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Antiproteinuric Effect of Ciclosporin A in Adriamycin Nephropathy in Rats

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Abstract
Ciclosporin A (CsA) can reduce proteinuria in various forms of human and experimental glomerulopathies. This antiproteinuric effect can be the result of a decrease of immunological damage, a decrease in the glomerular filtration rate (GFR), or a change in the permselective properties of the glomerular capillary wall. In this study we investigated the effect of CsA on Adriamycin-induced nephropathy in rats. A single intravenous injection of Adriamycin (5 mg/kg body weight) induced a severe nephrotic syndrome with a massive albuminuria (± 400 mg/24 h from 3 weeks onwards) and a hypoalbuminemia (± 7 mg/ml after 5 weeks). The IgG/albumin selectivity index was 0.16 ± 0.05, indicating a preferential loss of albumin. A 5-day treatment with CsA reduced the albumin excretion by almost 50% (from 336 ± 91 to 178 ± 58 mg/24 h; p = 0.002) and induced an increase in the serum albumin level (from 7.1 ± 4.1 to 12.8 ± 3.2 mg/ml; p = 0.002) in contrast to the vehicle olive oil (OO). CsA also decreased the GFR by 40% (from 0.74 ± 0.11 to 0.41 ± 0.11 mg/ml/100 g body weight; p = 0.002). Albuminuria corrected for the GFR (fractional excretion of albumin, FE_{alb}) was still significantly lower in CsA-treated than in OO-treated animals (FE_{alb} CsA: 1.35 ± 0.88, FE_{alb} OO: 3.17 ± 2.29%; p = 0.0005). This suggests that other factors are also involved in the reduction of albuminuria. To exclude that CsA has an effect on the tubular reabsorption of albumin, we evaluated the blockade of the tubular reabsorption by lysine and found no difference in albuminuria between the CsA- and OO-treated groups. These experiments suggest that the antiproteinuric effect of CsA is not (only) due to a decrease in the GFR, but also to a decrease of the enhanced permeability of the glomerular capillary wall for albumin.

Introduction

The antiproteinuric effect of ciclosporin A (CsA) has been described in several studies, both in patients with various glomerular diseases and in experimental animal models [1, 2]. As CsA is a potent immunosuppressive drug, it is possible that this antiproteinuric effect is due to inhibition of the glomerular inflammatory process. However, although the immunosuppressive effect might contribute to the observed antiproteinuric effect, there are

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several arguments which indicate that other mechanisms are also involved, since CsA is also effective in nonimmune glomerular diseases like Alport syndrome [3]. The antiproteinuric effect of CsA could be due to the drug-induced reduction of the glomerular filtration rate (GFR), which leads to a decrease of the filtered load of protein. If this mechanism is responsible, the fractional excretion of albumin (FE_{alb}) should be identical before and after treatment. However, as we [4, 5] and others [3, 6] have shown, the FE_{alb} was significantly lower during CsA treatment, which suggests that the reduction in GFR is not solely responsible for the observed antiproteinuric effect. Furthermore, in patients with focal glomerulosclerosis and membranoproliferative glomerulonephritis, CsA induced a significant drop in GFR and effective renal plasma flow, but had no effect on proteinuria [6]. This discrepancy between the hemodynamic and antiproteinuric effects of CsA raises the possibility that CsA may influence the permselective properties of the glomerular capillary wall (GCW). Recently, we did indeed find in a passive model of murine anti-glomerular basement membrane nephritis that CsA still had an antiproteinuric effect after pharmacological prevention with phenoxybenzamine of the CsA-induced drop of the GFR [4]. This study underlines that the antiproteinuric effect of CsA might be due to mechanisms other than GFR reduction. The results of in vitro glomerular permeability studies also show an effect of CsA. Isolated glomeruli of animals treated with CsA for 2–3 weeks [7] or glomeruli exposed to CsA in vitro [8] displayed in vitro a significantly lower ultrafiltration coefficient K_f and a lower hydraulic conductivity L_p than control glomeruli. Theoretically, CsA could increase the tubular reabsorption of albumin, although this explanation is not very likely in the face of the known tubulotoxic effects of CsA.

One of the most widely accepted indications for CsA treatment in the nephrotic syndrome is minimal change disease (MCD). Proteinuria decreases after the introduction of CsA in the majority of patients with MCD [9–12]. It has been argued that CsA may be effective in MCD by inhibiting the production of certain cytokines [9] or circulating cations [13] which are thought to be responsible for disturbing the charge-selective permeability of the GCW. We decided to study the antiproteinuric effect of CsA in Adriamycin nephropathy (ADR-NP). Because of the disappearance of the glomerular polyanion, ADR-NP is, although not uniformly, regarded as an experimental model of MCD [14, 15]. A second argument to study CsA in ADR-NP is that this experimental model has a nonimmunological genesis. This circumvents possible effects of CsA on the evolution of the nephrotic syndrome. In this model we investigated in animals with a heavy and stable proteinuria the antiproteinuric effect of CsA, and we correlated this with the CsA-induced changes in GFR. To exclude the possibility that CsA influences the tubular reabsorption of albumin, we analyzed the effect of intravenously administered lysine on the albumin excretion in CsA-treated and control rats.

Materials and Methods

Animals

For all experiments we used male Wistar rats that were bred in our animal laboratory and weighed ± 200 g at the start of the experiments. The animals were fed standard food and tap water ad libitum.

Adriamycin Nephropathy

Adriamycin® (Adriablastina; Farmitalia, Milan, Italy) was administered as a single intravenous injection via the tail vein under ether anesthesia. In an initial experiment, the optimal dose of ADR was determined. To this end, either 2.5, 5, or 7.5 mg ADR/kg body weight (BW) was injected into 5 rats/group. This experiment revealed that with 5 or 7.5 mg ADR/kg BW a stable albuminuria was achieved 3 weeks after the injection, whereas after 2.5 mg/kg the ensuing albuminuria was less severe and still increasing after 3 weeks. Since a dose of 5 mg/kg was not associated with any direct morbidity or mortality, in contrast to the 7.5 mg/kg dose, the 5 mg/kg dose was adopted for all further experiments.

Urine and serum samples were collected every week for 5 weeks to determine albumin and IgG concentrations. Urinary protein excretion was measured in urine collected for 24 h in metabolic cages. Urine and serum concentrations of albumin were determined by rocket immunoelectrophoresis [16], with goat anti-rat albumin and rat albumin as a standard (both from Nordic, Tilburg, The Netherlands). Urine and serum IgG concentrations were determined by means of a capture ELISA as previously described [17]. From these data, the albumin clearance and the FE_{alb} (clearance of albumin/GFR) were calculated with standard formulas. The selectivity index of the proteinuria was calculated as clearance IgG/clearance albumin.

CsA Treatment

To assess the antiproteinuric effect of CsA (Sandoz Nederland, Uden, The Netherlands), rats injected with 5 mg/kg BW ADR on day 0 received either CsA (n = 10) or the solvent olive oil (OO; n = 10). CsA was orally administered once daily at a dose of 20 mg/kg BW in 0.5 ml OO. On day 28, 24-hour urine was collected, a serum sample was drawn, and the GFR (see below) was measured on day 29. Thereafter, CsA or OO treatment was started on day 30. During CsA treatment, urinary protein excretion was determined on day 34 and GFR on day 35. At the end of the experiment, the animals were sacrificed.

GFR was measured by the single-shot ^{51}Cr-EDTA technique. In brief, after an intravenous injection of 10 µCi ^{51}Cr-EDTA (Amersham, UK), a single timed (60 min) blood sample was obtained from the retro-orbital plexus under ether anesthesia. A calibrated 200-µl

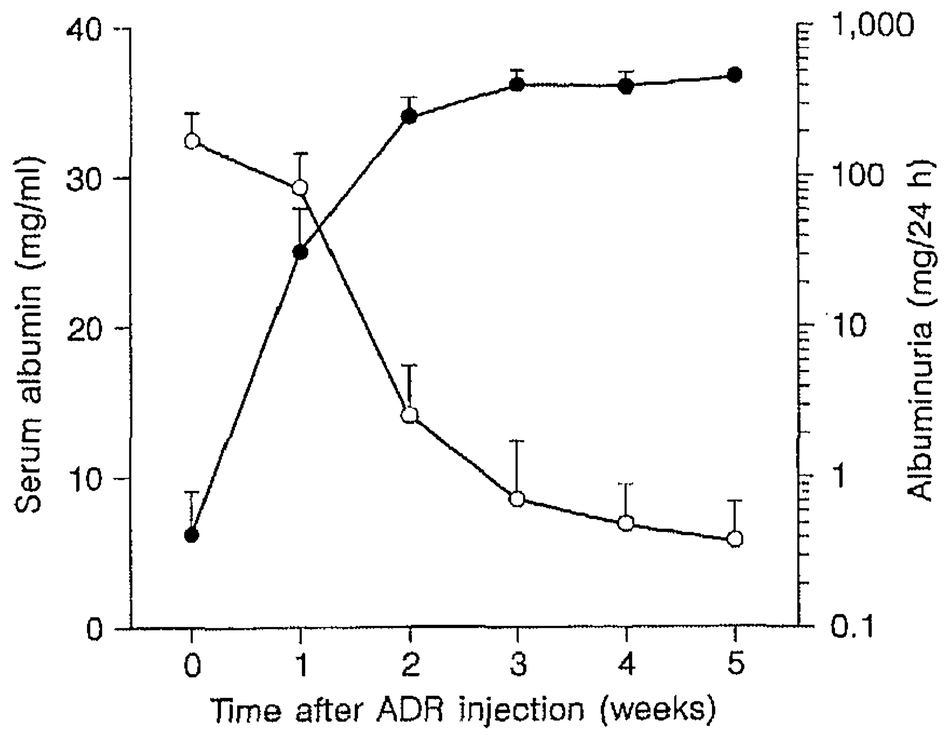

Fig. 1. Serum albumin and albuminuria over time in rats after a single intravenous injection of 5 mg ADR/kg BW. ○ = Serum albumin; ● = urinary albumin excretion.

plasma sample was counted in a gamma scintillation counter. GFR was calculated according to the formula: GFR = (V/t)·ln(P0/Pt), where V is the distribution volume of ^{51}Cr-EDTA (ml) and P0, Pt the plasma concentration of ^{51}Cr-EDTA at time zero and t min (cpm/ml). Pt is derived from the plasma sample at t = 60 min; P0 is calculated from P0 = I/V, where I is the injected amount of ^{51}Cr-EDTA (cpm), and V is calculated from the formula established by Provoost at al. [18]: V = (0.264·BW) – (1.92·10^{-4} BW^2) + 1.03.

To evaluate the effect of CsA on the tubular reabsorption of albumin, the effect of an intravenous bolus injection of lysine was investigated in a different group of animals. To this end, CsA or OO treatment (n = 10 for each group) was started on day 30 after injection of ADR and continued daily until the end of the experiment (day 36). On day 34 GFR was measured. On day 35 urinary albumin excretion was measured during 2 h, thereafter the animals received 600 mg/100 g BW lysine, and urinary albumin excretion was measured again for 2 h. To evaluate the effect of lysine on GFR, the next day the same dose of lysine and immediately thereafter ^{51}Cr-EDTA were administered to measure GFR.

Statistics

The Mann-Whitney U test was used for intergroup comparisons, and the Wilcoxon signed-rank test was used for intragroup comparisons. $p < 5\%$ was considered significant.

Results

Characteristics of ADR-NP

After an intravenous injection of 5 mg/kg BW ADR, a progressive albuminuria developed from 7 days onwards after the injection. At 3 weeks, this urinary albumin excretion stabilized at around 400 mg/24 h and thereafter remained at that level (fig. 1). Concomitant with the increase in urinary albumin excretion, there was a severe drop in serum albumin levels, reaching 5–7 mg/ml at 5 weeks. Despite this severe decrease in serum albumin concentration, there was an increasing clearance of albumin. To investigate whether this proteinuria was selective, we calculated the selectivity index of the proteinuria (clearance of IgG/clearance of albumin) at week 4. This revealed a selectivity index of 0.16 ± 0.05 (95% confidence interval 0.14–0.18), indicating a preferential urinary loss of albumin.

Antiproteinuric Effects of CsA

Treatment with CsA in the ADR model resulted in a significant decrease of albuminuria and an increase of serum albumin, in contrast to the OO-treated controls, in which these two parameters did not change (table 1). As expected, the GFR also decreased significantly in the CsA-treated animals, whereas in the OO-treated group the GFR remained stable. However, the increase in serum albumin together with the reduction in GFR cannot explain the reduction in albuminuria. When we calculated the albuminuria corrected for serum albumin and GFR (FE_{alb}), we still observed a significant decrease in the CsA-treated animals, whereas this parameter did not change in the OO-treated controls (table 1). If the GFR reduction was not (solely) responsible for the decrease in albuminuria, theoretically two other major mechanisms could be responsible for the antialbuminuric effect. CsA could either decrease the enhanced permeability of the GCW for proteins or increase the tubular reabsorption of albumin. Although this enhanced tubular reabsorption is unlikely in view of the known tubulotoxic effect of CsA,

Table 1. Effect of CsA or OO on albuminuria, serum albumin, GFR, and FE_{alb} in rats with ADR-NP

	CsA			OO		
	before	after	p	before	after	p
Albuminuria, mg/24 h	336±91	178±58	0.002	389±123	311±46	NS
Serum albumin, mg/ml	7.1±4.1	12.8±3.2	0.002	5.5±2.1	5.6±2.7	NS
GFR, ml/min/100 g BW	0.74±0.11	0.41±0.11	0.002	0.70±0.12	0.73±0.12	NS
FE_{alb}, %	2.43±1.26	1.35±0.88	0.002	4.06±3.29	3.17±2.29	NS

NS = Not significant.

we wanted to formally exclude this possibility. Therefore, we investigated whether the effect of blockade of the tubular albumin reabsorption by lysine differed between the two groups. We found no significant difference in the absolute amount of urinary albumin excretion before and after lysine infusion in both groups (table 2). However, lysine induced both in the CsA- and the OO-treated animals a significant decrease in the GFR and thereby in the filtered load of albumin. This lysine-induced decrease in GFR was also seen in control rats (table 2). Therefore, we calculated the ratio between the 2-hour excretion of albumin/GFR before and after lysine administration, assuming that the serum albumin concentration did not change during this short observation period. In CsA-treated animals, this ratio was 1.5 ± 0.6 and in the OO-treated rats 1.8 ± 0.9. From these ratios it is clear that lysine induced in both groups a significant increase of the FE_{alb} due to a blockade of tubular albumin reabsorption. This increase in the FE_{alb} was not statistically different between the two groups ($p = 0.6$).

Table 2. Effect of lysine on albuminuria, GFR, and albuminuria/GFR ratio in ADR-NP in rats treated with either CsA or OO

	Lysine		
	before	after	p
CsA			
Albuminuria, mg/2 h	11.2±3.9	11.3±3.0	NS
GFR, ml/min	0.89±0.20	0.64±0.20	0.012
Albuminuria/GFR ratio	13.3±4.9	20.0±3.8	0.03
Albuminuria/GFR ratio after/before lysine	1.5±0.6		–[a]
OO			
Albuminuria, mg/2 h	15.9±7.1	19.9±5.3	NS
GFR, ml/min	1.44±0.35	1.22±0.28	0.008
Albuminuria/GFR ratio	11.5±5.0	17.0±4.7	0.06
Albuminuria/GFR ratio after/before lysine	1.8±0.9		–[a]
Control rats			
GFR, ml/min	2.14±0.16	1.94±0.05	0.012

NS = Not significant.
[a] The ratio between CsA and OO is not significantly different.

Discussion

In this study, we investigated the effect of CsA on ADR-NP which is regarded as a model for human MCD. The injection of 5 mg ADR/kg BW in rats induced a severe nephrotic syndrome with a massive albuminuria and an evident hypalbuminemia which was also found in previous studies [14, 15]. In the literature several contradictory mechanisms have been proposed for the induction of this ADR-associated nephrotic syndrome, either a decrease in the charge-dependent permeability [14, 19, 20] or an increase of the size-dependent permeability of the GCW [15, 21] or a combination of both mechanisms [22]. In our study, we found a rather selective proteinuria which is more in line with a charge-dependent alteration. This is corroborated by our finding that the heparan sulfate staining of the glomerular basement membrane decreases by 60% over a period of 4 weeks, as we described previously [23]. With different but less specific approaches, a reduced glomerular polyanion has been found in ADR-NP before. In the first detailed description of ADR-NP [14], it was already reported that with the use of colloidal iron the glomerular polyanion disappeared quickly and was totally absent for at least 4 weeks. With polyethyleneimine as a probe, a 25% reduction in the number of heparan sulfate-associated sites was found in

the lamina rara externa of rats with ADR-NP [24], and with cationic colloidal gold a 60% reduction of anionic sites in the GBM of rats with ADR-NP was observed [25]. Benjelloun et al. [26] found a reduced number of total glycosaminoglycans in the glomeruli and an increased urinary excretion of both total glycosaminoglycans and of heparan sulfate. Since heparan sulfate is not only a determinant for the charge-dependent permeability, but also for the size-dependent permeability [27], these alterations might explain the existing discrepancies with regard to the pathophysiology of proteinuria in ADR-NP. To investigate whether other mechanism than the GFR reduction were responsible for the antiproteinuric effect of CsA, we evaluated the effect of blockade of the tubular albumin reabsorption by lysine. We found a similar change in albuminuria after lysine treatment between the CsA- and OO-treated groups. Although the GFR decreased, there was no difference in the ratio of the 2-hour excretion of albumin before and after treatment with lysine between the CsA- and OO-treated groups. Therefore, we were able to exclude that CsA has its effect through a change in tubular reabsorption of albumin.

These experiments suggest that the antiproteinuric effect of CsA is not (only) due to a decrease in the GFR, but also to a decrease of the enhanced permeability of the GCW for proteins. How can one envision this effect of CsA on the glomerular permeability? It is known that ADR can induce the formation of reactive oxygen species [28]. Indeed, in ADR-NP, the glomerular cells produce reactive oxygen species which cause glomerular injury, leading to proteinuria [29]. Also in other experimental models, such as Heymann nephritis and puromycin aminonucleoside-induced nephrosis, it is established that the proteinuria critically depends on the formation of reactive oxygen species [30–32]. The normalization of the glomerular permeability might be due to the fact that CsA is able to inhibit formation of reactive oxygen species [33].

In conclusion, our study suggests that the antiproteinuric effect of CsA in ADR-NP is due to both a decrease in GFR and a reduction of the enhanced permeability of the GCW for albumin.

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