Effects of anti-proteinuric therapy with angiotensin-converting-enzyme inhibition on renal protein catabolism in the adriamycin-induced nephrotic rat

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ABSTRACT

A direct consequence of glomerular protein leakage is an increased exposure of proximal tubular cells to proteins. The aim of the present study was to examine whether chronic proteinuria affects the tubular handling of proteins and whether anti-proteinuric therapy by angiotensin-converting-enzyme (ACE) inhibition restores this tubular function. Renal uptake and catabolic rate of the low-molecular-weight protein (LMWP) myoglobin was determined in anaesthetized control and adriamycin-induced nephrotic rats by external counting after radiolabelling. Proteinuria correlated with the uptake as well as the catabolism of myoglobin. The higher the proteinuria, the lower was the renal uptake of myoglobin \( r = 0.72, P = 0.002 \). Also, the catabolic rate of myoglobin \( r = 0.80, P = 0.0002 \) was lower with increasing severity of proteinuria. During treatment with the ACE inhibitor lisinopril, proteinuria was lowered by 79 ± 9% (mean ± S.E.M.). Renal uptake and catabolic rate of the LMWP were not restored by ACE inhibition. The catabolic rate of myoglobin was even decreased further with 48 ± 5% compared with pretreatment levels. In summary, adriamycin-induced proteinuria is associated with a lower uptake and a lower catabolic rate of LMWP by external counting after radiolabelling. Proteinuria correlated with the uptake as well as the catabolism of myoglobin. The higher the proteinuria, the lower was the renal uptake of myoglobin \( r = 0.72, P = 0.002 \). Also, the catabolic rate of myoglobin \( r = 0.80, P = 0.0002 \) was lower with increasing severity of proteinuria. During treatment with the ACE inhibitor lisinopril, proteinuria was lowered by 79 ± 9% (mean ± S.E.M.). Renal uptake and catabolic rate of the LMWP were not restored by ACE inhibition. The catabolic rate of myoglobin was even decreased further with 48 ± 5% compared with pretreatment levels. In summary, adriamycin-induced proteinuria is associated with a lower uptake and a lower catabolic rate of LMWP by external counting after radiolabelling. 

INTRODUCTION

Proteins that are filtered in the glomerulus are reabsorbed by the proximal tubular cell and subsequently catabolized in the intracellular lysosomes [1,2]. Under normal physiological circumstances, mainly low-molecular-weight proteins (LMWPs) are handled by this system. During glomerular disease, however, larger proteins, such as albumin, leak through the glomerular filter, leading to an increased burden for the proximal tubular system.

Key words: adriamycin-induced nephrotic rat, angiotensin-converting-enzyme (ACE) inhibition, low-molecular-weight protein, protein catabolism, proteinuria.

Abbreviations: ACE, angiotensin-converting enzyme; LMWP, low-molecular-weight protein; NAG, N-acetyl glucosaminidase.

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This tubular protein overload affects the physiological state of the tubular cell and could well be the pathophysiological link between glomerular protein leakage and tubulointerstitial damage, leading to progressive renal function loss [3,4].

Angiotensin-converting-enzyme (ACE) inhibitors and angiotensin-II receptor antagonists decrease proteinuria and result in renal protection [5–7]. The mechanism by which this renal protection is afforded is still under debate. Certainly, the decrease in proteinuria could be involved directly [8,9], since it decreases the protein burden of the proximal tubular cells. However, protection may also be a direct result of interference in tubular function of protein uptake and/or protein catabolism.

The aim of the present study was to examine in vivo whether chronic experimental proteinuria changes the function of the proximal tubule both in uptake and/or tubular rate of catabolism of LMWPs, and whether ACE inhibition restores these functional tubular alterations. Adriamycin-induced nephrosis in the rat was chosen as a model of stable proteinuria with a wide range of protein excretion. The model is typical for proteinuria-induced focal sclerosis and is very well suited for the study of the effects of ACE inhibition, since proteinuria, glomerulosclerosis and interstitial fibrosis are effectively decreased [10]. Myoglobin was chosen as the study LMWP, because its tubular handling is at the same tubular section as that of the main leaking plasma proteins such as albumin [11–14]. For the determination of renal uptake and catabolism, a technique was used in which the renal handling of radiolabelled myoglobin was monitored by external counting in the intact rat [15,16]. Tubular toxicity was measured by monitoring the urinary excretion of N-acetyl glucosaminidase (NAG).

MATERIALS AND METHODS

Materials
Myoglobin (horse heart; molecular mass 17.8 kDa and pI 7.3) was obtained from Sigma (Axel, The Netherlands). Adriamycin hydrochloride (2 mg/ml injection solution) was purchased from Farmitalia Carlo Erba (Brussels, Belgium). Lisinopril was obtained from Merck, Sharp and Dohme (Rahway, NJ, U.S.A.).

Experimental set-up
The protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Committee for Animal Experiments of the State University of Groningen.

In the protocol for study 1, male Wistar rats (Harlan, Zeist, The Netherlands) weighing approx. 250 g were kept on a low-sodium diet (0.05% NaCl and 20% protein; Hope Farms, Woerden, The Netherlands). Eight rats were anaesthetized with isoflurane (2% in O₂, 500 ml/min) and injected with 1.5 mg/kg adriamycin via the dorsal penal vein to induce proteinuria. Six rats did not receive adriamycin and were used as non-proteinuric controls. Once a week, 24 h urine samples were collected to measure the course of proteinuria and NAG excretion. At weeks 3 and 5 after induction of proteinuria, the renal handling of myoglobin was studied. In the six non-proteinuric controls, the renal handling of myoglobin was studied once.

In the protocol for study 2, the adriamycin-induced nephrotic rats from study 1 were treated with the ACE inhibitor lisinopril (75 mg/l of drinking water) for 3 weeks, starting at week 7 after induction of proteinuria. The effect of ACE inhibition on proteinuria, NAG excretion and the renal handling of myoglobin was determined by comparing the effects before therapy (at week 5 after induction of proteinuria), after 3 weeks of lisinopril therapy (at week 10) and 3 weeks after withdrawal of therapy (at week 13).

LMWP myoglobin was radiolabelled and the renal handling studied by external counting using a γ-camera. γ-Camera studies were carried out as described previously [13]. Briefly, during anaesthesia (isoflurane: 2% in O₂, 500 ml/min), the rats were fixed on a low-energy collimator. A window was selected on the energy peak of 123I with a width of 250 keV. Body temperature was monitored and maintained on 37°C with a heat pad and a lamp. After stabilization for 10 min, approx. 2 MBq of 123I-labelled LMWP was injected within 15 s via the dorsal penal vein. The γ-camera recorded the radioactivity in 1 min frames for 1 h. The radioactivity time course of the right kidney, urinary bladder and total body was plotted after analysis of the respective regions of interest. Data on the left kidney were not analysed, because of cumulated radioactivity in the stomach region possibly due to accumulation of free iodine [17]. Because of that, the amount of radioactivity in the right kidney was multiplied by two to determine the renal amount of tracer. The amount of tracer in the right kidney and urinary bladder were related to total body radioactivity as 100% of the injected dose, corrected for counting efficiency [15].

Radiolabelling
Labelling of LMWP with 123I was performed by the chloramine-T method of Hunter and Greenwood [18]. Briefly, 50 μl of protein (10 mg/ml) and 10 μl of chloramine-T (5 mg/ml) were added to 40 MBq (myoglobin) Na123I in 100 μl of sodium phosphate buffer (0.2 M, pH 7.4) and mixed for 60 s. The reaction was stopped by addition of 25 μl of sodium metabisulphite (2.5 mg/ml). Free iodine was removed by separation on a Sephadex-G25 column. The labelling efficiency was 42%. The protein fraction was kept on ice until use. At the start and end of the study day, 81% and 75% of...
Angiotensin-converting-enzyme inhibition and tubular protein catabolism

Figure 1  Typical example of time-curve of radioactivity in the right kidney
After an intravenous injection of $^{123}$I-labelled myoglobin, the amount of radioactivity in the kidney increases due to uptake of the radiolabelled protein. After reaching maximal uptake, the amount of radioactivity declines due to catabolism of the protein, followed by excretion of the radioactive breakdown products.

$^{123}$I respectively, was bound to the protein. All rats were randomly studied during a single day.

Urinary protein and NAG
Urinary protein concentration was measured by the Biuret method [18a]. Urinary NAG concentration was measured as described by Tucker [19].

Data analyses
Figure 1 shows a typical example of a time course of radioactivity in the right kidney after administration of $^{123}$I-labelled myoglobin. Renal uptake of the $^{123}$I-labelled LMPW was defined as the percentage of administered radioactivity that is present in the two kidneys (actually, the right kidney value was multiplied by two) at the time that the amount of radioactivity in the right kidney is maximal. Rate of renal catabolism of the $^{123}$I-labelled LMPW is expressed in fraction/min, and is calculated from the difference in renal radioactivity between the time of maximal amount of radioactivity in the kidneys and 30 min later. These arbitrary values were used instead of the ‘real’ values obtained by curve-fitting, because of the good correlation between the two methods while the determination of the arbitrary data was more accurate.

The individual data from weeks 3 and 5 were handled as independent values for inter-individual comparison in study 1 (each rat was thus included twice).

The relationship between the degree of proteinuria and renal uptake or renal rate of catabolism was analysed by linear regression.

Early excretion of radioactivity into the urinary bladder was indicated from the amount of radioactivity in the urinary bladder in the first 10 min after injection of the radiolabelled myoglobin intravenously.

All comparisons were made using the Student’s $t$ test. A paired analysis was used when individual changes were compared. Data are presented as means ± S.E.M.

RESULTS

Pretreatment

Proteinuria
Adriamycin induced chronic proteinuria that developed gradually and stabilized after 5 weeks. At week 5, the proteinuria ranged between 140 and 1010 mg·day$^{-1}$, with a mean of 560 ± 113 mg·day$^{-1}$.

Amount of renal LMWP uptake
In the non-proteinuric controls, 62 ± 2% of the injected dose of myoglobin was taken up by the kidney. In the proteinuric rats, the amount of myoglobin uptake was not significantly different from the non-proteinuric control values, with an average of 58 ± 2% of the injected dose. However, the severity of proteinuria correlated significantly with the amount of myoglobin uptake (Figure 2). The higher the proteinuria, the lower was the renal uptake of myoglobin ($r = 0.72$, $P = 0.002$). Additionally, the data suggest that tubular protein uptake
of myoglobin is determined by a proteinuria threshold of approx. 600 mg · day⁻¹. Above this proteinuria, the renal uptake of myoglobin was definitely affected.

Another indication of a threshold in tubular uptake was obtained by comparison of the amount of radioactivity, presumably intact myoglobin, excreted into the urinary bladder in the first 10 min after myoglobulin administration. In the first 10 min, only 1.7 ± 0.5 % of the radioactivity was excreted in the group with a proteinuria of less than 600 mg · day⁻¹, whereas 4.3 ± 0.7 % was recovered in the group with a proteinuria of more than 600 mg · day⁻¹ (P = 0.01).

Renal LMWP catabolism
In the non-proteinuric controls, 0.0160 ± 0.0007 min⁻¹ of myoglobin was catabolized by the kidney. In the proteinuric rats, the rate of myoglobin catabolism was 19 % lower (0.013 ± 0.001 min⁻¹; no significant difference) compared with that of the non-proteinuric control.

Interestingly, the severity of proteinuria inversely correlated strongly with the rate of myoglobin catabolism (Figure 3): the higher the proteinuria, the lower the catabolic rate of myoglobin was (r = 0.80, P = 0.0002).

NAG excretion
NAG is an enzyme located in the lysosomes of the proximal tubular cells. Since it is recovered in the urine in case of disruption of the cells, NAG excretion is used as a marker for tubular toxicity. In the non-proteinuric controls, NAG excretion was 0.6 ± 0.1 µmol/24 h. In the adriamycin-induced nephrotic rats, NAG excretion rose steadily in parallel with proteinuria and stabilized after 5 weeks (from 1.0 ± 0.2 µmol/24 h at week 1 to 2.8 ± 1.0 µmol/24 h at week 5; n = 16). Proteinuria and NAG excretion at week 5 was significantly correlated (r = 0.76, P = 0.002). Like proteinuria, NAG excretion was also negatively correlated with the uptake and catabolism of myoglobin (r = 0.71, P = 0.003, and r = 0.80, P = 0.0002 respectively).

Treatment

Proteinuria
Figure 4 shows the individual data for proteinuria before treatment (at week 5 after proteinuria induction), after 3 weeks of lisinopril therapy and 3 weeks after withdrawal of therapy. Proteinuria was decreased effectively by oral lisinopril therapy. The degree of decrease in proteinuria by lisinopril treatment was −80 ± 9 % (range −20 % to −95 %). Three weeks after withdrawal of therapy, proteinuria returned to pretreatment levels (89 ± 7 % of pretreatment level).

Renal LMWP uptake
Lisinopril treatment did not affect the amount of myoglobin that was taken up by the kidney. Mean renal uptake of myoglobin was 56 ± 2 %, 59 ± 2 % and 59 ± 3 % before, during and after withdrawal of treatment respectively. During treatment, the residual amount of proteinuria did not correlate with the renal uptake of myoglobin.
Figure 5  Effect of ACE inhibition on rate of renal catabolism of myoglobin
Pre, 5 weeks after induction of proteinuria; ACE inhibition, after 3 weeks of treatment and 10 weeks after proteinuria induction; post, 3 weeks after removal of treatment, 13 weeks after induction of proteinuria. Data points are from individual rats.

Renal LMWP catabolism
Since a higher proteinuria was associated with a lower catabolic rate of myoglobin (Figure 5), we speculated its catabolic rate to be increased during anti-proteinuric therapy. However, despite an 80 % decrease in proteinuria, the renal catabolic rate of myoglobin did not increase during lisinopril treatment. Surprisingly, the catabolic rate was even decreased further (Figure 5). During lisinopril treatment, the rate of myoglobin catabolism was decreased further by 48 $\pm$ 5 % ($P = 0.0005$ compared with pretreatment) with a range between 25 % and 64 %. Three weeks after withdrawal of therapy, the rate of catabolism returned to 89 $\pm$ 5 % of pretreatment levels (no significant difference compared with pretreatment). During treatment, the residual amount of proteinuria and renal rate of myoglobin catabolism remained negatively correlated ($r = 0.77$, $P = 0.02$).

NAG excretion
In contrast with the tubular catabolic function of the proximal tubular cell, lisinopril affected NAG excretion as expected. As higher proteinuria was associated with an increased NAG excretion, NAG excretion was expected to be decreased during anti-proteinuric therapy. Indeed, NAG excretion was lowered by lisinopril to 29 $\pm$ 16 %, whereas withdrawal of therapy resulted in a return of NAG excretion to 102 $\pm$ 23 % of pretreatment levels. To determine whether the effect of lisinopril on tubular protein catabolism was someway reflected in NAG excretion, the degree of NAG excretion during lisinopril treatment was compared with that in the pretreatment period. There was no significant difference in NAG excretion between pretreatment and lisinopril treatment ($0.7 \pm 0.1 \mu \text{mol}/24 \text{ h}$ for pretreatment and $0.8 \pm 0.1 \mu \text{mol}/24 \text{ h}$ during lisinopril) in urine samples, with a comparable low degree of proteinuria ($59 \pm 8 \text{ mg}/24 \text{ h}$ during lisinopril and $58 \pm 6 \text{ mg}/24 \text{ h}$ during pretreatment).

DISCUSSION
The present study shows the effect of proteinuria and decrease in proteinuria by ACE inhibition on the proximal tubular function of protein uptake and catabolism. The study shows that proteinuria is associated with a decreased renal uptake and catabolic rate of the LMWP myoglobin, indicating a damaging effect of filtered proteins on the proximal tubules. Surprisingly, ACE inhibition did not restore these tubular functions in spite of an effectively decreased proteinuria. In fact, the catabolic rate of the LMWP was even decreased further.

The validity of the results in the present study depends heavily on the method used for measuring tubular protein uptake and breakdown function. Several different methods [20–22] are available each with their advantages and disadvantages, as discussed previously [15,16]. We developed a technique based on that reported previously by Bianchi et al. [23]. By external counting, the renal time course of radioactivity was monitored after intravenous injection of a LMWP labelled with $^{123}$I. In two previous reports [15,16], the method has been extensively validated, showing that both uptake and catabolism are qualitatively and quantitatively reproducible and accurate.

In agreement with an experimental [24] and clinical [25] study, uptake of the radiolabelled LMWP myoglobin appeared to be minimally affected by the degree of proteinuria. If anything, it was affected at high levels of proteinuria. This could be due to a phenomenon of tubular threshold, indicating that the proximal tubule can reabsorb a certain amount of protein without affecting its uptake function. Above a certain threshold, the proximal tubule starts losing its full capacity. In agreement with this, the amount of radioactivity in the urinary bladder within the first 10 min after protein administration, presumably intact myoglobin, was higher during proteinuria. These findings are certainly compatible with the tubular uptake function for other proteins [1,12,24]. However, our data should be confirmed and explored further before a definite conclusion can be made.

With respect to renal LMWP catabolism, in patients with normal renal function but heavy proteinuria, LMWP catabolism appeared to be higher compared with
non-proteinuric patients with normal renal function [25]. On the other hand, in patients with chronic renal failure, LMWP catabolism was lower than in proteinuric patients with normal renal function [26]. Rustom et al. [25] explained these differences in metabolic state by a difference in the amount of functional tubular cells. Whereas LMWP catabolism in patients with chronic renal failure was lower, correction for glomerular filtration loss yielded values even higher than in proteinuric patients with normal renal function [26]. In our experimental model of proteinuria, we observed that the higher the adriamycin-induced proteinuria, the lower the rate of LMWP catabolism was. The present study is the first report showing a correlation between the degree of proteinuria and the LMWP catabolic rate in the kidney.

Which factors could be responsible for a proteinuria-associated decrease in renal catabolism of the LMWP as found in the present study? Firstly, LMWP catabolism could be decreased through competition of the LMWP with plasma proteins for the intracellular trafficking system of the proximal tubular cell. During proteinuria, an increased amount of plasma proteins leak through the glomerular filter and are taken up by the proximal tubular cell. As a result, the endosomal trafficking system in the proximal tubular cell becomes saturated, as indicated from histological studies showing protein droplets in the cytosol of proximal tubular cells of adriamycin-induced nephrotic rats [27]. Since the LMWP traffics through the same endosomal system, the rate of LMWP trafficking into the proteolytically active lysosomes will also be affected and, consequently, the catabolic rate of the LMWP will be decreased. Secondly, the overall LMWP catabolism in the kidney could be decreased through death of nephrons or individual tubular cells, as indicated from the urinary excretion of the tubular marker of toxicity NAG during proteinuria. However, the degree of damage must be rather low because, in the adriamycin model, glomerular filtration was unaffected at the time of our measurements. This could be concluded from the fact that myoglobin is solely excreted into the kidney via glomerular filtration, together with the observation that the initial renal uptake of myoglobin was rapid in all rats independent of the degree of proteinuria.

Next, we questioned whether anti-proteinuric therapy by ACE inhibition restores these functional alterations of the proximal tubular cells. In patients with proteinuria, ACE inhibition does not affect tubular LMWP uptake [28]. In the present study, we also did not find any effect of lisinopril on the amount of LMWP taken up by the tubules, despite the fact that proteinuria was decreased effectively. These data indicate that tubular LMWP uptake is only affected by severe proteinuria and that ACE inhibition does not have a pronounced direct effect on tubular LMWP uptake.

The catabolic function of the proximal tubular tubule was not restored by ACE inhibition, despite a 90% decrease in proteinuria. In fact, the catabolic rate was even decreased further. The residual amount of proteinuria and the rate of LMWP catabolism remained negatively correlated, indicating that proteinuria and LMWP catabolism are indeed causally related.

How can a further decrease in LMWP catabolism by ACE inhibition be explained? Firstly, through a decrease in proteinuria, ACE inhibition may abolish hypermetabolism in the remaining functional tubular cells, which will be reflected in an overall decrease in myoglobin catabolism [13,14,29]. However, it seems rather unlikely that this effect fully explains the large decrease of 45% in catabolic rate during ACE inhibition. Therefore we speculate that, additive to that, ACE inhibition has a direct inhibiting effect on protein metabolism in the proximal tubular cell. This idea is strengthened by the observation that, in healthy rats, the catabolic rate was also lower during lisinopril treatment (results not shown). In the proteinuric situation, lisinopril may affect protein metabolism via inhibition of brush-border ACE, since brush-border ACE (and angiotensinogen) is up-regulated during proteinuria [30]. Alternatively, lisinopril may exhibit a direct inhibiting effect on protein catabolism in the lysosomes. In the present study, a direct tubular involvement of ACE inhibition could not be confirmed by an additive effect of the treatment on tubular toxicity, since ACE inhibition decreased both proteinuria and NAG excretion effectively to control levels. Further studies are required to study these hypotheses.

In summary, adriamycin-induced proteinuria is associated with an increased NAG excretion and a lower uptake and a lower catabolic rate of LMWP in the proximal tubule. ACE inhibition lowers proteinuria and NAG excretion, but does not restore the affected LMWP uptake and rate of catabolism. The rate of LMWP catabolism is even decreased further. Taken together, the data suggest that proteinuria is toxic for the proximal tubular cells and that ACE inhibition protects the remaining functional tubular cells directly against destruction through decreasing hypercatabolism.

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