Renal purine efflux and xanthine oxidase activity during experimental nephrosis in rats: difference between puromycin aminonucleoside and adriamycin nephrosis

FABRIZIO GINEVRI*, ROSANNA GUSMANO*, ROBERTA OLEGGINI*, SILVIA ACERBO*, ROBERTA BONTELLI*, FRANCESCO PERFUMO*, GIOVANNI CERCIGNANI†, SIMONE ALLEGRI*, FRANCO D’ALLEGRI‡ AND GIANMARCO GHIGGERI*

*Department of Nephrology, G. Gaslini Institute, Genoa, †Department of Physiology and Biochemistry, University of Pisa, Pisa, and ‡Institute of Internal Medicine, University of Genoa, Genoa, Italy.

SUMMARY

1. The hypothesis was tested that the renal xanthine oxidase system provides a source of oxygen free radicals in puromycin aminonucleoside and adriamycin experimental nephrosis by generating uric acid from hypoxanthine and xanthine.

2. The concentrations in renal tissue of the putative intermediary products of puromycin aminonucleoside metabolism, hypoxanthine and xanthine, and of their precursors, adenosine and inosine, were lower in rats treated with puromycin aminonucleoside than in normal controls, whereas concentrations of the metabolites were normal after adriamycin intoxication. Their daily urinary excretion was lower in the 24 h after puromycin aminonucleoside administration compared with the baseline values and returned to near normal levels within 5 days. After adriamycin, the 24 h urinary excretion of xanthine and uric acid was double the baseline levels (P<0.001).

3. When equimolar amounts of hypoxanthine were injected instead of puromycin aminonucleoside, the concentration of all bases increased slightly in renal tissue and their urinary efflux was double the baseline level: allantoin, uric acid, the unmodified nucleotide and xanthine were the most represented compounds in urine.

4. The enzymatic activities relative to xanthine oxidase (EC 1.1.3.22) and xanthine dehydrogenase (EC 1.1.1.204) in renal tissues were unchanged 1 day after puromycin aminonucleoside or hypoxanthine intoxication and only moderately increased in both groups at 13 days (the time of appearance of heavy proteinuria in the puromycin aminonucleoside-treated group). In contrast, xanthine oxidase and xanthine dehydrogenase activities were higher in adriamycin-treated rats at 1 and 15 days after the treatment (P<0.001).

5. Feeding rats with normoprotein diets containing tungsten induced a marked and constant decrease of renal xanthine oxidase and xanthine dehydrogenase activities to 20% of the baseline values in both puromycin aminonucleoside- and adriamycin-treated rats. Inhibition of renal xanthine oxidase and xanthine dehydrogenase activities by tungsten was associated with a marked reduction (P<0.001) of proteinuria in adriamycin-treated rats and the same occurred with allopurinol, a specific inhibitor of xanthine oxidase activity. In contrast, tungsten treatment did not reduce the proteinuria associated with puromycin aminonucleoside, which reached a maximum 13 days after puromycin aminonucleoside intoxication. Hypoxanthine-treated rats were normoproteinuric after 2 months of observation.

6. These data demonstrate an activation of renal xanthine oxidase and xanthine dehydrogenase after adriamycin intoxication which is relevant to the induction of proteinuria. They also argue against the involvement of the renal xanthine oxidase system as a source of free radicals in puromycin aminonucleoside nephrosis and suggest that the nucleotide cycle is not a normal route for puromycin aminonucleoside degradation. Other metabolic pathways for free radical generation from puromycin aminonucleoside must be considered.

Key words: adriamycin, proteinuria, puromycin aminonucleoside, xanthine oxidase.

Abbreviations: ADM, adriamycin; AMP, adenosine monophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetate; NAD, nicotinamide–adenine dinucleotide; PAN, puromycin aminonucleoside; PMSF,
hydrogenase; XO, xanthine oxidase. 

INTRODUCTION

Puromycin aminonucleoside (PAN) and adriamycin (ADM) nephrosis in rats are two different experimental models of proteinuria classically considered the animal model of the same human disease, i.e. minimal change nephropathy [1, 2]. Though little is currently known of the metabolic pathways for renal metabolism of these drugs and of the mechanism responsible for renal lesions [3-6], recent research has led to hypotheses that reactive oxygen metabolites serve as final mediators for both PAN and ADM renal toxicity [7-10]. In preliminary studies by Shah et al. [10], ADM was found to enhance the generation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) by renal cortical microsomes. In PAN nephrosis, proteinuria was reduced by allopurinol and superoxide dismutase [7], supporting the idea of the OH- radical in PAN- and ADM-induced nephrosis. Evidence to support the aforementioned hypothesis derives from data recently reported by Thakur et al. [8], who demonstrated that a marked protection against PAN renal toxicity is provided by the OH- scavenger dimethylthiourea and by deferoxamine which is able to inhibit OH- production. Though all these data suggest an important role for \( \text{O}_2^- \) and the OH- radical in PAN- and ADM-induced nephrotic syndrome, a number of crucial aspects of this possibility need to be investigated further. First, an increased supply to the kidney of candidates for conversion to hypoxanthine after PAN intoxication should be documented, in the light of the fact that other PAN analogues such as adenine, adenosine, monomethyladenosine and others not only have no nephrotoxic effect but even protect the kidney from PAN-induced changes [11-13]. Secondly, because the model proposed by Diamond et al. [7] calls for physical enzyme modification via uncharacterized mechanisms (proteolysis, thiol reactions, etc.), it is important to stress that physical changes in the enzyme do accompany changes in dehydrogenase and oxidase activities within the tissue. Finally, XO as a potential source of \( \text{O}_2^- \) generation after ADM treatment requires further investigation. All these aspects were the topics of the present study. First, XO and XDH activities were assayed in renal homogenates after ADM and PAN administration. Second, the concentration of purines which are substrates for XO action (hypoxanthine and xanthine) and of their precursors adenosine and inosine were determined in renal tissues and in urine, together with the end products, uric acid and allantoin. The same investigations were finally performed in rats given equimolar amounts of hypoxanthine in order to mimic the hypothesized effect of PAN and in rats in which renal XO and XDH activities were chronically suppressed by using diets enriched with tungsten [14].

MATERIALS AND METHODS

Materials

ADM was a gift from Farmitalia Carlo Erba (Milan, Italy) and allopurinol sodium salt (Zyloric) was from Wellcome (London). PAN, adenosine deaminase (EC 3.5.4.4), purine-nucleoside phosphorylase (EC 2.4.2.1), XO, ethylenediaminetetra-acetate (EDTA), trypsin inhibitor, dithiothreitol (DTT), allantoin, uric acid, adenosine 5'-phosphate (AMP), adenosine 5'-pyrophosphate, adenosine 5'-triphosphate and phenylhydrazine were obtained from Sigma (St Louis, MO, U.S.A.); Coomassie G-250 was from Fluka (Buchs, Switzerland); NAD, potassium ferricyanide, 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazine were from Merck (Darmstadt, F.R.G.); \( \text{N}_2\text{N}'\text{N}'\text{N}'\text{-tetramethylendiamine} \), \( \text{N}_2\text{N}'\text{N}'\text{N}'\text{-methylenesicylamlde} \), and ammonium persulphate were from BioRad (Richmond, CA, U.S.A.). Amicon PM 30 membranes were from Amicon (Danvers, MA, U.S.A.); reverse-phase chromatography columns RP18 were from Waters (Milford, MA, U.S.A.). All other reagents were of the purest grade available.

Animal experiments

Sprague-Dawley/CD male rats (Charles River, Calco, Italy) weighing 200-260 g at the start of the experiment were used. PAN experimental nephrosis was induced by a single injection of PAN (7.5 mg/100 g, dissolved in 0.5 ml of 150 mmol/l NaCl) through the tail vein of non-anaesthetized animals. Hypoxanthine (7.5 mg/100 g dissolved in 0.5 ml of 150 mmol/l NaCl) was injected by the same route. Groups of five to ten rats were killed at 1 and 13 days after PAN or hypoxanthine injection for determination of XO and XDH activity in renal homogenates and the nucleotide content of the renal tissue. ADM nephrosis was induced by a single injection of ADM (5 mg/kg, dissolved in 0.5 ml of 150 mmol/l NaCl) through the tail vein. Control rats were given 150 mmol/l NaCl. Groups of ten to 19 rats were killed 1 and 15 days after ADM injection for determination of XO and XDH activities in renal homogenates. Allopurinol (80 mg/kg, dissolved in 0.5 ml of H2O) was injected into the anacannulated tail vein 3 h before and 1 min after ADM injection. Groups of four to 15 rats were killed 1 and 15 days after ADM and allopurinol injection for determination of renal XO and XDH activities. In all cases rats were fed ad libitum a standard diet containing 20% (w/w) casein and 2% (w/v) trace elements (not containing tungsten) and providing 3400 cal (14200 J/kg) (Altromin-Rieper, Vandoies, Italy), and were allowed free access to water. The same diet containing sodium tungsten (0.7 g/kg) was in some cases used to exert a chronic inhibitory effect on renal XO. In this case the diet containing tungsten was started 1 week before the treatment with ADM or PAN.
Tissue preparation

Each step was performed at 4°C. Kidneys were surgically excised from anaesthetized rats (Ketalar; Parke-Davis, Milan, Italy) after perfusion for 5 min with 60 ml of 150 mmol/l NaCl containing 1 mmol/l EDTA and freshly added 0.2 mmol/l PMSF and were immediately freeze-clamped. They were then maintained in liquid N2 until homogenized. For the XO and XO+XDH assay, frozen samples were rinsed in cold 0.01 mol/l Tris-HCl buffer, pH 7.8 containing 1 mmol/l EDTA, 1 mmol/l DTT, trypsin inhibitor (5 mg/l), 0.2 mmol/l PMSF and kallikrein (40.000 units/l) to remove residual blood elements and were homogenized at 4°C in the same buffer containing 0.25 mol/l sucrose with a Polytron disruptor. Homogenates were then processed with seven cycles of freezing-thawing, centrifuged at 27 000 g for 30 min and the pellet was discarded. The supernatant fraction was ultrafiltered in the cold with Amicon PM-30 membranes in the same buffer containing antiproteolytic substances, in order to remove endogenous substrates, and the residual fraction was utilized for the enzyme assay and for protein determination. PMSF was added periodically (every hour) during sample preparation, following the instructions given by Gordan [15]. Failure to do so was found to increase XO activity in comparison with XDH. PMSF in higher concentration was insoluble in water. Freeze-clamped kidneys maintained under N2 were utilized for analysis of uric acid, xanthine and hypoxanthine, and their nucleoside precursors adenosine and inosine. Weighed tissue samples were homogenized in 5 ml of 10% (v/v) perchloric acid/g of wet tissue. After centrifugation at 2000 g in the cold for 15 min, the supernatants were neutralized with adequate aliquots of 3.6 mol/l K2HPO4 and the filtered samples were maintained in liquid nitrogen until assayed.

Enzyme and protein assay

XO and XDH activities were assayed by measuring uric acid formation at 293 nm using xanthine as substrate in the absence and presence of NAD as described by Waud & Rajagopalan [16]. All activity measurements were performed at room temperature in 0.05 mol/l potassium phosphate buffer, pH 7.8, containing 1 mmol/l EDTA, 1 mmol/l DTT, trypsin inhibitor (5 mg/l) and 0.2 mmol/l PMSF. In some cases the same buffer deprived of DTT was employed in order to determine the reversible fraction of XO. Sample mixtures for assaying XO activity were prepared by mixing 25 µl of renal homogenate in phosphate buffer containing 0.15 mmol/l xanthine, in a final volume of 1 ml. XO+XDH was measured in a separate, albeit similar, assay in the presence of 1 mmol/l NAD. The enzyme activity unit is the amount of enzyme required to convert 1 µmol of xanthine to uric acid/min. Specific activity is defined as units/mg of protein. Proteins were determined with the Coomassie G-250 dye binding assay as modified by Read & Northcote [17] using serum albumin as standard.

Enzymatic assay for purines

The assay for xanthine, hypoxanthine, inosine and adenosine was performed as follows. A portion (1 ml) of the filtered sample was placed in a quartz cuvette and its absorbance at 293 nm was read in DU-7 Beckman spectrophotometer (Beckman Instruments, Palo Alto, CA, U.S.A.). XO (3 µl) was then added to the sample and the total absorbance increment due to uric acid formation was used to determine xanthine and hypoxanthine concentration. Purine nucleoside phosphorylase (2 µl) was then added to determine inosine from the phosphate-dependent increase in absorbance at the same wavelength. Adenosine was similarly determined by the further addition of 1 µl of adenosine deaminase. The renal levels of these nucleosides and bases were calculated by taking into account the amount of tissue corresponding to 1 ml of filtered sample, and expressed as µmol/g of frozen tissue.

H.p.l.c. analysis of nucleosides and bases

An h.p.l.c. system was used to separate hypoxanthine, xanthine, inosine and adenosine in urine and to determine their concentrations. All the standards and samples were deproteinized by precipitation with 12% trichloroacetic acid (1:5, v/v) at 4°C for 15 min, followed by protein removal by centrifugation at 2000 g (15 min at 4°C). After neutralization, 20 µl aliquots of supernatants were injected into an h.p.l.c. column (Waters RP18) working at room temperature. A three-step mobile-phase system was used to separate every single component: (1) constant 0.025 mol/l KH2PO4 (pH 5) with a flow rate of 1 ml/min for 15 min, (2) a gradient between phase 1 and 30% (v/v) methanol for 20 min with a 1 ml/min flow rate and (3) constant 0.25 mol/l KH2PO4–30% (v/v) methanol for 25 min. Nucleosides and bases corresponding to the eluted peaks were identified by (1) comparison with known standards and (2) enzymatic analysis of the eluates. In these cases the eluted peak was incubated for 1 h at 37°C with the corresponding enzyme, i.e. XO for xanthine and hypoxanthine, purine nucleoside phosphorylase for inosine, etc. After incubation, the sample was immediately deproteinized and analysed. A variable visible-u.v. wavelength detector was employed to analyse the spectral characteristics of eluted nucleotides whose concentrations were calculated by comparison with a known standard sample.

Assays for uric acid and allantoin

Uric acid in serum, renal tissue and urine was determined spectrophotometrically after its conversion by uricase into allantoin and H2O2. With this method, enzymatically produced H2O2 in turn reacts with 3,5-dichloro-2-hydroxybenzenesulphonic acid in the presence of 4-aminophenazone to give a red quinomine with a maximum absorbance at 520 nm. Renal and urinary allantoin was determined by the Rimini–Schrayer reaction [18, 19] as follows: NaOH (0.5 mol/l, 100 µl) was added to 500 µl of diluted urine (1:200, v/v) and the mixture was heated in
a bath of boiling water for 10 min to hydrolyse allantoin to allantoate. After the initial hydrolysis, the sample was treated with 125 μl of 0.5 mol/l HCl followed by 100 μl of 0.33% (w/v) phenylhydrazine and heating at 100°C was continued for 5 min. In this final stage, allantoin is hydrolysed to glyoxylic acid, which reacts with phenylhydrazine to form its 2,4-dinitrophenylhydrazine derivative, which is finally oxidized by ferricyanide and its absorbance read at 520 nm. Free glyoxylic acid was determined by the same method, by omitting the two initial steps (alkaline and acid hydrolysis) and its concentration was subtracted from the acid–alkaline hydrolysat.

Gel electrophoresis

Non-dissociating discontinuous gradient electrophoresis was performed in polyacrylamide gels (1 mm) (total monomer concentration T = between 8 and 22%, percentage of cross-linker C = 2.5%) by a modification of the original technique described by Davis [20]. Gradients of polyacrylamide solutions were obtained by mixing equal volumes of solution containing 8% and 22% polyacrylamide, constant 7.5% (w/v) ammonium persulphate and 10 μl (v/v) N,N,N',N'-tetramethylethylenediamine in 0.375 mol/l Tris-HCl, pH 8.8. The stacking gel, with a constant polyacrylamide concentration (T = 4.6%, C = 2.5%), was cast in 0.06 mol/l Tris–HCl, pH 6.2. Electrophoresis was carried out overnight (until the tracking dye had migrated to the bottom of the gel) applying 180 V constantly. Protein samples were suspended in 0.062 mol/l Tris–HCl, pH 6.8 containing 10% (v/v) glycerol and 2.5% (v/v) β-mercaptoethanol. Conventional gradient sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in discontinuous buffers was performed as described by Laemmli [20a]. Gels were stained by the silver method for proteins and for enzymatic activity by immersion in 0.05 mol/l potassium phosphate, pH 7.8 containing 0.02% tetrazolium nitroblue and 1.35 mmol/l xanthine. Low- and high-molecular-weight proteins were used as standards to calibrate the gel in the silver-stained experiment.

RESULTS

Renal serum and urinary purines in hypoxanthine- and PAN-treated rats

The renal concentrations of inosine and hypoxanthine plus xanthine were slightly higher in hypoxanthine-treated rats compared with the control group (Table 1). By contrast, PAN-treated rats had low levels of these metabolites in renal tissue, a difference that was statistically significant when compared with the hypoxanthine-treated group (Table 1). The renal levels of adenosine and uric acid were very low in every group; allantoin was comparably low and was not influenced by PAN and hypoxanthine injection. The serum concentration of uric acid, which is the main circulating transformation product of hypoxanthine and xanthine, remained constant, compared with the pretreatment levels, 2 and 24 h after PAN and hypoxanthine treatment (Table 2). In Figs. 1(a) to 1(d), the daily urinary excretion of the purines studied is shown. In hypoxanthine-treated rats, all metabolites considered, i.e. hypoxanthine, xanthine, uric acid and allantoin, were initially increased over the baseline levels but returned to the pretreatment level within a few days from the time of intoxication. The greatest increase was for allantoin, whose 24 h urinary excretion in the first day after hypoxanthine treatment was higher by a factor of 1.5 compared with the baseline (20 ± 2 vs 31 ± 3.6 mg/day), followed by hypoxanthine (0.15 ± 0.05 vs 0.4 f 0.06 mg/day). Adenosine and inosine were undetectable in urine. On the basis of the mean daily increment of each metabolite in urine, it was calculated that about 15% of hypoxanthine was excreted in its unmodified form, whereas 75% was excreted as the sum of xanthine, allantoin and uric acid. On the contrary, PAN-treated rats did not show any increase in the daily urinary excretion of purines apart from a modest, statistically insignificant, variation in xanthine (0.17 f 0.05 vs 0.4 f 0.06 mg/day). The other metabolites were even decreased compared with the baseline values, but the difference was nil when the urinary concentration was calculated per amount of urinary creatinine, thus demonstrating an influence of the decrease in renal function induced by PAN (Fig. 1e). Feeding rats with tungsten resulted in a marked decrease in the baseline daily urinary excretion of all metabolites, without significant change after PAN intoxication (Figs. 1a–d).

Renal XO and XO+XDH in hypoxanthine- and PAN-treated rats

The enzymatic activities of XO and XO+XDH in renal homogenates 1 and 13 days after PAN and hypoxanthine treatment are reported in Fig. 2. Compared with normal rats, no variation in the enzymatic activities was observed in either group after 1 day. An increase in XO+XDH activity, however, was observed in both PAN- and hypoxanthine-treated rats 13 days after the intoxication. In these cases, however, renal XO was unchanged, which suggests that the percentage of XO relative to the
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sum of XO and XDH activities remained constant or even decreased at any of the points considered. Feeding rats with a tungsten-enriched diet induced a marked decrease in renal enzymatic activities of both XO and XO + XDH to 20% of the normal level.

Proteinuria in hypoxanthine- and PAN-treated rats

The protein excretion in PAN- and hypoxanthine-treated rats and in PAN-treated, tungsten-fed rats is shown in Fig. 3. PAN-treated rats gradually became proteinuric 5 days after PAN injection and reached a peak value after 11 days. The same pattern was observed in PAN-treated, tungsten-fed rats, which presented the same levels of proteinuria as the PAN-treated group (387 ± 43 vs 355 ± 66 mg/day). On the contrary, hypoxanthine-treated rats remained constantly non-proteinuric for the whole period and continued to be normoproteinuric over the next 2 months.

Renal and urinary purines in ADM-treated rats

The renal concentrations of inosine, adenosine, hypoxanthine, xanthine, uric acid and allantoin in renal
Renal XO and XO + XDH in ADM-treated rats

As shown in Fig. 5, 24 h after ADM injection renal XO activity had doubled with respect to control rats (0.85 ± 0.11 vs. 0.49 ± 0.04 m-units; P < 0.01), whereas XO + XDH activity had increased by 40% (1.63 ± 0.08 vs. 1.16 ± 0.07 m-units; P < 0.05). Fifteen days after ADM administration, XO and XO + XDH activities in renal homogenates were further increased compared with normal rats and with the first day of treatment (XO 1.14 ± 0.18 m-units; XO + XDH 2.22 ± 2.24 m-units). On XO staining, electrophoresis of renal crude homogenates revealed a single band with an apparent molecular weight of 130 kDa corresponding to unmodified XO (not shown). Both XO and XO + XDH activities were markedly decreased in ADM-treated, tungsten-fed rats and in rats treated with allopurinol. As shown in Fig. 5, the inhibition of renal XO and XO + XDH was marked and persistent in tungsten-fed rats; allopurinol induced a time-dependent inhibition that was very marked on the first day but rats given allopurinol showed normal renal XO and XO + XDH activities after 15 days (Fig. 5).

Proteinuria in ADM-treated rats

The protein excretion in ADM-treated rats is shown in Fig. 6. Proteinuria began to appear 10 days after the treatment and was maximal after 15 days (432.32 ± 44.4 mg/day). In both tungsten-fed and allopurinol-treated rats, the levels of proteinuria were markedly decreased compared with ADM alone during the whole period of the study including the maximal level 15 days after ADM treatment (tungsten-fed rats 147.2 ± 27.7 mg/day, P < 0.001; allopurinol 163.13 ± 32.1 mg/day, P < 0.001), in spite of unchanged kidney weight and creatinine clearance (see Table 3).

DISCUSSION

This study was performed to focus on a possible implication of the XO system in the pathogenesis of two types of experimental nephrosis in rats (i.e. PAN and ADM) which are generally considered the animal models of the same human disease, namely minimal change nephropathy [1, 2]. In both cases, a role for XO, which generates O$_2^-$ from xanthine, can be hypothesized on the basis of the data in the literature [7–10], but a clear demonstration of this is still lacking. Our experimental approach was to determine XO and XDH activities in renal homogenates at various periods after PAN or ADM intoxication and to determine
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Fig. 4. Daily urinary excretion of hypoxanthine (a), xanthine (b), uric acid (c), allantoin (d) and AMP (e) after ADM treatment in rats fed a standard diet (●) and in tungsten-fed rats (▲). Results are means ± SEM. Numbers in parentheses are n values. Statistical significance: *P< 0.01, **P< 0.001 vs before treatment.

the renal and urinary levels of the companion nucleosides and bases involved in XO activities. Any relationship between XO and proteinuria was elucidated by inhibiting renal XO by feeding rats with tungsten-enriched diets or (for ADM) with allopurinol. Tungsten prevents the incorporation of molybdenum into XO and XDH [14], resulting in the synthesis of catabolically inactive enzymes. As pointed out by other studies, tungsten-treated rats provide a convenient model for blocking tissue XO, since the effect of this metal in rats is specific for molybdenum-dependent enzymes (XO, aldehyde oxidase and sulphite oxidase) and does not involve other enzymes. Since tungsten-treated rats lack any signs and symptoms of toxicity from the metal itself [14, 21, 22], tungsten has been successfully employed to prevent tissue damage in several organs where XO is implicated, such as ischaemia of the heart and brain [23, 24]. On the whole, this work documents a marked difference between PAN and ADM nephrosis with regard to the implication of XO, and, owing to the complexity of the subject, a separate discussion seems necessary.

PAN nephrosis

A role for toxic oxygen metabolites in PAN experimental nephrosis has been recently demonstrated on the
Table 1. Concentrations in renal homogenates of xanthine plus hypoxanthine, their precursors adenosine and inosine, and their end-products uric acid and allantoin, 1 and 13 days after infusion of PAN (7.5 mg/100 g) or hypoxanthine (7.5 mg/100 g), and 1 and 15 days after infusion of ADM (5 mg/kg), in a single dose

Results are means ± SEM. Statistical significance: *P<0.001 vs normal rats; †P<0.001 vs hypoxanthine-treated rats.

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<tr>
<th></th>
<th>Adenosine (µmol/g)</th>
<th>Inosine (µmol/g)</th>
<th>Xanthine + hypoxanthine (µmol/g)</th>
<th>Uric acid (µmol/g)</th>
<th>Allantoin (µmol/g)</th>
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<tr>
<td><strong>PAN-treated (n = 5)</strong></td>
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<tr>
<td>Day 1</td>
<td>&lt;0.1</td>
<td>0.53±0.04*</td>
<td>0.33±0.04†</td>
<td>&lt;0.1</td>
<td>0.12±0.01</td>
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<td>Day 13</td>
<td>&lt;0.1</td>
<td>0.58±0.05*</td>
<td>0.46±0.02</td>
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<td>Day 1</td>
<td>&lt;0.1</td>
<td>1.12±0.06</td>
<td>0.81±0.05</td>
<td>&lt;0.1</td>
<td>0.13±0.01</td>
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<td>Day 13</td>
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<td>0.71±0.02</td>
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<td>Day 15</td>
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<td>&lt;0.1</td>
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<td>0.76±0.03</td>
<td>&lt;0.1</td>
<td>0.13±0.02</td>
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Table 2. Serum uric acid concentration in rats receiving PAN (7.5 mg/100 g) or hypoxanthine (7.5 mg/100 g) intravenously in a single dose

Results are means ± SEM.

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<thead>
<tr>
<th></th>
<th>Serum uric acid (µg/ml)</th>
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<td>Before treatment</td>
<td>After treatment 2 h 24 h</td>
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<tr>
<td>PAN (n = 5)</td>
<td>1.01±0.3 0.96±0.01 0.96±0.06</td>
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<td>Hypoxanthine (n = 5)</td>
<td>1.06±0.1 1.01±0.1 0.98±0.03</td>
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Table 3. Kidney weight at the time of killing and creatinine clearance in ADM-treated rats and ADM-treated, tungsten-fed rats

Results are means ± SEM.

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<th>Creatinine clearance (ml/mg)</th>
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<td>Day 1 Day 15</td>
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<td>ADM+tungsten (n = 4)</td>
<td>1.26±0.02 1.0±0.2 1.1±0.1</td>
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basis of a protective effect on proteinuria of superoxide dismutase [7], and of other general scavengers such as sodium benzoate and dimethylthiourea [8], and of poly(ethylene glycol)-coupled catalase [9]. In addition, the iron chelator deferoxamine also provides marked protection against PAN-induced proteinuria [8], implying that OH· is produced through the Haber–Weiss reaction, during which O2·− and H2O2 may interact to generate OH· with iron as a catalyst [25]. The putative metabolic pathway for free-radical production in PAN nephrosis has been hypothesized by Diamond et al. [7] on the basis of the inhibitory effect on proteinuria of allopurinol, which suggests that renal XO plays a central role. According to this hypothesis, hypoxanthine, i.e. one putative minor intermediary product of PAN [26], can serve as a substrate for OH· production via the XO system, assuming the presence of sufficient amounts of enzyme in the kidney, where it is largely present in the dehydrogenase form [27]. There are at least three assumptions in the general theory of PAN nephrotoxicity and the related metabolic pathway that need to be more directly verified. The first implies that PAN is converted into hypoxanthine in renal tissue, since, due to the very short half-life of oxygen free radicals, their production at any other site (e.g. liver) could not be toxic for the kidney. A second assumption is that, after PAN administration, renal XDH is converted into XO or that a sufficient amount of XO is already present in the kidney [27, 28]. Finally, a central point in the theory is the correlation of the antiproteinuric effect of allopurinol to its inhibitory effect on XO, assuming no other collateral action. Considering that very few data on the renal and urinary concentrations of nucleosides and related compounds in rats are available, we first attempted to develop a model of kidney nucleotide overloading by giving rats an equimolar amount of hypoxanthine in place of PAN. After this, practically the whole amount of hypoxanthine was recovered in urine within 24 h as a sum of intact base (15–20%) and of the transformation products xanthine, uric acid and allantoin, whose net daily increment in this period approached the dose of hypoxanthine given to the rats. The kidney was in fact the main site of transformation of hypoxanthine into uric acid, since no increase in the serum concentration of uric acid was observed, whereas the substrates (given as hypoxanthine plus xanthine) were slightly elevated in renal tissues. No changes were further
observed in the renal concentration of inosine and adenosine, indicating no collateral efflux of hypoxanthine from the main metabolic pathway. In spite of a clear overloading of renal nucleotide metabolism, rats given hypoxanthine did not become proteinuric. The same study performed in PAN-treated rats led to quite opposite conclusions. In this case, the renal content of hypoxanthine plus xanthine was far below the normal levels, and the same was true for inosine. Moreover, no changes were observed in the urinary excretion of uric acid and allantoin, which were, if anything, lower than the baseline level. The second assumption of the theory of PAN nephrotoxicity was tested by determining the enzymatic activities of XO and XDH in renal homogenates shortly after PAN administration (1 day) and at the time of massive proteinuria (13 days). The results demonstrated that stable changes in enzymatic activity cannot be detected in PAN-treated rats compared with the hypoxanthine group and that in no case did PAN induce the transformation of the dehydrogenase form of the enzyme into the oxidase one. Taken together, these data cast doubt on the original idea that free radicals in PAN nephrosis are generated via the conversion of PAN into hypoxanthine and then into uric acid via the XO system. A final and decisive demonstration that renal XO is not involved as a mechanism of PAN nephrotoxicity derives from the experiment performed on a group of rats in which renal XO and XDH were chronically inhibited by feeding tungsten-enriched chow. Rats given tungsten in the diet and treated with PAN became massively proteinuric, in spite of a marked and stable decrease in renal XO and XDH activities. In this case, the urinary concentrations of both uric acid and allantoin and of the precursors hypoxanthine and xanthine were very low. Inhibition of renal XO by tungsten is a more physiological model compared with allopurinol, since this latter substance, a chemical analogue of xanthine, could compete for the cellular uptake of nucleotides and related compounds such as PAN. It is noteworthy that other molecular analogues of PAN such as adenine [12], adenosine, 6-methyladenosine and the dimethylated forms [13] are able to inhibit the development of proteinuria in PAN-treated rats through a competitive mechanism, and also exert an anticytotoxic effect on glomerular epithelial cells in vitro [3]. Since adenosine and its mono- and di-methylated forms do not serve as substrates for XO and XDH, it seems very improbable that their protective effect results from competition with PAN for this enzyme. While these data document no change in renal purine efflux after PAN and also argue against a role of the hypoxanthine-xanthine and renal XO system as a mediator of nephrotoxicity, they give virtual no information about the source of oxygen free radicals in PAN nephrosis. Therefore, other unexplored pathways for O$_2^-$ production must be hypothesized. Glomerular damage by oxygen free radicals, however, is not caused only by their enhanced production, but also by an impairment of the cellular enzymatic defence. Although no data are currently available as to the glomerular content of enzyme involved in free radical defence in PAN nephrosis, several metabolic effects of PAN on protein synthesis and metabolism, including RNA synthesis, have been well documented [3]. Thus, among the various possibilities, RNA and protein synthesis must be considered with regard to a defect in cellular defence against free radical nephrotoxicity.
ADM nephrosis

Although the mechanism(s) responsible for ADM nephrotoxicity in rats is so far unknown, circumstantial evidence suggesting a role for free radicals in ADM toxicity in other models or organs, such as murine cancer cells (line L 1210) and the heart, is available [29-31]. In general, it is believed that in biological systems the drug is reduced to an unstable semiquinone derivative by the action of enzymes such as xanthine oxidase and nicotinamide–adenine dinucleotide phosphate (reduced)-cytochrome P-450 reductase [32, 33], a step that is followed by the production of O$_2^-$, H$_2$O$_2$ and finally OH$^-$ by way of the so-called 'metal-catalysed Haber–Weiss reaction'. ADM also enhances the generation of O$_2$ and H$_2$O$_2$ production by renal cortical microsomes [10]. Our results demonstrate that a direct stimulation of renal XO and XDH by ADM is relevant in inducing the renal toxicity of the drug in rats as revealed by the appearance of proteinuria. Proof for this is that a significant induction of renal XO and XDH activity is evident 1 day after ADM treatment and increases 15 days after the drug administration. Furthermore, the block of renal XO and XDH by a tungsten-enriched diet significantly reduced proteinuria in ADM-treated rats. As already stated, tungsten is a specific inhibitor of XO, aldehyde oxidase and sulphite oxidase by interfering with their molybdenum-dependent active site [14, 21-22]. Allopurinol is a potent specific inhibitor of XO and XDH but not of sulphite oxidase and aldehyde oxidase. Allopurinol may nevertheless exert some competition with ADM itself for the renal uptake and studies are now in progress in order to verify this aspect. Measurable inhibition of renal XO and XDH by either tungsten and allopurinol together with the decrease in proteinuria focuses on XO and/or XDH as a potential cause of renal toxicity due to ADM. Owing to the well-known capacity of XO to generate oxidants, the first possibility deriving from these data is that ADM exerts its proteinuric effect by stimulating the activity of endogenous oxidant-generating pathways. Secondly, it is also possible that XO, whose renal expression is increased by ADM, contributes to the oxidation of ADM to give the unstable semiquinone derivative, i.e. the putative effector of ADM toxicity on other cells. These mechanisms are not, however, unique in determining ADM nephrotoxicity, since, as hereby demonstrated, rats treated with allopurinol and/or tungsten develop mild proteinuria even in the absence of XO and XDH activities in renal tissue. This fact suggests that other metabolic pathways for transformation of ADM into a semiquinone molecule not involving the XO system and/or other mechanisms may be still active in the presence of allopurinol and/or tungsten and account for the partial maintenance of the proteinuric effect of ADM. The observed induction by ADM of renal XO posed an additional problem, the identification of the mechanism(s) responsible for the enzymatic induction. With regard to this, the increased activity of XO could derive from a direct stimulatory effect of ADM on renal tissue, or alternatively, the drug could increase the levels of the XO substrates, namely hypoxanthine and xanthine. The high levels of xanthine in urine, together with normal or slightly higher levels of this substrate in the kidney, support this possibility, but other mechanisms cannot be ruled out.

Conclusion

Hypoxanthine and renal XO are not implicated as a source of O$_2^-$ in the pathogenesis of PAN nephrosis, and other mechanisms for free-radical generation of PAN must be hypothesized. By contrast, XO activity in renal homogenates increases markedly after ADM intoxication and high levels of the products of hypoxanthine metabolism (xanthine and uric acid) are present in urine. This seems to act as a mechanism of renal toxicity due to ADM, since proteinuria is reduced in those cases where the enzymatic activity was inhibited. No direct evidence for the intervention of O$_2^-$ in ADM nephrosis is available, but other studies are now assessing this aspect.

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REFERENCES

15. Waud, W.R. & Rajagopalan, K.V. Purification and properties of the NAD-dependent (Type D) and O2-dependent (Type O) form of rat liver xanthine dehydrogenase. Arch. Biochem. Biophys. 1976; 172, 3540–64.