

Original Article

Early proximal tubule injury in experimental aristolochic acid nephropathy: functional and histological studies

Catherine Lebeau¹, Frédéric D. Debelle^{1,2}, Volker M. Arlt³, Agnieszka Pozdzik¹, Eric G. De Prez¹, David H. Phillips³, Monique M. Deschodt-Lanckman¹, Jean-Louis Vanherweghem² and Joëlle L. Nortier^{1,2}

¹Laboratory for Research on Peptide Metabolism, Faculty of Medicine, ²Department of Nephrology, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium and ³Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton, Surrey, UK

Abstract

Background. Aristolochic acid (AA), the plant extract of *Aristolochia* species, is involved in the onset of progressive tubulointerstitial renal fibrosis in humans. Clinical and *in vitro* findings have previously suggested that the proximal tubule was the target of AA.

Methods. Using a rat model of AA nephropathy, the proximal tubular lesions induced by daily subcutaneous injections of AA for 35 or 5 days were characterized biochemically and histologically. Urinary excretion of proteins, albumin, low molecular weight proteins, *N*-acetyl- β -D-glucosaminidase, α -glutathione *S*-transferase, leucine aminopeptidase and neutral endopeptidase (NEP) was determined and related to histological conventional findings and immunostainings of NEP and megalin.

Results. In both protocols, an acute phase of release of urinary markers was observed within the first 3 days of AA treatment in parallel with a significant increase of specific AA-related DNA adducts reflecting early tubular intoxication. A dramatic loss of the proximal tubule brush border was histologically confirmed, while the expression of megalin decreased at the damaged apical epithelium (mainly of the S3 segment).

Conclusion. Proximal tubule injury occurs early after AA intoxication in rats, with a link between specific AA–DNA adduct formation, decreased megalin expression and inhibition of receptor-mediated endocytosis of low molecular weight proteins, bringing *in vivo* confirmation of previous *in vitro* studies.

Keywords: aristolochic acid nephropathy; low molecular weight proteins; megalin; neutral endopeptidase; proximal tubule injury; tubular proteinuria

Introduction

Chinese-herb nephropathy (CHN), a rapidly progressive tubulointerstitial renal fibrosis associated with a high risk of urothelial cancer, was reported in patients after ingestion of nephrotoxic and carcinogenic aristolochic acid (AA) contained in Chinese herbal remedies [1–3]. Exposure to AA was confirmed by the detection of AA–DNA adducts in kidney tissue samples [3,4].

Pathological examinations of kidneys from human cases showed extensive interstitial fibrosis with atrophy and loss of tubules. These lesions progressed from the superficial to the deep cortex [5]. Structural impairment of the proximal tubule epithelium in CHN patients was attested by a progressive decrease in urinary neutral endopeptidase (neprilysin, NEP) levels, reflecting the loss of the brush border and an abnormal urinary release of the lysosomal enzyme *N*-acetyl- β -D-glucosaminidase (NAG), another marker of tubular damage [6]. These alterations led to a defect of tubular reabsorption of filtered microproteins such as Clara cell protein, retinol-binding protein, β_2 -microglobulin and α_1 -microglobulin, indicating tubular dysfunction [6,7].

Experimentally, the toxicity of AA to the proximal tubule was confirmed by investigation on a well-established opossum kidney (OK) proximal tubule cell line. Following exposure of OK cell monolayers to AA, the reabsorption of albumin and β_2 -microglobulin was

Correspondence and offprint requests to: Catherine Lebeau, Laboratory for Research on Peptide Metabolism, Université Libre de Bruxelles, Campus Erasme CP 603, Route de Lennik 808, B-1070 Brussels, Belgium. Email: clebeau@ulb.ac.be

significantly inhibited and the expression of megalin decreased, demonstrating alterations of the receptor-mediated endocytosis process [8].

An experimental rat model of AA-induced nephropathy has been developed in our laboratory [9,10]. Daily subcutaneous (s.c.) AA injections for 35 days resulted in renal failure, interstitial fibrosis and tubular atrophy. A reduction of urinary excretion of leucine aminopeptidase (LAP) was already observed at day 10, suggesting proximal tubular alterations caused by AA.

In numerous clinical and experimental situations, urinary excretion rates of enzymes and low molecular weight proteins have been studied as early signs of structural and/or functional insult of tubular epithelial cells [11]. Enzymes may be released from different cellular parts of the tubular epithelium: NAG is of lysosomal origin, α -glutathione *S*-transferase (α -GST) is a cytosolic enzyme, and LAP and NEP are present at the apical brush border of proximal tubule. In physiological conditions, low molecular weight proteins and albumin are freely filtered across the glomerular capillary wall and almost completely reabsorbed by the proximal tubular cells. Their excretion level into urine is increased in the case of reduced reabsorption by these cells, which is due to an increased reabsorptive load, or to functional/structural damage. Increased excretion rates of urinary enzymes or proteins are typically observed before elevation of other parameters of renal function, such as plasma creatinine level, and are to be considered as subclinical markers of tubular insult.

According to this background, the experimental observations of AA effects prompted us to investigate in greater detail the early structural and functional impairments of the proximal tubule in the rat model of AA-induced nephropathy.

Subjects and methods

Experimental protocols

Long-term protocols (35 days). In a first series of experiments, 72 male Wistar rats, 4 weeks of age (Elevage Janvier, Le Genest Saint-Isle, France), were housed under a controlled temperature, humidity and light environment in the animal care facility of the Faculty of Medicine, Université Libre de Bruxelles (Brussels, Belgium). Animals were allowed free access to food and water throughout the experiment.

After 1 week acclimatization (at day 0), weight-matched rats were randomly assigned to two groups. One group ($n = 36$) received s.c. injections of AA (10 mg/kg body weight) (mixture containing 40% AAI and 60% AAI, Acros Organics Co., Geel, Belgium) daily for 35 days. AA was dissolved in polyethylene glycol (PEG) 400 (Fluka Chemie, Buchs, Switzerland) to a final concentration of 10 mg/ml and diluted in distilled water before injection. The control group ($n = 36$) was treated with a v/v solution of PEG 400 and distilled water only. Body weights were measured weekly for adjustment of drug dosages.

Six rats per group were anaesthetized on days 3, 7, 10, 14, 18 and 35 with an intraperitoneal (i.p.) injection of a mixture

containing ketamine (Imalgen, LEO Pharma Belgium, Wilrijk, Belgium) and xylazine (Rompun, Bayer, Brussels, Belgium). Rats were dissected to collect blood by intracardiac puncture with a syringe containing potassium-EDTA, and the kidneys were excised and immediately fixed. Before sacrifice, rats were placed in rodent metabolic cages for urine collection.

A second series of experiments performed as detailed above included the follow-up of urinary parameters during 35 days. Briefly, four male Wistar rats were injected daily with AA mixture (10 mg/kg body weight) and three control rats with PEG solution for 35 days. Urine collection was performed on days 0, 3, 5, 7, 10, 13, 15, 18, 22, 24, 27, 31 and 35 for each rat.

Short-term protocol (5 days). With respect to the same experimental conditions as described above, 60 male Wistar rats were divided into two groups. One group ($n = 30$) was injected daily with AA mixture (10 mg/kg body weight) and another group (control, $n = 30$) with PEG solution for up to 5 days. Six rats per group were anaesthetized on days 1, 2, 3, 4 and 5 and dissected to collect blood from the heart, and the kidneys were excised. Before sacrifice, urine was collected at days 0, 1, 2, 3, 4 and 5.

All protocols were approved by the local Ethical Committee for Animal Care (Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium).

Urine, blood and kidney samples

After collection, urine samples were stored at -20°C . Blood samples were centrifugated at 1600 *g* for 15 min at 4°C and the plasma was stored at -20°C until assayed.

Immediately after bilateral nephrectomy, half of a kidney was fixed in 4% buffered formaldehyde for histological analyses of paraffin-embedded sections. Before freezing kidney tissues for cryostat sections, the remaining half of a kidney was fixed in 4% phosphate-buffered paraformaldehyde and then cryoprotected by successive immersion in 10, 20 and 30% sucrose.

Renal cortex was dissected, frozen in liquid nitrogen and stored at -80°C for further detection of specific AA-DNA adducts.

Plasma and urinary creatinine assays

Plasma creatinine levels were measured by a sensitive accurate high-performance liquid chromatography method adapted from Xue *et al.* as described previously [9] and expressed in $\mu\text{mol/l}$.

Urinary creatinine values were determined by a pseudo-kinetic Jaffé's method using a Creatinine Diagnostic Kit (Sigma-Aldrich, Bornem, Belgium) and expressed in mmol/l.

Urinary excretion of proteins and enzymes

Total proteinuria was quantified by the Bradford binding assay as detailed previously [9]. Briefly, Bradford reagent was added to standards or urine samples. The optical density was measured at 620 nm and reported to a bovine serum albumin standard curve (BSA; Sigma-Aldrich, Bornem, Belgium), and expressed in mg/mmol creatinine.

Urinary excretion of LAP was measured by a spectrofluorimetric assay as detailed previously [9] and expressed

Early proximal tubule impairment in aristolochic acid nephropathy in $\mu\text{mol AMC}$ (7-amino-4-methyl-coumarin) produced/mmol creatinine/h.

Urinary excretion of NEP was determined by a spectrofluorimetric assay as detailed previously [6] and expressed in $\mu\text{g}/\text{mmol creatinine}$.

Urinary excretion of lysosomal NAG was measured by a colorimetric assay following the manufacturer's protocol (Roche Diagnostics Belgium, Brussels, Belgium) and expressed in $\text{mU}/\text{mmol creatinine}$.

Urinary excretion of cytosolic α -GST was measured by an enzyme immunoassay following the protocol of the manufacturer (Biotrin, Herman Biognostics, Wommel, Belgium) and expressed in $\mu\text{g}/\text{mmol creatinine}$.

Electrophoresis of urinary proteins

Urinary proteins were precipitated with v/v 10% trichloroacetic acid solution, centrifuged at 4000 g for 10 min, washed with 100% ethanol and dried in a Speed-Vac evaporator. The pellets were resuspended in Laemmli sample buffer (31.2 mM Tris-HCl, 5% glycerol, 1% SDS, 0.5% β -mercaptoethanol, bromophenol blue). Protein samples (20 μg) were heated at 95°C for 2 min and separated by ready gel Tris-HCl 4–20% (Bio-Rad Laboratories, Nazareth-Eke, Belgium). The gels were coloured with Coomassie blue solution (30% methanol, 10% acetic acid, 60% H₂O, 0.1% Coomassie blue), then incubated with a decoloration solution (40% methanol, 10% acetic acid, 50% H₂O, 0.5% glycerol) and dried.

The relative urinary contents of low molecular weight proteins (LMWPs) were evaluated semi-quantitatively by scanning (CanoScan N676U, Canon Inc., Tokyo, Japan) followed by analysis of the electrophoretic gels with a computer-assisted image analyser system (Scion Image version b4.0.2, Scion Corporation, MA). The quantity of LMWPs was reported relative to total proteins levels and expressed in $\text{mg}/\text{mmol creatinine}$.

Albumin assay

Urinary and plasma albumin were measured by using the rat albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, IMTEC Diagnostics, Antwerpen, Belgium). Briefly, plates were coated with a capture rabbit anti-rat albumin affinity-purified antibody (1:100). After blocking, standards, urine or plasma samples (dilution $\times 1000$ or $\times 2 \times 10^6$, respectively) were added, then detected by a rabbit anti-rat albumin-horseradish peroxidase (HRP)-conjugated antibody (1:50 000) and revealed by the enzyme substrate 3,3',5,5'-tetramethylbenzidine, which reacted with peroxidase. The reaction was stopped with sulfuric acid (1 N) and the resulting yellow end-product was read at 450 nm. The data were related to a standard rat albumin curve and expressed in $\text{mg}/\text{mmol creatinine}$.

Histological studies

Conventional microscopy. For brush border integrity studies, fixed kidney tissues embedded in paraffin were cut at 5 μm and stained with periodic acid-Schiff (PAS). Briefly, tissue sections were dewaxed, rehydrated, incubated for

10 min with 1% periodic acid, washed and incubated for 15 min with Schiff reagent. After washing, sections were counterstained with Mayer's haematoxylin.

Neutral endopeptidase immunostaining. Immunostaining of the brush border NEP (neprilysin) was performed on paraffin-embedded kidney sections. After dewaxing and hydration, endogenous peroxidase activity was inhibited by incubation of tissue sections in methanol containing 0.3% H₂O₂ for 30 min. Sections were blocked for 20 min with goat normal blocking serum (Vectastain Elite[®] ABC kit Rabbit IgG, Vector Laboratories, Labconsult, Brussels, Belgium), then with avidin D solution and with biotin solution (Avidin/biotin blocking kit, Vector Laboratories). After overnight incubation at room temperature with the primary rabbit anti-neprilysin polyclonal antibody (1:2000; Chemicon International, Biognost, Heule, Belgium), sections were treated for 30 min with biotinylated secondary goat anti-rabbit IgG antibody. Then, after 30 min incubation with avidin and biotinylated HRP complex, staining was revealed by incubation in 3,3'-diaminobenzidine (DAB) solution (DAB substrate kit for peroxidase, Vector Laboratories). Finally, sections were counterstained with Mayer's haematoxylin.

Semi-quantitation of NEP immunostaining was performed by two independent observers (C.L. and A.P.) counting the NEP-positive proximal tubules by field at 200 \times magnification. Ten fields were examined per kidney section, corresponding to a mean population of ~ 85 proximal tubules per field. The final result for each time point was expressed as a percentage of total proximal tubules.

Megalin immunostaining. Immunostaining of megalin was performed on kidney cryostat sections of 8 μm . After incubation in Tris-buffered (10 mM, pH 7.6) saline (0.9%), the endogenous peroxidase activity was inhibited by incubation of tissue sections in methanol containing 0.3% H₂O₂ for 30 min. Sections were blocked for 20 min with horse normal blocking serum (Vectastain Elite[®] ABC kit Mouse, Vector Laboratories), then with avidin D solution and with biotin solution. After overnight incubation at room temperature with the primary mouse monoclonal anti-megalin antibody (1:1000; kindly provided by Dr V. Marshansky, Program in Membrane Biology and Renal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA), sections were treated for 30 min with biotinylated secondary horse anti-mouse antibody. Then, after 30 min incubation with avidin and biotinylated HRP complex, staining was revealed by incubation in DAB solution. Finally, sections were counterstained with Mayer's haematoxylin.

DNA adduct analysis from renal cortex of AA-treated rats

DNA isolation from kidney samples was performed by a standard phenol extraction method. ³²P-Post-labelling analysis using nuclease P1 enrichment, chromatography on polyethyleneimine-cellulose thin-layer plates (Machery and Nagel, Düren, Germany), autoradiography using a Packard Instant Imager (Canberra Co., Dowers Grove, IL) and quantitation were performed as described previously [12]. Results were expressed as DNA adducts per 10⁸ normal nucleotides.

Table 1. Biological parameters for AA-treated rats and controls

Measurements	Groups	Day 3	Day 7	Day 10	Day 14	Day 18	Day 35
Plasma creatinine ($\mu\text{mol/l}$)	AA	14.6 \pm 0.3	17.5 \pm 0.8	19.9 \pm 1.0	21.5 \pm 0.6	19.7 \pm 0.7	31.1 \pm 3.5*
	Control	15.8 \pm 1.1	18.3 \pm 1.1	17.4 \pm 0.7	22.5 \pm 2.6	20.2 \pm 2.0	22.0 \pm 1.3
Total urinary protein (mg/mmol creatinine)	AA	91.3 \pm 9.3	119 \pm 6.4**	224 \pm 9.8**	318 \pm 40.9**	204 \pm 15.8**	359 \pm 22.2**
	Control	73.2 \pm 6.4	78.0 \pm 11.1	107 \pm 9.5	155 \pm 10.4	122 \pm 11.0	147 \pm 8.1
Urinary albumin (mg/mmol creatinine)	AA	20.4 (4.1–45.5)**	4.6 (2.7–28.6)**	8.1 (4.3–57.3)	7.9 (2.3–164)	6.9 (4.7–31.3)*	74.6 (19.3–156)**
	Control	3.7 (2.9–7.2)	2.3 (1.7–2.6)	4.7 (2.2–6.7)	3.3 (2.3–5.1)	3.2 (1.7–8.1)	3.2 (2.1–5.0)
Urinary LMWPs (mg/mmol creatinine)	AA	64.8 (31.8–75.6)	96.7 (82.4–103)	185 (163–213)**	258 (160–298)**	185 (137–225)**	238 (222–312)**
	Control	54.9 (44.3–73.4)	64.1 (40.6–106)	86.4 (67.6–140)	143 (122–187)	115 (70.1–141)	138 (117–179)
Urinary NAG (mU/mmol creatinine)	AA	413 (214–616)	753 (124–986)	1077 (384–2116)**	1116 (511–2834)**	758 (470–1321)*	2035 (1275–3677)**
	Control	177 (34–525)	257 (155–414)	188 (172–552)	287 (82–410)	320 (152–752)	366 (245–506)

Results are presented as the mean \pm SEM for parameters normally distributed (plasma creatinine and urinary proteins), and as median (minimum–maximum) for other markers ($n=6$). Significant levels are * $P<0.05$ and ** $P<0.01$ as compared with control values. AA = aristolochic acid; LMWPs = low molecular weight proteins; NAG = *N*-acetyl- β -D-glucosaminidase.

Statistical analysis

Data are presented as mean \pm SEM. Comparisons between controls and AA-treated rats for each time point and between AA-treated rats from consecutive days were performed by the non-parametric Mann–Whitney test. Correlations between structural and functional markers were evaluated by the Spearman non-parametric test. Statistical significance was set at $P<0.05$.

Results

Biological markers of proximal tubule injury

Long-term protocols. In rats injected with vehicle only (PEG 400), no alteration of biochemical parameters was detected. In rats treated with AA for 35 days, plasma creatinine was increased significantly on day 35 relative to controls (Table 1).

Proteinuria increased significantly from day 7 to day 35. Albuminuria was enhanced significantly as early as day 3 and remained high. Plasma levels of albumin remained normal during the whole observation period (data not shown). Semi-quantitative analyses of urinary proteins by electrophoresis showed a significant release of LMWPs, with doubling values at day 10 and progressive increase from day 10 to day 35. In parallel, NAG enzymuria increased by day 3, reaching a statistically significant level from day 10 to day 35 (Table 1).

Using individual biochemical data for each time-point, relationships between structural and functional impairment of the proximal tubule were found: NAG

enzymuria correlated tightly with proteinuria ($r=0.80$, $n=36$, $P<0.0001$), with albuminuria ($r=0.54$, $n=36$, $P=0.0007$) and with plasma creatinine levels ($r=0.65$, $n=36$, $P<0.0001$).

Bearing these data in mind, it was interesting to perform sequential urinary measurements. Urinary parameters of three control rats and four AA-treated rats were analysed after 0, 3, 5, 7, 10, 13, 15, 18, 22, 24, 27, 31 and 35 days. Total urinary protein, urinary albumin, NAG, α -GST, LAP and NEP were not different from basal values during 35 days in control rats (data not shown). For all the four AA-treated rats, a rapid and transient increase of proteinuria and albuminuria was detected around day 5 (from day 3 to day 7) and a second one around day 27 (from day 25 to day 30) (Figure 1A). In parallel, urinary NAG and α -GST increased dramatically during the first days of AA intoxication, with a maximum at days 5 or 7, then decreased and finally increased to a lesser extent during the last days (Figure 1B). A similar pattern of excretion was observed for urinary LAP and NEP, two membrane-bound brush border enzymes, with the earliest release at days 3 and 7 (Figure 1C).

Short-term protocol. Considering early abnormalities in urinary markers, a short-term protocol was performed to relate biochemical measurements to histological findings.

Total proteinuria increased significantly in AA-treated rats relative to controls as early as day 1, and extended to day 5 (Figure 2A). Albuminuria significantly increased from day 2 (73.2 \pm 43.3 vs 3.6 \pm 1.0 mg/mmol creatinine; $P<0.01$) to day 5

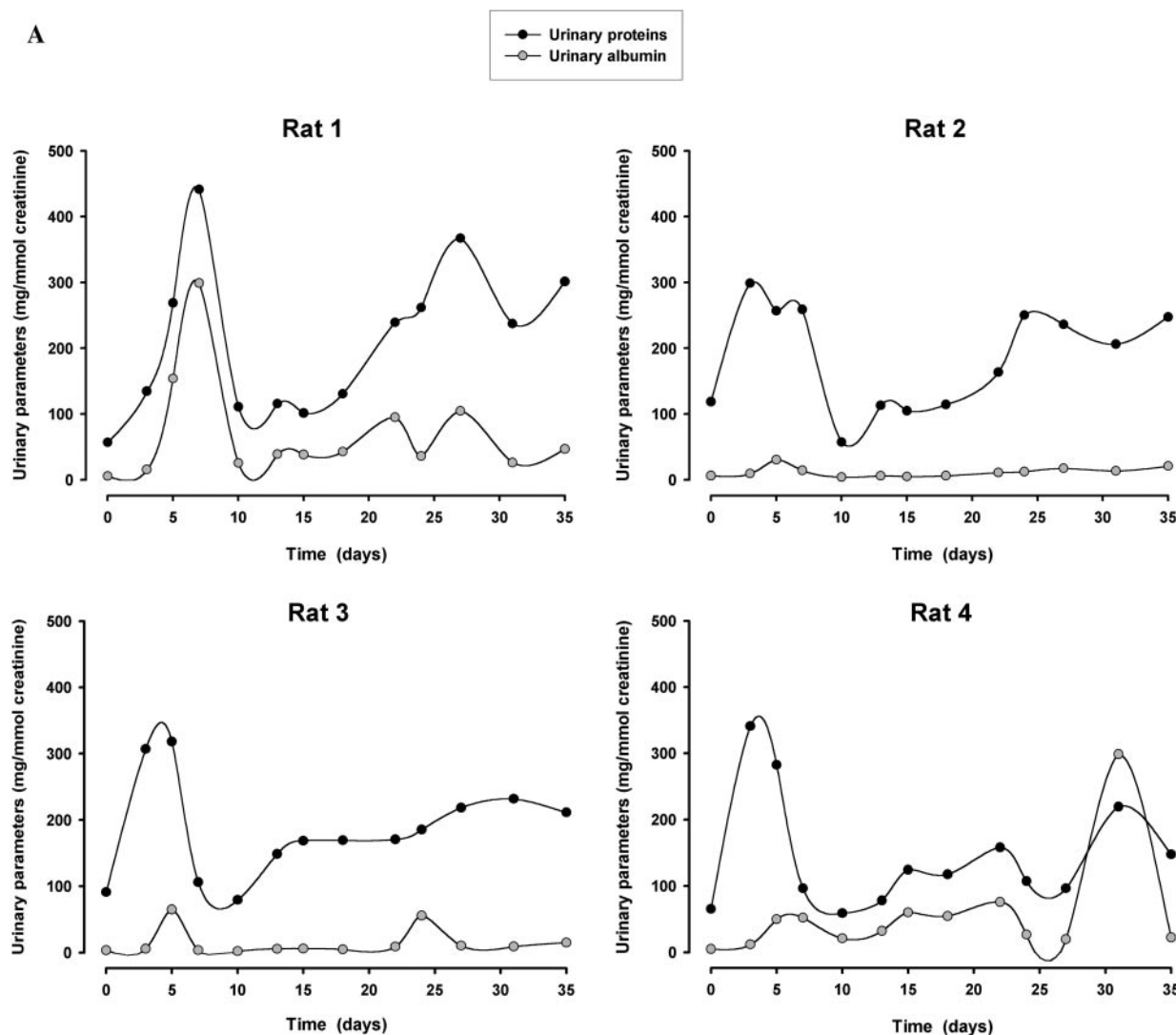


Fig. 1. Time course of urinary parameters from four AA-treated rats ($n=4$). (A) Total proteinuria (black circles) and albuminuria (grey circles). (B) *N*-acetyl- β -D-glucosaminidase (NAG; black squares), α -glutathione S-transferase (α -GST; grey squares). (C) Leucine aminopeptidase (LAP; black triangles) and neutral endopeptidase (NEP; grey triangles). AMC = 7-amino-4-methyl-coumarin.

(213.5 ± 46.6 vs 3.0 ± 0.3 mg/mmol creatinine; $P < 0.01$). Plasma albumin levels did not change with time (data not shown). In parallel with total proteinuria, NAG enzymuria increased significantly from day 2 to day 5 (Figure 2B). The time course of NAG enzymuria followed the progressive increase of plasma creatinine, reflecting acute renal failure (Figure 2C).

Structure–function correlations were found: NAG enzymuria was correlated closely with proteinuria ($r=0.71$, $n=30$, $P < 0.0001$), with albuminuria ($r=0.81$, $n=30$, $P < 0.0001$) and with plasma creatinine ($r=0.50$, $n=30$, $P=0.0044$).

Detection of AA–DNA adducts

Specific AA–DNA adducts were detected only in renal tissue samples from AA-treated rats for each day using the short-term protocol. As described previously, the typical AA–DNA adduct pattern on thin-layer chromatography plates consisted of three major

adduct spots: 7-(deoxyadenosin- N^6 -yl)-aristolactam I (dA-AAI, spot 1), 7-(deoxyguanosin- N^2 -yl)-aristolactam I (dG-AAI, spot 2) and 7-(deoxyadenosin- N^6 -yl)-aristolactam II (dA-AAII, spot 3) [12].

Quantitative analysis of these adducts revealed an almost linear increase in DNA binding for each of the individual AA–DNA adducts during the first 2 days, reaching a steady-state level after that time (Figure 3). The long-term protocol showed that the steady-state level persisted to day 35 (total AA–DNA binding at day 35: 1049 ± 377 adducts per 10^8 nucleotides, with mean values of individual AA–DNA adducts of 280, 405 and 365 adducts per 10^8 nucleotides for spots 1, 2 and 3, respectively).

Histological findings

Conventional microscopy. In control rats injected with PEG 400 solution only, renal tissue samples were

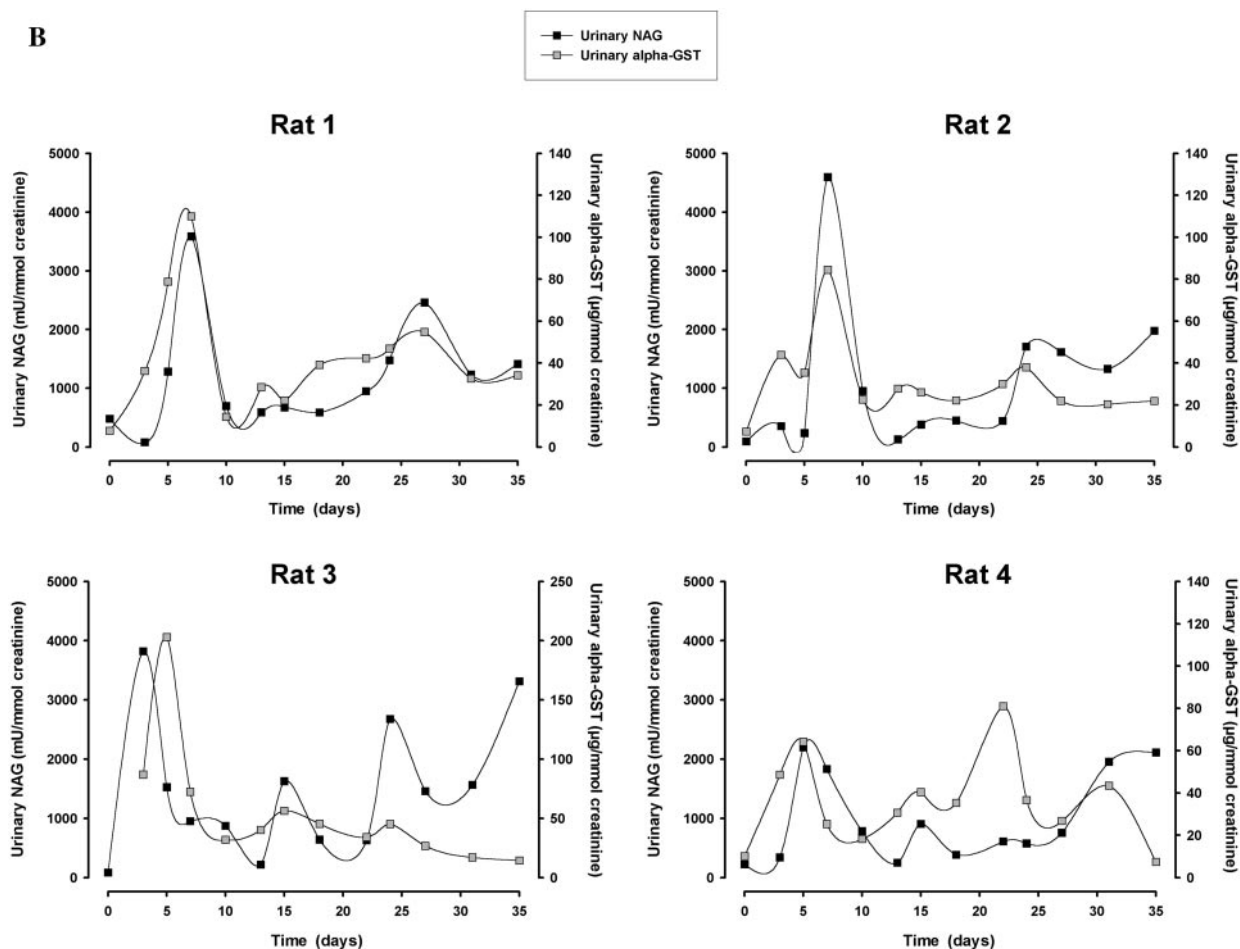


Fig. 1. Continued.

histologically normal. As early as day 1, PAS staining revealed swelling of tubular cells compared with controls (Figure 4A and B). Progressive loss of tubular brush border was observed from day 2 to day 5 (Figure 4C–F). In the lumen of these proximal tubules, necrotic cells and cellular fragments were already found on day 2 (Figure 4C), becoming massive on day 5 (Figure 4F). These morphological alterations were observed in the outer stripe of the outer medulla and in the medullary rays. No glomerular lesions were found.

Neutral endopeptidase immunostaining. To assess further these observations of brush border loss, immunostaining of NEP was performed and evaluated semi-quantitatively (Figure 5). As shown in Figure 5A, NEP staining clearly delineated the apical brush border of proximal tubules from control rats. This staining was already reduced on day 2 in AA-treated rats (Figure 5B). The proportion of NEP-positive proximal tubules decreased significantly from day 2 to day 5 relative to controls (Figure 5C). At day 35, almost all proximal tubules had lost their apical membrane epithelium, as reflected by the disappearance of NEP immunostaining (Figure 5D and E).

Megalin immunostaining. To investigate functional impairment of proximal tubules, immunostaining of megalin, involved in the receptor-mediated endocytosis process, was performed. Megalin staining was observed at the brush border of proximal tubules in control rats and was more pronounced for proximal tubules from S1 and S2 segments than from the S3 segment (Figure 6A and B). Megalin staining partially or totally disappeared in tubules from the S3 segment from day 2 to 4 (Figure 6C and D) and remained detectable in the proximal tubule population from the S1 and S2 segments.

Discussion

Experimental AA nephrotoxicity was previously induced both orally and intravenously. After a single dose of AA (orally 120–300 mg/kg and intravenously 38–110 mg/kg), severe acute tubular necrosis was observed within a few days [13]. Rats treated by AA (orally 10–100 mg/kg) exhibited within 3 days increased levels of plasma creatinine, urinary protein and NAG enzymuria [14]. Interstitial fibrosis and chronic renal failure were induced in female

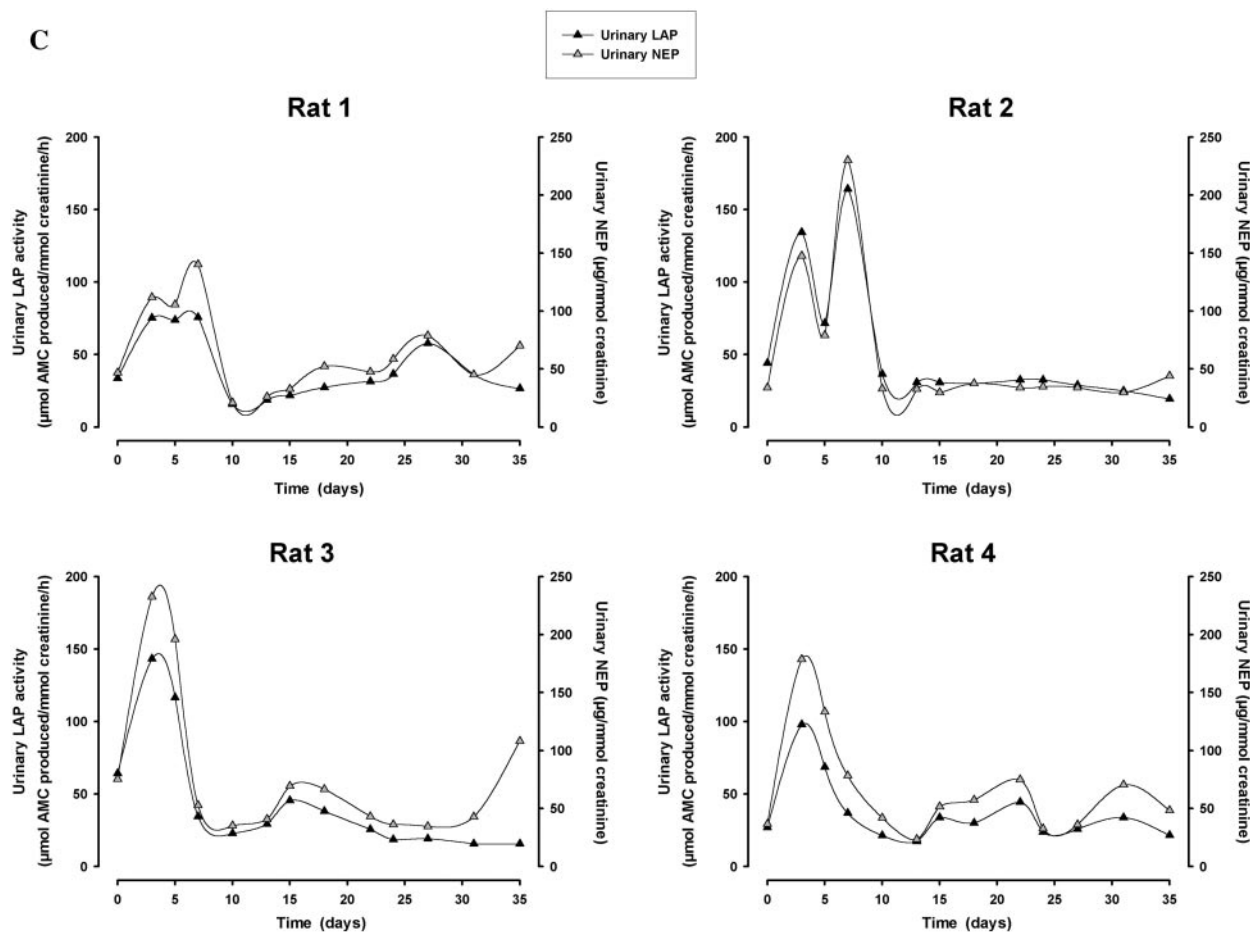


Fig. 1. Continued.

New Zealand White rabbits by i.p. AA injections (0.1 mg/kg, 5 days/week, for 17–21 months) [15]. Our group reproduced similar renal insults by s.c. AA injections (10 mg/kg, for 35 days) in Wistar rats that were subjected to either a salt-depleted or a normal diet [9,10]. In view of signs of proximal tubular damage in experimental models, the authors suggested that interstitial renal fibrosis had been preceded by an acute tubular dysfunction [9,15].

The relevance of the animal model as well as the dose of AA used have already been described in the initial experimental study [9]. The choice of AA dose was based on our clinical experience in CHN patients [1–3]. The total amount of AA ingested by some patients was 10 mg/kg body weight, which represents a daily dose of 0.29 mg AA/kg body weight for 35 days. The dose of AA used in the experimental model was then 30 times higher than that for CHN patients. The impact of the dose seems to be crucial to develop nephrotoxicity, as we previously showed that 1 mg/kg body weight of AA instead of 10 mg/kg body weight injected into rats did not result in the onset of renal failure and interstitial fibrosis [9].

At the time of the set-up of the rat model of AA nephropathy [9], preliminary experiments had already

shown progressive increase of urinary parameters as well as plasma creatinine in control rats. This observation could be related to the growth of the animals rather than to a possible toxicity of the solution used to dissolve AA. Indeed, rats injected with water or PEG solution did not demonstrate any alteration of biochemical parameters and renal tissue samples were histologically normal.

The present study was thus carried out to characterize in the rat model of AA nephropathy early proximal tubular injury induced by AA intoxication, in particular the respective structural and functional impairments. We demonstrated a biphasic evolution of renal function and morphological alterations. Early proximal tubule dysfunction and structural abnormalities occurred within the first 3 days, reflecting an acute phase of proximal tubular injury. Indeed, biochemical measurements showed that tubular proteinuria, NAG, α -GST, LAP and NEP enzymuria, already increased within the first 3 days of AA intoxication, returned to basal levels, and finally were enhanced again, indicating that tubular impairment occurred in two phases and presented an acute phase during the first days of AA treatment. Differences in the design of the two long-term protocols should be

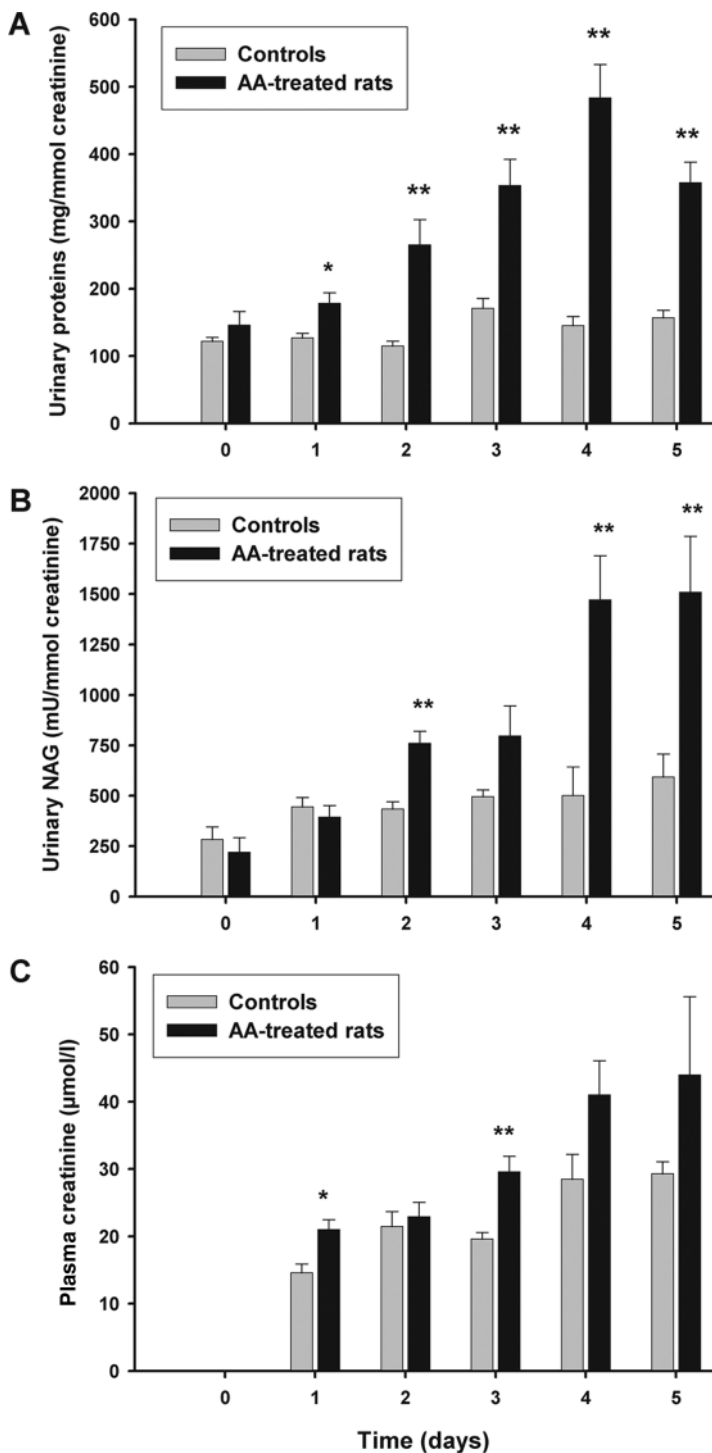


Fig. 2. Total proteinuria (A), *N*-acetyl- β -D-glucosaminidase (NAG) enzymuria (B) and plasma creatinine (C) from control (grey columns) and AA-treated rats (black columns) from day 0 to 5. Results are presented as the mean \pm SEM ($n=6$, except for urinary measurement on day 3 with $n=5$). Significant levels are * $P < 0.05$, ** $P < 0.01$ as compared with control values.

considered to explain the absence of biphasic evolution in this first long-term protocol. In the first series of experiments, urine samples were collected at fixed time points before the sacrifice of the animals (days 3, 7, 10, 14, 18 and 35). In contrast, in the second series of experiments, individual urine collections were analysed more regularly all throughout the protocol

(days 0, 3, 5, 7, 10, 13, 15, 18, 22, 24, 27, 31 and 35). In addition to the heterogeneity between samples from different animals from one time point to another, the probability of missing the peaks of urinary enzymes and proteins is far greater in the fixed time points' long-term protocol than in the longitudinal follow-up.

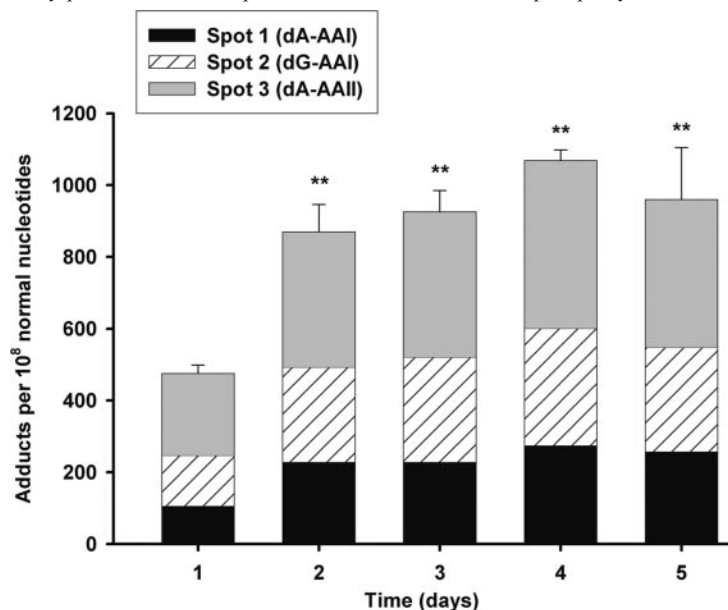


Fig. 3. Time-dependent formation of DNA adducts in the renal tissue of AA-treated rats from day 1 to day 5. Results are presented as the mean \pm SEM ($n=6$); each DNA sample was post-labelled once. Spot 1, 7-(deoxyadenosin- N^6 -yl)-aristolactam I (dA-AAI: black column); spot 2, 7-(deoxyguanosin- N^2 -yl)-aristolactam I (dG-AAI: hatched column); and spot 3, 7-(deoxyadenosin- N^6 -yl)-aristolactam II (dA-AAII: grey column). Significant levels are $**P < 0.01$ as compared with values at day 1.

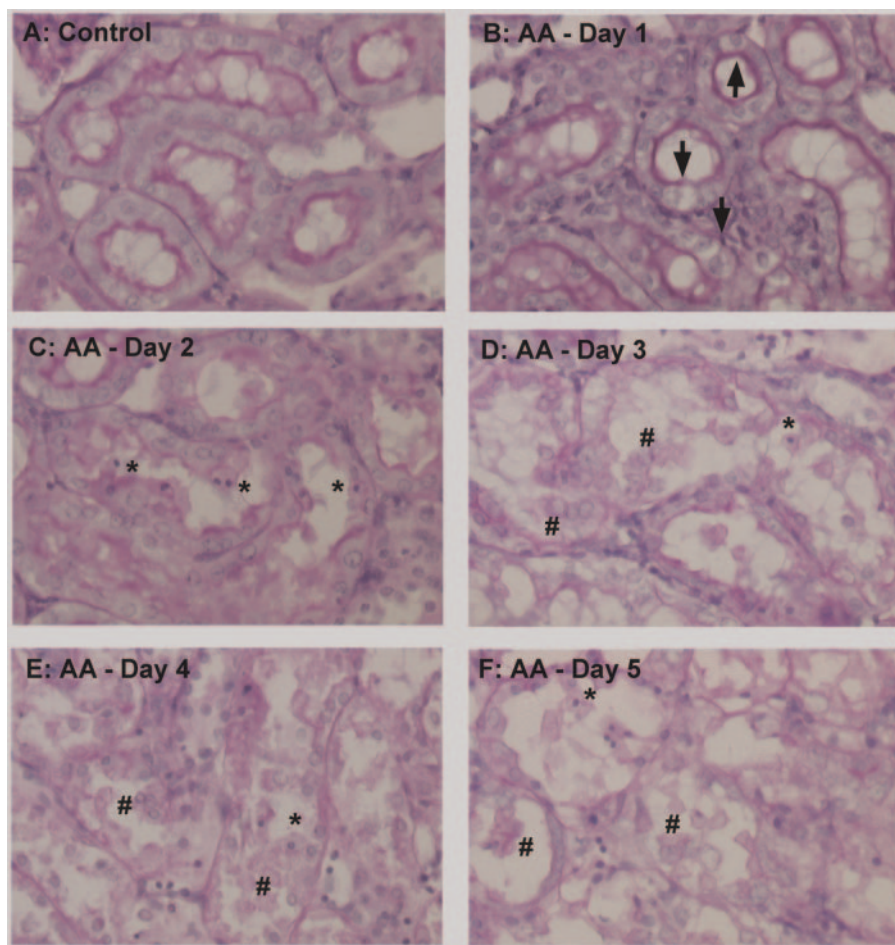


Fig. 4. PAS-stained proximal tubules of control rats (A) and AA-treated rats after 1–5 days of intoxication (B–F). Note the tubular cell swelling (B, arrow), the irregularity of the brush border and necrotic cells (*) with cellular fragments (#) in the lumen (D–F). Magnification $\times 400$.

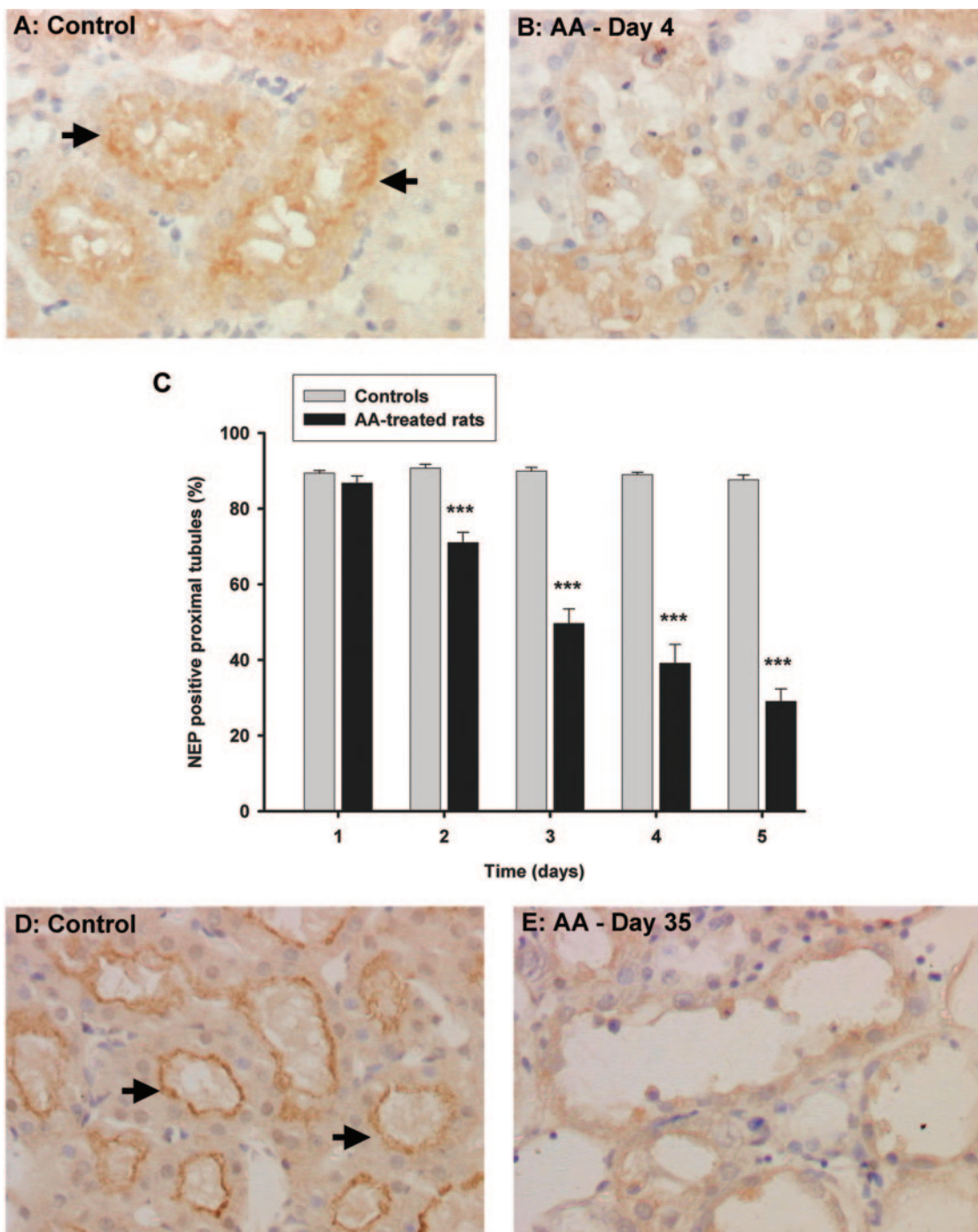


Fig. 5. (A) Typical neutral endopeptidase (NEP) staining at the apical brush border of tubular epithelium in a control rat (arrow). (B) Partial disappearance of NEP expression after 4 days of AA exposure. (C) Semi-quantitative analysis of NEP immunostaining for control (grey columns) and AA-treated rats (black columns) on days 1, 2, 3, 4 and 5. Results are presented as the mean \pm SEM ($n=6$). Significant levels are *** $P < 0.0001$ as compared with control values. (D) Typical NEP staining in a control rat at day 35 (arrow). (E) Total loss of NEP expression after 35 days of AA treatment. Magnification $\times 400$.

In a recent study in rats, acute renal failure was noticed after 5 days of AA treatment (4 mg/day) but recovery of renal function was observed by day 14 after withdrawal of AA [16]. Signs of regeneration of tubular epithelium were present but tubular dilatations still remained severe. In another study using FVB mice, daily i.p. AA injections (5 mg/kg body weight) for

2 weeks induced tubular degeneration and renal failure. Regeneration of tubular epithelium with moderate peritubular fibrosis was seen 4 weeks after cessation of AA treatment [17]. In the present model of AA intoxication, the progression to chronic renal failure and interstitial fibrosis after an early acute phase can probably be related to repeated s.c. AA injections to

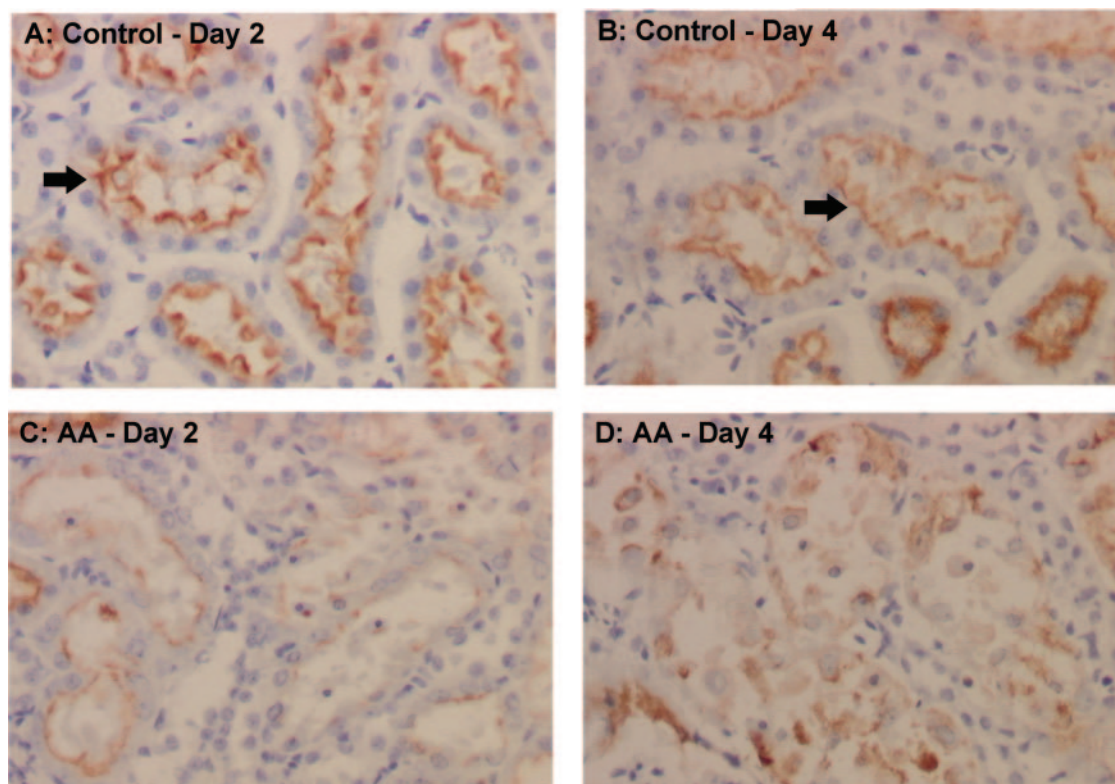


Fig. 6. Megalin immunostaining at the apical membrane of proximal tubules in control rats (arrow) (A and B). Progressive loss of megalin expression in AA-treated rats after 2 and 4 days of intoxication (C and D). Magnification $\times 400$.

rats for 35 days. Interestingly, similar observations of acute tubular necrosis with consecutive renal failure were reported recently in a patient after ingestion of AA for 10 days [18].

It should be noted that early signs of impairment can be related to a rapid intense intoxication of the cells. Indeed, AA-related DNA adducts were detected as early as day 1 and reached a steady-state level at day 2, which remained almost constant up to day 35, as previously observed [12]. In these first days of intoxication, a significant increase of plasma creatinine was observed, suggesting early renal dysfunction.

From a histological point of view, structural lesions of proximal tubule epithelium associated with acute renal failure are known to be of ischaemic or toxic origin. Epithelial cells of the proximal tubule S3 segment typically show early morphological changes, including the formation of blebs in the apical membrane, with loss of brush border [19]. Our histological observations are in agreement with these descriptions: cell swelling after the first day of AA injection followed by necrosis 3 days later, progressive loss of tubular brush border from the second day, and reduction of NEP immunostaining along the S3 segment of the proximal tubule. This enzyme is located in the pars recta of that proximal tubule [20]. Furthermore, the expression of megalin, a receptor involved in receptor-mediated endocytosis, was also reduced early (on day 2) at the apical epithelium of this segment. However, the progression of the tubular atrophy

process was so severe that megalin immunostaining studies were not justified beyond day 10.

Interestingly, the early reduction of megalin expression paralleled previous *in vitro* observations in OK cells: after 24 h intoxication with AA, as reflected by the presence of specific AA-related DNA adducts, the receptor-mediated endocytosis process of LMWP reabsorption was inhibited and the expression of megalin significantly decreased [8].

In conclusion, structural and functional impairments of the S3 segment proximal tubules occur rapidly in experimental AA nephropathy. These data thus confirm *in vivo* that AA administration induces a very early proximal tubular dysfunction. They might explain the Fanconi syndrome described in some forms of human AA intoxication [21]. A more pronounced intoxication leads, on the other hand, to severe tubular atrophy and interstitial fibrosis.

Acknowledgements. C.L. is a research fellow of Université Libre de Bruxelles (Brussels, Belgium). This study was supported by grants from the Fonds de la Recherche Scientifique Médicale and the Banque Nationale, Brussels, Belgium, and by Cancer Research, UK. We thank Dr I. Salmon and Professor J.-P. Brion as well as Mrs N. Vanmuylder for their helpful scientific advice. The excellent technical assistance of Mrs C. Husson, Mrs M. Authélet and Mr T. Gras is highly appreciated. This work was presented in part in November 12–17, 2003, at the American Society of Nephrology 36th annual meeting, San Diego, California, USA.

Conflict of interest statement. None declared.

References

1. Vanherweghem JL, Depierreux M, Tielemans C *et al.* Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *Lancet* 1993; 341: 387–391
2. Vanhaelen M, Vanhaelen-Fastre R, But P, Vanherweghem JL. Identification of aristolochic acid in Chinese herbs. *Lancet* 1994; 343: 174
3. Nortier JL, Martinez MC, Schmeiser HH *et al.* Urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). *N Engl J Med* 2000; 342: 1686–1692
4. Schmeiser HH, Bieler CA, Wiessler M, van Ypersele de Strihou C, Cosyns JP. Detection of DNA adducts formed by aristolochic acid in renal tissue from patients with Chinese herbs nephropathy. *Cancer Res* 1996; 56: 2025–2028
5. Depierreux M, Van Damme B, Vanden Houte K, Vanherweghem JL. Pathologic aspects of a newly described nephropathy related to the prolonged use of Chinese herbs. *Am J Kidney Dis* 1994; 24: 172–180
6. Nortier JL, Deschodt-Lanckman MM, Simon S *et al.* Proximal tubular injury in Chinese herbs nephropathy: monitoring by neutral endopeptidase enzymuria. *Kidney Int* 1997; 51: 288–293
7. Kabanda A, Jadoul M, Lauwerys R, Bernard A, van Ypersele de Strihou C. Low molecular weight proteinuria in Chinese herbs nephropathy. *Kidney Int* 1995; 48: 1571–1576
8. Lebeau C, Arlt VM, Schmeiser HH *et al.* Aristolochic acid impedes endocytosis and induces DNA adducts in proximal tubule cells. *Kidney Int* 2001; 60: 1332–1342
9. Debelle FD, Nortier JL, De Prez EG *et al.* Aristolochic acids induce chronic renal failure with interstitial fibrosis in salt-depleted rats. *J Am Soc Nephrol* 2002; 13: 431–436
10. Debelle FD, Nortier JL, Husson CP *et al.* The renin–angiotensin system blockade does not prevent renal interstitial fibrosis induced by aristolochic acids. *Kidney Int* 2004; 66: 1815–1825
11. D’Amico G, Bazzi C. Urinary protein and enzyme excretion as markers of tubular damage. *Curr Opin Nephrol Hypertens* 2003; 12: 639–643
12. Debelle F, Nortier J, Arlt VM *et al.* Effects of dexfenfluramine on aristolochic acid nephrotoxicity in a rat model for Chinese-herb nephropathy. *Arch Toxicol* 2003; 77: 218–226
13. Mengers U. Acute toxicity of aristolochic acid in rodents. *Arch Toxicol* 1987; 59: 328–331
14. Mengers U, Stotzem CD. Renal toxicity of aristolochic acid in rats as an example of nephrotoxicity testing in routine toxicology. *Arch Toxicol* 1993; 67: 307–311
15. Cosyns JP, Dehoux JP, Guiot Y *et al.* Chronic aristolochic acid toxicity in rabbits: a model of Chinese herbs nephropathy? *Kidney Int* 2001; 59: 2164–2173
16. Liu MC, Maruyama S, Mizuno M *et al.* The nephrotoxicity of *Aristolochia manshuriensis* in rats is attributable to its aristolochic acids. *Clin Exp Nephrol* 2003; 7: 186–194
17. Okada H, Watanabe Y, Inoue T *et al.* Transgene-derived hepatocyte growth factor attenuates reactive renal fibrosis in aristolochic acid nephrotoxicity. *Nephrol Dial Transplant* 2003; 18: 2515–2523
18. Lo SH, Mo KL, Wong KS *et al.* Aristolochic acid nephropathy complicating a patient with focal segmental glomerulosclerosis. *Nephrol Dial Transplant* 2004; 19: 1913–1915
19. Bonventre JV, Weinberg JM. Recent advances in the pathophysiology of ischemic acute renal failure. *J Am Soc Nephrol* 2003; 14: 2199–2210
20. Ronco P, Pollard H, Galceran M *et al.* Distribution of enkephalinase (membrane metalloendopeptidase, E.C. 3.4.24.11) in rat organs. Detection using a monoclonal antibody. *Lab Invest* 1988; 58: 210–217
21. Krumme B, Endmeir R, Vanhaelen M, Walb D. Reversible Fanconi syndrome after ingestion of a Chinese herbal ‘remedy’ containing aristolochic acid. *Nephrol Dial Transplant* 2001; 16: 400–402

Received for publication: 6.4.05

Accepted in revised form: 5.7.05