peroxides degradation products, notably seven aldehydes and acetone, has been measured in this study to further evaluate their utility as a non-invasive indicator of radical-induced renal toxicity. In urine of rats treated with cisplatin, $HgCl_2$ and TFE-Nac, a strong increase in the urinary excretion of acetone was found, which parallelled the increased urinary

excretion in glucose (figures 3 and 9), which might be due to enhanced beta-oxidation, as also occurs in diabetes (Dhanakoti and Draper 1987).

In rats treated with cisplatin no increase was found in the urinary excretion of most of the measured products per 12 h. When expressed per mol creatinine, however, the urinary excretion of hexanal, pentanal, propanal and formaldehyde were found to be increased 10–18-fold when compared with control values (figures 6(B), 7(B) and 8(B)). These increases, however coincided with similar effects of the clinical chemical parameters in urine and did not exceed the decreases in creatinine excretion. Therefore, it was concluded that no effect was found on the urinary excretion of aldehydes in these rats.

The precise role of oxidative stress in cisplatin-induced nephrotoxicity is still controversial (Vermeulen and Baldew 1992). Although cisplatin has been shown to generate oxygen free radicals *in vitro*, it is not known at what stage in the development of the renal toxicity this may occur (Masuda *et al.* 1994). Depletion of glutathione by cisplatin might be a cause of the induced oxidative stress as was found both *in vitro* (Zhang and Lindup 1993) and in the rat *in vivo* (Somani *et al.* 1995) and may occur at a late stage in the development of toxicity. In agreement with the controversial role of free radical damage in cisplatin toxicity, no indication of free radical damage could be detected with the biomarkers used in this study.

Well established and widely utilized as a model of acute renal failure is the administration of mercury-containing compounds such as $HgCl_2$ (Weinberg 1993). The role of oxidative stress in $HgCl_2$-induced nephrotoxicity has been related to a massive generation of H_2O_2 by HgCl2 both *in vitro* and *in vivo* (Lund *et al.* 1993, Nath *et al.* 1996). Despite indications for an oxidative mechanism underlying the renal toxicity of HgCl2, in this study no significant increases in the urinary excretion of aldehydic lipid peroxides degradation products per 12 h could be detected. Instead, even a statistically significant decrease in the excretion of propanal, butanal, pentanal and MDA was found. This suggests that lipid peroxidation may be not a primary cause of the toxicity induced by HgCl2 and HgCl2 may not be able to induce radical formation by itself but only by indirect reactions, such as GSH depletion (Guillermina *et al.* 1989).

TFE-Nac was previously shown to cause nephrotoxicity without any effect on the liver and the mechanism of toxicity of this compound is thought to be covalent binding of the reactive intermediates formed by action of renal ß-lyase (Commandeur *et al.* 1988, 1995). The reactive metabolites formed were shown to inhibit glutathione peroxidase and glutathione reductase thus causing a decrease in the activity of the cellular antioxidant system, eventually leading to free radical damage (Van de Water *et al.* 1996). In the present study the effects on the urinary excretion of formaldehyde and pentanal per mol creatinine were only observed at a late timepoint, 60 h after treatment. Again, these increases did not exceed the decrease observed in the excretion of creatinine, thus no increase in the urinary excretion of aldehydes could be determined. These results are in agreement with earlier studies in which it was shown that free radical damage is only an indirect effect of TFE-Nac (Van de Water *et al.* 1996).

Several differences between the various nephrotoxins have been found, probably due to the different mechanisms and sites of toxicity of these compounds. This could be a result of differences in impaired kidney functions, but also of differences in the metabolism and disposition of the lipid peroxides products. Remarkably, the urinary excretion of MDA, the most used indicator of lipid

peroxidation, was decreased in all three treatments, when expressed per 12 h. This decrease was most pronounced in cisplatin-treated rats and in these rats even a decrease was found in the excretion of MDA expressed per mol creatinine, whereas all other lipid peroxidation products were increased when expressed per mol creatinine. Only in $HgCl_2$-treated animals was a slight increase found in the excretion of MDA per mol creatinine. A reason for this decrease in urinary excretion of MDA might be that MDA is faster metabolized than other aldehydes. It is known that MDA is extensively metabolized *in vivo* (Ekström *et al.* 1988). Another reason might be that the background excretion of MDA is highly due to food intake and the explanation for the decreased excretion is decreased food-intake or decreased excretion due to impaired kidney function.

Conclusions

It can be concluded that in cases of acute severe nephrotoxicity the use of urinary lipid peroxides degradation products as non-invasive biomarkers for free radical damage is complicated, which might, at least partly, be due to the highly impaired kidney functions. No convincing evidence of free radical damage could be found with the biomarkers for free radical damage used in this study. Therefore it may be concluded that there is no major role of free radical damage in the toxicity mechanism of the three nephrotoxic compounds used in the present study.

In order to be able to get a better insight in the relation of the excretion of the investigated biomarkers and the free radical damage of the three used nephrotoxic compounds, experiments with lower doses of these compounds may be useful. Futhermore, it would be very interesting to investigate if antioxidant treatment would reduce the nephrotoxicity as well as reduce the urinary excretion of biomarkers.

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