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ADRIAMYCIN AFFECTS GLOMERULAR RENAL FUNCTION: EVIDENCE FOR THE INVOLVEMENT OF OXYGEN RADICALS

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The early nephrotoxic effect of the antitumor drug adriamycin (ADR) is suggested to be related to the generation of oxygen free radicals. Therefore the O_2-dependence and the influence of free radical scavengers were studied in the model of the isolated perfused single glomerulus of *Myxine glutinosa* and by histochemical demonstration of the glomerular ATP-ase. In *Myxine*, the glomerular ATP-ase activity was decreased after injection of ADR (5 mg/kg, i.v.).

Both ADR-treated *Myxine* and controls were exposed for 48 h to an artificial atmosphere of 20% O_2/80% N_2 or 80% O_2/20% N_2, respectively. After 10 days a significant decrease of the hydraulic conductivity (k) was measured in the experimental group exposed to 80% O_2 (k-values expressed as nl/s·mm Hg·mm^2: controls (7): 0.059 ± 0.017; ADR (7): 0.033 ± 0.026). The reduction of k following the administration of ADR (20 mg/kg) could be prevented by the sulphydryl donor N-acetylcysteine (NAC). The sieving coefficient for albumin (φ) was significantly increased in ADR-treated animals, showing no O_2-dependence ($\varphi \times 10^{-2}$: controls (7) 1.3 ± 0.2; ADR 20% O_2 (8): 8.1 ± 9.6; ADR 80% O_2 (7): 6.9 ± 6.7). φ was not affected by NAC.

The lipid peroxide levels in liver, kidney and heart of *Myxine* increased after the administration of ADR, peaking by day 2 to 5. The circulation disorders of ADR-treated *Myxine* were not due to an accumulation of the drug in the heart, but rather to a lack of the intracellular antioxidant glutathione.

It is concluded that the early nephrotoxic effect of ADR, as reflected by a decreased glomerular ATP-ase activity, is mediated by free radical formation. Oxidative stress on membrane compounds seems to reduce the water permeability of the glomerular barrier, while the ADR-induced sieving defect may be due to oxygen independent pathological mechanisms.

KEY WORDS: Adriamycin, nephrotoxicity, glomerular ATP-ase, glomerular barrier function, free radical scavengers, lipid peroxidation.

INTRODUCTION

The anthracycline antibiotic adriamycin (ADR) is a potent cancer chemotherapeutic agent, but is limited in its clinical applicability because of severe cytotoxic side effects. The nephrotoxicity of ADR in rodents is documented in detail and described as a model for experimentally induced nephrotic syndrome.[1] The actual cellular targets and molecular mechanisms responsible for ADR nephrotoxicity, however, are not yet clearly defined. Several studies support the involvement of ADR semiquinone radicals generated by ADR bioreduction. In the presence of oxygen the semiquinone radicals

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rapidly give rise to reactive oxygen free radicals, which are thought to damage the cells by lipid peroxidation and cross linking of cellular thiols.[2–4] The intracellular thiol glutathione (GSH) is regarded to be the most vital endogenous defense against oxidative stress in eukaryotic cells.[5]

To further specify the nephrotoxic action of ADR with respect to its oxygen dependence and the influence of free radical scavengers functional and biochemical studies were performed, using the Atlantic hagfish *Myxine glutinosa* as an experimental model.

The Atlantic hagfish (*Myxine glutinosa*; Cyclostomata) is a vertebrate with a kidney that consists of about 70 segmentally arranged glomeruli drained via short neck segments into two parallel collecting ducts (Archinephric ducts = ANDs). Because of this arrangement and a morphology and fine structure that is similar to the kidney of higher vertebrates[6,7] the animal lends itself to studies of glomerular processes. The model of the isolated perfused single glomerulus of *Myxine* (IPSG)[8] was applied to measure ADR-induced permeability changes of the glomerular barrier for both proteins and water under different oxygen pressures. Glutathione (GSH) concentrations were measured in different tissues of *Myxine* and rats, since it has been shown that low endogenous GSH pools render cells more susceptible to free radical attack.[9] Oxidative stress on membrane compounds was investigated by the determination of tissue levels of malondialdehyde, reported as a final product of lipid peroxidation.[10]

As a very early marker of the ADR-induced glomerular lesions, preceeding the impairment of overall kidney function, a reduction of ATP-ase activity within glomeruli of rats could be demonstrated at the electron microscopical level as soon as 48 h after the injection of the drug.[11] In this study glomerular ATP-ase activity was demonstrated at the light microscopical level in untreated *Myxine glutinosa* and compared to hagfish treated with ADR.

The data presented suggest an involvement of free radical species in the nephrotoxicity of ADR.

MATERIAL AND METHODS

Animals

Hagfish (40–75 g, 33–39 cm long), caught either in the Oslo Fjord (Norway) or in Huntsman Bay (Canada), were kept up to two months in filtered and aerated seawater at 4–5°C in the dark without feeding. The animals were anesthetized with propylen phenoxetol (Nipa Laboratories, U.K., 2 ml/l seawater) for 90 minutes.[12]

Experimental Protocol

After the injection of ADR (5 mg/kg b.w.) into the caudal blood sinus the hagfish were exposed for 48 h to an artificial atmosphere of either 20% O_2/80% N_2, or 80% O_2/20% N_2 and kept under normal O_2 conditions for further 8 days. Controls received O_2 in a corresponding manner. N-acetylcysteine (NAC), Fluimucil, Inpharzam, Grafeling, FRG; 450 mg/kg) was injected i.v. 0.5 h prior to and 0.5 and 4 h after administration of ADR (20 mg/kg b.w.) under normal atmosphere.

The IPSG of Myxine Glutinosa

10 days after ADR administration a pressure controlled microperfusion of single, isolated glomeruli was performed essentially as described previously.[8] Briefly, the animals received a single bolus injection of 0.07 ml perfusate (Ringer's solution[13] containing 7.5 g/l bovine serum albumin, Sigma) supplemented with heparin (Liquemin 2500, Hoffmann La Roche, Switzerland; 25 U/ml) to prevent thrombosis. A double barreled cannula, inserted into the dorsal aorta, allowed a simultaneous perfusion and recording of the perfusion pressure, which was maintained at 9.1 ± 0.7 mm Hg (Precidor microinfusion pump, Infors AG, Switzerland). Pressure on the epithelial side of the glomerular capillary was measured by insertion of a PP 10 catheter proximal into the AND. Another catheter inserted distally allowed measurements of the single nephron GFR (SNGFR) by reading the advance of the ultrafiltrate. The complete arrangement was transferred into a small cooled chamber which contained a bathing solution identical with the perfusate. The hydrostastic pressures in the dorsal aorta and in the AND were received by a pressure transducer (Statham, P 23 Db, Gould, USA) and continuously recorded (Linseis L 6510, Selb, FRG).

As glomerular diameter of *Myxine glutinosa* is linearly correlated with the glomerular capillary surface area (Fels L., unpublished data), measurements of the glomerular size allowed the determination of the hydraulic conductivity (k). k is expressed as nl/s·mm Hg·mm^2.

Analytical Methods

In situ activity of glomerular ATP-ase within the glomerulus was histochemically demonstrated by using the cerium based method at the light microscopical level.[14] Glomeruli were fixed in acetone (−20°C) and subsequently embedded in glycomethacrylate plastic at −20°C according to the method of van Goor.[15] Plastic sections (2 μm) were incubated in 70 mM *tris*-maleate buffer (pH 7.2) with 1 mM $CeCl_3$, 5 mM $Mg(NO_3)_2$ and 2.3 mM ATP for 1 h at 37°C following 10 min preincubation in complete medium without ATP. To demonstrate reaction product by light microscopy, cerium phosphate was converted to cerium-perhydroxide in glycine-NaOH buffer (pH 8.5) with 0.5% H_2O_2 and contrasted with 3,3-diaminobenzidine according to the method of van Goor.[15]

Analysis of albumin in perfusate and filtrate was performed by rocket-immunoelectrophoresis.[16] ADR was extracted with $AgNO_3$ and isoamylalcohol from tissue homogenates 12 h after injection (7.5 mg/kg b.w.) and fluorometrically analyzed (Turner fluorometer model 112, excitation filter NP 460, emission filter SC 535, Gamma Analysentechnik, FRG).[17] Glutathione concentrations were determined in deproteinized tissue supernatants following the method of Ellman.[18] Lipid peroxide levels were measured spectrophotometrically with the thiobarbituric acid reaction using 1,1,3,3-tetraethoxypropane (Sigma) as external standard.[19]

Statistics

All values are presented as means of n experiments ± S.D. The significant differences were calculated according to the t-test with a level of significance of $p < 0.05$.

TABLE I
Influence of ADR (5 mg/kg b.w.) under different oxygen pressures on the hydraulic conductivity (k) and the sieving coefficient for albumin (φ) of the IPSG of Myxine glutinosa

	n	k (nl/s·mm Hg·mm^2)	$\varphi \times 10^{-2}$
controls (20% or 80% O_2)	7	0.059 ± 0.017	1.3 ± 0.2
ADR 20% O_2	8	0.046 ± 0.032	8.1 ± 9.6*
ADR 80% O_2	7	0.033 ± 0.026*	8.9 ± 6.7*

$\bar{x}$ ± S.D., *$p < 0.05$ against controls.

RESULTS

ADR (5 mg/kg) given to hagfish either under normoxic or hyperoxic conditions as indicated above caused disturbances of the glomerular barrier in the model of the IPSG (Table 1). An O_2-dependent reduction of the hydraulic conductivity (k) was measured, which was significant in the experimental group exposed to 80% O_2. The sieving coefficient (φ) for albumin significantly increased after the administration of ADR, but was independent of the O_2-pressure applied. The perfusate flow necessary to maintain the perfusion pressure was within the same order of magnitude in all groups under study.

ATP-ase activity was demonstrated within the glomeruli of *Myxine*. A reduction

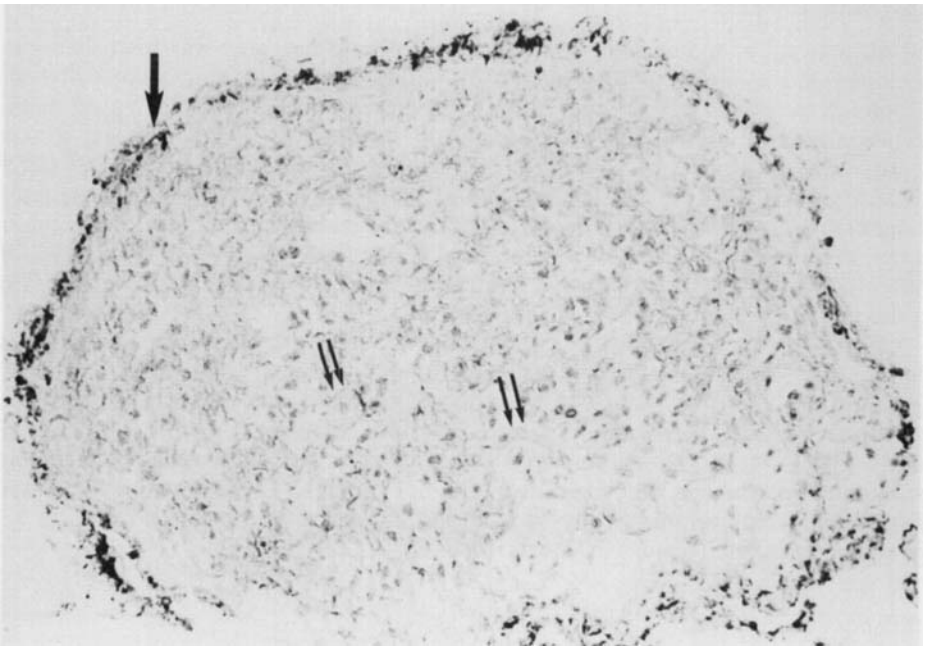

FIGURE 1a Part of capillary tuft of a glomerulus of an untreated hagfish, stained for ATP-ase activity. Reaction product is present along Bowman's capsule and along capillary walls (smaller arrows). Magnification, × 320.

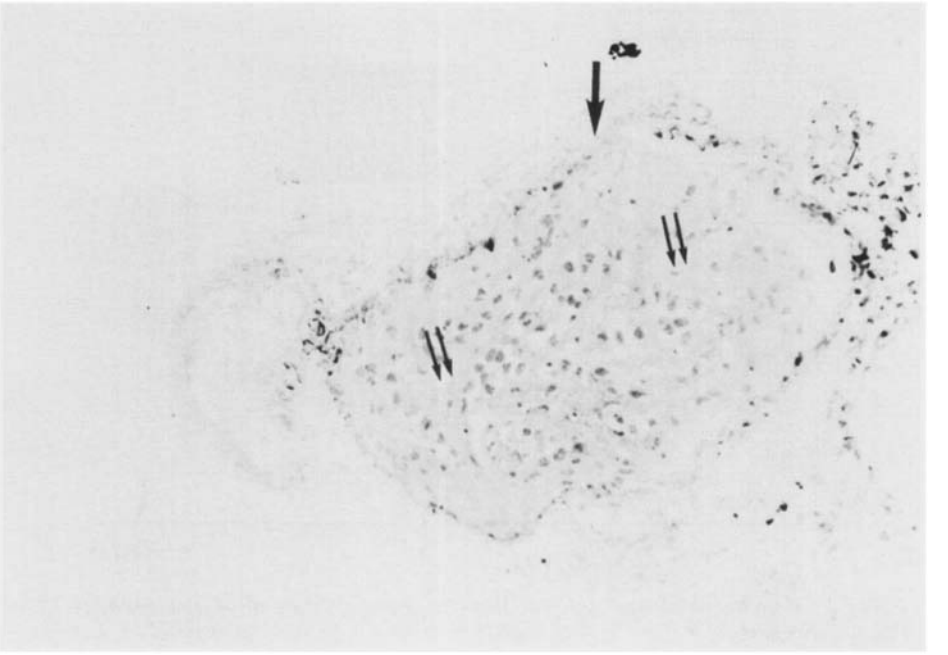

FIGURE 1b Part of capillary tuft of a hagfish glomerulus after ADR injection (5 mg/kg b.w.). A decreased amount of reaction product can be observed compared to Fig. 1a on both the Bowman's capsule and capillary walls. Magnification, × 320.

could be observed in glomerular ATP-ase activity after injection of 5 mg/kg ADR (Figure 1a, b).

The sulphydryl donor NAC had different effects on φ and k. The hydraulic conductivity, significantly ($p < 0.05$ against controls) reduced by 20 mg/kg ADR, did not differ from controls after a combined treatment of ADR plus NAC. The increase in albumin permeability, however, was not affected by NAC (increase of 167% and 134% after treatment with ADR or ADR + NAC, respectively. $p < 0.05$ against controls).

Halondialdehyde, a final product of the peroxidation of unsaturated fatty acids, was measured to determine the time course of lipid peroxide levels in different tissues of hagfish treated with 20 mg/kg ADR (Figure 2). The initial lipid peroxide concentrations, highest in the liver, increased after injection of ADR in all experimental animals. In the liver the maximal increase of 17% had occured by day 2. The lipid peroxide levels in kidney and heart were increased by 79% and 33%, respectively, on day 5.

After 10 days of ADR-treatment, hagfish revealed severe circulation disorders with intravasal thrombi, while O_2-exposed controls were in good condition. To examine a possible correlation between the concentration of ADR in different tissues and its cytotoxic activity ADR was measured 12 h after the injection of 7.5 mg/kg (Table 2). While an accumulation of ADR was found in the liver, there was no significant difference between the ADR concentrations in kidney and heart. However, the

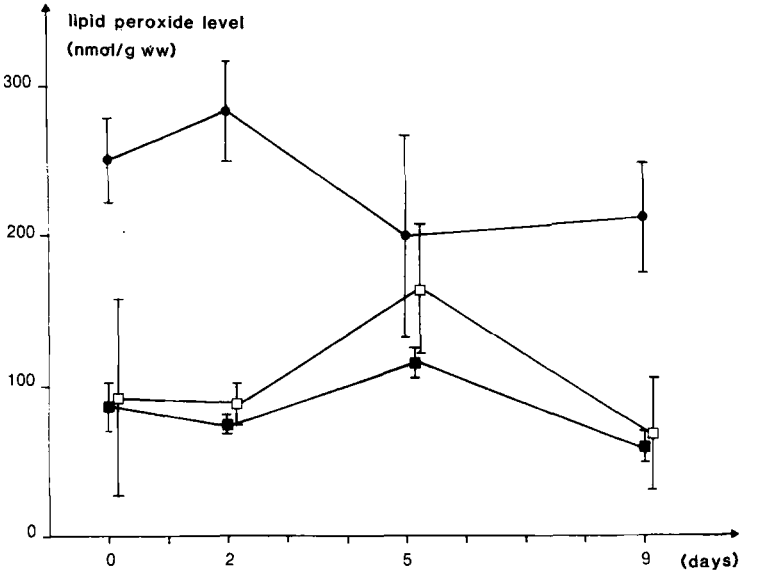

FIGURE 2 Time course of lipid peroxide levels in different tissues of Myxine glutinosa following injection of 20 mg/kg ADR. liver, kidney, heart. $\bar{x} \pm$ S.D., n = 94, 5, 6, on days 0, 2, 5, 9, respectively. ●—●, liver; □—□, kidney; ■—■, heart.

concentrations of the intracellular antioxidant GSH was remarkably low in the heart of *Myxine* as compared to other tissues (Table 3). Generally, the GSH concentrations in hagfish tissues were at the most half that in rat tissues (data not shown).

DISCUSSION

The mechanisms of ADR nephrotoxicity were investigated with respect to the involvement of oxygen free radicals. The present study focuses on histochemical (glomerular ATP-ase) and functional (glomerular permeability for water and protein) parameters of the glomerular filtration barrier of the Atlantic hagfish (Myxine glutimosa).

Because of its excellent accessibility the model of the isolated perfused glomerulus of Myxine glutinosa has been developed to study the acute effect of toxic compounds on single glomeruli.[8] The similarities between hagfish kidneys and those of higher

TABLE II
ADR concentrations (μg/g wet weight) in different tissues of Myxine 12 h after i.v. injection of 7.5 mg/kg b.w.

liver	kidney	heart	intestine
64.1 ± 27.3 (5)	18.1 ± 6.6 (6)	23.1 ± 1.7 (5)	6.3 ± 2.3 (6)

$\bar{x} \pm$ S.D., (n).

TABLE III
Glutathione concentrations (μmol/g wet weight) in different tissues of Myxine glutinosa

liver	kidney	heart
2.89 ± 0.93 (12)	1.58 ± 0.60 (12)	0.60 ± 0.24 (12)

x̄ ± S.D., (n).

vertebrates as revealed by morphological and electron microscopical studies[6,7] are further confirmed by enzyme histochemistry.

The enzyme ATP-ase is an intrinsic component of the glomerular basement membrane (GBM) of the rat kidney as demonstrated by enzyme histochemical methods at the light- and electron microscopical level.[14] The present study demonstrates ATP-ase activity in glomeruli of *Myxine glutinosa* as well by using enzyme histochemistry at the light microscopical level, and demonstrates further a reduced activity in ADR treated hagfish. This effect of ADR has also been observed by Bakker *et al.* within 48 h after ADR injection in rats,[11] showing glomerular ATP-ase activity as an early marker of ADR toxicity. ADR induced as well as by other O_2-generating systems (i.e. X-irradiation) induced inactivation of glomerular ATP-ase could be prevented by the O_2-scavenger superoxide dismutase suggesting that this early effect of ADR is mediated by oxygen free radicals.[20]

The observed intravascular thrombi in hagfish following ADR treatment is in line with the thrombotic tendency observed in rats following ADR treatment and suggests a major antithrombotic role for particular enzyme activities within the glomerular nucleoside polyphosphatase complex.[21]

With the experimental protocol used the hagfish showed no signs that ADR causes a reduced renal blood flow by affecting the renal vasculature. It is therefore assumed that ADR impairs the hydraulic conductivity (k) and protein permeability (sieving coefficient, φ) of the glomerular barrier. This is in accordance with the reduction of GFR and the increase of proteinuria found in ADR-nephrotic rats.[22]

Following the administration of 5 mg/kg ADR the hydraulic conductivity was significantly reduced in experimental hagfish exposed to 80% O_2, while animals under normoxic conditions did not differ from controls. This oxidative component of ADR-induced cytotoxicity is also reported for cardiac myocyte cultures, where lipid peroxidation could be detected only when the cells were exposed to ADR and hyperoxia.[23]

The injection of 20 mg/kg ADR resulted in a significant reduction of k already under normoxic conditions. This reduction could be prevented by the sulfhydryl donor NAC, known to be very reactive towards free radicals through non-enzymatic donation of hydrogen atoms.[9] These data indicate the involvement of oxygen free radicals in the early lesions of the final water barrier, suggested to be the epithelium. A probable damage of the epithelial membrane by lipid peroxidation is compatible with morphological studies of rodent kidneys, showing an ADR-induced loss of foot processes and replacement by epithelial cytoplasm.[24]

An oxidative stress on membrane compounds is also consistent with the increase in lipid peroxide levels in different tissues of ADR-treated hagfish. The initial lipid peroxide concentration was highest in the liver as the main catabolic organ for xenobiotics, further increasing by 17% 2 days after ADR-injection. The percentage change from the control was most pronounced in the kidney, reaching a 79% increase

in lipid peroxide level by day 5. The liver may counteract a further increase in lipid peroxides by its higher content of intracellular GSH (Table 3). In contrast, the lipid peroxide levels in different tissues of the mouse uniformly peaked by day 4 of ADR-treatment.[10]

The increased protein permeability of the ADR-impaired IPSG is best explained with a sieving defect of the GBM. Former studies of ADR nephrosis in rats indicated that the functional charge barrier remained intact and proteinuria derived from a size selective defect of the GBM.[25] As the drug-induced disorders of protein permeability were independent of O_2-supply and were not prevented by NAC, the sieving defect may be not mediated by oxygen free radicals. Instead it is suggested that ADR directly interferes with the metabolism of basement membrane compounds. This is in accordance with a proliferation of mesangial matrix in ADR nephrosis,[24] possibly reflected by an impaired turnover of the extracellular matrix protein fibronectin in ADR-treated rats (Soose M., unpublished data).

After 10 days of ADR treatment hagfish, unlike rats, revealed severe circulation disorders, which were not due to an accumulation of the drug in the heart. However, the GSH concentation found to be at the most half that of rat tissues, were extremely low in the heart of *Myxine*. This lack of intracellular antioxidant suggests that the cardiotoxicity of ADR in *Myxine* may be the consequence of oxidative stress, as is the most likely reason for cardiotoxicity in humans.[9] Presumably in both man and hagfish the cardiotoxicity is the limiting factor in the administration of ADR, nephrotoxicity becoming manifested by extending ADR concentrations beyond the cumulative cardiotoxic dose.[3] Though the GSH concentration in the hearts of rats was also lower as compared to kidney and liver (data not shown), the renal lesions were the early and more pronounced effects of ADR treatment in this species.[22] Possibly the renal blood flow of higher vertebrates exceeds that of poikilothermal *Myxine*, living in an environment of approximately 4°C. Alternatively, a minimal threshold of GSH, counteracting oxidative stress, can be assumed as previously reported for rat liver following GSH depletion *in vivo*.[26]

It is concluded that the early nephrotoxic effect of ADR, as reflected by a decreased ATP-ase activity, is mediated by free radical formation. Oxidative stress on membrane compounds seems to reduce the water permeability of the glomerular barrier, while the sieving defect for proteins is best explained by ADR-induced metabolic disturbances of structural proteins.

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