Vascular endothelial and myogenic function in renal disease

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Vascular endothelial and myogenic function in renal disease:

Focus on individual susceptibility to organ damage

Peter Ochodnický

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The cover photo depicts a bunch of individual kidney beans, symbolizing human kidneys coming from various individuals. Although unrecognizable from one another at a first glance, they are not the same. As this work suggests, individual kidneys differ in their susceptibility to chronic renal impairment.

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Chapter 1

General introduction and aims of the thesis

Introduction

In recent decades, cardiovascular medicine has witnessed a tremendous progress in understanding the mechanisms governing the regulation of vascular tone. Moreover, by discovering the crucial role of the endothelium in vasomotor control[1], the concept of endothelial dysfunction has been defined and the involvement of vasomotor mechanisms in the pathophysiology of cardiovascular disease and end-organ damage has been proposed[2-4]. Conditions characterized by chronic end-organ failure, such as cardiac or renal failure, are no more regarded as diseases of isolated organs, but rather as syndromes associated with multiple vascular changes. However, the role of peripheral and intraorgan vascular dysfunction in the initiation and progression of end-organ damage is far from being understood. Endothelial dysfunction has been shown to occur early in the course of cardiovascular disease and proposed to predict cardiovascular outcome[5-7]. This suggests that vascular dysfunction might be crucially involved in the development of end-organ damage. This thesis aims to investigate the role of intraorgan and peripheral vasoreactivity as a determinant of renal end-organ damage potentially representing novel renoprotective therapeutic strategy.

Vascular tone regulation

Small arteries (diameter < 500 μm) crucially regulate organ blood supply and are responsible for a major portion of the vascular resistance[8,9]. Vascular tone of small arteries is controlled by nervous, hormonal and local mechanisms. Reactivity of a given vascular bed is the result of interplay between vascular smooth muscle cells (VSMC) and locally produced endothelium-derived mediators. Pressurized vessels exhibit constrictive response against the intraluminal pressure, which determines the level of basal vascular tone. This reaction, termed myogenic response[10], is an intrinsic property of VSMC. VSMC sense mechanical stretch, which leads to the depolarization of the cell membrane, opening of voltage-sensitive Ca^{2+} channels, subsequent Ca^{2+} influx, and activation of the contractile apparatus. On top of the basal vascular tone, determined by myogenic reactivity, vascular diameter is controlled by additional local constrictive and dilatory mechanisms. Endothelium, an inner lining of the vessel is of major importance in the regulation of vascular tone. By releasing several vasoconstrictive and dilative substances (see **Chapter 2** of this thesis for details), it modulates the tone of the underlying VSMC. Endothelium-derived relaxing factors include cyclooxygenase (COX)-derived vasodilatory prostaglandins, nitric oxide synthase (NOS)-derived nitric oxide (NO), and the yet unidentified endothelium-derived hyperpolarizing factor (EDHF). Endothelial cells however also play an important role in multiple other processes, such as hemostasis, inflammation, permeability, and angiogenesis[11]. Improper functioning of endothelial regulation is reflected by altered release of endothelial vasodilative mediators and may be assessed by the vasodilatory response of the vessel to agonists, such as acetylcholine

(ACh). Impaired endothelium-mediated ACh-induced vasodilation is believed to be a marker of endothelial dysfunction, a condition associated with various aspects of cardiovascular disease[2,12]. Myogenic response and endothelium-mediated reactivity represent the principal local mechanisms controlling the tone of small arteries and as such are the major focus of investigation in the present thesis.

Myogenic response and endothelial function in small renal and mesenteric arteries

Myogenic reactivity sets the basal tone of vessels and thus co-determines the basal level of resistance in the vascular tree. It is pronounced in small arteries known to serve as resistance vessels, whereas mostly absent in large conduit vessels[10,13]. Small mesenteric artery, employed in our experiments, represents the prototype of a peripheral resistance artery. Increased peripheral resistance is a hallmark of several cardiovascular diseases, such as hypertension, cardiac or renal failure[14]. Several authors have proposed that excessive myogenic reactivity might be responsible for elevated peripheral resistance in spontaneous hypertension or heart failure, whereas not much is known about chronic renal failure[15,16]. However, in other organs such as the kidney and the brain, myogenic response may serve an additional function. In the kidney, myogenic reactivity of renal preglomerular arteries is responsible for renal autoregulation, a mechanism that keeps renal hemodynamics optimal under changes in systemic blood pressure. In particular, the glomerulus is protected from an increase in intraglomerular pressure and the induction of hyperfiltration[17]. Hyperfiltration represents one of the key events in the development of proteinuria and renal end-organ damage. Indeed, several lines of evidence suggest that myogenic reactivity of renal vessels may be impaired in chronic renal failure[18]. However, mechanisms underlying heterogeneous changes of myogenic tone between systemic and renal vasculature in chronic renal disease are not yet understood. Also, endothelial regulation may differ between intrarenal and extrarenal vascular beds. The contribution of endothelial mediators to endothelium-mediated vasodilation seems to be crucially dependent on location and the artery investigated. For instance, whereas in the large conduit arteries NO is the major endothelial vasodilator, in smaller vessels EDHF prevails[19,20]. Therefore, the physiological characteristics of vasomotor regulation in a given vascular bed might be important to define their role in the pathogenesis of cardiovascular and/or renal damage. Although heterogeneity in endothelial function between renal and systemic vessels may be anticipated, it has been proposed that the kidney can be regarded as an organ reflecting generalized vascular changes, such as in case of urinary leakage of small amounts of protein (microalbuminuria), as explained below.

Chronic kidney disease- a vasculopathic state

Chronic kidney disease (CKD) refers to a condition characterized either by a decline in glomerular filtration rate (GFR of less than 60 ml/min/1.73 m^2) or the presence of any other marker of renal damage, such as by histology confirmed renal injury or abnormal protein excretion[21]. The most evident outcome of CKD is chronic renal failure requiring treatment

by transplantation and/or dialysis. However, many more individuals suffer from early stages of CKD with only mildly decreased or even normal to increased GFR. Early and late stages of CKD are currently no longer regarded as a disease of an isolated organ, but rather a vasculopathic state with generalized vascular changes in multiple vascular beds. CKD is associated with increased prevalence of cardiovascular disease and in fact, patients with CKD have even higher chance to experience a cardiovascular event than to progress to renal failure[22]. Microalbuminuria predicts the rate of renal function decline as well as an increase in cardiovascular morbidity and mortality in several populations[23-26], giving rise to the hypothesis that excessive protein leakage in kidney is a reflection of generalized vascular or endothelial function[27]. Moreover, it suggests that vascular changes occur in early stages of the disease and might actively participate in the development of renal and cardiovascular end-organ damage. However, the mechanisms underlying the relation between renal and systemic vascular function and their role in CKD development remain incompletely characterized.

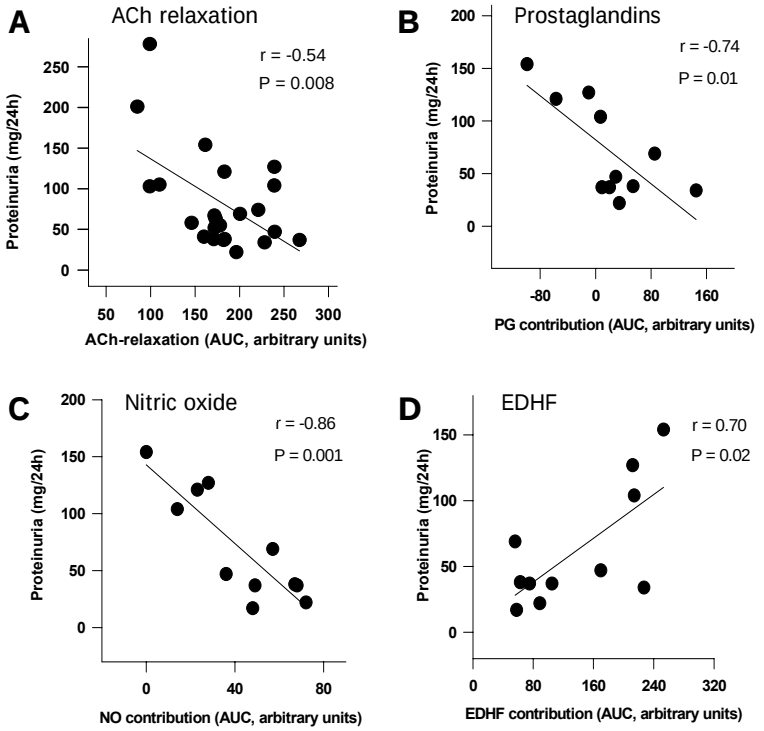

Figure 1. *Endothelial function of healthy rat predicts development of renal damage after 5/6 nephrectomy. Endothelium-dependent vasodilation to acetylcholine* ***(A)*** *and the contribution of endothelial vasodilatory mechanisms prostaglandins* ***(B)****, NO* ***(C)*** *and EDHF* ***(D)*** *measured in small renal arteries of healthy rats correlate with the severity of proteinuria after subsequent 5/6 nephrectomy. Adapted from*[28].

Predictive value of endothelial function in renal end-organ damage

The early occurrence of vascular dysfunction in the course of progressive renal disease leads to the question whether vascular function might condition the susceptibility of a healthy individual to renal damage. The severity of CKD varies considerably among patients with similar systemic risk factor profiles, such as hypertension and diabetes, and seems to be also dependent on intrinsic, probably genetically conditioned factors. Likewise, in experimental animal models of CKD, the individuals of outbred rat strains develop renal damage and renal function loss of highly variable severity after a relatively uniform injury, such as subtotal nephrectomy. In our laboratory, the hypothesis that variability in renal endothelial function among healthy animals might be responsible for the observed differences in susceptibility to end-organ damage was recently tested. To this end, endothelium-dependent vasodilation of small intrarenal arteries in vitro was measured in healthy rats, including the contribution of three principal endothelial dilatory mediators, e.g., NO, EDHF, and prostaglandins. Indeed, vascular function was remarkably variable among the individuals. Following these measurements, renal injury was induced by 5/6 nephrectomy. The endothelium-dependent vasodilation of small renal arteries predicted the subsequent development of end-organ damage, measured as proteinuria and decline in GFR. Thus, rats with more pronounced total endothelial relaxation, NO-mediated or prostaglandin-mediated vasodilation, were protected against the end-organ damage, whereas prevalence of EDHF was associated with worse renal outcome[28] *(Figure 1)*. These data suggest that variability in endothelial function among healthy individuals accounts for the differences in susceptibility to renal damage induced by a reduction in nephron number. However, it remains unknown, whether this prognostic value is specific for this particular type of renal injury or it might be extended to other forms of renal disease.

Aims of the thesis

The aim of this thesis was to investigate the role of vasomotor mechanisms in the development and progression of CKD and related systemic cardiovascular complications, providing novel potential therapeutic targets. More specifically, two principal mechanisms of vascular regulation in small arteries, namely myogenic and endothelium-mediated responses were tested in several experimental as well as spontaneous models of chronic renal disease. The following main research questions were addressed:

1. Does vascular function measured in the healthy individual (rat) predict the susceptibility of an individual to a renal insult?

2. If so, is this predictive value of vascular function dependent on the type of insult inflicted to the kidney?

3. Is renal vascular reactivity in CKD related to vasomotor function in peripheral vascular beds?

4. Does vascular function represent an early renal risk marker and a potential target for renoprotective preventive therapy?

Given the above, the following problems were studied in specific chapters of this thesis:
Chapter 2 summarizes the relation between microalbuminuria and endothelial function, providing a clinical basis for the experimental work in this thesis and suggesting potential preventive therapeutic strategies. **Chapters 3** and **4** investigate the hypothesis, that interindividual heterogeneity in renal endothelial function determines the susceptibility to experimentally-induced renal damage of various etiologies, namely myocardial infarction-induced and nephrotoxic renal damage. **Chapter 5** provides evidence of impaired renal vasomotor function in a model of spontaneous renal disease prior to the development of end-organ damage and explores the related vascular alterations in systemic vessels. **Chapter 6** addresses the role of peripheral myogenic and endothelial responses in a hypertensive CKD model. **Chapter 7** summarizes the current knowledge on renal and systemic endothelial changes in various stages of CKD, and provides experimental evidence for the predictive value of endothelial function with respect to the antiproteinuric therapeutic response by ACE inhibitors.

References

1. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288:373-376
2. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 2004; 15:1983-1992
3. Luscher TF, Yang ZH, Diederich D, Buhler FR. Endothelium-derived vasoactive substances: potential role in hypertension, atherosclerosis, and vascular occlusion. J Cardiovasc Pharmacol 1989; 14 Suppl 6:S63-S69
4. Vanhoutte PM. Endothelium and control of vascular function. State of the Art lecture. Hypertension 1989; 13:658-667
5. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 106:653-658
6. Heitzer T, Baldus S, von Kodolitsch Y, Rudolph V, Meinertz T. Systemic endothelial dysfunction as an early predictor of adverse outcome in heart failure. Arterioscler Thromb Vasc Biol 2005; 25:1174-1179
7. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroroberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial dysfunction in hypertensive patients. Circulation 2001; 104:191-196
8. Christensen KL, Mulvany MJ. Location of resistance arteries. J Vasc Res 2001; 38:1-12
9. Mulvany MJ, Aalkjaer C. Structure and function of small arteries. Physiol Rev 1990; 70:921-961
10. Davis MJ, Hill MA. Signaling mechanisms underlying the vascular myogenic response. Physiol Rev 1999; 79:387-423
11. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998; 91:3527-3561
12. Goligorsky MS. Endothelial cell dysfunction: can't live with it, how to live without it. Am J Physiol Renal Physiol 2005; 288:F871-F880
13. Davis MJ. Myogenic response gradient in an arteriolar network. Am J Physiol 1993; 264:H2168-H2179
14. Sleight P. Hemodynamics in hypertension and heart failure. Am J Med 1984; 76:3-13
15. Falcone JC, Granger HJ, Meininger GA. Enhanced myogenic activation in skeletal muscle arterioles from spontaneously hypertensive rats. Am J Physiol 1993; 265:H1847-H1855
16. Gschwend S, Henning RH, Pinto YM, de Zeeuw D, van Gilst WH, Buikema H. Myogenic constriction is increased in mesenteric resistance arteries from rats with

chronic heart failure: instantaneous counteraction by acute AT1 receptor blockade. Br J Pharmacol 2003; 139:1317-1325
17. Navar LG. Integrating multiple paracrine regulators of renal microvascular dynamics. Am J Physiol 1998; 274:F433-F444
18. Palmer BF. Impaired renal autoregulation: implications for the genesis of hypertension and hypertension-induced renal injury. Am J Med Sci 2001; 321:388-400
19. Nagao T, Illiano S, Vanhoutte PM. Heterogeneous distribution of endothelium-dependent relaxations resistant to NG-nitro-L-arginine in rats. Am J Physiol 1992; 263:H1090-H1094
20. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, Takeshita A. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. J Cardiovasc Pharmacol 1996; 28:703-711
21. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 2003; 139:137-147
22. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation 2003; 108:2154-2169
23. Hillege HL, Fidler V, Diercks GF, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, Gans RO, Janssen WM, Grobbee DE, de Jong PE. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. Circulation 2002; 106:1777-1782
24. Verhave JC, Gansevoort RT, Hillege HL, Bakker SJ, de Zeeuw D, de Jong PE. An elevated urinary albumin excretion predicts de novo development of renal function impairment in the general population. Kidney Int Suppl 2004; S18-S21
25. Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet 1982; 1:1430-1432
26. Wachtell K, Ibsen H, Olsen MH, Borch-Johnsen K, Lindholm LH, Mogensen CE, Dahlof B, Devereux RB, Beevers G, de Faire U, Fyhrquist F, Julius S, Kjeldsen SE, Kristianson K, Lederballe-Pedersen O, Nieminen MS, Okin PM, Omvik P, Oparil S, Wedel H, Snapinn SM, Aurup P. Albuminuria and cardiovascular risk in hypertensive patients with left ventricular hypertrophy: the LIFE study. Ann Intern Med 2003; 139:901-906

27. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage. The Steno hypothesis. Diabetologia 1989; 32:219-226
28. Gschwend S, Buikema H, Navis G, Henning RH, de Zeeuw D, van Dokkum RP. Endothelial dilatory function predicts individual susceptibility to renal damage in the 5/6 nephrectomized rat. J Am Soc Nephrol 2002; 13:2909-2915

Chapter 2

Microalbuminuria and endothelial dysfunction: Emerging targets for primary prevention of end-organ damage

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Abstract

A minor increase in urinary albumin excretion (microalbuminuria) is known to predict adverse renal and cardiovascular events in diabetic and hypertensive patients. Recent intriguing findings show that microalbuminuria is an early and sensitive marker of future cardiovascular events even in healthy subjects. The mechanisms linking microalbuminuria with end-organ damage have not been fully explained yet, however generalized endothelial dysfunction might play an important role. Prevailing experimental and clinical data suggest that generalized endothelial dysfunction, frequently characterized by decreased nitric oxide bioavailability, actually precedes the development of microalbuminuria. This review summarizes the current knowledge about the intricate relationship between microalbuminuria and endothelial dysfunction. Based on the current evidence we propose that microalbuminuria and endothelial dysfunction are an emerging target for primary prevention strategies in cardiovascular disease. In near future, dietary components improving nitric oxide bioavailability, such as cocoa -derived flavanols may play important role in these preventive strategies.

Introduction

End-organ damage associated with cardiovascular disease is the leading cause of morbidity and mortality in the Western world. Moreover, the costs related to the end-organ damage, such as chronic kidney or heart failure, constitute the majority of expenditures in the total health care budget[1]. Therefore, a shift is required from secondary prevention of renal and cardiac end-organ damage to primary prevention targeting the individuals with an increased risk profile at an early stage of the disease. Disclosure of novel markers for increased risk may help to identify specific individuals and assist in tailoring prevention according to their individual risk profile.

In addition to traditional cardiovascular risk factors, such as hypertension, dyslipidemia, central obesity, hyperglycemia and smoking, microalbuminuria has recently received a great deal of attention as a new, accessible and sensitive marker of renal and cardiovascular risk[2-4]. The mechanisms linking microalbuminuria to increased renal and cardiovascular risk are not fully understood, but it has been proposed that microalbuminuria is a reflection of generalized endothelial dysfunction[5]. This review summarizes the current knowledge about the intricate relationship between microalbuminuria and endothelial dysfunction. It focuses on the role of these two parameters as early markers of both renal and cardiovascular disease. Based on the current evidence we suggest that microalbuminuria and endothelial dysfunction represent emerging targets for primary prevention strategies. Since endothelial dysfunction is frequently characterized by decreased bioavailability of nitric oxide (NO), we propose that dietary components improving NO bioavailability, such as cocoa-derived flavanols, may play important role in these preventive strategies.

Microalbuminuria

Definition

Albumin is the major constituent of proteins excreted in urine. The widely used dipstick method detects only albumin excretion exceeding 300 mg per 24h, a range currently defined as macroalbuminuria or proteinuria. However, studies in diabetic patients demonstrated that much lower values of albumin excretion are associated with increased risk for the development of diabetic nephropathy[6,7] Currently, the range between 30 and 300 mg per 24h, or 20-200 μg/min measured overnight is defined as microalbuminuria. Methods for the measurements of urinary albumin, its definition and classification have been extensively summarized elsewhere[8,9]

Microalbuminuria is not rare in normal healthy population

Prevalence of microalbuminuria has been initially studied in individuals with diabetes and later in hypertensive cohorts. Still, a large variability is reported in distinct clinical trials, probably due to heterogeneity of the study population, regarding age, race, severity of

hypertension, coexistence of nephropathy, antihypertensive medication and associated lipid abnormalities. Methods of detection and sampling techniques might form an additional source of variation among trials. In general, a prevalence of 20-40% in patients with diabetes mellitus is reported in large studies[10-13]. In the individuals with essential hypertension the prevalence of microalbuminuria seems somewhat lower[14,15]. As an example, microalbuminuria was found in 23% of patients with essential hypertension and left ventricular hypertrophy included in the Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) study[16].

Increased incidence of microalbuminuria is not exclusively limited to the populations with elevated cardiovascular risk. An increasing number of large trials suggest that microalbuminuria is also common in a general, "healthy" population. In one of the largest cohorts studying a general population, the Prevention of Renal and Vascular End stage Disease (PREVEND) study, a prevalence of 7.2% was reported in 40.856 subjects and 6.6% after exclusion of the subjects with diabetes mellitus and hypertension[17]. Several other cross-sectional studies confirm 5-8% prevalence of microalbuminuria in the general population[18-20], suggesting that among healthy individuals a considerable variability in urinary albumin excretion already exists without the presence of any clinical condition. This phenomenon renders microalbuminuria a promising candidate as the integrated marker of cardiovascular risk in the general population.

Microalbuminuria predicts renal and cardiovascular outcome in diseased and healthy population

Considerable attention for microalbuminuria as a predictive parameter stemmed from the publication of *Viberti et al.* establishing the predictive value of microalbuminuria for nephropathy in insulin-dependent diabetes mellitus[7]. Since then several studies have confirmed elevated albumin excretion as a marker for the development of diabetic nephropathy and progressive renal failure both in patients with type I[6] and type II diabetes[21]. However, the predictive value of microalbuminuria in diabetics is not only limited to renal events, as microalbuminuria predicts total and especially cardiovascular mortality and morbidity in several studies in non-insulin-dependent diabetic populations[23] even after adjustment for other conventional cardiovascular risk factors. *Yudkin et al.* were the first to report this association also in non-diabetic subjects[24]. By now, it is well established that microalbuminuria identifies the individuals with adverse prognosis among hypertensive patients. In one of the largest longitudinal studies performed to investigate a predictive role of microalbuminuria, the Danish MONICA study, hypertensive subjects with albuminuria showed almost 4-fold increased risk of ischemic heart disease as compared to normoalbuminuric hypertensive subjects[25]. In the prospective LIFE trial in non-diabetic hypertensive patients with left ventricular hypertrophy, levels of albumin excretion at entry were predictive for composite end-point[26]. Since there was no threshold for the increased risk, correlation between albuminuria and risk exists also at albumin levels below the current definition of microalbuminuria.

Whereas microalbuminuria is clearly related to cardiovascular risk in high risk populations, it is important to mention that its predictive value also extrapolates to the general population. In the previously mentioned prospective Danish MONICA study, microalbuminuric subjects in general population were at increased risk for ischemic heart disease[27]. Furthermore, *Hillege et al.* demonstrated that microalbuminuria was independently associated with cardiovascular risk factors and morbidity in the general population, based on cross-sectional analysis of the baseline data from the PREVEND study[17]. In a more recent prospective analysis of this study, microalbuminuria independently predicted cardiovascular and all-cause mortality in the general population *(Figure 1)*[22]. Moreover, the relationship was already apparent at levels of albumin excretion considered to be normal. Within the same cohort, *Verhave et al.* showed that subjects with microalbuminuria at baseline had a higher chance to develop *de novo* impairment of renal function in a 4-year follow-up[28].

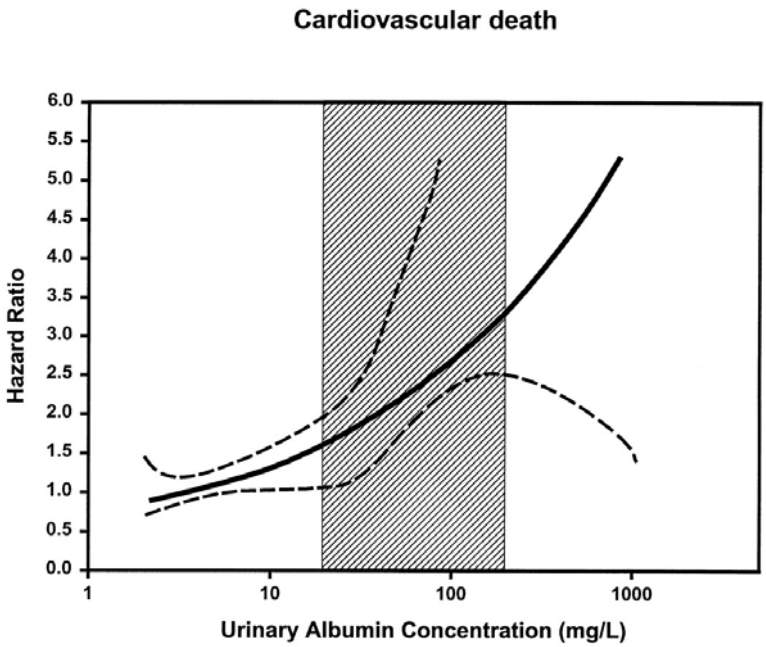

Figure 1. *Urinary albumin excretion predicts cardiovascular mortality in the general population; Relationship between urinary albumin excretion and hazard ratio. The dotted lines represent 95% confidence limits and the squared area indicates the definition of microalbuminuria. Reproduced from* [22]

Taken together, these data demonstrate the usefulness of microalbuminuria as a valuable and clinically relevant tool for the identification of individual patients at risk for the development of end-organ damage, e.g. renal as well as systemic cardiovascular disease. An important question is how such a relationship may be explained and what the link is between increased urinary excretion of albumin and end-organ damage. Currently, the evidence points towards the hypothesis that microalbuminuria is a reflection of *generalized systemic endothelial dysfunction*.

Endothelial dysfunction

The concept of endothelial dysfunction

The concept of endothelial dysfunction has emerged from cardiovascular research over the past 25 years, recognizing the principal role of the endothelium in regulation of vascular function in healthy individuals and its impairment in diseased states[29,30]. Endothelial dysfunction is now considered to play a principal role in the initiation and progression of atherosclerosis. Since endothelial dysfunction is also a common denominator for a wide variety of conditions such as hypertension, diabetes or chronic renal failure, it may provide a link to increased cardiovascular risk in above mentioned conditions.

Endothelial dysfunction may be defined as alterations in the normal properties of endothelium that are inappropriate for preservation of organ function[31]. Under physiological circumstances, the endothelium maintains homeostasis at the vascular wall. Normal healthy endothelium reduces vascular tone, regulates vascular permeability, limits platelets adhesion and aggregation, prevents activation of the coagulation cascade and restricts leukocyte adhesion. The specific term endothelial activation denotes the loss of endothelial anti-inflammatory properties characterized by elevated expression of adhesive molecules, such as E-selectin, intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and chemoattractant molecules e.g. monocyte chemotactic protein-1 (MCP-1), and consequently pronounced interaction with blood leukocytes. Functional properties of endothelium and involved mediators are summarized in *Table 1*.

One of the most important mediators released by endothelium is nitric oxide (NO)[32]. NO acts a potent vasodilator, inhibits inflammation, growth of vascular smooth muscle and aggregation of platelets. Altered production and/or bioavailability of NO are frequently reported in the conditions associated with endothelial dysfunction. However, the mechanisms responsible for the development of endothelial dysfunction are not yet completely understood. Probably the convergence of traditional and non-traditional risk factors, genetic predisposition and local, yet unknown mechanisms all contribute to endothelial perturbations[32]. Summary of the mechanisms playing a role in endothelial cell dysfunction is given in *Table 2*.

Several methods are available for investigation of endothelial function in humans, which however assess only certain aspects of endothelial function. Measurements of endothelium-

dependent vasodilation assess determines abilities of endothelium in coronary, forearm resistance or brachial arteries. Transcapillary escape rate of intravenously injected radioactive albumin is employed as a surrogate for endothelial microvascular permeability. Finally, plasma levels of endothelium-derived regulatory mediators, such as Von Willebrand factor (vWF), soluble thrombomodulin, tissue-type plasminogen-activator (tPA), plasminogen activator inhibitor-1 (PAI-1), soluble adhesive (selectins, ICAM-1, VCAM-1) and chemoattractant molecules (MCP-1) could be used to estimate systemic endothelial activation. The current evidence for the association of microalbuminuria with several aspects of endothelial dysfunction in humans is summarized below.

Table 1. *Endothelium-mediated mechanisms prevailing in health (beneficial) and disease (detrimental factors).*

Regulated process	***Beneficial factors***	***Detrimental factors***
Vascular tone	*Vasodilators* Nitric oxide (NO) Prostacycline Endothelium-derived hyperpolarizing factor (EDHF) C-natriuretic peptide (CNP) Bradykinine	*Vasoconstrictors* Endothelin-1 Thromboxane A_2 Prostaglandin H_2 Angiotensin II Reactive oxygen species
Permeability	Junctional proteins Cell surface glycocalix Extracellular matrix	Inflammatory molecules Endocytic receptors
Hemostasis	*Anticoagulant, antithrombotic, and fibrinolytic factors* Nitric oxide (NO) Prostacycline Thrombomodulin Tissue factor inhibitor (TFI) Tissue type plasminogen activator (t-PA)	*Procoagulant, prothrombotic and antifibrinolytic factors* Endothelin-1 Thromboxane A_2 Reactive oxygen species Tissue factor (TF) Von Willebrand factor (vWF) Fibrinogen Plasminogen activator inhibitor-1 (PA-1)
Inflammation		*Adhesion and chemoattractant molecules* E-selectin Intracellular adhesion molecule-1 (ICAM-1) Vascular cell adhesion molecule-1 (VCAM-1) Monocyte chemoattractant protein -1 (MCP-1)
Smooth muscle cell growth	*Antiproliferative factors* Nitric oxide (NO) Prostacyclin Transforming growth factor β (TGF β)	*Proliferative factors* Platelet-derived growth factor (PDGF) Angiotensin II Endothelin-1 Reactive oxygen species Vascular endothelial growth factor (VEGF)

Table 2. *Classical risk factors and putative mechanisms leading to endothelial dysfunction*

Classical risk factors
Aging
Hypertension
Smoking
Dyslipidemia
Obesity
Diabetes mellitus
Molecular mechanisms
Reduced bioavailability of nitric oxide
Oxidative stress
Hyperhomocysteinemia
Asymmetric Dimetylarginine
Angiotensin II
Advanced glycation end-products (AGE)

Endothelial dysfunction precedes the development of microalbuminuria

Increased vascular permeability

Increased permeability of albumin through the vascular wall is considered to be a marker of endothelial dysfunction. The evidence that increased albumin leakage in the glomerulus is associated with enhanced capillary permeability for albumin in the systemic vasculature comes from several studies testing ^{125}I-albumin escape rate in diabetic microalbuminuric patients. *Feldt-Rasmussen et al.* showed that patients with type I diabetes and microalbuminuria exhibit higher transcapillary leakage of albumin than those without microalbuminuria[33]. Similar findings were later reported in type II diabetic patients[34]. Furthermore, one study demonstrated that microalbuminuria was related to systemic vascular leakage even in healthy subjects[35]. Although microalbuminuria seems to be consistently associated with increased systemic leakage of albumin, some studies found increased albumin escape rate also in the hypertensive subjects with normoalbuminuria[36,37], suggesting that increased permeability of systemic microvessels is not always reflected in glomerular protein leakage. Although it is difficult to draw final conclusion from these findings, it might be suggested that in these states development of systemic vascular protein leakage actually precedes the appearance of microalbuminuria.

Impaired systemic endothelium-dependent vasodilation

Another aspect of systemic endothelial dysfunction, the loss of vasomotor control in the peripheral vessels has been repeatedly found in microalbuminuric patients. Most of the data support the hypothesis on generalized endothelial dysfunction in albuminuria. In patients with diabetes type I[38,39] and type II[40,41] the endothelium-dependent dilation of the peripheral arteries is impaired in microalbuminuric subjects when compared to normoalbuminuric or healthy subjects. Furthermore, the presence of microalbuminuria is inversely related to flow-mediated dilation of the brachial artery in insulin-dependent diabetic patients[42], in elderly individuals with or without diabetes[43] and in asymptomatic type II diabetics[44]. Intriguing is the fact that these findings could be extended from high risk populations to cohorts with clinically healthy subjects. *Clausen et al.* found that brachial artery flow-dependent dilation was significantly impaired in healthy individuals with microalbuminuria as compared to those with normoalbuminuria[45]. However, it should be noted that some studies failed to find direct relationship between endothelial dysfunction and urinary albumin excretion in essential hypertensives[46] and healthy subjects[47]. In fact, blunted systemic endothelium-mediated dilatory response is often found also in normoalbuminuric diabetic subjects[48-50], strongly suggesting that impaired endothelial function precedes the development of microalbuminuria.

Elevated plasma levels of pro-thrombotic and pro-inflammatory endothelial markers

Increased plasma levels of inflammatory and pro-thrombotic markers have also been reported from the various cohorts of patients with microalbuminuria suggesting that elevated urinary excretion of albumin is associated with generalized endothelial activation and a low-grade inflammatory state. Higher circulating levels of von Willebrand factor (vWF) were found in patients with hypertension and microalbuminuria as compared to those without microalbuminuria[51]. In addition to vWF, other markers of a procoagulant state, such as plasminogen activator inhibitor-1 (PAI-1) and coagulation factor VII are elevated in both insulin-dependent[52] and non-insulin dependent diabetic patients[53,54] with microalbuminuria. Therefore, systemic hemostatic dysfunction is frequently present in microalbuminuric patients. Elevations of vWF levels are also paralleled by increased markers of oxidative stress in type II diabetic microalbuminuric individuals[55-57]. Activation of endothelial cells is characterized by excessive levels of soluble adhesive molecules (ICAM-1, VCAM-1 and E-selectin) and is present in microalbuminuric type I[58,59] and type II[60] diabetics. Similar findings have also been reported in low-risk populations. *Agewall et al*. showed that plasma levels of PAI-1 were independently related to the level of urinary albumin in healthy subjects[61] in a cross-sectional manner.

Intriguing data from several groups provide compelling evidence that endothelial dysfunction might actually precede the occurrence of microalbuminuria. In a longitudinal study performed in patients with type I diabetes, *Stehouwer et al.* demonstrated that increases in vWF levels precede the appearance of microalbuminuria by approximately 3 years[62]. Similar findings were reported in prospective study by *Verrotti et al.* in children

with type I diabetes[63]. Furthermore, baseline levels of vWF were strongly related to the *de novo* development of microalbuminuria in the follow-up of non-insulin-dependent diabetic cohort[64]. Comparable data have also been found in a low-risk population. In a 4-year prospective study performed in healthy subjects, baseline elevated levels of vWF and tissue plasminogen activator (t-PA) predicted the development of increased urinary albumin excretion[65]. Simultaneously to impaired endothelium-dependent dilation, increased levels of soluble adhesive molecules are already present in normoalbuminuric diabetic subjects, a finding consistent with the hypothesis on the endothelial dysfunction appearing prior to microalbuminuria[66].

In conclusion, microalbuminuria is associated with increased in systemic albumin permeability, impaired endothelium-vasodilation of systemic vasculature and elevated levels of pro-inflammatory and pro-thrombotic endothelium-derived mediators. This is not only true in diabetic and hypertensive patients, but also in healthy subjects. Prevailing evidence suggests that in some of these states endothelial dysfunction actually precedes the development of microalbuminuria.

Prognostic value of endothelial function for cardiovascular risk

Present data consistently confirm that increased urinary excretion of albumin might be a useful integrated early marker of renal and cardiovascular risk. On the other hand, prognostic value of endothelial function is still a matter of debate. Since recognition of principal role of endothelial dysfunction in the development of atherosclerosis, several studies have been designed to investigate prognostic value of endothelial dysfunction for cardiovascular outcome. However, reports from microalbuminuric cohorts are scarce.

Most of the studies investigated endothelial dilatory reactivity as a marker of endothelial dysfunction. Coronary or peripheral endothelium-mediated response in patients with mild, moderate or established coronary artery disease predicted the adverse event rates in several studies[67-70]. Prognostic value of peripheral endothelial vasodilation has been also shown in patients with end-stage renal disease[71] and chronic heart failure[72]. The predictive value of endothelial function in patients with normal coronary angiograms[73], cohort of essential hypertensives[74] and hypertensive postmenopausal women[75] might suggest that this measurement could identify patients at risk at very early stage of the cardiovascular disease.

Alternatively, the prognostic value of endothelium-derived regulatory markers in plasma has been reported from several studies. Once more, the majority of studies was performed in patients with known coronary disease. In these cases, vWF, t-PA[76], PAI-1[77], soluble ICAM-1[78] and endothelin[79] were predictors of cardiovascular events. In one of the few studies with a low risk population, *Ridker et al*. found elevated plasma levels of soluble ICAM-1 to predict the risk for future myocardial infarction in healthy men[80].

Studies investigating the predictive value of endothelial dysfunction in patients with microalbuminuria are sporadic and the relation between these two parameters in predicting the cardiovascular outcome is not straightforward. *Jager et al*. have found plasma levels of VCAM-1 being predictive for cardiovascular outcome in type II diabetics independently

from microalbuminuria[81], suggesting that increased plasma levels of VCAM-1 might reflect a different aspect of endothelial dysfunction than microalbuminuria. The same group showed that cardiovascular risk predicted by microalbuminuria is modified by presence of elevated levels of vWF and retinopathy in type II diabetics, favouring the conclusion, that "benign" microalbuminuria (without concomitant presence of endothelial dysfunction) has more favourable prognosis than "malign" microalbuminuria (with endothelial dysfunction)[82,83].

In conclusion, predictive value of the endothelial dysfunction for cardiovascular outcome has been shown mostly in high risk patients. Although some data suggest that this relationship exists in low risk, healthy or microalbuminuric populations, further studies will be needed to establish it conclusively.

Microalbuminuria and endothelial dysfunction as therapeutic target

Presence of microalbuminuria is consistently associated with worse cardiovascular outcome in several diseased conditions and in the general population. Therefore, it is of importance to explore, whether limiting of microalbuminuria provides benefit for decreased cardiovascular risk. Given the early occurrence of endothelial dysfunction in microalbuminuric patients, modulation of endothelial function might provide an additional strategy to limit adverse cardiovascular events. Furthermore, the both parameters emerge as therapeutic targets for primary prevention in the general population.

Lowering of albumin excretion is associated with reduction of cardiovascular risk

Several therapeutic approaches reverse the excessive urinary excretion of proteins. Strict glucose control may prevent the development of microalbuminuria in diabetics[84]. Furthermore, several studies showed that angiotensin-converting enzyme inhibitors (ACEi)[85-87], angiotensin II AT1 receptor blockers (ARB)[88,89], lipid-lowering drugs, such as statins[90,91] or fibrates[92] and recently also oral glycosaminoglycane sulodexide[93,94] all reduce or even regress microalbuminuria in patients with type I or type II diabetes. Tight blood pressure control is required to halt the progression of microalbuminuria in hypertensive patients, however drugs interfering with renin-angiotensin-aldosterone system (RAAS) might provide more benefit than diuretic, beta-blocking agents or calcium channel blockers in lowering albuminuria[95].

Importantly, available evidence suggests, that specific lowering of microalbuminuria translates in the reduction of renal and cardiovascular adverse events in several populations. *Parving et al.* showed that lowering of albuminuria with ARB irbesartan is dose-dependently associated with reduced progression to diabetic nephropathy in hypertensive type II diabetics independent of blood pressure control[88]. Comparably, several other studies demonstrated efficacy of ACEi in preventing diabetic nephropathy in diabetic microalbuminuric patients[85,96]. In the LIFE study among hypertensive patients with left

ventricular hypertrophy, a reduction in albumin excretion was inversely related to the risk of cardiovascular mortality and morbidity[97]. It seems that drugs interfering with the RAAS are superior to other antihypertensives, also in reducing cardiovascular events in microalbuminuric subjects. This is however largely based on the data from hypertensive diabetic populations, which are known to have high incidence of microalbuminuria[98-100].

Recent compelling evidence for microalbuminuria as a justified target for primary prevention comes from the PREVEND-IT study[101]. Healthy individuals with microalbuminuria, but without hypertension or hypercholesterolemia, were treated either with placebo or the ACEi fosinopril. At 4-year's follow-up, the microalbuminuria was effectively reduced by ACEi treatment, which was associated with a 44% reduction in cardiovascular events.

In conclusion, lowering of urinary albumin excretion, preferably by RAAS inhibitory agents substantially reduces the number of cardiovascular events in both high risk and healthy population.

Table 3. *Intervention strategies leading to reversal of endothelial dysfunction in humans*

General interventions
Physical activity
Smoking cessation
Lipid-lowering therapy
Glycemic control in diabetes
Pharmaceuticals
Angiotensin converting enzyme inhibitors
Angiotensin receptor blockers
Statins
Peroxisome proliferator-activated receptor-γ activators
Estrogens
Dietary supplements
n-3 fatty acids
Folate
Tetrahydrobiopterin
L-Arginine
Vitamin C
Vitamin E
Dietary flavonoids

Modulation of endothelial dysfunction in microalbuminuric patients

A wide spectrum of treatments *(Table 3)* has been shown to improve endothelial dysfunction in several conditions. However, the hypothesis, that reversal of endothelial dysfunction is associated with risk reduction has not been directly tested. Nevertheless, some of endothelium-protective therapeutic strategies, such as ACEi[102], ARB[103], and statins[104] have been consistently shown to reduce cardiovascular events in multiple populations. It is however unclear to what extent the improvement in endothelial function governs cardiovascular risk reduction.

A limited number of studies is available on reversing endothelial dysfunction in patients with microalbuminuria. Strikingly, all therapeutic approaches associated with lowering microalbuminuria are also known to improve endothelial function. Therefore it is tempting to speculate that improvement of endothelial function plays role in the beneficial effects of these drugs on albumin urinary excretion and probably in cardiovascular risk reduction. However, the current data from diabetic patients do not allow such conclusion on this issue. Several trials investigated the effect of ACEi and ARB treatment on the peripheral endothelial function in diabetic microalbuminuric patients. While in type I diabetics *Arcaro et al.*[105] found ACEi to improve endothelium-dependent vasodilation of femoral artery without affecting microalbuminuria, *Schalkwijk et al.* reported unchanged peripheral endothelium-dependent dilation after short-term therapy with ACEi quinapril[106]. However, in the latter study ACEi reduced plasma levels of soluble E-selectin, suggesting that some aspects of endothelial dysfunction were selectively improved. Similarly, reversal of elevated VCAM-1 levels paralleled the decrease of microalbuminuria by fosinopril in hypertensive type II diabetics[107]. In contrast, a low dose of ARB losartan, which did not affect blood pressure, did not have any impact on peripheral endothelial dilation, while it reduced microalbuminuria[108]. Overall, studies with RAAS interfering agents suggest that these drugs may improve several aspects of endothelial function in microalbuminuric diabetic subjects, but it is not clear whether these effects play role in their anti-albuminuric action. Factors such as duration of treatment, population and agent characteristics might underlie these discrepancies.

In addition to ACEi and ARB, one study investigated the effect of atorvastatin on brachial artery flow-mediated dilation in microalbuminuric type I diabetics. Six weeks treatment improved vasodilation, but had no effect on albumin excretion, probably due to the short duration of the treatment[109]. Therefore also statins may prove beneficial in reversing endothelial dysfunction in microalbuminuric diabetic patients.

In conclusion, the available data provide evidence that lowering microalbuminuria especially by RAAS modulating agents in diabetics, hypertensives and even in healthy subjects might provide benefits in terms of reduced cardiovascular events. Majority of the agents efficiently lowering microalbuminuria might also reverse endothelial dysfunction. However a role of the endothelial modulation in risk reduction remains unclear. Nevertheless, experimental evidence suggesting that endothelial dysfunction precedes microalbuminuria and that variability in endothelial dysfunction among healthy individuals

determines the end-organ damage renders endothelial dysfunction the important modifiable factor for primary prevention.

Future potential of dietary flavonoids in primary prevention of microalbuminuria

Data from the PREVEND-IT study clearly show that reduction of microalbuminuria among healthy subjects might prevent future cardiovascular events. Although modulation of microalbuminuria and endothelial dysfunction might be the most efficiently achieved by ACEi or ARB, additional strategies might prove useful in primary prevention. Furthermore, as reported from PREVEND-IT cohort, in otherwise-healthy microalbuminuric population only 63% of subjects were compliant to ACEi treatment. Therefore, for primary prevention, dietary supplements might provide more acceptable and inexpensive alternative to pharmaceutical compounds.

Recently, attention has been drawn to several nutritional factors in prevention of cardiovascular disease. Majority of the research concentrated on n-3 fatty acids, antioxidant vitamins, L-arginine, folic acid and plant-derived polyphenols. The latter can be present in relatively high concentrations in certain plant-based foods and beverages, such as red wine, tea, grapefruit juice or cocoa-based products[110]. This makes them potentially interesting candidates for primary prevention. Several studies performed both in high and low risk populations suggest beneficial effect of these supplements on cardiovascular outcome[111,112]. These benefits seem to be mediated by improved endothelial function. Indeed, plant polyphenols, especially flavonoids have been shown to improve endothelial function in experimental and human studies[113,114]. Even more important for primary prevention is the fact that endothelium-protective characteristics of flavonoids, such as increase in nitric oxide bioavailability due to antioxidant properties or stimulating effects on endothelial nitric oxide synthase, have been found in healthy individuals. This suggests the potential of flavonoids to modify healthy endothelial function and thereby modulate individual sensitivity to cardiovascular injury.

Although the flavonoids content is variable among the various dietary products and is also dependent on food processing practices, it appears that raw cocoa might contain the concentrations of specific flavonoids substantially exceeding most other known sources[115]. Beneficial vascular effects of cocoa-based products, probably attributed to subclass of flavonoids known as flavanols, has been recently extensively reported[116,117]. Impressive studies in healthy humans have shown that ingestion of flavanol-rich cocoa is associated with increased NO-dependent vasodilation[118], reduced wave reflections[119], decrease in blood pressure and even an increase in insulin sensitivity[120]. Beneficial effects on endothelial function and insulin sensitivity were also confirmed in smokers[121] and hypertensives[122]. Although the effects of cocoa flavanols on renal vasculature and albumin excretion await further investigation, dietary strategies utilizing flavanol-rich cocoa hold a promise as a primary preventive approach in subjects with microalbuminuria.

Conclusion

In conclusion, microalbuminuria is an early and sensitive marker of renal and cardiovascular risk in both high and low risk patients. Most likely, it reflects a state of generalized endothelial dysfunction. Modulation of microalbuminuria and endothelial function might provide beneficial effects on future cardiovascular outcome even in the general or healthy population. Therefore, targeting microalbuminuria and endothelial dysfunction by several agents, such as ACE inhibitors or angiotensin receptor blockers might provide an effective strategy for primary prevention of renal and cardiovascular disease. Inexpensive and well tolerated dietary strategies utilizing plant-based products such as flavanol-rich cocoa might prove useful for primary prevention of end-organ damage in the general population.

References

1. Collins AJ, Li S, Gilbertson DT, Liu J, Chen SC, Herzog CA. Chronic kidney disease and cardiovascular disease in the Medicare population. Kidney Int Suppl 2003; S24-S31
2. de Jong PE, Brenner BM. From secondary to primary prevention of progressive renal disease: the case for screening for albuminuria. Kidney Int 2004; 66:2109-2118
3. de Zeeuw D. Albuminuria, just a marker for cardiovascular disease, or is it more? J Am Soc Nephrol 2005; 16:1883-1885
4. Diercks GF, van Boven AJ, Hillege JL, de Jong PE, Rouleau JL, van Gilst WH. The importance of microalbuminuria as a cardiovascular risk indicator: A review. Can J Cardiol 2002; 18:525-535
5. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage. The Steno hypothesis. Diabetologia 1989; 32:219-226
6. Mogensen CE, Christensen CK. Predicting diabetic nephropathy in insulin-dependent patients. N Engl J Med 1984; 311:89-93
7. Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet 1982; 1:1430-1432
8. Marshall SM. Screening for microalbuminuria: which measurement? Diabet Med 1991; 8:706-711
9. Toto RD. Microalbuminuria: definition, detection, and clinical significance. J Clin Hypertens (Greenwich) 2004; 6:2-7
10. Savage S, Nagel NJ, Estacio RO, Lukken N, Schrier RW. Clinical factors associated with urinary albumin excretion in type II diabetes. Am J Kidney Dis 1995; 25:836-844
11. Tapp RJ, Shaw JE, Zimmet PZ, Balkau B, Chadban SJ, Tonkin AM, Welborn TA, Atkins RC. Albuminuria is evident in the early stages of diabetes onset: results from the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). Am J Kidney Dis 2004; 44:792-798
12. Schmitz A, Vaeth M. Microalbuminuria: a major risk factor in non-insulin-dependent diabetes. A 10-year follow-up study of 503 patients. Diabet Med 1988; 5:126-134
13. Gerstein HC, Mann JF, Pogue J, Dinneen SF, Halle JP, Hoogwerf B, Joyce C, Rashkow A, Young J, Zinman B, Yusuf S. Prevalence and determinants of microalbuminuria in high-risk diabetic and nondiabetic patients in the Heart Outcomes Prevention Evaluation Study. The HOPE Study Investigators. Diabetes Care 2000; 23 Suppl 2:B35-B39
14. Bigazzi R, Bianchi S, Campese VM, Baldari G. Prevalence of microalbuminuria in a large population of patients with mild to moderate essential hypertension. Nephron 1992; 61:94-97
15. Pontremoli R, Sofia A, Ravera M, Nicolella C, Viazzi F, Tirotta A, Ruello N, Tomolillo C, Castello C, Grillo G, Sacchi G, Deferrari G. Prevalence and Clinical Correlates of Microalbuminuria in Essential Hypertension : The MAGIC Study. Hypertension 1997; 30:1135-1143
16. Wachtell K, Olsen MH, Dahlof B, Devereux RB, Kjeldsen SE, Nieminen MS, Okin PM, Papademetriou V, Mogensen CE, Borch-Johnsen K, Ibsen H. Microalbuminuria in hypertensive patients with electrocardiographic left ventricular hypertrophy: the LIFE study. J Hypertens 2002; 20:405-412

17. Hillege HL, Janssen WM, Bak AA, Diercks GF, Grobbee DE, Crijns HJ, van Gilst WH, de Zeeuw D, de Jong PE. Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. J Intern Med 2001; 249:519-526
18. Atkins RC, Polkinghorne KR, Briganti EM, Shaw JE, Zimmet PZ, Chadban SJ. Prevalence of albuminuria in Australia: the AusDiab Kidney Study. Kidney Int Suppl 2004; S22-S24
19. Garg AX, Kiberd BA, Clark WF, Haynes RB, Clase CM. Albuminuria and renal insufficiency prevalence guides population screening: results from the NHANES III. Kidney Int 2002; 61:2165-2175
20. Romundstad S, Holmen J, Kvenild K, Aakervik O, Hallan H. Clinical relevance of microalbuminuria screening in self-reported non-diabetic/non-hypertensive persons identified in a large health screening--the Nord-Trondelag Health Study (HUNT), Norway. Clin Nephrol 2003; 59:241-251
21. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. The New England Journal of Medicine 1984; 310:356-360
22. Hillege HL, Fidler V, Diercks GF, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, Gans RO, Janssen WM, Grobbee DE, de Jong PE. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. Circulation 2002; 106:1777-1782
23. Dinneen SF. The association of microalbuminuria and mortality in non-insulin-dependent diabetes mellitus. A systematic overview of the literature. Archives of Internal Medicine 1997; 157:1413-1418
24. Yudkin JS. Microalbuminuria as predictor of vascular disease in non-diabetic subjects. Islington Diabetes Survey. The Lancet 1988; 2:530-533
25. Jensen J, Feldt-Rasmussen B, Strandgaard S, Schroll M, Borch-Johnsen K. Arterial Hypertension, Microalbuminuria, and Risk of Ischemic Heart Disease. Hypertension 2000; 35:898-903
26. Wachtell K, Ibsen H, Olsen MH, Borch-Johnsen K, Lindholm LH, Mogensen CE, Dahlof B, Devereux RB, Beevers G, de Faire U, Fyhrquist F, Julius S, Kjeldsen SE, Kristianson K, Lederballe-Pedersen O, Nieminen MS, Okin PM, Omvik P, Oparil S, Wedel H, Snapinn SM, Aurup P. Albuminuria and cardiovascular risk in hypertensive patients with left ventricular hypertrophy: the LIFE study. Ann Intern Med 2003; 139:901-906
27. Borch-Johnsen K, Feldt-Rasmussen B, Strandgaard S, Schroll M, Jensen JS. Urinary albumin excretion. An independent predictor of ischemic heart disease. Arterioscler Thromb Vasc Biol 1999; 19:1992-1997
28. Verhave JC, Gansevoort RT, Hillege HL, Bakker SJ, de Zeeuw D, de Jong PE. An elevated urinary albumin excretion predicts de novo development of renal function impairment in the general population. Kidney Int Suppl 2004; S18-S21
29. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation 2004; 109:III27-III32
30. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 2004; 15:1983-1992
31. Stehouwer CD. Endothelial dysfunction in diabetic nephropathy: state of the art and potential significance for non-diabetic renal disease. Nephrol Dial Transplant 2004; 19:778-781
32. Goligorsky MS. Endothelial cell dysfunction: can't live with it, how to live without it. Am J Physiol Renal Physiol 2005; 288:F871-F880

33. Feldt-Rasmussen B. Increased transcapillary escape rate of albumin in type 1 (insulin-dependent) diabetic patients with microalbuminuria. Diabetologia 1986; 29:282-286
34. Nannipieri M, Rizzo L, Rapuano A, Pilo A, Penno G, Navalesi R. Increased transcapillary escape rate of albumin in microalbuminuric type II diabetic patients. Diabetes Care 1995; 18:1-9
35. Jensen JS, Borch-Johnsen K, Jensen G, Feldt-Rasmussen B. Microalbuminuria reflects a generalized transvascular albumin leakiness in clinically healthy subjects. Clin Sci (Lond) 1995; 88:629-633
36. Nannipieri M, Penno G, Rizzo L, Pucci L, Bandinelli S, Mattei P, Taddei S, Salvetti A, Navalesi R. Transcapillary escape rate of albumin in type II diabetic patients. The relationship with microalbuminuria and hypertension. Diabetes Care 1997; 20:1019-1026
37. Pedrinelli R, Penno G, Dell'Omo G, Bandinelli S, Giorgi D, Di Bello V, Navalesi R, Mariani M. Microalbuminuria and transcapillary albumin leakage in essential hypertension. Hypertension 1999; 34:491-495
38. Zenere BM, Arcaro G, Saggiani F, Rossi L, Muggeo M, Lechi A. Noninvasive detection of functional alterations of the arterial wall in IDDM patients with and without microalbuminuria. Diabetes Care 1995; 18:975-982
39. Dogra G, Rich L, Stanton K, Watts GF. Endothelium-dependent and independent vasodilation studies at normoglycaemia in type I diabetes mellitus with and without microalbuminuria. Diabetologia 2001; 44:593-601
40. Yu Y, Suo L, Yu H, Wang C, Tang H. Insulin resistance and endothelial dysfunction in type 2 diabetes patients with or without microalbuminuria. Diabetes Res Clin Pract 2004; 65:95-104
41. Papaioannou GI, Seip RL, Grey NJ, Katten D, Taylor A, Inzucchi SE, Young LH, Chyun DA, Davey JA, Wackers FJ, Iskandrian AE, Ratner RE, Robinson EC, Carolan S, Engel S, Heller GV. Brachial artery reactivity in asymptomatic patients with type 2 diabetes mellitus and microalbuminuria (from the Detection of Ischemia in Asymptomatic Diabetics-brachial artery reactivity study). Am J Cardiol 2004; 94:294-299
42. Poredos P, Kek A. Relation of blunted dilation of the brachial artery in insulin-dependent diabetes mellitus to microalbuminuria. Am J Cardiol 2000; 86:364-367
43. Stehouwer CD, Henry RM, Dekker JM, Nijpels G, Heine RJ, Bouter LM. Microalbuminuria is associated with impaired brachial artery, flow-mediated vasodilation in elderly individuals without and with diabetes: further evidence for a link between microalbuminuria and endothelial dysfunction--the Hoorn Study. Kidney Int Suppl 2004; S42-S44
44. Papaioannou GI, Seip RL, Grey NJ, Katten D, Taylor A, Inzucchi SE, Young LH, Chyun DA, Davey JA, Wackers FJ, Iskandrian AE, Ratner RE, Robinson EC, Carolan S, Engel S, Heller GV. Brachial artery reactivity in asymptomatic patients with type 2 diabetes mellitus and microalbuminuria (from the Detection of Ischemia in Asymptomatic Diabetics-brachial artery reactivity study). Am J Cardiol 2004; 94:294-299
45. Clausen P, Jensen JS, Jensen G, Borch-Johnsen K, Feldt-Rasmussen B. Elevated urinary albumin excretion is associated with impaired arterial dilatory capacity in clinically healthy subjects. Circulation 2001; 103:1869-1874
46. Taddei S, Virdis A, Mattei P, Ghiadoni L, Sudano I, Arrighi P, Salvetti A. Lack of correlation between microalbuminuria and endothelial function in essential hypertensive patients. J Hypertens 1995; 13:1003-1008

47. Diercks GF, Stroes ES, van Boven AJ, van Roon AM, Hillege HL, de Jong PE, Smit AJ, Gans RO, Crijns HJ, Rabelink TJ, van Gilst WH. Urinary albumin excretion is related to cardiovascular risk indicators, not to flow-mediated vasodilation, in apparently healthy subjects. Atherosclerosis 2002; 163:121-126
48. Dogra G, Rich L, Stanton K, Watts GF. Endothelium-dependent and independent vasodilation studies at normoglycaemia in type I diabetes mellitus with and without microalbuminuria. Diabetologia 2001; 44:593-601
49. Lim SC, Caballero AE, Smakowski P, LoGerfo FW, Horton ES, Veves A. Soluble intercellular adhesion molecule, vascular cell adhesion molecule, and impaired microvascular reactivity are early markers of vasculopathy in type 2 diabetic individuals without microalbuminuria. Diabetes Care 1999; 22:1865-1870
50. Meeking DR, Cummings MH, Thorne S, Donald A, Clarkson P, Crook JR, Watts GF, Shaw KM. Endothelial dysfunction in Type 2 diabetic subjects with and without microalbuminuria. Diabet Med 1999; 16:841-847
51. Pedrinelli R, Giampietro O, Carmassi F, Melillo E, Dell'Omo G, Catapano G, Matteucci E, Talarico L, Morale M, De Negri F, . Microalbuminuria and endothelial dysfunction in essential hypertension. Lancet 1994; 344:14-18
52. Gruden G, Cavallo-Perin P, Bazzan M, Stella S, Vuolo A, Pagano G. PAI-1 and factor VII activity are higher in IDDM patients with microalbuminuria. Diabetes 1994; 43:426-429
53. Kario K, Matsuo T, Kobayashi H, Matsuo M, Sakata T, Miyata T. Activation of tissue factor-induced coagulation and endothelial cell dysfunction in non-insulin-dependent diabetic patients with microalbuminuria. Arterioscler Thromb Vasc Biol 1995; 15:1114-1120
54. Yu Y, Suo L, Yu H, Wang C, Tang H. Insulin resistance and endothelial dysfunction in type 2 diabetes patients with or without microalbuminuria. Diabetes Res Clin Pract 2004; 65:95-104
55. Collier A, Rumley A, Rumley AG, Paterson JR, Leach JP, Lowe GD, Small M. Free radical activity and hemostatic factors in NIDDM patients with and without microalbuminuria. Diabetes 1992; 41:909-913
56. Ibrahim HA, El Meligi AA, Abdel-Hamid M, Elhendy A. Relations between von Willebrand factor, markers of oxidative stress and microalbuminuria in patients with type 2 diabetes mellitus. Med Sci Monit 2004; 10:CR85-CR89
57. Kashiwazaki K, Hirano T, Yoshino G, Kurokawa M, Tajima H, Adachi M. Decreased release of lipoprotein lipase is associated with vascular endothelial damage in NIDDM patients with microalbuminuria. Diabetes Care 1998; 21:2016-2020
58. Clausen P, Jacobsen P, Rossing K, Jensen JS, Parving HH, Feldt-Rasmussen B. Plasma concentrations of VCAM-1 and ICAM-1 are elevated in patients with Type 1 diabetes mellitus with microalbuminuria and overt nephropathy. Diabet Med 2000; 17:644-649
59. Schmidt AM, Crandall J, Hori O, Cao R, Lakatta E. Elevated plasma levels of vascular cell adhesion molecule-1 (VCAM-1) in diabetic patients with microalbuminuria: a marker of vascular dysfunction and progressive vascular disease. Br J Haematol 1996; 92:747-750
60. Lim SC, Caballero AE, Smakowski P, LoGerfo FW, Horton ES, Veves A. Soluble intercellular adhesion molecule, vascular cell adhesion molecule, and impaired microvascular reactivity are early markers of vasculopathy in type 2 diabetic individuals without microalbuminuria. Diabetes Care 1999; 22:1865-1870

61. Agewall S, Lindstedt G, Fagerberg B. Independent relationship between microalbuminuria and plasminogen activator inhibitor-1 activity (PAI-1) activity in clinically healthy 58-year-old men. Atherosclerosis 2001; 157:197-202
62. Stehouwer CD, Fischer HR, van Kuijk AW, Polak BC, Donker AJ. Endothelial dysfunction precedes development of microalbuminuria in IDDM. Diabetes 1995; 44:561-564
63. Verrotti A, Greco R, Basciani F, Morgese G, Chiarelli F. von Willebrand factor and its propeptide in children with diabetes. Relation between endothelial dysfunction and microalbuminuria. Pediatr Res 2003; 53:382-386
64. Stehouwer CD, Nauta JJ, Zeldenrust GC, Hackeng WH, Donker AJ, den Ottolander GJ. Urinary albumin excretion, cardiovascular disease, and endothelial dysfunction in non-insulin-dependent diabetes mellitus. Lancet 1992; 340:319-323
65. Clausen P, Feldt-Rasmussen B, Jensen G, Jensen JS. Endothelial haemostatic factors are associated with progression of urinary albumin excretion in clinically healthy subjects: a 4-year prospective study. Clin Sci (Lond) 1999; 97:37-43
66. Lim SC, Caballero AE, Smakowski P, LoGerfo FW, Horton ES, Veves A. Soluble intercellular adhesion molecule, vascular cell adhesion molecule, and impaired microvascular reactivity are early markers of vasculopathy in type 2 diabetic individuals without microalbuminuria. Diabetes Care 1999; 22:1865-1870
67. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 106:653-658
68. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 2001; 104:2673-2678
69. Neunteufl T, Heher S, Katzenschlager R, Wolfl G, Kostner K, Maurer G, Weidinger F. Late prognostic value of flow-mediated dilation in the brachial artery of patients with chest pain. Am J Cardiol 2000; 86:207-210
70. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 2000; 101:1899-1906
71. London GM, Pannier B, Agharazii M, Guerin AP, Verbeke FH, Marchais SJ. Forearm reactive hyperemia and mortality in end-stage renal disease. Kidney Int 2004; 65:700-704
72. Heitzer T, Baldus S, von Kodolitsch Y, Rudolph V, Meinertz T. Systemic endothelial dysfunction as an early predictor of adverse outcome in heart failure. Arterioscler Thromb Vasc Biol 2005; 25:1174-1179
73. Schindler TH, Hornig B, Buser PT, Olschewski M, Magosaki N, Pfisterer M, Nitzsche EU, Solzbach U, Just H. Prognostic value of abnormal vasoreactivity of epicardial coronary arteries to sympathetic stimulation in patients with normal coronary angiograms. Arterioscler Thromb Vasc Biol 2003; 23:495-501
74. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroroberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial dysfunction in hypertensive patients. Circulation 2001; 104:191-196
75. Modena MG, Bonetti L, Coppi F, Bursi F, Rossi R. Prognostic role of reversible endothelial dysfunction in hypertensive postmenopausal women. J Am Coll Cardiol 2002; 40:505-510
76. Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris.

European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. N Engl J Med 1995; 332:635-641
77. Hamsten A, de Faire U, Walldius G, Dahlen G, Szamosi A, Landou C, Blomback M, Wiman B. Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. Lancet 1987; 2:3-9
78. Haim M, Tanne D, Boyko V, Reshef T, Goldbourt U, Leor J, Mekori YA, Behar S. Soluble intercellular adhesion molecule-1 and long-term risk of acute coronary events in patients with chronic coronary heart disease. Data from the Bezafibrate Infarction Prevention (BIP) Study. J Am Coll Cardiol 2002; 39:1133-1138
79. Omland T, Lie RT, Aakvaag A, Aarsland T, Dickstein K. Plasma endothelin determination as a prognostic indicator of 1-year mortality after acute myocardial infarction. Circulation 1994; 89:1573-1579
80. Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 1998; 351:88-92
81. Jager A, van Hinsbergh VW, Kostense PJ, Emeis JJ, Nijpels G, Dekker JM, Heine RJ, Bouter LM, Stehouwer CD. Increased levels of soluble vascular cell adhesion molecule 1 are associated with risk of cardiovascular mortality in type 2 diabetes: the Hoorn study. Diabetes 2000; 49:485-491
82. Fioretto P, Stehouwer CD, Mauer M, Chiesura-Corona M, Brocco E, Carraro A, Bortoloso E, van Hinsbergh VW, Crepaldi G, Nosadini R. Heterogeneous nature of microalbuminuria in NIDDM: studies of endothelial function and renal structure. Diabetologia 1998; 41:233-236
83. Jager A, van Hinsbergh VW, Kostense PJ, Emeis JJ, Nijpels G, Dekker JM, Heine RJ, Bouter LM, Stehouwer CD. Prognostic implications of retinopathy and a high plasma von Willebrand factor concentration in type 2 diabetic subjects with microalbuminuria. Nephrol Dial Transplant 2001; 16:529-536
84. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993; 329:977-986
85. Randomised placebo-controlled trial of lisinopril in normotensive patients with insulin-dependent diabetes and normoalbuminuria or microalbuminuria. The EUCLID Study Group. Lancet 1997; 349:1787-1792
86. Ravid M, Brosh D, Levi Z, Bar-Dayan Y, Ravid D, Rachmani R. Use of enalapril to attenuate decline in renal function in normotensive, normoalbuminuric patients with type 2 diabetes mellitus. A randomized, controlled trial. Ann Intern Med 1998; 128:982-988
87. Ruggenenti P, Fassi A, Ilieva AP, Bruno S, Iliev IP, Brusegan V, Rubis N, Gherardi G, Arnoldi F, Ganeva M, Ene-Iordache B, Gaspari F, Perna A, Bossi A, Trevisan R, Dodesini AR, Remuzzi G. Preventing microalbuminuria in type 2 diabetes. N Engl J Med 2004; 351:1941-1951
88. Parving HH, Lehnert H, Brochner-Mortensen J, Gomis R, Andersen S, Arner P. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. N Engl J Med 2001; 345:870-878
89. Viberti G, Wheeldon NM. Microalbuminuria reduction with valsartan in patients with type 2 diabetes mellitus: a blood pressure-independent effect. Circulation 2002; 106:672-678
90. Nakamura T, Ushiyama C, Hirokawa K, Osada S, Shimada N, Koide H. Effect of cerivastatin on urinary albumin excretion and plasma endothelin-1 concentrations in

type 2 diabetes patients with microalbuminuria and dyslipidemia. Am J Nephrol 2001; 21:449-454
91. Tonolo G, Melis MG, Formato M, Angius MF, Carboni A, Brizzi P, Ciccarese M, Cherchi GM, Maioli M. Additive effects of Simvastatin beyond its effects on LDL cholesterol in hypertensive type 2 diabetic patients. Eur J Clin Invest 2000; 30:980-987
92. Smulders YM, van Eeden AE, Stehouwer CD, Weijers RN, Slaats EH, Silberbusch J. Can reduction in hypertriglyceridaemia slow progression of microalbuminuria in patients with non-insulin-dependent diabetes mellitus? Eur J Clin Invest 1997; 27:997-1002
93. Dedov I, Shestakova M, Vorontzov A, Palazzini E. A randomized, controlled study of sulodexide therapy for the treatment of diabetic nephropathy. Nephrol Dial Transplant 1997; 12:2295-2300
94. Gambaro G, Kinalska I, Oksa A, Pont'uch P, Hertlova M, Olsovsky J, Manitius J, Fedele D, Czekalski S, Perusicova J, Skrha J, Taton J, Grzeszczak W, Crepaldi G. Oral sulodexide reduces albuminuria in microalbuminuric and macroalbuminuric type 1 and type 2 diabetic patients: the Di.N.A.S. randomized trial. J Am Soc Nephrol 2002; 13:1615-1625
95. Bianchi S. Microalbuminuria in patients with essential hypertension: effects of several antihypertensive drugs. The American Journal of Medicine 1992; 93:525-528
96. Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. Heart Outcomes Prevention Evaluation Study Investigators. Lancet 2000; 355:253-259
97. Ibsen H, Olsen MH, Wachtell K, Borch-Johnsen K, Lindholm LH, Mogensen CE, Dahlof B, Devereux RB, de Faire U, Fyhrquist F, Julius S, Kjeldsen SE, Lederballe-Pedersen O, Nieminen MS, Omvik P, Oparil S, Wan Y. Reduction in albuminuria translates to reduction in cardiovascular events in hypertensive patients: losartan intervention for endpoint reduction in hypertension study. Hypertension 2005; 45:198-202
98. Estacio RO, Jeffers BW, Hiatt WR, Biggerstaff SL, Gifford N, Schrier RW. The effect of nisoldipine as compared with enalapril on cardiovascular outcomes in patients with non-insulin-dependent diabetes and hypertension. N Engl J Med 1998; 338:645-652
99. Pahor M, Psaty BM, Alderman MH, Applegate WB, Williamson JD, Furberg CD. Therapeutic benefits of ACE inhibitors and other antihypertensive drugs in patients with type 2 diabetes. Diabetes Care 2000; 23:888-892
100. Tatti P, Pahor M, Byington RP, Di Mauro P, Guarisco R, Strollo G, Strollo F. Outcome results of the Fosinopril Versus Amlodipine Cardiovascular Events Randomized Trial (FACET) in patients with hypertension and NIDDM. Diabetes Care 1998; 21:597-603
101. Asselbergs FW, Diercks GF, Hillege HL, van Boven AJ, Janssen WM, Voors AA, de Zeeuw D, de Jong PE, van Veldhuisen DJ, van Gilst WH. Effects of fosinopril and pravastatin on cardiovascular events in subjects with microalbuminuria. Circulation 2004; 110:2809-2816
102. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med 2000; 342:145-153
103. Dahlof B, Devereux RB, Kjeldsen SE, Julius S, Beevers G, de Faire U, Fyhrquist F, Ibsen H, Kristiansson K, Lederballe-Pedersen O, Lindholm LH, Nieminen MS, Omvik P, Oparil S, Wedel H. Cardiovascular morbidity and mortality in the Losartan

Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 2002; 359:995-1003
104. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 1994; 344:1383-1389
105. Arcaro G, Zenere BM, Saggiani F, Zenti MG, Monauni T, Lechi A, Muggeo M, Bonadonna RC. ACE inhibitors improve endothelial function in type 1 diabetic patients with normal arterial pressure and microalbuminuria. Diabetes Care 1999; 22:1536-1542
106. Schalkwijk CG, Smulders RA, Lambert J, Donker AJ, Stehouwer CD. ACE-inhibition modulates some endothelial functions in healthy subjects and in normotensive type 1 diabetic patients. Eur J Clin Invest 2000; 30:853-860
107. Gasic S, Wagner OF, Fasching P, Ludwig C, Veitl M, Kapiotis S, Jilma B. Fosinopril decreases levels of soluble vascular cell adhesion molecule-1 in borderline hypertensive type II diabetic patients with microalbuminuria. Am J Hypertens 1999; 12:217-222
108. Tan KC, Chow WS, Ai VH, Lam KS. Effects of angiotensin II receptor antagonist on endothelial vasomotor function and urinary albumin excretion in type 2 diabetic patients with microalbuminuria. Diabetes Metab Res Rev 2002; 18:71-76
109. Dogra GK, Watts GF, Chan DC, Stanton K. Statin therapy improves brachial artery vasodilator function in patients with Type 1 diabetes and microalbuminuria. Diabet Med 2005; 22:239-242
110. Stoclet JC, Chataigneau T, Ndiaye M, Oak MH, El Bedoui J, Chataigneau M, Schini-Kerth VB. Vascular protection by dietary polyphenols. Eur J Pharmacol 2004; 500:299-313
111. Mukamal KJ, Maclure M, Muller JE, Sherwood JB, Mittleman MA. Tea consumption and mortality after acute myocardial infarction. Circulation 2002; 105:2476-2481
112. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 1993; 342:1007-1011
113. Duffy SJ, Keaney JF, Jr., Holbrook M, Gokce N, Swerdloff PL, Frei B, Vita JA. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. Circulation 2001; 104:151-156
114. Flesch M, Schwarz A, Bohm M. Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. Am J Physiol 1998; 275:H1183-H1190
115. Lee KW, Kim YJ, Lee HJ, Lee CY. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J Agric Food Chem 2003; 51:7292-7295
116. Fisher ND, Hollenberg NK. Flavanols for cardiovascular health: the science behind the sweetness. J Hypertens 2005; 23:1453-1459
117. Keen CL, Holt RR, Oteiza PI, Fraga CG, Schmitz HH. Cocoa antioxidants and cardiovascular health. Am J Clin Nutr 2005; 81:298S-303S
118. Fisher ND, Hughes M, Gerhard-Herman M, Hollenberg NK. Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. J Hypertens 2003; 21:2281-2286
119. Vlachopoulos C, Aznaouridis K, Alexopoulos N, Economou E, Andreadou I, Stefanadis C. Effect of dark chocolate on arterial function in healthy individuals. Am J Hypertens 2005; 18:785-791

120. Grassi D, Lippi C, Necozione S, Desideri G, Ferri C. Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. Am J Clin Nutr 2005; 81:611-614
121. Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, Kelm M. Vascular effects of cocoa rich in flavan-3-ols. JAMA 2003; 290:1030-1031
122. Grassi D, Necozione S, Lippi C, Croce G, Valeri L, Pasqualetti P, Desideri G, Blumberg JB, Ferri C. Cocoa Reduces Blood Pressure and Insulin Resistance and Improves Endothelium-Dependent Vasodilation in Hypertensives. Hypertension 2005; 49: 398–405

Chapter 3

Endothelial function predicts the development of renal damage after combined nephrectomy and myocardial infarction

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Abstract

Introduction: We have demonstrated that individual renal endothelial dilatory function of the healthy rat predicts susceptibility to subsequent renal damage induced by 5/6 nephrectomy. Additionally, we reported that myocardial infarction (MI) performed upon unilateral nephrectomy (UnX) induced highly variable renal damage. Therefore, we studied whether the variability in renal damage after MI could be explained by the variation in individual renal endothelial function prior to the induction of injury.

Methods: Endothelium-dependent relaxation to acetylcholine was investigated in in vitro in small arteries isolated from the extirpated kidney at UnX. MI was induced one week after UnX by ligation of the left coronary artery. Proteinuria and systolic blood pressure (SBP) were evaluated weekly for 16 weeks thereafter using metabolic cages and the tail-cuff method, respectively. Upon termination of the study, focal glomerulosclerosis (FGS) was evaluated by histology as additional marker of renal damage.

Results: After MI nephrectomized male Wistar rats (n=15) gradually developed variable proteinuria, ranging from 20 to 507 mg/24h at week 16, with an average SBP of 131 ± 7 mmHg. The individual renal endothelial function of the healthy animals predicted the extent of renal damage in terms of proteinuria ($r=-0.62$, $p=0.008$) and FGS ($r=-0.70$, $p=0.003$).

Conclusions: Individual level of renal endothelial function in the healthy rat is able to predict the severity of renal damage induced by MI. Further exploration of the underlying mechanisms may lead to discovery of preventive renoprotective therapies.

Introduction

The susceptibility of the individuals to develop proteinuria and subsequent renal damage shows large variability. Some individuals develop the renal damage, while others do not. This interindividual variability can not be explained by variation in blood pressure levels[1] or by differences in the degree of injury, such as subtotal nephrectomy[2]. Therefore, the variation in susceptibility has been proposed to be intrinsic to the kidney itself[3]. Indeed, we previously we demonstrated individual differences in the renal endothelial function of the healthy animal to predict severity of renal impairment after subtotal (5/6) nephrectomy[4].
Recently, we have reported that myocardial infarction (MI) leads to the development of proteinuria in the rat after unilateral nephrectomy (UnX)[5]. Although the large infarcts induced more pronounced proteinuria than the small ones, proteinuria still varied to a great extent among the rats with relatively uniform MI sizes.
Given the above, we hypothesize that the variation in the renal endothelial function among healthy animals predicts their susceptibility to develop proteinuric renal damage after UnX+MI. We employed the rat model of cardiorenal interaction, in which MI is performed following unilateral nephrectomy (UnX). In the healthy kidneys removed at nephrectomy endothelial function of small renal arteries was investigated. Subsequently, this baseline endothelial function was related to markers of renal damage (proteinuria, glomerulosclerosis) measured in the remaining kidney 16 weeks after MI.

Methods

Experimental animals

Animal experimentation was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethical Committee of the University of Groningen. Male Wistar rats (250-275 g, n=15, Harlan, Zeist, The Netherlands) were housed under standard conditions. Rats underwent surgical procedures for UnX (followed by *in vitro* measurements of renal endothelial function) and MI. Subsequently, the animals were followed for 16 weeks. Then the left kidney and the heart were removed under anesthesia, weighted and analyzed for the markers of organ damage.

Surgical procedures

Unilateral nephrectomy (UnX)

Rats underwent unilateral nephrectomy (UnX) of the right kidney by laparotomy and under anesthesia with isoflurane 3% in N_2O/O_2 (1:2). The removed kidney was weighted, put into cold Krebs solution and one small renal (interlobar) artery per kidney was immediately prepared for measurement of renal endothelial function in vitro.

Myocardial infarction (MI)

One week after nephrectomy, rats were intubated, ventilated (Amsterdam Infant Ventilator, Hoek/Loos, Schiedam, The Netherlands), and anesthetized by the administration of isoflurane 3% in N_2O/O_2 (1:2). Myocardial infarction was induced by ligation of the left ascending coronary artery (LAD) as described previously[5]. Variation in the infarct sizes was limited by the standard localization of the suture around LAD. Subsequently, the wound was closed and anesthetics replaced by 100% oxygen for a short while until the rat was able to breathe sufficiently on its own.

In vitro measurements of the renal endothelial function

Endothelial function of isolated small renal (interlobar) arteries was investigated in an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously[4] and measured as endothelium-dependent relaxation to cumulative doses of acetylcholine (ACh; 10^{-8} mol/L - 10^{-4} mol/L) in the vessels preconstricted to 45-50% by phenylephrine ($3x10^{-7}$ - 10^{-6} mol/L). We previously established that endothelial function measured in this way does not differ between arteries of left and right kidney of the same animal[4].

Markers of the renal damage

Urinary protein excretion

Urinary protein excretion was determined weekly by nephelometry (Dade Behring III, The Netherlands) by placing the rats in metabolic cages for 24h (Tecniplast, Buggugiate, Italy).

Focal glomerulosclerosis (FGS)

The left kidneys removed at autopsy were cut longitudinally, fixed and processed for paraffin embedding according to standard procedures. Section of 3 μm were stained with periodic acid Schiff (PAS) and microscopically evaluated for the incidence of FGS as described previously[5].

Cardiovascular parameters

Systolic blood pressure (SBP) was measured weekly in restrained awake animals by means of the tail-cuff method (IITC Inc, USA). Infarct sizes were measured in hearts removed at autopsy. Mid-sagittal slices of the left ventricle were fixed in Bouin's solution, embedded in paraffin and stained with 0.1% Fast Green FCF. Infarct sizes were determined by computerized planimetric measurements as described previously[5].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Concentration-response curves to acetylcholine (ACh) were expressed as percentage of preconstriction to phenylephrine. The Area Under each individual Curve (AUC) was determined (Sigma Plot, Jandell Scientific) and expressed in arbitrary units. The AUC was used to represent renal

endothelial function of the individual animals. The relationship between the baseline endothelial function and markers of renal damage 16 weeks afterwards were determined by Kendall non-parametric correlation test using regression analysis (SPSS).

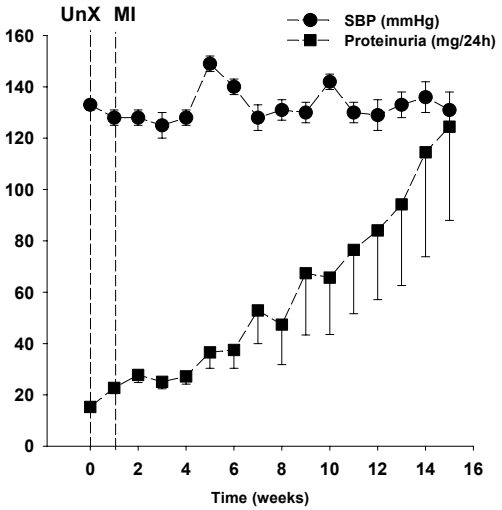

Figure 1. *The time course of systolic blood pressure (SBP, mmHg) and proteinuria (mg/24h) in rats undergoing unilateral nephrectomy (UnX, week 0) and myocardial infarction (MI, week 1). Values are given as means ± SEM.*

Results

Survival

Out of 15 rats undergoing MI operation, 4 died due to acute heart failure within 24 hours after MI (27%). These rats were excluded from further analysis. Remaining 11 rats were followed during the entire period of 16 weeks.

Endothelial function in the healthy nephrectomized kidney

The renal arteries isolated at the time point 0 from the healthy nephrectomized kidney responded to acetylcholine to a variable extent. The endothelium-dependent relaxation characterized by the Area Under the acetylcholine Curve (AUC) averaged 175.6 ± 6.0 arbitrary units with individual values ranging from 155.0 to 216.9 arbitrary units.

Renal damage induced by MI

After UnX and MI, proteinuria gradually increased with time from the mean value of 15 ± 3 mg/24h at week 0 up to 125 ± 37 mg/24h at week 16 *(Figure 1, $p<0.001$)*. Individual values

showed large variation (factor 25.4), ranging from 20 to 507 mg/24h. The renal damage induced by MI in the remaining left kidney was characterized by an increased FGS incidence (17.7 ± 3.4 % compared with the baseline value of the right healthy kidney 0.8 ± 0.5 %, p<0.001). Further, a marked increase of renal mass/body weight ratio was observed at the end of the study in the left kidney (5.1 ± 0.1 x 10^{-3}) as compared with the baseline value of the right healthy kidney (4.1 ± 0.1 x 10^{-3}, p<0.01).

Cardiovascular parameters

Systolic blood pressure (SBP) did not increase with time, as with the values amounting 133 ± 3 mmHg at week 0 and 131 ± 7 mmHg at week 16 *(Figure 1)*. Histologically assessed MI sizes averaged 25 ± 2%.

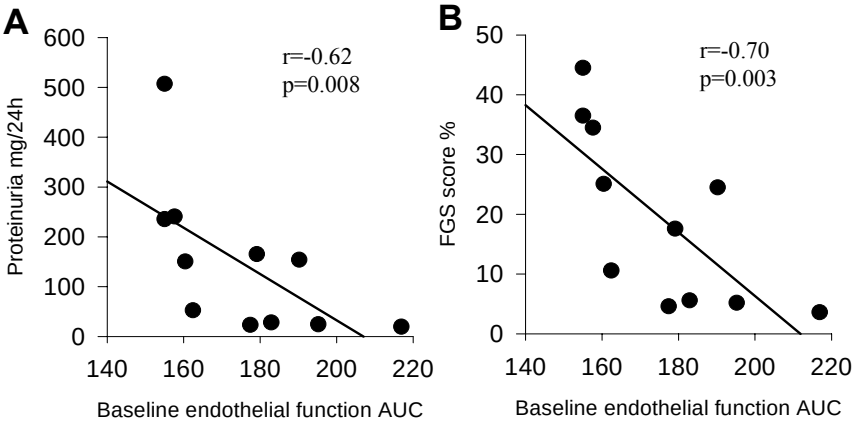

Figure 2. *Correlation between the baseline renal endothelial function measured in the healthy nephrectomized kidney and the development of* ***A)*** *proteinuria,* ***B)*** *incidence of focal glomerular sclerosis 16 weeks after the unilateral nephrectomy and myocardial infarction. FGS- focal glomerular sclerosis, AUC- Area Under the acetylcholine Curve in arbitrary units*

Correlation analysis

The endothelial function measured in the healthy kidney removed by UnX predicted the renal damage inflicted on the remaining kidney by myocardial infarction. The baseline endothelial function negatively correlated with the proteinuria at week 16 (*Figure 1A*, r=-0.62, p=0.008), indicating that rats with more pronounced endothelial function developed less proteinuria after UnX and MI. A similar correlation was found between the baseline renal endothelial function and focal glomerulosclerosis (FGS) at week 16 (*Figure 1B*, r=-0.70, p=0.003). Finally, the weight of the kidney measured at autopsy at week 16 was also

predicted by the renal endothelial function at the baseline (r=-0.59, p=0.01). Consecutively, all the parameters of renal damage were interrelated (proteinuria *versus* FGS r=0.96, p<0.001; proteinuria *versus* kidney weight r=0.72, p=0.01, FGS *versus* kidney weight r=0.60, p=0.01). In contrast, baseline renal endothelial function and the parameters of renal damage did not correlate with either SBP or the infarct sizes (data not shown).

Discussion

In the current study, we found that myocardial infarction (MI) of similar size induces a highly variable damage in the remaining kidney of rats with unilateral nephrectomy (UnX). The severity of the renal damage was predicted by the renal endothelial function measured in isolated interlobular arteries prepared from the healthy kidney prior to the MI.

It is intriguing, that myocardial infarction of relatively uniform size induces highly variable renal damage characterized by both proteinuria and focal glomerulosclerosis. The considerable variation in the proteinuria and the renal damage has been shown also for the other models of progressive renal disease in spite of the relative stable and uniform injury, such as subtotal nephrectomy[4], adriamycin-induced nephrosis[6] or hypertensive-induced renal damage[7].

The mechanisms responsible for the development of renal damage after MI and UnX are largely unexplored. Interestingly, no proteinuria or histological damage occurs in animals with MI or UnX alone[5]. Unilateral nephrectomy (UnX) already represents a state of mild renal damage as the hemodynamic adaptations occur in the remaining glomeruli to compensate for the nephron loss. Several mechanisms by which MI accelerates mild renal damage may be hypothesized, including neurohumoral activation occurring after the MI and subsequent heart failure[8,9] the endothelial dysfunction of the systemic arteries[10,11] or the generalized inflammatory reaction associated with acute MI. Our data, however, indicate that the renal effects of MI depend, at least partially, on intrarenal factors that are present previous to the injury (MI) triggering renal damage. These factors are reflected by the intrinsic variation in the endothelial dilatory capacity of renal arteries of healthy individuals, as the renal endothelial function predicted the renal outcome after MI. Consequently, the animals with pronounced endothelial dilatory capacity of renal arteries seem to be more protected against proteinuric renal damage induced by an MI.

One of the possible explanations for the relationship between pronounced renal endothelial dilation and protection from injury may be that such vessels have spare capacity to deal with the deleterious hemodynamic alterations associated with neurohumoral activation. The endothelial function of the healthy renal arteries has been already shown to predict proteinuria in the model of subtotal (5/6) nephrectomy[4], in which the glomerular hemodynamic adaptation has also been implicated. It may be suggestive to speculate that similar hemodynamic mechanisms operate in the development of proteinuria in both 5/6Nx and UnX+MI model. Alternatively, the pronounced vasodilatory function may reflect

different endothelial protective properties which deal with other deleterious systemic factors. e.g. the inflammatory response[12,13]. Finally, even the model of combined UnX and MI represents the complex relation between heart and kidney. Proteinuric disease itself is associated with worsening of cardiovascular prognosis[14,15] and thus has an impact on the cardiac damage[16]. Indeed, Dikow et al. has shown increased infarct size in uremic rats[25]. Although in our study, care was taken to limit the variability of infarct size at the operation, the infarct size was measured at the end of the study and thus under the influence of renal disease. However, the resulting variation was relatively small and the individual infarct sizes did not correlate with either proteinuria or renal endothelial function. Furthermore, another cardiovascular extrarenal parameter, systolic blood pressure, did not change throughout the study and did not correlate with the renal damage. Therefore it is likely that an intrarenal factor was responsible for the variation in renal damage.

In this study we demonstrated, that the extent of renal impairment induced by MI upon subclinical renal dysfunction is predicted by the intrarenal endothelial function prior to the induction of MI. This observation would imply that measurements of intrarenal endothelial function may be used as a tool to identify the individuals prone to the renal impairment. Further, should renal endothelial function actually determine the sensitivity of the kidney to deleterious events, the modulation of the renal endothelial function may provide protection against the progressive renal damage. Finally, the progression of the renal damage is most likely (co)-dependent on an intrarenal mechanism, emphasizing further the need for renoprotection in patients with cardiovascular disease. Further exploration of the intrarenal mechanism(s) of the individual susceptibility to renal damage may lead to improved prevention of both cardiovascular mortality and renal function loss.

References

1. Cusi D, Tripodi G, Casari G, Robba C, Bollini P, Merati G, Bianchi G. Genetics of renal damage in primary hypertension. Am J Kidney Dis 1993; 21:2-9
2. Gretz N, Waldherr RSM. The remnant kidney model. In: Gretz, N. and Strauch M. (editors). Experimental and genetic rat models of chronic renal failure. Basel: Karger; 1993. pp. 1-28.
3. Churchill PC, Churchill MC, Bidani AK, Griffin KA, Picken M, Pravenec M, Kren V, Lezin ES, Wang JM, Wang N, Kurtz TW. Genetic Susceptibility to Hypertension-induced Renal Damage in the Rat . Evidence Based on Kidney-specific Genome Transfer. J Clin Invest 1997; 100:1373-1382
4. Gschwend S, Buikema H, Navis G, Henning RH, de Zeeuw D, van Dokkum RPE. Endothelial Dilatory Function Predicts Individual Susceptibility to Renal Damage in the 5/6 Nephrectomized Rat. J Am Soc Nephrol 2002; 13:2909-2915
5. van Dokkum RPE, Eijkelkamp WBA, Kluppel ACA, Henning RH, van Goor H, Citgez M, Windt WAKM, van Veldhuisen DJ, de Graeff PA, de Zeeuw D. Myocardial Infarction Enhances Progressive Renal Damage in an Experimental Model for Cardio-Renal Interaction. J Am Soc Nephrol 2004; 15:3103-3110
6. De Boer E, Navis G, Tiebosch AT, De Jong PE, De Zeeuw D. Systemic factors are involved in the pathogenesis of proteinuria-induced glomerulosclerosis in adriamycin nephrotic rats. J Am Soc Nephrol 1999; 10:2359-2366
7. St.Lezin E, Griffin KA, Picken M, Churchill MC, Churchill PC, Kurtz TW, Liu W, Wang N, Kren V, Zidek V, Pravenec M, Bidani AK. Genetic Isolation of a Chromosome 1 Region Affecting Susceptibility to Hypertension-Induced Renal Damage in the Spontaneously Hypertensive Rat. Hypertension 1999; 34:187-191
8. Eiskjaer H, Bagger JP, Danielsen H, Jensen JD, Jespersen B, Thomsen K, Sorensen SS, Pedersen EB. Mechanisms of sodium retention in heart failure: relation to the renin-angiotensin-aldosterone system. Am J Physiol Renal Physiol 1991; 260:F883-F889
9. Mento PF, Maita ME, Wilkes BM. Renal hemodynamics in rats with myocardial infarction: selective antagonism of angiotensin receptor subtypes. Am J Physiol Heart Circ Physiol 1996; 271:H2306-H2312
10. Drexler H, Lu W. Endothelial dysfunction of hindquarter resistance vessels in experimental heart failure. Am J Physiol Heart Circ Physiol 1992; 262:H1640-H1645
11. Ontkean M, Gay R, Greenberg B. Diminished endothelium-derived relaxing factor activity in an experimental model of chronic heart failure. Circ Res 1991; 69:1088-1096
12. Galle J, Quaschning T, Seibold S, Wanner C. Endothelial dysfunction and inflammation: what is the link? Kidney Int Suppl 2003; S45-S49
13. Kaysen GA, Eiserich JP. Characteristics and Effects of Inflammation in End-Stage Renal Disease. Seminars In Dialysis 2003; 16:438-446

14. Hillege HL, Janssen WMT, Bak AAA, Diercks GFH, Grobbee DE, Crijns HJGM, Van Gilst WH, De Zeeuw D, De Jong PE. Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. Journal of Internal Medicine 2001; 249:519-526
15. Hillege HL, Fidler V, Diercks GFH, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, Gans ROB, Janssen WMT, Grobbee DE, de Jong PE, for the Prevention of Renal and Vascular End Stage Disease (PREVEND) Study Group. Urinary Albumin Excretion Predicts Cardiovascular and Noncardiovascular Mortality in General Population. Circulation 2002; 106:1777-1782
16. Kennedy D, Omran E, Periyasamy SM, Nadoor J, Priyadarshi A, Willey JC, Malhotra D, Xie Z, Shapiro JI. Effect of Chronic Renal Failure on Cardiac Contractile Function, Calcium Cycling, and Gene Expression of Proteins Important for Calcium Homeostasis in the Rat. J Am Soc Nephrol 2003; 14:90-97
17. Dikow R, Kihm LP, Zeier M, Kapitza J, Tornig J, Amann K, Tiefenbacher C, Ritz E. Increased Infarct Size in Uremic Rats: Reduced Ischemia Tolerance? J Am Soc Nephrol 2004; 15:1530-1536

Chapter 4

Renal endothelial function and blood flow predict the individual susceptibility to adriamycin-induced renal damage

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Abstract

Background: Susceptibility to renal injury varies among individuals. Previously, we found that individual baseline endothelial function of healthy renal arteries *in vitro* predicts severity of renal damage after 5/6 nephrectomy. Here we sought to establish this relation in adriamycin-induced nephropathy and questioned whether this predictive value might be detected *in vivo* as well. We hypothesized that individual differences in endothelial function and renal perfusion predict the severity of adriamycin-induced renal damage.

Methods: Total endothelial relaxation and the contribution of its dilatory mediators prostaglandins, nitric oxide (NO) and endothelium-dependent hyperpolarizing factor (EDHF) were evaluated in small renal arteries isolated from healthy rat kidneys (n=16) using pressurized vessel set-up. In an additional group of healthy spontaneously voiding rats (n=16), baseline glomerular filtration rate- GFR and effective renal plasma flow- ERPF was measured as clearance of iothalamate and para-amino hippuric acid, respectively. Following functional measurements, adriamycin (1.75 mg/kg i.v.) was injected and subsequent renal damage after 6 weeks was related to baseline parameters.

Results: Animals developed highly variable renal damage. Pronounced individual baseline total endothelial and EDHF-mediated relaxation, as well as baseline ERPF was correlated with more severe proteinuria 6 weeks after injection ($r= 0.51$, $p= 0.04$; $r= 0.68$, $p= 0.01$ and $r= 0.66$, $p= 0.005$, respectively). In contrast, baseline NO-mediated dilation was inversely correlated with proteinuria ($r= -0.71$, $p= 0.006$).

Conclusion: Individuals with pronounced baseline endothelial dilatory ability measured *in vitro* and high renal blood flow *in vivo* are vulnerable to renal damage after adriamycin injection. Therefore interindividual variability in renal hemodynamics might be crucially involved in susceptibility to renal damage.

Introduction

The development and progression of chronic renal damage is largely variable among individuals both in experimental and clinical settings. Environmental systemic factors, such as severity of diabetes or hypertension, cannot fully explain this variation, suggesting some individuals might be intrinsically predisposed to develop renal impairment[1]. Several specific animal strains spontaneously develop renal function loss[2,3], indicating that predisposition of an individual to renal damage involves genetically conditioned factors[4]. However, variable susceptibility to renal damage could also be observed among individuals within a given animal strain. For instance, after a standardized nephrotoxic challenge, such as 5/6 nephrectomy (5/6Nx), outbred Wistar rats develop renal impairment of highly variable severity[5]. Seeking for the factors responsible for this variability we previously observed that *in vitro* measured endothelium-dependent dilatory capacity of small renal arteries in healthy Wistar rats predicts the severity of subsequent renal damage inflicted by 5/6 nephrectomy (5/6Nx)[6]. This indicates that intrinsic variability in renal vascular function might be responsible for variable susceptibility to renal injury. However, at present it is unclear whether this finding is specific for hemodynamically-induced renal impairment, such as seen in 5/6Nx, or whether variability in renal vascular function might also be involved in other types of renal injury.

Therefore, in the present study we investigated whether the concept of predictive value of renal endothelial function is valid in a model of nephropathy induced by the nephrotoxic drug adriamycin. In adriamycin nephropathy, a single injection of cytostatic agent adriamycin leads to progressive renal damage with proteinuria, glomerulosclerosis and interstitial damage[7-9]. Remarkably, this relatively uniform challenge (standard adriamycin injection), results in largely variable renal damage among individuals[10,11], indicating that some individuals might be more susceptible to adriamycin challenge than others. To elucidate the factors responsible for this variability we measured total endothelium-mediated and specific endothelial mediators (e.g. nitric oxide- NO, endothelium-dependent hyperpolarizing factor- EDHF, prostaglandins- PGs)-dependent relaxation of small intrarenal arteries prior to the administration of adriamycin and related this baseline *in vitro* vascular reactivity to the severity of subsequent renal damage. We also explored whether the concept of renal vascular function predictive value might be confirmed *in vivo.* Therefore, in an additional study we sought to determine whether variability in renal hemodynamic function (GFR, ERPF) measured in conscious healthy rats predicts the nephrotoxic effect of subsequent adriamycin administration. We here report that both renal endothelium-dependent reactivity measured *in vitro* and renal blood flow (ERPF) measured *in vivo* in healthy individuals predict the development of adriamycin-induced nephropathy.

Materials and Methods

The experiments were performed using outbred male Wistar rats (300-350 g, Harlan, Zeist, The Netherlands) housed under standardized conditions in animal facilities of the University of Groningen with free access to food and drinking water. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

Study I: Relation between baseline in vitro endothelial function and the severity of adriamycin-induced nephropathy

To investigate the relationship between individual renal endothelial function *in vitro* and subsequent renal damage in adriamycin-induced nephropathy, rats (n=16) underwent unilateral nephrectomy (UnX) under 3% isoflurane in N_2O/O_2 anesthesia. Small renal arteries isolated from nephrectomized kidney were employed for *in vitro* measurements of vascular function as described below. Following UnX, a single i.v. injection of adriamycin in tail vein in a dose of 1.75 mg/kg (Pharmachemie BV, Haarlem, The Netherlands) was administered to induce renal damage. Subsequently, the animals were followed for 6 weeks, during which systolic blood pressure (SBP) was measured weekly in restrained awake animals by means of the tail-cuff method (IITC Inc, Ithaca, USA). Urinary protein excretion was determined weekly by nephelometry (Dade Behring III, Mannheim, Germany) in 24-hour urine samples obtained by putting the animals in metabolic cages. At the end of the study, animals were sacrificed under anesthesia. Remaining kidney and blood samples were harvested for assessment of renal functional and structural damage. Plasma creatinine was measured by means of a colorimetric assay with the Jaffé method without deproteinization (Chema Diagnostica, Jesi, Italy). Focal glomerulosclerosis (FGS) score was determined according to standard procedures in kidneys removed at nephrectomy and autopsy and subjected to fixation and embedding in paraffin. Sections of 3 μm were stained with periodic acid Schiff (PAS) and microscopically evaluated for the incidence of FGS as described previously[12].

Measurements of baseline renal endothelial function in vitro

Small renal (interlobar) arteries (250-350 μm) were isolated from the nephrectomized kidney and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously[6]. Artery segments were cannulated on glass micropipettes and intraluminal pressure was held constant at 70 mmHg. The vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO_2 in O_2) Krebs solution with a pH of 7.4 (120.4 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 NaH_2PO_4, 11.5 mmol glucose). An inverted light microscope

attached to a video camera and video dimension analyzer was used to continuously register lumen diameter.

Following 40 minutes equilibration period, arteries were pre-constricted submaximally with phenylephrine (PE, $3x10^{-7}$- 10^{-6} mol/l) and studied for endothelium-dependent and endothelium-independent relaxation by administering cumulative doses of acetylcholine (ACh; $3x10^{-8}$ mol/l- $3x10^{-5}$ mol/l) and sodium nitroprusside (SNP, 10^{-9}- 10^{-4} mol/l) to the recirculating bath, respectively. ACh-induced relaxation was also studied in the same artery in the presence of either indomethacin (10^{-5} mol/l, to inhibit prostaglandins- PGs); indomethacin and N^{ω}-monomethyl-L-arginine (L-NMMA, 10^{-4} mol/l, to additionally inhibit nitric oxide- NO) or indomethacin plus L-NMMA and a combination of charybdotoxin (chtx, 10^{-7} mol/l) and apamin (apa, $5x10^{-7}$ mol/l), applied into the lumen of the artery as well as to the superfusion medium (to additionally inhibit endothelium-derived hyperpolarizing factor- EDHF). ACh and SNP concentration-response curves were successfully obtained in all 16 animals, whereas curves in the presence of all inhibitors were obtained in 13 individuals. Previously, we established that endothelial dilatory function did not differ between renal arteries within the used diameter range isolated from the same kidney and therefore ACh-induced relaxation of one artery can be considered representative[6].

Study II: Relation between baseline in vivo renal hemodynamic function and severity of adriamycin-induced nephropathy

To investigate whether *in vivo* determinants of renal hemodynamics in the healthy rat predict subsequent renal damage in adriamycin nephropathy measurements of renal function were performed prior to the injection of adriamycin (1.75 mg/kg) in an additional group of rats (n=16). Following the injection, SBP and urinary protein excretion were measured weekly in the same way as in the first experimental study.

Measurements of baseline renal hemodynamic function in vivo

Glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were measured by clearance of simultaneously infused iothalamate and para-amino hippuric acid (PAH) respectively, in freely moving and spontaneously voiding rats as described previously[13]. Briefly, the rats instrumented with a jugular and carotid catheter were infused with a bolus of iothalamate (9 mg/kg) and PAH (12 mg/kg) followed by continuous intra-arterial infusion of these markers (iothalamate 0.9 mg/h and PAH 4.5 mg/h). Following an initial equilibration period of two hours, clearance period was determined by the urine collection depending on spontaneous voiding of the rat and a blood sample was drawn via jugular catheter after each urine collection. Plasma and urine levels of iothalamate and PAH in the samples were determined by HPLC[14]. ERPF and GFR were calculated as a plasma clearance of PAH and urinary clearance of iothalamate respectively, according to the method described by *Donker et al.*[15] in man, which was adapted for rats at our laboratory by *de Vries et al.*[13]. Mean arterial pressure (MAP) was measured continuously during the

experiment by connecting the carotid catheter to a pressure transducer (Baxter Healthcare, Irvine, USA). Renal vascular resistance was calculated as the ratio of MAP and ERPF.

Table 1. *Clinical parameters of animals in both studies measured prior to the administration of adriamycin (baseline) and 6 weeks thereafter and in vivo measurements of renal hemodynamics at baseline in study II.*

	Study I		**Study II**	
	Baseline	**Week 6**	**Baseline**	**Week 6**
Clinical parameters				
Body weight (g)	320 ± 15	406 ± 22	328 ± 14	410 ± 24
SBP (mmHg)	135 ± 5	136 ± 11	132 ± 4	135 ± 8
Proteinuria (mg/24h)	21 ± 6	430 ± 195*	22 ± 4	420 ± 241*
FGS (%)	0 ± 1	24 ± 10*	ND	ND
Plasma creatinine (μmol/l)	46 ± 16	69 ± 22*	ND	ND
Renal hemodynamic measurements				
ERPF (ml/min/100g BW)	-	-	2.85 ± 0.89	-
GFR (ml/min/100g BW)	-	-	0.53 ± 0.15	-
MAP (mmHg)	-	-	114 ± 15	-
RVR (mmHg.min/ml)	-	-	15 ± 7	-

SBP- systolic blood pressure, FGS- focal glomerulosclerosis, BW- body weight, ERPF- effective renal plasma flow, GFR- glomerular filtration rate, RVR- renal vascular resistance, MAP- mean arterial pressure, ND- not determined; data are presented as mean ± SD; ***** $p<0.05$ *versus* baseline.

Statistical analysis

Data are expressed as mean ± standard deviation (SD), unless stated otherwise. In the analysis of vascular experiments, concentration-response curves to ACh or SNP were expressed in percentage of pre-constriction to PE. The concentrations of drugs causing half-maximal responses were expressed as negative logarithm of the molar concentration (pD_2 values). The Area Under each individual acetylcholine Curve (AUC) was determined (Sigma Plot, SPSS Inc.) and expressed in arbitrary units. The contribution of three endothelial mediators (PGs, NO and EDHF) to endothelial relaxation was calculated as a difference between corresponding AUCs (*Figure 1A*). Statistical comparisons between

parameters at the baseline and at the end of the study were performed by a Student's paired t-test. The characteristics of the concentration-response curves were compared by one-way ANOVA or ANOVA for repeated measures when appropriate. Significance was accepted at $p<0.05$. The relationship between individual endothelial or renal function and renal damage was calculated using Pearson's parametric or Spearman's non-parametric correlation test (SPSS), where appropriate.

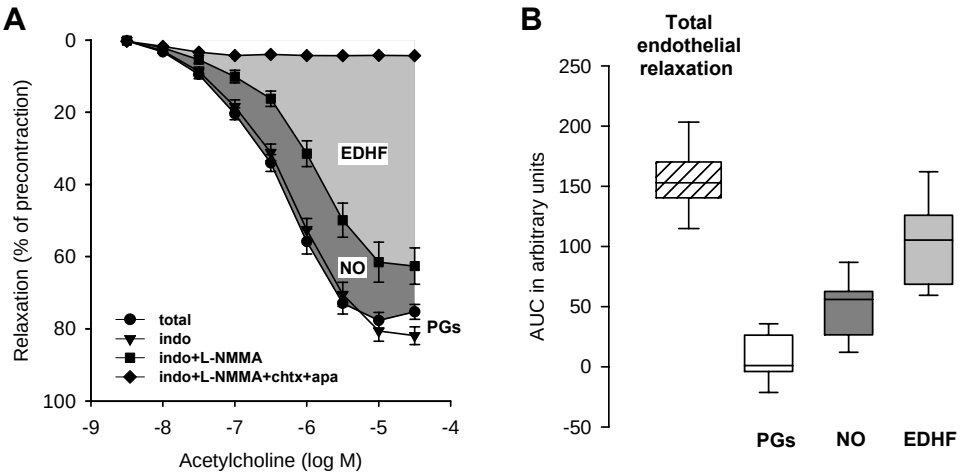

Figure 1. A) *Concentration-response curves to endothelium-dependent vasodilator acetylcholine (ACh) in small renal arteries isolated before the administration of adriamycin. The curves were constructed in absence of any inhibitor (total), in presence of indomethacin (indo, 10^{-5} mol/l), in additional presence of L-NMMA (10^{-4} mol/l) and in additional presence of charybdotoxin (chtx, 10^{-7} mol/l) and apamin (apa, $5x10^{-7}$ mol/l). Data are given as mean ± SEM.* ***B)*** *Variability in total endothelium-dependent relaxation, prostaglandins (PGs)-, nitric oxide (NO)- and EDHF-mediated relaxation of small renal arteries isolated from healthy rats before the administration of adriamycin. AUC- Area Under Curve expressed in arbitrary units; box whisker plot: central box encloses middle 50% of all the data, horizontal line inside the box represents median and the whiskers encompass 5 to 95 percentiles.*

Results

Markers of renal damage in adriamycin nephropathy

Clinical characteristics of the rats used in both studies are presented in *Table 1*.

Six weeks after injection of adriamycin, rats in the first study developed overt nephropathy, characterized by elevated proteinuria, FGS and increased plasma creatinine when compared to values measured in healthy animals before the injection. SBP remained stable over the

entire experimental period. Interestingly, despite the standardized injection of adriamycin, proteinuria varied considerably among individual rats, similarly ranging from 145 to 883 mg/24h in the first and from 124 to 869 mg/24 h in the second experimental group. Both plasma creatinine and FGS score correlated positively with proteinuria ($r= 0.56$, $p< 0.01$ and $r= 0.63$, $p= 0.01$ respectively), suggesting that proteinuria adequately reflects renal damage in this model.

Table 2. *Baseline characteristics of the concentration-response curves to endothelium-dependent vasodilator acetylcholine and endothelium-independent vasodilator sodium-nitroprusside (SNP) in small renal arteries isolated from healthy rats prior to the administration of adriamycin. The effect of endothelial vasodilatory pathway inhibitors on the acetylcholine concentration-response curve is also shown.*

	E_{max}	pD_2
Endothelium-dependent relaxation		
Acetylcholine total	80 ± 8	6.4 ± 0.2
+ indomethacin	82 ± 9	6.2 ± 0.2
+ indomethacin + L-NMMA	64 ± 19[a,b]	6.0 ± 0.2[a,b]
+ indomethacin + L-NMMA + chtx+apa	3 ± 2[a,b,c]	-
Endothelium-independent relaxation		
SNP	88 ± 9	6.9 ± 0.4

Data are means ± SD; E_{max}- maximal relaxation to acetylcholine in % of precontraction to phenylephrine, pD_2- negative logarithm of molar concentration of acetylcholine causing half of maximal response (EC_{50}), chtx + apa: charybdotoxin + apamin, [a] $p<0.05$ versus control, [b] $p<0.05$ versus indomethacin, [c] $p<0.05$ versus indomethacin + L-NMMA

Study I: endothelial function predicts renal damage in adriamycin nephropathy

Variability of renal endothelial function in healthy rat

ACh induced concentration-dependent relaxation of small renal arteries. The average group response and curve characteristics are presented in *Figure 1A* and *Table 2*. Relaxation to ACh was highly variable prior to injection of adriamycin in these –at that time- healthy

animals *(Figure 1B)* and was independent from the level of PE-induced pre-constriction or endothelium-independent relaxation to SNP. Blockade of PGs by indomethacin resulted in variable small changes of the ACh curve in individual rats, however on average not being significantly different *(Figure 1A, Table 2).* Additional administration of the NO inhibitor consistently decreased endothelium-dependent relaxation *(Figure 1A, Table 2),* however to a highly variable extent in individual animals *(Figure 1B).* As a result, the remaining EDHF-mediated relaxation also displayed considerable variability *(Figure 1B).* This relaxation was completely blocked by the combination of indomethacin, L-NMMA and chtx+apa *(Figure 1A, Table 2)*.

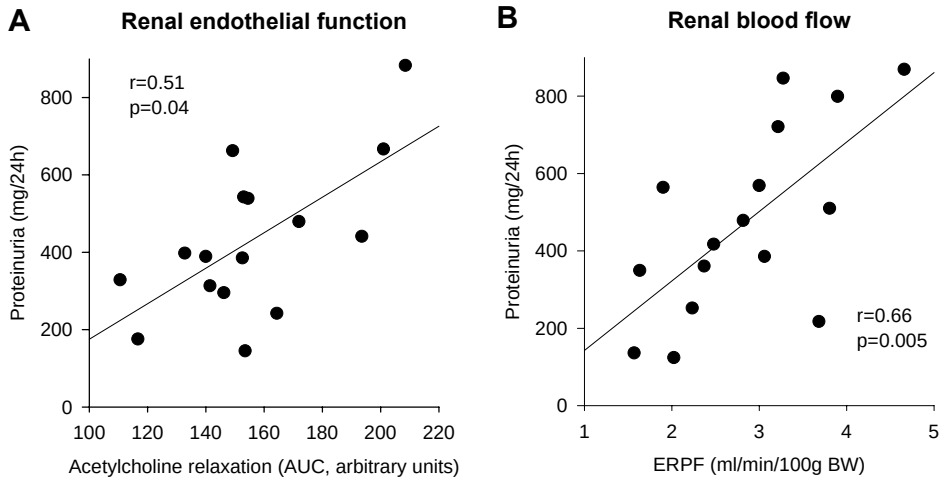

Figure 2. A) *Correlation between individual total endothelium-dependent relaxation of small renal arteries measured prior to the administration of adriamycin and proteinuria determined 6 weeks after the administration of adriamycin (n=16).* ***B)*** *Correlation between individual relative effective renal plasma flow (ERPF) measured prior to the administration of adriamycin and proteinuria determined 6 weeks after adriamycin injection (n=16). AUC- Area Under Curve expressed in arbitrary units, BW- body weight.*

Correlation analysis

Correlation analysis was performed to investigate the relation between baseline endothelial dilatory function and the level of renal damage 6 weeks after the administration of adriamycin. ACh-induced relaxation (expressed as AUC) positively correlated with the level of proteinuria *(Figure 2A).* Endothelium-dependent relaxation also predicted plasma creatinine levels in individual animals ($r= 0.68$, $p< 0.01$). Thus, the rats with a pronounced endothelium-dependent dilation at baseline developed more severe renal damage after adriamycin injection. There was no correlation between the individual level of PE pre-

constriction or endothelium-independent relaxation by SNP on one hand and renal damage on the other.

Additionally, we studied the relation between the endothelial mediators of relaxation and proteinuria. PGs-mediated relaxation tended to correlate positively with proteinuria, but this was of marginal statistical significance *(Figure 3C).* A positive correlation was also found between the individual EDHF-mediated relaxation and proteinuria *(Figure 3B).* In contrast, individual NO-mediated relaxation was inversely correlated with the level of proteinuria (*Figure 3A)* as well as FGS (r= -0.69, p= 0.01), suggesting that individuals with a large NO-mediated relaxation are protected from the development of renal damage in adriamycin nephropathy.

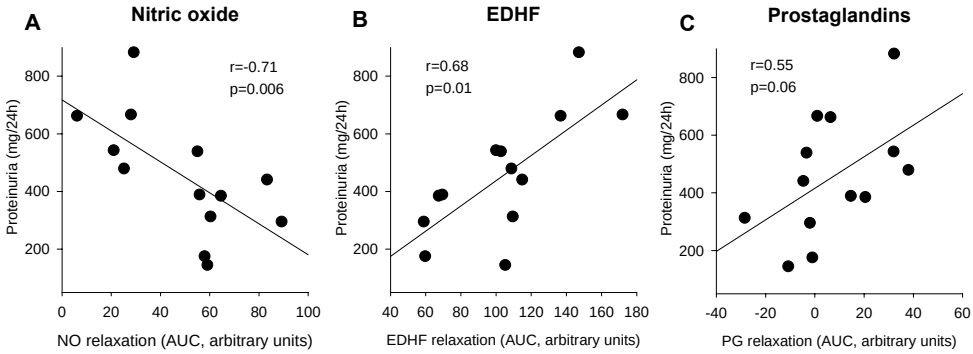

Figure 3. *Correlation between individual nitric oxide* ***(A)****-, EDHF* ***(B)****- and prostaglandins* ***(C)****- mediated endothelium-dependent relaxation of small renal arteries measured prior to the administration of adriamycin and proteinuria determined 6 weeks after administration of adriamycin (n=13 each). AUC- Area Under Curve expressed in arbitrary units.*

Study II: renal blood flow predicts renal damage in adriamycin nephropathy

Baseline values of renal functional parameters have been included in *Table 1.* GFR, ERPF and RVR displayed considerable variability in healthy animals prior to adriamycin injection.

Individual ERPF just prior to adriamycin injection markedly correlated with the proteinuria 6 weeks after the induction of the disease (*Figure 2B*), indicating that individuals with highly perfused kidney at the time of adriamycin administration developed more renal damage. Additionally, a negative correlation was found between individual RVR and proteinuria 6 weeks after adriamycin injection (r= -0.65, p= 0.007). In contrast, no correlation was found between individual baseline GFR and proteinuria at week 6 (r= 0.24, p= NS).

Discussion

In the present study we found that the individual level of renal endothelial function of healthy rats measured *in vitro* predicts their susceptibility to renal damage after injection of adriamycin. Additionally, baseline level of renal blood flow and renal vascular resistance measured *in vivo* were related to adriamycin-induced renal damage as well. These data indicate that state of renal vasculature measured both *in vitro* and *in vivo* might predispose certain healthy individuals to a more severe course of toxic renal damage.

Endothelium-dependent relaxation of intrarenal arteries varies considerably in healthy animals of an outbred rat strain, which allows to test for the predictive value of this parameter in the development of renal damage. In the model of adriamycin nephropathy employed in our study, animals progressively developed highly variable renal damage indicated by increased urinary protein excretion. As shown both in present and previous experiments[10], proteinuria represents a good indicator of renal damage severity in this model, since it remains relatively stable after 6 weeks and it is correlated with other structural and functional markers of renal damage.

The animals with pronounced baseline acetylcholine-induced relaxation in small renal arteries developed more severe proteinuria after adriamycin injection. This finding might seem surprising since acetylcholine-induced relaxation is considered to be a measure of protective abilities of vascular endothelium[16] and preserved endothelial relaxation has been shown to be associated with lower rate of future cardiovascular events in high risk populations[17,18]. Furthermore, in contrast to our present data, we previously observed that individuals with large renal endothelial relaxation were protected against renal injury after 5/6Nx[6]. It should be noted, however, that the nature of renal injury crucially differs between adriamycin nephropathy and 5/6Nx, involving direct nephrotoxicity in the former and hyperfiltration of remnant nephrons in the latter model[5]. A crucial role of the specific etiology of renal injury in adriamycin nephropathy is also suggested by the striking finding of our *in vivo* study. Enhanced baseline level of renal blood flow and reduced renal vascular resistance measured in conscious rats prior to the administration of adriamycin were associated with more severe renal outcome. It has been previously shown that transient clipping of the renal artery for only several minutes prevents adriamycin-induced renal damage[10], indicating that the acute cytotoxic effect of adriamycin in the kidney is responsible for the initial renal injury. Therefore it seems conceivable, that highly perfused kidneys, such as seen in individuals with high ERPF and low RVR, may be exposed to a larger amount of the toxic agent leading to more adverse renal damage. Since endothelium crucially participates in the regulation of renal hemodynamics[19,20], *in vivo* data might provide a hemodynamic explanation for predictive value of *in vitro* acetylcholine-induced relaxation. However, neural, humoral and local mechanisms other than endothelial participate in the regulation of blood flow *in vivo*. This fact might also contribute to a stronger relation we observed between blood flow and proteinuria than between endothelial

function and proteinuria. Overall, the relation between endothelial function and blood flow on one hand and severity of renal damage on the other hand indicates that the basal state of the renal vasculature, measured both in conscious animals and in isolated preparations, may reflect the susceptibility to renal damage in adriamycin nephropathy. Combined results of our previous (in 5/6Nx)[6] and present (adriamycin nephropathy) data suggest that variability in renal endothelial function is involved in the susceptibility to renal injury under various experimental conditions, however its exact role might be critically dependent on type of renal injury and etiology of progressive renal damage.

To further elucidate contrasting findings of endothelial prediction in diverse experimental models of renal disease we investigated the role of specific mechanisms underlying endothelial relaxation. Acetylcholine-mediated relaxation such as measured in this experiment reflects the sum of functional activity of prostaglandins (PGs), nitric oxide (NO), and EDHF, as evidenced by complete blockade of endothelial response by the combination of respective inhibitors of these pathways indomethacin, L-NMMA and charybdotoxin plus apamin. The view of a protective role of endothelial relaxation is largely based on the concept of beneficial activity of NO[16]. Interestingly, when addressing NO-mediated vasodilation specifically, the individuals with large NO relaxation were protected from adriamycin-induced renal damage. A similar relation was also found in rats subjected to 5/6Nx[6], suggesting a protective role of NO in the development of renal injury with various etiologies. Beneficial effects of NO might go far beyond its vasodilatory abilities: NO limits inflammation, proliferation, leucocyte adhesion and other processes involved in the progression of renal damage[21]. Several authors propose a crucial role of NO also in the initiation and development of renal injury. *Erdely et al.* report that mild nitric oxide synthase (NOS) inhibition might convert a rat strain resistant to renal injury after 5/6Nx into a model of rapidly progressing renal disease[22]. In humans, several studies have found an association between endothelial NOS polymorphism and end-stage renal disease[23,24]. Collectively, these data indicate that variation in NO bioactivity might be crucially involved in interindividual susceptibility to renal injury.

Contribution of additional endothelial mediator, termed EDHF, predicted the development of proteinuria in agreement with total acetylcholine-induced relaxation. EDHF exerts its effects by hyperpolarization of underlying smooth muscle cells, however its identity remains elusive[25]. Therefore its role is more difficult to interpret than that of NO. However, one intriguing aspect of EDHF is its putative inverse relationship with NO. Based on this assumption, it was proposed that EDHF might serve as a backup dilatory mechanism under circumstances when NO production is decreased[26,27]. Indeed, in present study, individual NO contribution inversely correlated with contribution of EDHF to endothelium-mediated relaxation, thus possibly reflecting the lack of NO-mediated protection. Relaxation mediated by yet another endothelial mechanism, release of prostaglandins (PGs) tended to predict the severity of renal damage. We previously proposed that despite a minimal net effect of cyclooxygenase blockade on acetylcholine-induced relaxation, interindividual variation in the proportion of the relaxing and constrictive PGs might still be detected[6]. The

reason for the potential protective role of vasoconstrictive PGs in adriamycin nephropathy remains unclear. One might speculate that predominant release of PGs with constrictive properties in preglomerular vessels may protect glomeruli against a deleterious increase in intraglomerular pressure and hyperfiltration, or could be involved in the regulation of renal blood flow during adriamycin injection. Overall, these data suggest that measurement of endothelium-dependent relaxation attributed to specific mediators, such as NO, EDHF and PGs, might provide additional important information on the interindividual susceptibility to renal damage.

Perspectives

If consistently confirmed, the predictive value of renal vascular function might have broad potential clinical implications. Measurements of baseline endothelial or hemodynamic function and organ blood flow might identify individuals with increased risk for future adverse renal outcomes. More important, one may speculate on interventional strategies to prevent end-organ damage, for instance by specifically targeting NO, EDHF and/or cyclooxygenase pathways. Additionally, findings in a model of adriamycin nephropathy might have potential implications for adriamycin-induced long-term organ toxicity associated with chemotherapy in humans. In addition to nephrotoxicity, severe cardiomyopathy often manifests after adriamycin treatment in humans[28]. Whether interindividual differences in endothelial function and/or organ perfusion predict the extent of damage also in this condition, needs to be investigated further. If so, then lowering blood flow to a specific organ at the time of drug administration may provide a protection from unwanted toxicity.

Conclusion

In this study, we showed that both baseline endothelial function of isolated renal vessels and renal blood flow measured *in vivo* in conscious animals predict the severity of renal damage imposed by subsequent administration of a nephrotoxic drug. Together with previous findings in other experimental models of renal damage, these data suggest that interindividual variability in baseline renal hemodynamics might be responsible for susceptibility to renal impairment. The predictive value of total renal endothelial function seems to be critically dependent on the etiology of renal injury, whereas nitric oxide-dependent relaxation provides the consistent information in different experimental models. Further investigation into the nature of renal vascular variability may help us to reveal the mechanisms involved in the development of progressive renal disease.

References

1. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 2003; 139:137-147
2. Provoost AP. Spontaneous glomerulosclerosis: insights from the fawn-hooded rat. Kidney Int Suppl 1994; 45:S2-S5
3. Schulz A, Litfin A, Kossmehl P, Kreutz R. Genetic dissection of increased urinary albumin excretion in the munich wistar fromter rat. J Am Soc Nephrol 2002; 13:2706-2714
4. Bowden DW. Genetics of kidney disease. Kidney Int Suppl 2003; S8-12
5. Gretz N, Waldherr R, Strauch M. The remnant kidney model. In: Gretz, N. and Strauch, M. (editors). Experimental and genetic models of chronic renal failure. Basel: Karger; 1993. pp. 1-28.
6. Gschwend S, Buikema H, Navis G, Henning RH, de Zeeuw D, Van Dokkum RP. Endothelial dilatory function predicts individual susceptibility to renal damage in the 5/6 nephrectomized rat. J Am Soc Nephrol 2002; 13:2909-2915
7. Bertani T, Poggi A, Pozzoni R, Delaini F, Sacchi G, Thoua Y, Mecca G, Remuzzi G, Donati MB. Adriamycin-induced nephrotic syndrome in rats: sequence of pathologic events. Lab Invest 1982; 46:16-23
8. Bertani T, Cutillo F, Zoja C, Broggini M, Remuzzi G. Tubulo-interstitial lesions mediate renal damage in adriamycin glomerulopathy. Kidney Int 1986; 30:488-496
9. Okuda S, Oh Y, Tsuruda H, Onoyama K, Fujimi S, Fujishima M. Adriamycin-induced nephropathy as a model of chronic progressive glomerular disease. Kidney Int 1986; 29:502-510
10. de Boer E, Navis G, Tiebosch AT, de Jong PE, de Zeeuw D. Systemic factors are involved in the pathogenesis of proteinuria-induced glomerulosclerosis in adriamycin nephrotic rats. J Am Soc Nephrol 1999; 10:2359-2366
11. Rook M, Lely AT, Kramer A, van Goor H, Navis G. Individual differences in renal ACE activity in healthy rats predict susceptibility to adriamycin-induced renal damage. Nephrol Dial Transplant 2005; 20:59-64
12. Van Dokkum RP, Eijkelkamp WB, Kluppel AC, Henning RH, van Goor H, Citgez M, Windt WA, van Veldhuisen DJ, de Graeff PA, de Zeeuw D. Myocardial infarction enhances progressive renal damage in an experimental model for cardio-renal interaction. J Am Soc Nephrol 2004; 15:3103-3110
13. Marcel de Vries PA, Navis G, de Boer E, de Jong PE, de Zeeuw D. A method for accurate measurement of GFR in conscious, spontaneously voiding rats. Kidney Int 1997; 52:244-247

14. Bell RR, Bombardt PA, DuCharme DW, Kolaja GJ, Packwood WH, Bothwell BE, Satoh PS. A non-radioactive iothalamate and p-aminohippuric acid high-performance liquid chromatographic method for simultaneously measuring glomerular filtration rate and renal blood flow in the rat. Biomed Chromatogr 1994; 8:224-229
15. Donker AJ, van der Hem GK, Sluiter WJ, Beekhuis H. A radioisotope method for simultaneous determination of the glomerular filtration rate and the effective renal plasma flow. Neth J Med 1977; 20:97-103
16. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 2004; 15:1983-1992
17. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 106:653-658
18. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 2000; 101:1899-1906
19. Baylis C, Qiu C. Importance of nitric oxide in the control of renal hemodynamics. Kidney Int 1996; 49:1727-1731
20. Beierwaltes WH, Sigmon DH, Carretero OA. Endothelium modulates renal blood flow but not autoregulation. Am J Physiol 1992; 262:F943-F949
21. Blantz RC, Munger K. Role of nitric oxide in inflammatory conditions. Nephron 2002; 90:373-378
22. Erdely A, Wagner L, Muller V, Szabo A, Baylis C. Protection of wistar furth rats from chronic renal disease is associated with maintained renal nitric oxide synthase. J Am Soc Nephrol 2003; 14:2526-2533
23. Buraczynska M, Ksiazek P, Zaluska W, Nowicka T, Ksiazek A. Endothelial nitric oxide synthase gene intron 4 polymorphism in patients with end-stage renal disease. Nephrol Dial Transplant 2004; 19:2302-2306
24. Noiri E, Satoh H, Taguchi J, Brodsky SV, Nakao A, Ogawa Y, Nishijima S, Yokomizo T, Tokunaga K, Fujita T. Association of eNOS Glu298Asp polymorphism with end-stage renal disease. Hypertension 2002; 40:535-540
25. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. Trends Pharmacol Sci 2002; 23:374-380
26. Sofola OA, Knill A, Hainsworth R, Drinkhill M. Change in endothelial function in mesenteric arteries of Sprague-Dawley rats fed a high salt diet. J Physiol 2002; 543:255-260
27. Waldron GJ, Ding H, Lovren F, Kubes P, Triggle CR. Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. Br J Pharmacol 1999; 128:653-658
28. Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. N Engl J Med 1998; 339:900-905

Chapter 5

Selective impairment of myogenic constriction and endothelial function of small renal arteries precedes the development of renal damage in the hypertensive Fawn-Hooded rat

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Abstract

Background: Chronic kidney disease is associated with abnormal regulation of arterial tone throughout various vascular beds. It is however unknown whether generalized vascular dysfunction precedes the development of kidney disease. We studied myogenic constriction and endothelium-mediated dilatory responses in two inbred Fawn-Hooded (FH) rat strains, one of which (FHH) spontaneously develops hypertension, proteinuria and glomerulosclerosis, whereas other (FHL) does not.

Methods: Small renal, mesenteric resistance arteries and aorta isolated from FH rats prior to (7 weeks old) and following the development of mild proteinuria (12 weeks old), were mounted in perfused and isometric set-ups, respectively. Myogenic response, acetylcholine-induced endothelium-dependent relaxation and the contribution of nitric oxide (NO), cyclooxygenase (COX)-derived prostaglandins and endothelium-derived hyperpolarizing factor (EDHF) were studied using the inhibitors L-NMMA, indomethacin and charybdotoxin+apamin, respectively.

Results: In small renal arteries, markedly impaired myogenic reactivity and endothelial dysfunction due to excessive COX1-mediated production of constrictive prostaglandins were selectively present in FHH as compared to FHL even prior to the development of proteinuria. In contrast, myogenic reactivity was intact in mesenteric resistance artery of FHH. In addition, meseneteric endothelial dysfunction was observed attributed to the loss of EDHF. Renal myogenic and peripheral EDHF alterations were further attenuated after the development of proteinuria. Aortic reactivity did not differ between FHL and FHH at the time points studied.

Conclusion: The present study shows that vascular dysfunction in both small renal and systemic arteries precedes renal end-organ damage in a spontaneous model of hypertension-associated renal damage. Renal and peripheral vasomotor mechanisms are affected heterogeneously and differentially modulated by renal disease. These early vascular changes might be potentially involved in the increased susceptibility of FHH rats to renal injury.

Introduction

In chronic kidney disease, a progressive deterioration in renal function is associated with abnormal regulation of arterial tone, both on the level of endothelium and vascular smooth muscle[1,2]. A growing body of evidence indicates that even patients with microalbuminuria, an earliest marker of renal structural damage, exhibit generalized endothelial dysfunction in both renal and systemic vascular beds[3-5]. In fact, it has been suggested that endothelial dysfunction might precede the development of microalbuminuria[6,7], thus potentially representing a determinant of renal disease progression. Endothelium-dependent relaxant responses, a surrogate of endothelial function, are mediated by the release of vasoactive factors, such as nitric oxide (NO), cyclooxygenase-derived prostaglandins (PGs) and the yet unidentified endothelium-derived hyperpolarizing factor (EDHF)[8]. We have previously shown, that the interindividual variability in endothelium-dependent reactivity of intrarenal arteries, including NO-, PGs- and EDHF-mediated responses, among healthy rats of an outbred Wistar rat strain predicts their susceptibility to subsequent renal damage induced by renal mass reduction[9] or nephrotoxic drug[10]. This suggests that endothelial function might be one of the factors governing the susceptibility to experimental renal end-organ damage.

In addition to experimental models, specific inbred animal strains have been described spontaneously developing progressive renal disease[11]. Fawn-hooded (FH) rat provides a genetically well-defined model comprised of two inbred strains with different occurrence of renal injury. Hypertensive fawn-hooded rats (FHH) spontaneously develop moderate hypertension, proteinuria and severe glomerulosclerosis at a young age, subsequently followed by progressive renal failure[12-15]. In contrast, Fawn-hooded rats with low blood pressure (FHL) seem to be resistant to the development of hypertension and renal damage[14]. It has been proposed that altered vascular-smooth muscle-mediated reactivity to intraluminal pressure, termed myogenic response, in preglomerular arteries might be responsible for different sensitivity of these strains to renal injury[16]. Yet, the data comparing renal myogenic response in FHL and FHH rats provide inconsistent results[16,17], whereas the role of endothelial reactivity is unknown.

In the present study we aim to define the role of vasomotor changes in the course of spontaneous hypertension-associated renal disease. First, we explored whether renal vascular dysfunction precedes the development of renal damage. To this end, in animals prone (FHH) and resistant (FHL) to renal damage, we compared endothelium-mediated (NO-, PGs and EDHF-dependent) and myogenic responses of small renal arteries at early age, prior to the appearance of renal damage. In addition, we explored whether renal vascular changes reflect generalized vasomotor dysfunction by studying endothelial and myogenic responses in small resistance (mesenteric) and large conduit arteries (aorta). Finally, we evaluated the the observed vascular changes in time, by investigating vasomotor reactivity in animals at the older age, when mild proteinuria had already developed.

Materials and methods

Animals and in vivo measurements

Experiments were performed in young male FHL and FHH rats at week 7 and week 12 after birth (n= 9-12 per strain and per time point). In FHH rats, this time frame represents the ages in which none and minor proteinuria is detected, respectively. All animals were bred at the animal facilities of Erasmus University, Rotterdam, the Netherlands and housed under standard conditions in animal facility of University of Groningen, the Netherlands receiving food and water *ad libitum*. Short before reaching their target age, animals were put in metabolic cages to measure fluid intake and urine output. Urinary protein excretion was determined by nephelometry (Dade Behring III, Mannheim, Germany) in 24-hour urine samples. Subsequently, animals were anesthetized by 2% isoflurane in N_2O/O_2 (2:1), the right carotid artery was cannulated, and systolic and diastolic blood pressure was measured by a pressure transducer catheter (Millar Instruments, Germany) in the aortic root. Following these measurements, blood was drawn via abdominal aorta. Subsequently, mesenterium, kidneys and thoracic aorta were harvested for the analysis of vascular function and end-organ damage. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

Determination of renal damage

Plasma and urine creatinine was measured by means of a photometric assay with the Jaffé method without deproteinization (DiaSys Diagnostic Systems, Holzheim, Germany) and creatinine clearance was calculated as creatinine clearance= (urine creatinine x urine flow) / (plasma creatinine x bodyweight). Paraffin embedded kidneys were cut in 3 μm sections and stained with periodic acid Schiff (PAS) and the incidence of focal glomerulosclerosis (FGS) score was microscopically evaluated according to standard procedures as described previously[18].

Vascular reactivity of small renal and systemic resistance arteries

Small renal (interlobar) arteries and third-order branches of superior mesenteric arteries were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously[9]. Artery segments were cannulated on glass micropipettes and the vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO_2 in O_2) Krebs solution with a pH of 7.4. An inverted light microscope attached to a video camera and video dimension analyzer was used to continuously register lumen diameter.

Myogenic reactivity of small renal and systemic resistance arteries

Intraluminal pressure was set at 80 mmHg, arteries were allowed to equilibrate for 40 minutes and checked by a single dose of phenylephrine (PE, $3x10^{-7}$ mol/l) and

acetylcholine (ACh; $3x10^{-5}$ mol/l), for smooth muscle and endothelium viability, respectively. Following a wash out, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each pressure step was maintained for 5 minutes to reach the stable contractile response. Following the myogenic protocol, preparations were washed with Krebs solution and employed for investigation of endothelial function as described below. Thereafter, calcium containing Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether)tetraacetic acid (EGTA, 2 mmol/l) and passive pressure-diameter curves were obtained over the same 20-160 mmHg pressure range.

To explore the role of endothelium in myogenic tone regulation, the effect of endothelium removal was investigated in additional renal arteries (n=10 for each strain) isolated from 12 weeks old animals. Endothelium was removed by perfusing the preparation with 5 ml of air and endothelial removal was confirmed by the absence of dilative response to ACh ($3x10^{-5}$ mol/l) following a submaximal pre-constriction with PE ($3x10^{-7}$ mol/l). Subsequently to the wash-out, active and passive myogenic curves were recorded as described above.

Endothelium-dependent relaxation of small renal and systemic resistance arteries

Following the measurement of active myogenic curves, intraluminal pressure was set to 80 mmHg and arteries were washed and stabilized for 20 minutes. Because the level of spontaneous tone was not sufficient for the subsequent relaxation studies, arteries were pre-constricted with phenylephrine ($3x10^{-7}$- 10^{-6} mol/l) to 50-60% of initial baseline diameter. Endothelium-dependent relaxation was assessed by administering cumulative doses of acetylcholine (ACh; 10^{-9}- $3x10^{-5}$ mol/l) to the recirculating bath. After the construction of a full ACh concentration-response curve and wash out, the response to ACh was studied in the same artery in the presence of indomethacin (10^{-5} mol/l) to inhibit prostaglandins (PGs) production. Subsequently, the same procedure was repeated in the combined presence of indomethacin and the nitric oxide (NO) production inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA, 10^{-4} mol/l). In some arteries of both vascular types (FHL and FHH, n=4 each), we found that this remaining relaxation in the presence of indomethacine and L-NMMA was caused by the release of endothelium-derived hyperpolarizing factor (EDHF), as it was completely attenuated by the combination of the potassium channels blockers charybdotoxin (chtx, 10^{-7} mol/l) and apamin (apa, $5x10^{-7}$ mol/l). By analyzing the above-mentioned protocols of endothelium-dependent relaxation, the contribution of all three mediators (PGs, NO and EDHF) to endothelial relaxation was calculated as a difference between Area Under the Curve (AUC) of respective ACh-concentration-response curves.

Involvement of cycloxygenase (COX) pathway in endothelium-dependent contractions of small renal arteries

To investigate the underlying mechanisms of prostanoid-dependent endothelium-mediated contractions observed in small renal arteries of FHH rats, in separate set of arteries (n=6),

ACh-concentration responses were obtained in a presence of either the COX-1 selective inhibitor valeryl salicylate (VAS, 10^{-4} mol/l), the COX-2 selective inhibitor NS398 (10^{-6} mol/l), the TXA_2/PGH_2 receptor antagonist SQ29548 (10^{-6} mol/l) or the superoxide scavenger superoxide dismutase (SOD, 50 U/ml).

General smooth muscle reactivity of small renal and systemic arteries

Additional arteries were used to control for the potential variation in depolarization-and receptor-mediated smooth muscle reactivity. After a stabilization period, concentration-response contractile curves were obtained using KCl (20- 120 mmol/l) and phenylephrine (10^{-8}- 10^{-5} mol/l) with a wash out period between the protocols. Additionally, concentration-response curves to the direct smooth muscle vasodilator sodium nitroprusside (SNP, 10^{-9}- $3x10^{-5}$ mol/l) were constructed after submaximal preconstriction with phenylephrine ($3x10^{-7}$- 10^{-6} mol/l).

Vascular reactivity of isolated aortic rings

The thoracic aorta was cleaned from the connective tissue and cut into 2 mm rings, which were mounted in isotonic contraction organ baths filled with aerated, warmed Krebs solution and subjected to 14 mN preload. After one hour stabilization period, arteries were stimulated by KCl (60 mmol/l) to check their viability, washed out and pre-constricted submaximally by 10^{-6} mol/l phenylephrine. Endothelium-dependent relaxation was investigated similarly to the protocol performed in perfused small arteries, e.g. concentration-response curves to acetylcholine (10^{-9}- 10^{-4} mol/l) were obtained in absence and subsequently in presence of indomethacin (10^{-5} mol/l) and indomethacin+L-NMMA (10^{-4} mol/l) to investigate the contribution of PGs, NO and EDHF to endothelium-mediated relaxation. In other rings, dose-response curves to phenylephrine (10^{-9}- 10^{-5} mol/l) were followed by measurements of reactivity to sodium nitroprusside (10^{-10}- 10^{-5} mol/l).

Chemicals

Krebs solution had a following composition (mM): 120.4 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 NaH_2PO_4, 11.5 glucose. All these compounds were purchased from Merck (Darmstadt, Germany). VAS, NS398 and SQ29548 were purchased from Cayman Chemical (Ann Harbor, MI, USA). All other drugs were obtained from Sigma-Aldrich Chemie, the Netherlands. They were dissolved either in ethanol (VAS, NS398, SQ29548) or in de-ionized water and diluted with Krebs solution. Stock solution for indomethacin was prepared in 96 mmol $NaHCO_3$.

Statistical analysis and calculations

Data are expressed as mean ± standard error of means (SEM). Myogenic tone, describing myogenic behaviour of an artery at a given pressure, was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same

pressure in calcium-free/EGTA solution, i.e., myogenic tone (%)= 100 [($D_{Ca\text{-}free}$– D_{Ca})/$D_{Ca\text{-}free}$], where D is the diameter in calcium-free ($D_{Ca\text{-}free}$) or calcium-containing (D_{Ca}) Krebs.

Table 1. *In vivo characteristics of FHL and FHH rats showing either no (7 weeks of age) or minor (12 weeks of age) renal damage.*

	No renal damage		**Minor renal damage**	
	FHL	**FHH**	**FHL**	**FHH**
Body weight (g)	215 ± 3	211 ± 3	308 ± 4#	307 ± 5#
SBP (mmHg)	118 ± 3	130 ± 4*	123 ± 3	132 ± 3*
DBP (mmHg)	76 ± 3	86 ± 3*	78 ± 3	87 ± 3*
Fluid intake (ml/24h)	33 ± 5	40 ± 3	36 ± 3	39 ± 2
Urine output (ml/24 h)	19 ± 2	23 ± 2	24 ± 1	28 ± 1
Proteinuria (mg/ 24 h)	19 ± 1	17 ± 2	28 ± 1#	46 ± 4*#
Plasma creatinine (μmol/l)	51 ± 3	50 ± 2	41 ± 1#	43 ± 1#
Creatinine clearance (ml/min/100g body weight)	7.4 ± 0.4	6.2 ± 0.2	9.7 ± 0.3#	9.2 ± 0.4#
Kidney weight (g)	1.1 ± 0.03	1.02 ± 0.03	1.28 ± 0.02#	1.31± 0.03#
FGS score (%)	1 ±1	1 ±1	1 ±1	2 ±1

* p<0.01 *versus* FHL of the same age
# p<0.01 *versus* the same strain at age of 7 weeks
SBP- systolic blood pressure, DBP- diastolic blood pressure, FGS- focal glomerulosclerosis

The myogenic index, describing myogenic reactivity of an artery in response to a pressure change, i.e. the slope of active pressure-diameter relationship, was calculated for every 20 mmHg pressure step (ΔP) as a percentage change in corresponding active diameter D_{Ca}, i.e. myogenic index (%/mmHg) = 100 [($\Delta D_{Ca}/D_{Ca}$)/ΔP]. For each individual artery maximal myogenic tone and peak myogenic index were determined from all the pressures and pressure steps studied, respectively. Concentration-response curves to the vasoconstrictors

KCl and phenylephrine (PE) were calculated as a percentage change from baseline artery diameter and from maximal KCl response for small arteries and aorta, respectively. Concentration-response curves of the vasodilators ACh and SNP were expressed in percentage of pre-constriction to PE. The curves were characterized by the maximal relaxation (E_{max}) and the negative logarithm of acetylcholine molar concentration causing half-maximal relaxation (pD_2). Area Under ACh concentration-response curve (AUC) was determined (Sigma Plot, SPSS Inc., Chicago, IL, USA) and expressed in arbitrary units. The contribution of three endothelial mediators (PGs, NO and EDHF) to endothelial relaxation was calculated as a difference between corresponding AUCs. Full myogenic and concentration-response curves of ACh and SNP were compared by ANOVA for repeated measures followed by Bonferroni *post hoc* test for multiple comparisons. Group-comparison of animal and vascular parameters was performed by unpaired Student's t-test. Differences were considered significant at $p<0.05$ (two-tailed).

Results

Animal characteristics

Characteristics of FHL and FHH rats in both experimental periods are given in *Table 1*. In 7 weeks old FHL and FHH rats, no renal damage was present, as evidenced by similar levels of proteinuria, FGS, plasma creatinine and creatinine clearance. Both SBP and DBP were marginally elevated in FHH rats as compared to FHL. Five weeks later, FHH rats developed significant proteinuria, without an increased incidence of FGS or loss of renal function. This suggests that in 12 weeks old FHH rats, mild renal damage is present. In contrast to proteinuria, blood pressure did not increase in both strains as compared to week 7. Additional animals (n=10 each strain) were followed for 26 weeks to confirm development of renal damage. At this age, hypertension, profound proteinuria and structural damage were present in FHH rats, but not in FHL rats (data not shown).

Myogenic reactivity is selectively impaired in the renal vasculature of FHH rats prior to the development of renal end-organ damage

At the age of 7 weeks, passive diameters of small renal arteries did not differ between FHL and FHH rats in the pressure range studied (*Figure 1A*). However, as evidenced by the differences in active curves *(Figure 1A),* renal arteries isolated from kidneys of FHH rats developed significantly lower myogenic tone as compared to FHL (*Figure 2A*). Consequently, young FHH demonstrated reduced maximal myogenic tone (22 ± 4.8 *versus* 10.8 ± 2.0 %, p= 0.03) and the peak myogenic index (-6.9 ± 4.8 *versus* 0.6 ± 0.8 %/mmHg, p= 0.07 for FHL *versus* FHH, respectively). In contrast to small renal arteries, active myogenic curves obtained in mesenteric arteries isolated from 7 weeks old rats did not differ between both strains (*Figure 1C*), demonstrating a similar level of systemic myogenic tone in FHL and FHH rats (*Figure 2C*). Therefore, before any renal end-organ

damage is present, myogenic response seems impaired selectively in renal vasculature of FHH rats.

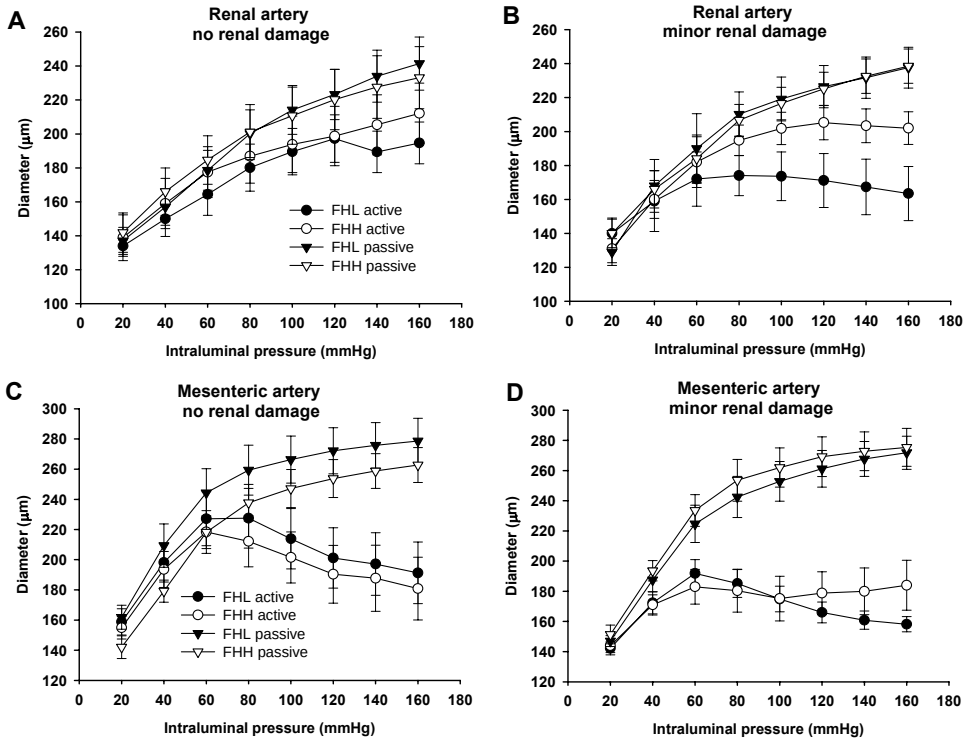

Figure 1. *Reactivity of small renal* ***(A, B)*** *and small mesenteric* ***(C, D)*** *arteries to increase of intraluminal pressure. Curves were recorded in presence (active) and in absence (passive) of extracellular calcium. Arteries were isolated from FHL and FHH rats showing either no (7 weeks of age;* ***A, C****) and or minor (12 weeks of age;* ***B, D****) renal damage.*

Selective renal impairment of myogenic reactivity is more pronounced after the development of proteinuria

At 12 weeks of age, small renal arteries from FHL rats showed marked difference between active and passive myogenic curves *(Figure 1B)*, developing more pronounced myogenic tone *(Figure 2B)* when compared to 7 weeks old animals *(Figure 2A).* In contrast, the level of myogenic tone in 12 weeks old FHH rats remained minimal and significantly different from FHL animals *(Figure 2B),* as reflected by markedly impaired maximal myogenic tone and peak myogenic index in FHH rats as compared to FHL *(Table 2).* In small mesenteric arteries at 12 weeks, active myogenic curves *(Figure 1D)* and myogenic tone *(Figure 2D)* development were comparable in FHL and FHH. As a result, maximal myogenic tone and

peak myogenic index in mesenteric arteries *(Table 2)* did not differ between FHL and FHH rats. Therefore, selective impairment of myogenic reactivity in small renal arteries is even more pronounced after the development of renal damage.

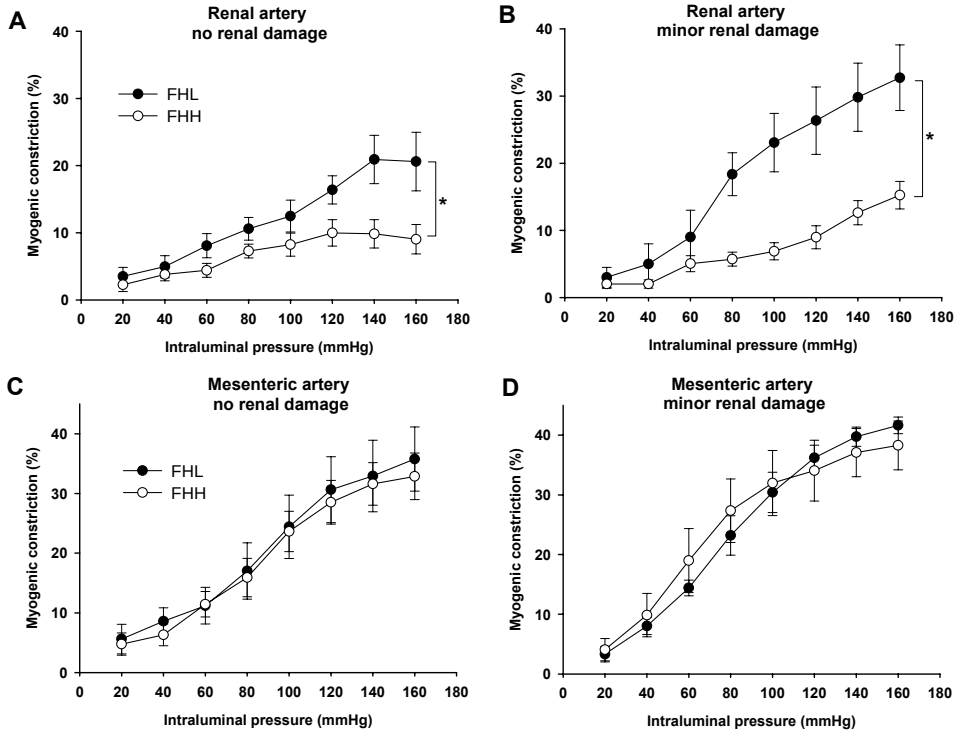

***Figure 2**. Changes in myogenic tone of small renal **(A, B)** and small mesenteric **(C, D)** arteries in FHL and FHH rats showing either no (7 weeks of age; **A, C**) or minor (12 weeks of age; **B, D**) renal damage. * $p<0.05$*

To investigate whether impaired contractile ability of small arteries is confined to myogenic stimuli or a general impairment of vascular contraction, we investigated depolarization- and receptor-mediated contraction to KCl and PE, respectively. Depolarization- and receptor-mediated contractile ability in both vascular beds studied were similar between FHL and FHH rats, as evident from the characteristics of the concentration-response curves to KCl and PE shown in *Table 2*,. To explore whether the endothelium plays a role in the impairment of myogenic tone in renal arteries of FHH rat, we repeated the myogenic protocol after removal of the endothelium. As shown in *Figure 3*, removal of the endothelium did not attenuate the differences in myogenic reactivity between FHL and FHH rats.

***Table 2**. Characteristics of vasoreactivity of small renal, small mesenteric arteries and aorta isolated from FHL and FHH rats at the age of 12 weeks:*
*parameters of response curves to potassium chloride (KCl), α-agonist phenylephrine (PE), increased intraluminal pressure (myogenic tone), endothelium-dependent vasodilator acetylcholine (ACh) and endothelium-independent vasodilator sodium nitroprusside (SNP); E_{max}- maximal contractile response in % of baseline vascular diameter (contractility) or in % of pre-constriction (relaxation), pD_2- negative logarithm of the molar concentration of agonist causing half of the maximal responses, PMI- peak myogenic tone- maximal slope of active myogenic curve (%/mmHg), * $p<0.05$*

			FHL	**FHH**
Renal artery				
Contractility	*KCl*	E_{max} (%)	68 ± 4	71 ± 3
		pD_2	1.33 ± 0.08	1.40 ± 0.04
	PE	E_{max} (%)	68 ± 4	70 ± 3
		pD_2	6.6 ± 0.1	6.8 ± 0.2
	Myogenic	E_{max} (%)	34 ± 5	16 ± 2*
		PMI (%/mmHg)	-3.2 ± 1.0	-0.9 ± 0.4*
Relaxation	*ACh*	E_{max} (%)	66 ± 4	60 ± 4
		pD_2	6.5 ± 0.1	6.8 ± 0.2
	SNP	E_{max} (%)	88 ± 3	86 ± 3
		pD_2	6.9 ± 0.2	6.9 ± 0.2
Mesenteric artery				
Contractility	*KCl*	E_{max} (%)	83 ± 4	82 ± 3
		pD_2	1.37 ± 0.06	1.39 ± 0.04
	PE	E_{max}	81 ± 4	82 ± 4
		pD_2	6.5 ± 0.1	6.7 ± 0.1
	Myogenic	E_{max} (%)	42 ± 1	39 ± 4
		PMI (%/mmHg)	-4.3 ± 0.8	-3.1 ± 0.9
Relaxation	*ACh*	E_{max} (%)	100 ± 1	97 ± 1*
		pD_2	7.2 ± 0.1	6.6 ± 0.1*
	SNP	E_{max} (%)	98 ± 2	97 ± 2
		pD_2	7.3 ± 0.2	7.3 ± 0.2
Aorta				
Contractility	*PE*	$E_{max\ (\%\ of\ KCl)}$	75 ± 5	78 ± 5
		$pD_{2\ (\%\ of\ KCl)}$	6.7 ± 0.1	6.7 ± 0.1
Relaxation	*ACh*	E_{max} (%)	56 ± 4	57 ± 8
		pD_2	6.6 ± 0.1	6.5 ± 0.1
	SNP	E_{max} (%)	98 ± 1	100 ± 1
		pD_2	7.9 ± 0.1	7.7 ± 0.2

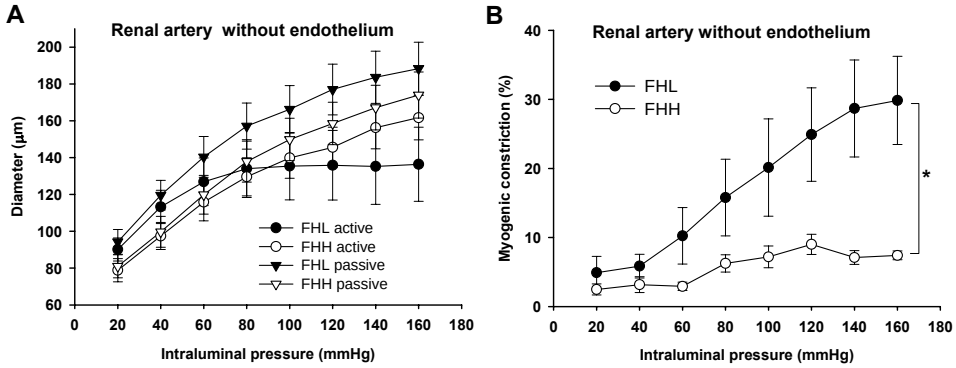

Figure 3. *The influence of endothelium removal on myogenic reactivity of small renal arteries of FHL and FHH rats with minor renal damage.* ***A)*** *Passive and active curves recorded in presence or absence of extracellular calcium, respectively.* ***B)*** *myogenic constriction upon increase of intraluminal pressure in % of passive diameters.* * $p<0.05$

Renal and systemic endothelial dysfunction in FHH rats prior to the development of renal end-organ damage

Acetylcholine (ACh) relaxed small renal arteries isolated from 7 weeks old FHL rats, resulting in monophasic concentration-response curve (*Figure 4A*). In FHH rats however, a biphasic curve was observed as higher concentrations of ACh induced a contractile response (*Figure 4A*). In contrast to renal artery, ACh incubation resulted in monophasic concentration-response curve in both FHL and FHH rats in small mesenteric arteries, although the curve was shifted to the right in FHH rats (*Figure 4C)*. Unlike in small arteries, no difference between ACh-induced relaxations of the two strains was observed in aortic rings (*Figure 4E*). All observed alterations specifically indicated the presence of endothelial dysfunction, since no differences were found in the reactivity to endothelium-independent vasodilator SNP in any vascular bed between FHL and FHH rats (*Table 2*).

Progression of systemic endothelial dysfunction in FHH rats after the development of proteinuria

At the age of 12 weeks, animals showed a similar pattern of endothelial dysfunction in the investigated vascular beds as observed at the age of 7 weeks. In 12 weeks old animals, endothelial dysfunction was present in small renal (*Figure 4B*) and mesenteric artery (*Figure 4D*), but not in aorta (*Figure 4F*). In small mesenteric artery, endothelial dysfunction in FHH was aggravated when compared to 7 weeks old rats, as evidenced by a further shift of the response curve to the right *(Figure 4D).* SNP-induced relaxation remained unchanged in all vascular beds.

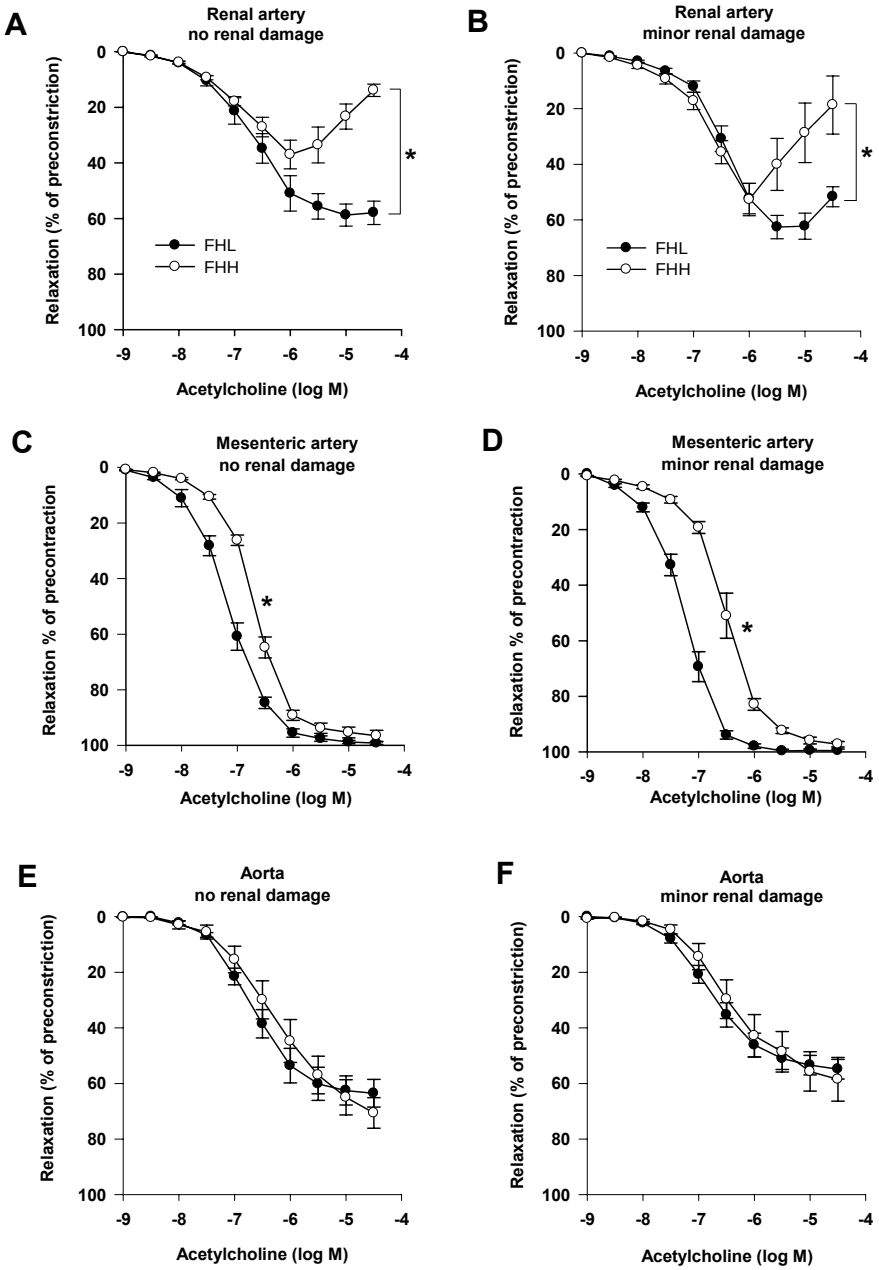

Figure 4. *Endothelial dysfunction in various vascular beds of FH rats. Endothelium-mediated vasodilation to acetylcholine measured in small renal arteries* ***(A, B)****, small mesenteric arteries* ***(C, D)*** *and aorta* ***(E, F)*** *isolated from FHL and FHH rats showing either no (7 weeks of age;* ***A, C, E****) or minor (12 weeks of age;* ***B, D, F****) renal damage.* * *$p<0.05$*

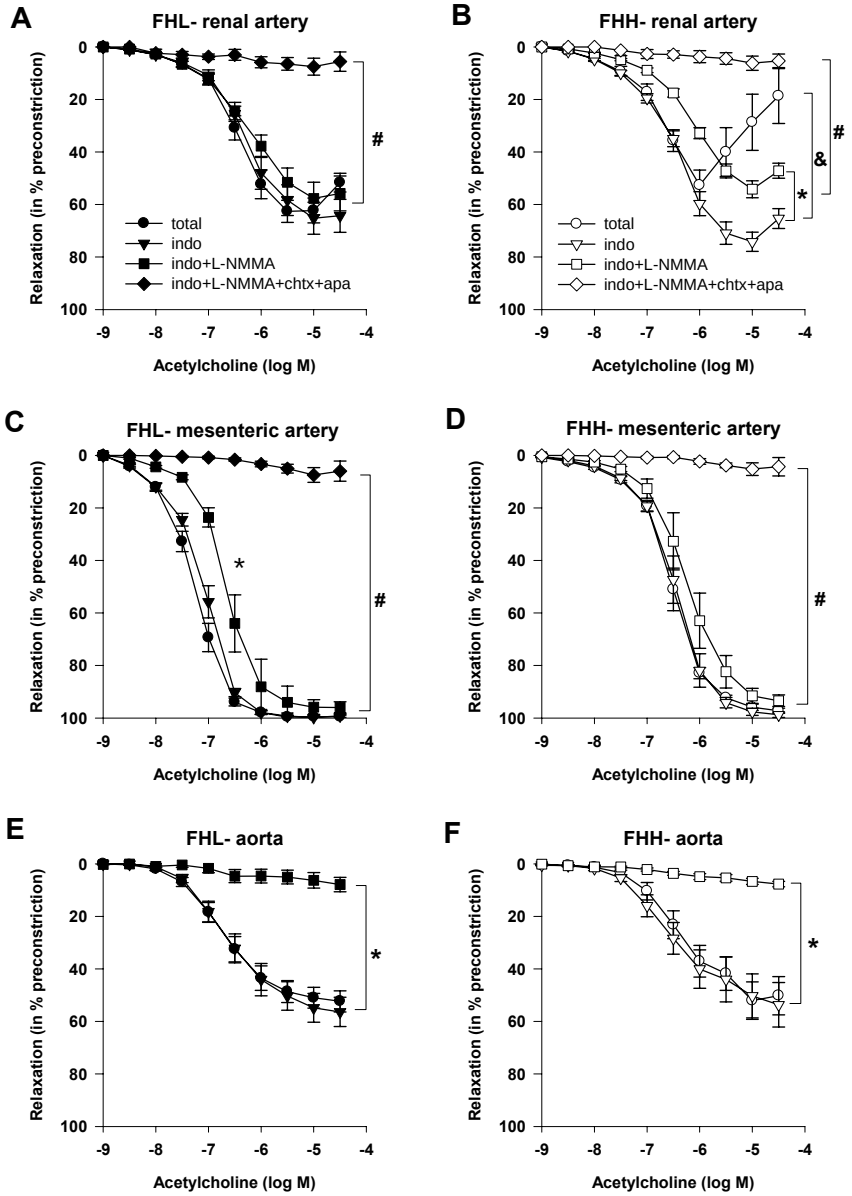

Figure 5. *Heterogeneous mechanisms of endothelial dysfunction between 12 weeks old FHL* ***(A, C, E)*** *and FHH* ***(B, D, F)*** *rats in small renal* ***(A, B)****, small mesenteric arteries* ***(C, D).****and aorta* ***(E, F).*** *Endothelium-mediated vasodilation to acetylcholine in absence (total) of any inhibitors and in the presence of indomethacin (indo, 10^{-5} mol/l), combination of indomethacin and L-NMMA (10^{-4} mol/l) and eventually in additional presence of charybdotoxin (chtx, 10^{-7} mol/l) and apamin (apa, $3x10^{-7}$ mol/l). * $p<0.05$ indo versus indo+L-NMMA, # $p<0.05$ indo+L-NMMA+chtx+apa versus all other curves, & $p<0.05$ indo versus total.*

Heterogeneous mechanisms underlying endothelial dysfunction in FHH rats

To explore the mechanisms responsible for the endothelial dysfunction in studied vascular beds, we constructed ACh concentration response-curve in presence of the inhibitors of endothelium-derived vasodilatory mediators in both 7 and 12 weeks old animals. Since similar curves were obtained at both time points, for reasons of clarity only data from week 12 are presented in *Figure 5*. Indomethacin, an inhibitor of cyclooxygenase (COX), completely reversed endothelium-dependent contractions associated with higher doses of ACh in small renal arteries of FHH rats (*Figure 5B*), while it had no significant effect in FHL rats (*Figure 5A*). Additional blockade of NO by L-NMMA resulted in significant attenuation of ACh-vasodilation in renal arteries of FHH rats (*Figure 5B*), while affecting the relaxation to a lesser extent in FHL animals (*Figure 5A*). The remaining ACh-relaxation was completely blocked by the combined application of potassium channels inhibitors charybdotoxin and apamin suggesting this response is mediated by EDHF *(Figure 5A, B)*.

Indomethacin did not affect the endothelial dilation in either FHL or FHH rats in small mesenteric artery (*Figure 5C, D*). Additional block of NO led to a small right-shift of the curve in FHL (*Figure 5C*), with even less pronounced effect in FHH (*Figure 5D*). Similar to renal vessels, the remaining relaxation was completely inhibited by charybdotoxin and apamin *(Figure 5C, D)*.

In aorta, the ACh-induced response was not altered in the presence of indomethacin, whereas it was almost completely attenuated by NO blockade. No differences were observed between FHL and FHH rats *(Figure 5E, F)*.

Based on these observations, the contribution of the principal endothelial mediators (PGs, NO, EDHF) to endothelial function was calculated. The release of constrictive PGs seems responsible for endothelial dysfunction in small renal arteries of FHH prior to the development of renal damage, as indicated by negative value of PGs contribution in *Figure 6A*. At the same time, PGs do not play any role in endothelial dysfunction in the mesenteric artery, in which rather a reduction in EDHF is found (*Figure 6C*). In aorta, no changes in endothelial mediators could be detected *(Figure 6E)*.

After the development of renal damage, endothelial dysfunction persists in FHH rats with similar mechanisms involved. Renal arteries showed comparable production of contractile PGs (*Figure 6B*) as observed prior to the development of renal damage *(Figure 6A)*. In contrast, mesenteric arteries displayed even more pronounced loss of EDHF-mediated relaxation compared to 7 weeks old FHH rats (*Figure 6D*). No significant changes were observed in the responses of aorta *(Figure 6F)*. Additionally, small renal arteries of FHH rats with renal damage rely relatively more on NO-mediated vasodilation than FHL rats (*Figure 6B*).

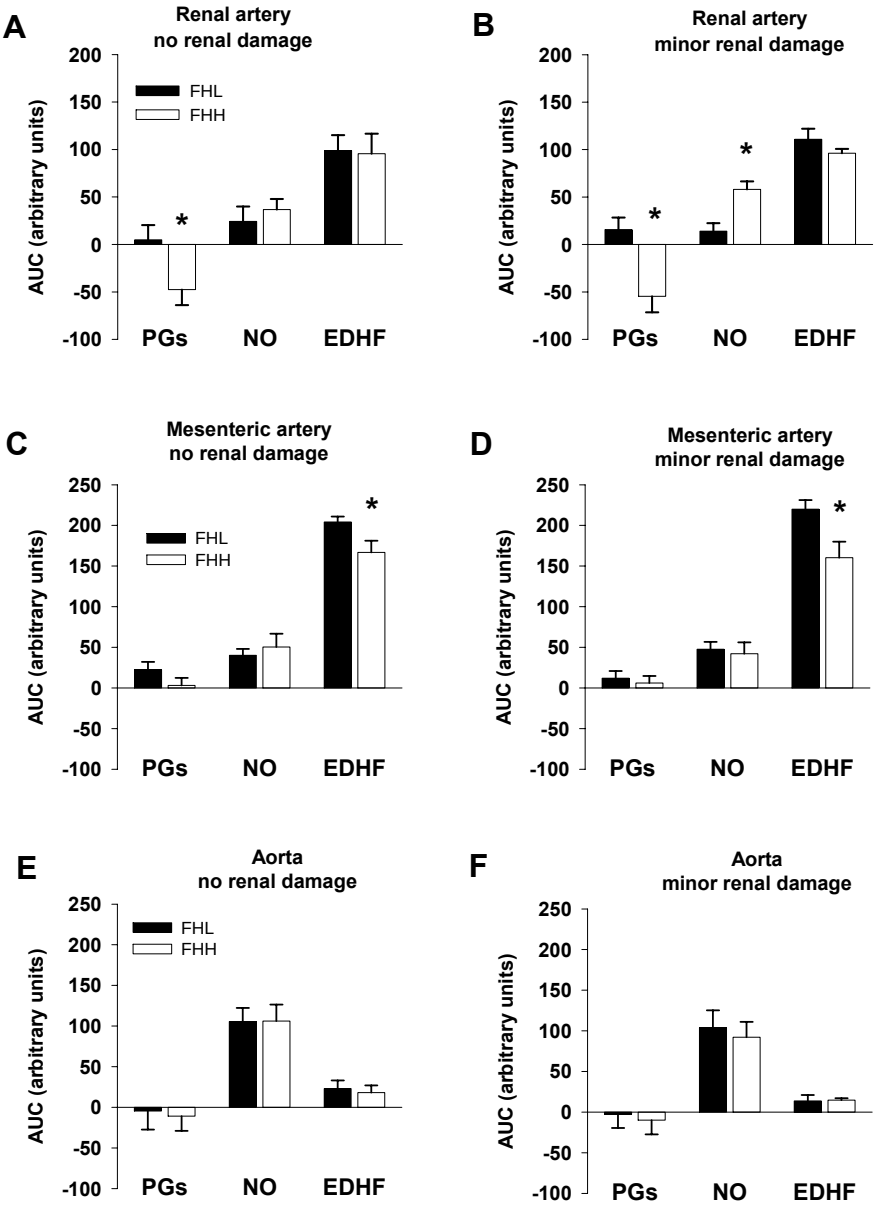

Figure 6. *Heterogeneous contribution of endothelial mediators prostaglandins (PGs), nitric oxide (NO) and endothelium-dependent hyperpolarizing factor (EDHF) to endothelium-dependent relaxation shown in arbitrary units of Area Under acetylcholine concentration-response curve (AUC) in small renal* ***(A, B)****, small mesenteric arteries* ***(C, D)*** *and aorta* ***(E, F)*** *of FHL and FHH rats showing either no (7 weeks of age,* ***A, C, E****) or minor (12 weeks of age;* ***B, D, F****) renal damage.* * $p<0.05$

Mechanisms underlying endothelium-mediated contractions in renal arteries of FHH rats

To identify the mechanisms underlying ACh-mediated indomethacin-sensitive contractile response in renal arteries of FHH rats, additional experiments were performed in 12 week old rats. As shown in *Figure 7*, endothelium-dependent contractions to ACh are reversed to relaxations in the presence of COX-1 inhibitors, but unaffected by COX-2 inhibitors. Furthermore, the contractions were blunted by a TXA_2/PGH_2 receptor antagonist and by superoxide dismutase, identifying the target receptor of the COX1-derived prostanoids and suggesting involvement of reactive oxygen species in this response, respectively.

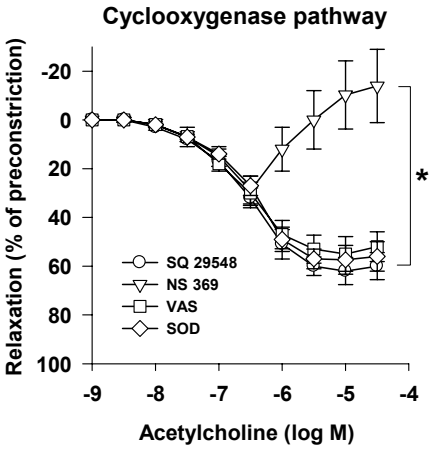

Figure 7. *The effect of cyclooxygenase pathway inhibitors on prostanoid-mediated endothelium-dependent contractions in small renal arteries of 12 weeks old FHH rats. VAS- valeryl salicylate (10^{-4} mol/l)- COX-1 selective inhibitor, NS398 (10^{-6} mol/l)- COX-2 selective inhibitor, SQ29548 (10^{-6} mol/l)- TXA_2/PGH_2 receptor antagonist, SOD- superoxide dismutase (50 U/ml). * $p<0.05$ NS398 versus all other inhibitors.*

Discussion

The present study reports a severely impaired myogenic contractility of small renal arteries in an inbred rat model of spontaneous renal disease (FHH) when compared to its disease-resistant counterpart (FHL). Moreover, FHH rats displayed a COX-1-dependent endothelial dysfunction. These renal vascular changes were detected prior to the manifestation of renal damage, thus being potentially involved in the increased susceptibility of FHH rats to renal injury. Furthermore, these alterations were selectively observed in small renal, but not in small mesenteric arteries, in which only endothelial dysfunction due to impaired EDHF-mediated vasodilation was found. Finally, impaired vascular reactivity was further

aggravated during the course of spontaneous renal damage. Therefore, heterogeneous vascular dysfunction is present in various vascular beds in animal models of spontaneous renal disease prior to the development of end-organ damage.

A progressive pattern of spontaneous hypertension-associated renal disease in young Fawn-Hooded rats as observed in our study is in agreement with previous reports[19,20]. Seven weeks old FHH rats showed minor elevation in blood pressure as compared to age-matched FHL counterparts. However, blood pressure of both strains remained in the normotensive range at that age. At this time point, markers of renal damage did not differ between experimental strains. Although blood pressure did not increase further in FHH rats over the following 5 weeks, a marked elevation in urinary protein excretion was observed. Renal damage progresses rapidly in FHH strain and eventually leads to end-stage renal failure-related death before the age of 70 weeks[21], whereas FHL rats do not develop early hypertension and renal lesions[14]. Therefore, FHH rats studied at an early age provide a useful model to investigate the role of vascular changes in the development of spontaneous renal damage and systemic vascular complications.

Our observation of marked attenuation of myogenic reactivity in small renal arteries of FHH rat as compared to FHL strain is in agreement with observations of *van Dokkum et al.*[16], who reported significantly lower myogenic constriction in renal interlobular artery of proteinuric FHH rats. We extend these data by showing that the difference in myogenic reactivity between FHL and FHH is detected prior to development of proteinuria. In addition, we found that myogenic reactivity increases with age in FHL, whereas this increase is absent in FHH. rats. This observation suggests that the myogenic mechanism in FHH rats fails to adapt to increased hemodynamic load during the progression of hypertension or associated renal disease. Altered myogenic reactivity may result in an impaired autoregulatory ability of the kidney, leading to elevated glomerular capillary pressure, subsequent hyperfiltration and progressive proteinuria. Although we were not able to detect hyperfiltration, as calculated creatinine clearance was similar in both studied groups, *Simons et al.* demonstrated an elevation of intraglomerular capillary pressure in FHH rats at the age of 7 weeks[15], i.e. before the development of proteinuria. Furthermore, an attenuated autoregulation of renal blood flow has been reported in 12 weeks old FHH rats as compared to FHL[22]. Recently, it has been shown that autoregulation is restored and proteinuria reduced by the transfer of a specific region of chromosome 1 from Brown-Norway rats to FHH[23], indicating that altered autoregulation may be responsible for the genetic susceptibility of FHH strain to renal disease. Collectively, these data indicate that impaired myogenic reactivity may represent the mechanism rendering a given animal strain susceptible to renal injury.

Interestingly, the impairment of myogenic constriction is specific for the renal vasculature, since no differences in myogenic reactivity between FHH and FHL rats were found in the systemic resistance arteries. This suggests that myogenic response of various vascular beds is heterogeneously affected in FHH rats and that differential mechanisms underlie myogenic reactivity in renal and mesenteric arteries. However, only few studies reported

heterogeneity of myogenic mechanisms among vascular beds[24] and differential mechanisms have not been characterized yet. Generally, myogenic constriction is a result of intrinsic reactivity of smooth muscle cells associated with membrane depolarization and increase in intracellular calcium levels. Several mechanosensitive ion channels and integrins may be involved in a stretch-induced mechanotransduction, whereas other factors have been implicated in the myogenic signal transduction, including activation of calmodulin/ myosin light chain kinase pathway, PKC, MAP kinases, cytochrome P450-derived metabolites of arachidonic acid (20-HETE) and several potassium channels[25]. Altered activity of any of these components may underlie the impairment of renal myogenic constriction FHH rats, although several of these mechanisms participate also in receptor- or depolarization-mediated contraction. Since these contractions were intact, the affected mechanism in FHH seems rather specific for the stretch-induced reactivity, such as mechanotransduction. Moreover, the defect is independent of basal reactivity of endothelium, since impairment of myogenic constriction in FHH persisted after removal of the endothelium. Thus, reduced myogenic reactivity in small renal arteries may explain the susceptibility of FHH rats to renal damage. In addition, its selective impairment in renal arteries indicates that different mechanisms are involved in the generation of myogenic responses across different vascular beds.

We have previously shown that variation in renal endothelial function of healthy rats from an outbred Wistar strain predicts their susceptibility to the development of renal end-organ damage after renal mass reduction[9] and combined unilateral nephrectomy and myocardial infarction[26]. Healthy individuals with pronounced endothelium-dependent relaxation developed less severe renal damage. The current finding that endothelial function in FHH is already impaired at an early age prior to the development of renal damage is in agreement with these observations in outbred strains and suggests that endothelium actively participates in the susceptibility to end-organ damage.

The primary mechanism underlying endothelial dysfunction in renal arteries of FHH rats is COX-1-mediated endothelium-dependent contraction, in which the production of vasoconstrictive endoperoxides and/or thromboxanes and superoxide radical were involved. In a previous report from our lab, we showed that pronounced production of endothelial contractile prostanoids in healthy rats was associated with the excessive renal damage induced by subsequent renal mass reduction. Interestingly, early hyperfiltration in FHH rats was reported to coincide with an excessive urinary excretion of contractile prostaglandins[27]. These observations may suggest the role of prostaglandins in the development of spontaneous renal injury. However, spontaneously hypertensive rat (SHR), a strain relatively resistant to the development of renal injury also displays COX-mediated renal endothelial dysfunction at an early, pre-hypertensive stage[28]. Therefore alternatively, renal vasoconstrictive prostaglandins may play the critical role in the development of spontaneous hypertension[29]. Interestingly, while endothelium-dependent contraction is confined exclusively to renal vasculature in FHH, it is found also in systemic resistance arteries and aorta in SHR[30-32]. However, the implications of this observation remain unclear

and further research is needed to clarify the role of early renal endothelium-dependent contractions in the pathophysiology of hypertension-associated renal damage in FHH rats.
Reduced bioavailability of nitric oxide (NO) is considered to be a key mechanism for renal endothelial dysfunction in various experimental models of hypertension and renal damage[33,34]. We previously reported that pronounced renal NO-mediated vasodilation is associated with protection against the development of renal damage in several models of renal injury[9,10]. However, in the present study, no difference was found in NO-mediated vasodilation between FHL and FHH rats prior to the development of renal damage. Moreover, in proteinuric FHH animals, the NO-dependent vasodilation is increased rather than reduced, suggesting that NO bioactivity is modified during the course of renal disease. Likewise, increased NO activity has been described in early stages of diabetic nephropathy and has been linked to hyperfiltration[35,36]. One might speculate about a similar mechanism in FHH rats, leading to elevated glomerular pressure and the development of proteinuria. Alternatively, increased NO release might represent a compensatory mechanism in response to the production of COX-derived reactive oxygen species. Interestingly, increased constitutive nitric oxide synthase-1 (NOS-1) expression has been described in macula densa of young FHH rats[37], however it is unclear whether NO signaling in preglomerular arteries is affected. NO-related changes in FHH rats seem to be limited to the renal vasculature and was not observed in systemic vascular beds, even though acetylcholine-induced relaxation almost entirely relies on NO in the aorta. Taken together, these data indicate that reduced NO-mediated relaxation does not seem to represent a major mechanism of early vascular dysfunction in spontaneous renal disease.
In small arteries, such as renal or mesenteric artery, the majority of endothelial relaxation under physiological condition is mediated by EDHF[9,38,39]. Interestingly, this mechanism was intact in small renal, but impaired in mesenteric resistance artery of FHH rats at both time points investigated. This heterogeneity might be explained by the variable identity of EDHF in different vascular beds[40-44]. Nevertheless, it is well established that in all vascular beds activation of the endothelial calcium-regulated potassium K_{Ca} channels is critical for EDHF-response, as evidenced in our study by the complete blockade of EDHF-mediated vasodilation in the different arteries by charybdotoxine and apamin, inhibitors of the large/intermediate (BK_{Ca}/IK_{Ca}) and small conductance (SK_{Ca}) channels, respectively. Selectively altered EDHF-mediated relaxation in systemic arteries appears before the development of proteinuria, suggesting that generalized endothelial dysfunction may precede renal injury. EDHF reduction in resistance arteries may possibly be related to the minor blood pressure increase in FHH rats. However, it seems unlikely, because during the course of the study EDHF further progressed with proteinuria increase, despite unchanged blood pressure. Moreover, impaired EDHF-mediated relaxation observed in various vascular beds following experimental renal disease induced by renal mass reduction[45,46] is independent from blood pressure increase. Changes in the expression of endothelial SK_{Ca} and IK_{Ca} channels underlying the EDHF loss in this model[45], might be also involved in endothelial dysfunction in FHH. Collectively, impaired EDHF-mediated relaxation

represents the earliest systemic vascular alterations in spontaneous hypertension-associated renal disease.

In conclusion, the present study shows that vascular dysfunction in both small renal and systemic arteries precedes renal damage in FHH rats, a model of spontaneous hypertension-associated damage. This study confirms the early presence of vascular impairment throughout various vascular beds in individuals susceptible to renal disease, however it reports marked heterogeneity in affected vasomotor mechanisms between small renal, resistance and conduit arteries. Future research should be warranted to define the role of endothelial and smooth muscle reactivity in the development of renal end-organ damage.

References

1. Morris ST, Jardine AG. The vascular endothelium in chronic renal failure. J Nephrol 2000; 13:96-105
2. Ochodnicky P, Vettoretti S, Henning RH, Kluppel CA, de Zeeuw D, Van Dokkum RP. Concomitant decrease of systemic arterial myogenic tone and EDHF-mediated dilation in subtotally nephrectomized rat [Abstract]. J Am Soc Nephrol 2005; 16:185A-
3. Clausen P, Jensen JS, Jensen G, Borch-Johnsen K, Feldt-Rasmussen B. Elevated urinary albumin excretion is associated with impaired arterial dilatory capacity in clinically healthy subjects. Circulation 2001; 103:1869-1874
4. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage. The Steno hypothesis. Diabetologia 1989; 32:219-226
5. Pistrosch F, Herbrig K, Kindel B, Passauer J, Fischer S, Gross P. Rosiglitazone improves glomerular hyperfiltration, renal endothelial dysfunction, and microalbuminuria of incipient diabetic nephropathy in patients. Diabetes 2005; 54:2206-2211
6. Clausen P, Feldt-Rasmussen B, Jensen G, Jensen JS. Endothelial haemostatic factors are associated with progression of urinary albumin excretion in clinically healthy subjects: a 4-year prospective study. Clin Sci (Lond) 1999; 97:37-43
7. Stehouwer CD, Fischer HR, van Kuijk AW, Polak BC, Donker AJ. Endothelial dysfunction precedes development of microalbuminuria in IDDM. Diabetes 1995; 44:561-564
8. Vanhoutte PM, Mombouli JV. Vascular endothelium: vasoactive mediators. Prog Cardiovasc Dis 1996; 39:229-238
9. Gschwend S, Buikema H, Navis G, Henning RH, de Zeeuw D, Van Dokkum RP. Endothelial dilatory function predicts individual susceptibility to renal damage in the 5/6 nephrectomized rat. J Am Soc Nephrol 2002; 13:2909-2915
10. Ochodnicky P, Henning RH, Buikema H, de Zeeuw D, Van Dokkum RP. Renal endothelial function predicts the individual susceptibility to adriamycin-induced renal damage [Abstract]. J Am Soc Nephrol 2005; 16:800A-
11. Korstanje R, DiPetrillo K. Unraveling the genetics of chronic kidney disease using animal models. Am J Physiol Renal Physiol 2004; 287:F347-F352
12. De Keijzer MH, Provoost AP, Molenaar JC. Proteinuria is an early marker in the development of progressive renal failure in hypertensive fawn-hooded rats. J Hypertens 1989; 7:525-528
13. Kuijpers MH, Provoost AP, de Jong W. Development of hypertension and proteinuria with age in fawn-hooded rats. Clin Exp Pharmacol Physiol 1986; 13:201-209
14. Provoost AP. Spontaneous glomerulosclerosis: insights from the fawn-hooded rat. Kidney Int Suppl 1994; 45:S2-S5

15. Simons JL, Provoost AP, Anderson S, Troy JL, Rennke HG, Sandstrom DJ, Brenner BM. Pathogenesis of glomerular injury in the fawn-hooded rat: early glomerular capillary hypertension predicts glomerular sclerosis. J Am Soc Nephrol 1993; 3:1775-1782
16. Van Dokkum RP, Sun CW, Provoost AP, Jacob HJ, Roman RJ. Altered renal hemodynamics and impaired myogenic responses in the fawn-hooded rat. Am J Physiol 1999; 276:R855-R863
17. van Rodijnen WF, van Lambalgen TA, Tangelder GJ, Van Dokkum RP, Provoost AP, ter Wee PM. Reduced reactivity of renal microvessels to pressure and angiotensin II in fawn-hooded rats. Hypertension 2002; 39:111-115
18. Van Dokkum RP, Eijkelkamp WB, Kluppel AC, Henning RH, van Goor H, Citgez M, Windt WA, van Veldhuisen DJ, de Graeff PA, de Zeeuw D. Myocardial infarction enhances progressive renal damage in an experimental model for cardio-renal interaction. J Am Soc Nephrol 2004; 15:3103-3110
19. Simons JL, Provoost AP, Anderson S, Rennke HG, Troy JL, Brenner BM. Modulation of glomerular hypertension defines susceptibility to progressive glomerular injury. Kidney Int 1994; 46:396-404
20. Van Dokkum RP, Jacob HJ, Provoost AP. Difference in susceptibility of developing renal damage in normotensive fawn-hooded (FHL) and August x Copenhagen Irish (ACI) rats after N(omega)-nitro-L-arginine methyl ester induced hypertension. Am J Hypertens 1997; 10:1109-1116
21. Verseput GH, Provoost AP, Braam BB, Weening JJ, Koomans HA. Angiotensin-converting enzyme inhibition in the prevention and treatment of chronic renal damage in the hypertensive fawn-hooded rat. J Am Soc Nephrol 1997; 8:249-259
22. Van Dokkum RP, Alonso-Galicia M, Provoost AP, Jacob HJ, Roman RJ. Impaired autoregulation of renal blood flow in the fawn-hooded rat. Am J Physiol 1999; 276:R189-R196
23. Lopez B, Ryan RP, Moreno C, Sarkis A, Lazar J, Provoost AP, Jacob HJ, Roman RJ. Identification of a QTL on chromosome 1 for impaired autoregulation of RBF in fawn-hooded hypertensive rats. Am J Physiol Renal Physiol 2006; 290:F1213-F1221
24. Lagaud GJ, Skarsgard PL, Laher I, van Breemen C. Heterogeneity of endothelium-dependent vasodilation in pressurized cerebral and small mesenteric resistance arteries of the rat. J Pharmacol Exp Ther 1999; 290:832-839
25. Davis MJ, Hill MA. Signaling mechanisms underlying the vascular myogenic response. Physiol Rev 1999; 79:387-423
26. Ochodnicky P, de Zeeuw D, Henning RH, Kluppel CA, Van Dokkum RP. Endothelial function predicts the development of renal damage after combined nephrectomy and myocardial infarction. J Am Soc Nephrol 2006; 17:S49-S52
27. De Keijzer MH, Provoost AP, Zijlstra FJ. Enhanced urinary excretion of eicosanoids in fawn-hooded rats. Nephron 1992; 62:454-458

28. Dai FX, Skopec J, Diederich A, Diederich D. Prostaglandin H2 and thromboxane A2 are contractile factors in intrarenal arteries of spontaneously hypertensive rats. Hypertension 1992; 19:795-798
29. Vanhoutte PM, Feletou M, Taddei S. Endothelium-dependent contractions in hypertension. Br J Pharmacol 2005; 144:449-458
30. Fu-Xiang D, Jameson M, Skopec J, Diederich A, Diederich D. Endothelial dysfunction of resistance arteries of spontaneously hypertensive rats. J Cardiovasc Pharmacol 1992; 20 Suppl 12:S190-S192
31. Iwama Y, Kato T, Muramatsu M, Asano H, Shimizu K, Toki Y, Miyazaki Y, Okumura K, Hashimoto H, Ito T, . Correlation with blood pressure of the acetylcholine-induced endothelium-derived contracting factor in the rat aorta. Hypertension 1992; 19:326-332
32. Yang D, Feletou M, Levens N, Zhang JN, Vanhoutte PM. A diffusible substance(s) mediates endothelium-dependent contractions in the aorta of SHR. Hypertension 2003; 41:143-148
33. Hayakawa H, Hirata Y, Suzuki E, Sugimoto T, Matsuoka H, Kikuchi K, Nagano T, Hirobe M, Sugimoto T. Mechanisms for altered endothelium-dependent vasorelaxation in isolated kidneys from experimental hypertensive rats. Am J Physiol 1993; 264:H1535-H1541
34. Ozawa Y, Hayashi K, Kanda T, Homma K, Takamatsu I, Tatematsu S, Yoshioka K, Kumagai H, Wakino S, Saruta T. Impaired nitric oxide- and endothelium-derived hyperpolarizing factor-dependent dilation of renal afferent arteriole in Dahl salt-sensitive rats. Nephrology (Carlton) 2004; 9:272-277
35. Veelken R, Hilgers KF, Hartner A, Haas A, Bohmer KP, Sterzel RB. Nitric oxide synthase isoforms and glomerular hyperfiltration in early diabetic nephropathy. J Am Soc Nephrol 2000; 11:71-79
36. Sugimoto H, Shikata K, Matsuda M, Kushiro M, Hayashi Y, Hiragushi K, Wada J, Makino H. Increased expression of endothelial cell nitric oxide synthase (ecNOS) in afferent and glomerular endothelial cells is involved in glomerular hyperfiltration of diabetic nephropathy. Diabetologia 1998; 41:1426-1434
37. Weichert W, Paliege A, Provoost AP, Bachmann S. Upregulation of juxtaglomerular NOS1 and COX-2 precedes glomerulosclerosis in fawn-hooded hypertensive rats. Am J Physiol Renal Physiol 2001; 280:F706-F714
38. Gschwend S, Pinto-Sietsma SJ, Buikema H, Pinto YM, van Gilst WH, Schulz A, de Zeeuw D, Kreutz R. Impaired coronary endothelial function in a rat model of spontaneous albuminuria. Kidney Int 2002; 62:181-191
39. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, Takeshita A. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. J Cardiovasc Pharmacol 1996; 28:703-711

40. Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH. K+ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature 1998; 396:269-272
41. Jiang F, Li CG, Rand MJ. Mechanisms of nitric oxide-independent relaxations induced by carbachol and acetylcholine in rat isolated renal arteries. Br J Pharmacol 2000; 130:1191-1200
42. Wang D, Borrego-Conde LJ, Falck JR, Sharma KK, Wilcox CS, Umans JG. Contributions of nitric oxide, EDHF, and EETs to endothelium-dependent relaxation in renal afferent arterioles. Kidney Int 2003; 63:2187-2193
43. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. J Clin Invest 2000; 106:1521-1530
44. Sandow SL, Tare M, Coleman HA, Hill CE, Parkington HC. Involvement of myoendothelial gap junctions in the actions of endothelium-derived hyperpolarizing factor. Circ Res 2002; 90:1108-1113
45. Kohler R, Eichler I, Schonfelder H, Grgic I, Heinau P, Si H, Hoyer J. Impaired EDHF-mediated vasodilation and function of endothelial Ca-activated K channels in uremic rats. Kidney Int 2005; 67:2280-2287
46. Koobi P, Kalliovalkama J, Jolma P, Rysa J, Ruskoaho H, Vuolteenaho O, Kahonen M, Tikkanen I, Fan M, Ylitalo P, Porsti I. AT1 receptor blockade improves vasorelaxation in experimental renal failure. Hypertension 2003; 41:1364-1371

Chapter 6

Altered myogenic constriction and EDHF-mediated relaxation in small mesenteric arteries of hypertensive subtotally nephrectomized rats

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Abstract

Objectives: Chronic renal failure (CRF) is associated with altered systemic arterial tone and hypertension. Myogenic constriction and endothelium-derived hyperpolarizing factor (EDHF)-dependent relaxation represent major vasoregulatory mechanisms in small systemic arteries. Elevated myogenic response and impaired EDHF might participate in the development of essential hypertension, however their role in CRF-related hypertension is unknown. We investigated whether myogenic response and EDHF are altered in subtotally nephrectomized (sNX) rats and whether these changes are modifiable by chronic treatment with ACE inhibitor.

Methods: In a pressure arteriograph, myogenic constriction and EDHF-mediated relaxation were evaluated in small mesenteric arteries isolated from male Wistar rats 15 weeks after either SHAM operation (n=7), sNX (n=12) or sNX followed by 9 week treatment with lisinopril (sNX+LIS, 2.5 mg/kg, n=13).

Results: Surprisingly, myogenic response was reduced in hypertensive CRF rats (maximal myogenic tone: 37±2 and 18±4%, $p<0.01$; peak myogenic index: -0.80±0.08 and -0.40±0.12 %/mmHg, $p<0.05$ in SHAM and sNX respectively). At the same time EDHF-mediated relaxation was impaired as well (maximal response: 92±2 and 77±5%, $p<0.01$; pD_2: 6.9±0.1 and 5.3±0.1, $p<0.05$). Both myogenic response and EDHF were inversely related to the severity of renal failure and restored by treatment with lisinopril to levels found in SHAM animals.

Conclusion: Major constrictive (myogenic) and dilatory (EDHF) mechanisms of small systemic arteries are impaired in hypertensive CRF rats. These alterations do not seem to participate in the development hypertension, being rather directly related to the severity of renal impairment. Both systemic vascular changes might be restored by renoprotective treatment with ACE inhibitor.

Introduction

Chronic renal failure (CRF) is associated with increased cardiovascular morbidity and mortality[1,2]. Structural and functional alterations of peripheral blood vessels are likely to be involved in many of the cardiovascular complications occurring in CRF. Abnormal function of large conduit arteries characterized by reduced arterial compliance may result in increased pulse pressure and the development of left ventricular hypertrophy, both independent prognostic factors for cardiovascular mortality in patients with end-stage renal disease[3,4]. On the other hand, compromised reactivity of small systemic arteries may contribute to the increased peripheral resistance and development of hypertension in CRF. In fact, hypertension, the ultimate cardiovascular risk factor, occurs in 80-90% of patients with end-stage renal disease[5].

Several alterations in peripheral vasomotor mechanisms are implicated in CRF, among which, endothelial dysfunction have received a great deal of attention. Endothelial dysfunction due to oxidative stress-mediated loss of nitric oxide (NO) vasodilation, was proposed to account for hypertension in rats subjected to subtotal nephrectomy (sNX)[6,7]. However, apart from NO, other endothelium-derived mediators are involved in the regulation of peripheral vascular tone. Yet unknown factor termed endothelium-derived hyperpolarizing factor (EDHF) relaxes the underlying vascular smooth muscle cells by opening potassium channels and hyperpolarizing the cell membrane[8]. Interestingly, in small resistance arteries such as small mesenteric artery, EDHF-mediated relaxation represents the principal endothelial vasomotor mechanism accounting for vast majority of endothelium-dependent vasodilation[9-11]. Several authors have shown that EDHF might be compromised in large conduit arteries of rats with sNX[12,13]. However EDHF-relaxation in small peripheral vasculature during hypertensive CRF has not been addressed thoroughly.

In addition to decreased local vasodilatory capacity, exaggerated contractile abilities of peripheral vessels have been reported in various forms of hypertension. Excessive contractile response of small arteries to increases in intravascular pressure, termed myogenic constriction[14] might contribute to elevated peripheral resistance in spontaneously hypertensive rats (SHR)[15-17]. It is unknown whether a similar mechanism occurs in hypertension associated with sNX. Interestingly, we previously found that the level of myogenic constriction may be inversely related to EDHF-mediated dilation, suggesting that both mechanisms share common signaling pathways, probably interacting at the level of vascular Ca^{2+}-regulated potassium channel[18]. Therefore, the aim of the present study was to assess changes in myogenic contractility and EDHF-mediated vasodilation of small systemic arteries in rats with experimental CRF induced by sNX. We hypothesized that small mesenteric arteries of rats with experimental CRF display increased myogenic reactivity and decreased EDHF-mediated dilation as these mechanisms could be inversely related. Since systemic vascular alterations might play a role in the development of hypertension and cardiovascular damage in CRF our secondary aim was to explore whether

these mechanisms are modifiable by renoprotective treatment with the angiotensin-converting enzyme inhibitor (ACEi) lisinopril.

Methods

Animals

Male Wistar rats, 12-13 weeks of age (n=40, Harlan, Zeist, The Netherlands) were housed under standard conditions at the animal facilities of the University of Groningen. Animals had free access to food (standard rat chow containing 0.3% sodium, Hope Farms, Woerden, The Netherlands) and drinking water throughout the study. Experiments were approved by the local Animal Ethical Committee.

Surgery

Under anaesthesia with isoflurane 3% in N_2O/O_2 (2:1), sNX was performed by excision of the right kidney and subsequent infarction of approximately 2/3 of contralateral kidney by ligation of two to three branches of the left renal artery. SHAM animals underwent the same procedure and were closed after manipulation and decapsulation of the kidneys without being nephrectomized. Postoperatively, the rats were allowed to recover for one week.

Experimental design and in vivo measurements

Following surgery, systolic blood pressure (SBP) and urinary protein excretion were determined weekly. 6 weeks after the operation, nephrectomized rats were stratified based on proteinuria and divided in untreated (sNX, n=12) and treated (lisinopril; sNX+LIS, n=13) rats. SHAM rats (n=7) were left untreated. Lisinopril was dissolved in the drinking water at a dose of 2.5 mg/kg body weight. Subsequently, rats were followed up to 15 weeks after surgery. Shortly before sNX (baseline), at 6 weeks (stratification) and 15 weeks after sNX (termination), blood samples were collected for the determination of plasma creatinine. SBP was measured as the mean of three consecutive measurements in awake, restrained animals by means of the tail-cuff method (IITC Inc., Woodland Hills, CA, USA). Urinary protein excretion was determined by placing the rats in metabolic cages for 24 hours and protein concentration was analyzed by TCA (Nephelometer Analyzer II, Dade Behring, Marburg, Germany). Creatinine concentration was measured colorimetrically (Chema Diagnostica, Jesi (AN), Italy). Upon termination under isoflurane in N_2O/O_2, the remnant kidney and heart were removed and weighted. The mesenteric arterial bed was excised for investigation of vascular function.

Preparation and cannulation of small mesenteric arteries

Third-order branches in mesenteric arterial bed were isolated, cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System

Instrumentation, Burlington, VT, USA). Each artery was cannulated at both ends with glass micropipettes, secured, and the lumen of the vessel was filled with Krebs solution through the micropipettes. Intraluminal pressure was set at 60 mmHg and was held constant (without flow) by a pressure servo system (Living System Instrumentation, Burlington, VT, USA). The vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO_2 in O_2) Krebs solution with a pH of 7.4. Subsequently, the chamber was transferred to an inverted light microscope with a video camera attached to a viewing tube. Lumen diameter was continuously registered by a video dimension analyzer (Living System Instrumentation, Burlington, VT, USA).

Determination of arterial reactivity and experimental protocol

Prior to the experiments, arteries were allowed to equilibrate for 40 minutes. To test for viability of smooth muscle cells and endothelium, arteries were pre-constricted with a thromboxane A_2 analogue (U46619, 30 nmol/L) and relaxed with a single dose of acetylcholine (100 µmol/L). Following wash-out, myogenic reactivity was investigated by constructing active pressure-diameter curves in normal Krebs solution. These were obtained over a pressure range of 20–160 mmHg in steps of 20 mmHg. After each stepwise increase in intraluminal pressure, lumen diameter was registered when a stable diameter (contraction) was reached (~ 5 min). Subsequently, the pressure was set back to 60 mmHg and the arteries were allowed to stabilize for 20 minutes before the EDHF-mediated vasodilation was investigated. To address EDHF-dependent relaxation, the vessel was pre-contracted with U46619 (30 nmol/L). Thereafter, the concentration-response curve to acetylcholine (3.10^{-8}- 3.10^{-5} mol/l) was determined in the presence of indomethacin (10 µmol/L) and N^{ω}-monomethyl-L-arginine (L-NMMA, 100 µmol/L) in order to block cyclooxygenase and nitric oxide synthase, respectively. Previously, we showed that the remaining relaxation under these conditions is mediated by EDHF, since it is completely abrogated either by calcium-regulated potassium channels inhibitors charybdotoxin (100 nmol/L) and apamin (500 nmol/L)[11,18] or by high concentration (40 mmol) of KCl[9]. Finally, after perfusing the system with calcium-free Krebs solution supplemented with ethylene glycol-bis-(b-amino ethyl ether) tetraacetic acid (EGTA, 2 mmol/L), passive pressure-diameter step curves were obtained over a pressure range of 20-160 mmHg.

Solutions and drugs

Vessel segments were perfused with Krebs solution of the following composition (in mmol/L): NaCl 120.4, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11.5, $NaHCO_3$ 25.0). Lisinopril was purchased from Astra-Zeneca (The Netherlands). All other compounds were purchased from Sigma (St. Louis, MO, USA). Stock solution of indomethacin was prepared in 64 mmol/l $NaHCO_3$.

Data analysis

Data are expressed as mean ± SEM; n values represent the number of investigated rats as well as the number of investigated arteries since one artery segment per rat was used for the same protocol. To characterize myogenic responsiveness, the following parameters were calculated from the pressure-diameter curve of each individual artery:

1. Myogenic tone, describing myogenic behaviour of an artery at a given pressure, was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same pressure in calcium-free/EGTA solution, i.e., myogenic tone (%) = 100 [($D_{Ca\text{-}free} - D_{Ca}$)/$D_{Ca\text{-}free}$], where D is the diameter in calcium-free ($D_{Ca\text{-}free}$) or calcium-containing (D_{Ca}) Krebs. For every individual artery segment maximal myogenic tone was determined as the maximal value over the studied pressure range.

2. Myogenic index, describing myogenic reactivity of an artery in response to a pressure change, e.g. the slope of active pressure-diameter relationship, was calculated for every 20 mmHg pressure step (ΔP) as a percentage change in corresponding active diameter D, i.e. myogenic index (%/mmHg) = 100 [(ΔD/D)/ΔP]. A negative value indicates active luminal reduction in response to an increase in pressure and provides the evidence of myogenic behaviour independently from passive diameters of the vessel[19]. For each individual artery peak myogenic index denotes the largest value of all the pressure steps studied.

EDHF-dependent concentration-response curves to acetylcholine were expressed in percentage of pre-constriction to thromboxane A_2 analogue U46619. The curves were characterized by maximal relaxation (E_{max}) and negative logarithm of acetylcholine molar concentration causing half-maximal relaxation (pD_2). Statistical differences for vascular parameters, proteinuria, systolic blood pressure, creatinine, body and organ weights, water intake and urinary output were determined by Student's independent t-test. Differences in myogenic and EDHF curves among experimental groups were tested using Bonferroni post hoc multiple comparisons applied to ANOVA for repeated measures. The relationships between vascular reactivity and *in vivo* data were calculated using Spearman's nonparametric correlation. Differences were considered significant at $p<0.05$ (two-tailed).

Results

Survival

Following the nephrectomy 6 rats died because of uremia before the stratification, and 2 animals of the sNX group died later. Consequently 32 rats completed the study and were eligible for the full protocol analysis at termination, (sNX, n=12; sNX+LIS, n=13 and SHAM, n=7). Prior to surgery, baseline values (week 0) of body weight, water intake, urinary output and plasma creatinine, proteinuria and blood pressure *(Table 1)* were similar in the three experimental groups.

Table 1. *Clinical characteristics of experimental animals (SHAM-operated, subtotally nephrectomized- sNX and subtotally nephrectomized rats treated with lisinopril 2.5 mg/kg- sNX+LIS) measured at the baseline (week 0), stratification (week 6) and termination (week 15 after the operation).*

Variable		SHAM	sNX	sNX+LIS
Body Weight (g)				
Baseline	week 0	321±4	338±17	330±8
Stratification	week 6	427±9	419±12	393±14
Termination	week 15	512±10	490±15	472±14
Water intake (ml/24h)				
Baseline	week 0	21±2	28±3	34±2
Stratification	week 6	26±2	45±3*	42±3*
Termination	week 15	24±1	47±4*	47±4*
Urine output (ml/24h)				
Baseline	week 0	10±1	14±2	17±3
Stratification	week 6	14±2	23±2*	24±2*
Termination	week 15	13±1	33±4*	32±4*
Proteinuria (mg/24h)				
Baseline	week 0	20±3	20±2	30±7
Stratification	week 6	26±2	86±20*	117±25*
Termination	week 15	39±9	354±38*	183±42*,#
Plasma creatinine (μmol/l)				
Baseline	week 0	46±5	44±2	47±4
Stratification	week 6	47±4	77±4*	77±8*
Termination	week 15	57±5	100±16*	77±16
Systolic blood pressure (mmHg)				
Baseline	week 0	124±6	122±3	124±3
Stratification	week 6	134±4	163±9*	155±6*
Termination	week 15	136±8	173±6*	139±9#
Left ventricle weight (mg/g body weight)				
Termination	week 15	2.1±0.1	2.7±0.2*	2.4±0.1*
Wet kidney weight (mg/g body weight)				
Termination	week 15	3.3±0.1	4.7±0.2*	4.7±0.3*

* $p<0.05$ compared with SHAM at the same time point

# $p<0.05$ compared with sNX at the same time point

In vivo data

Within 6 weeks after sNX, the animals from both nephrectomized groups (sNX and sNX+LIS) developed severe renal failure characterized by increased SBP (*Figure 1A*), proteinuria (*Figure 1B*), elevated plasma creatinine levels and when compared to SHAM operated rats *(Table 1, stratification).* Additionally, water intake and urine output were higher in sNX and sNX+LIS group compared to SHAM animals (*Table 1, stratification*). At this time point, no differences were found in any of the measured parameters between the sNX and sNX+LIS groups.

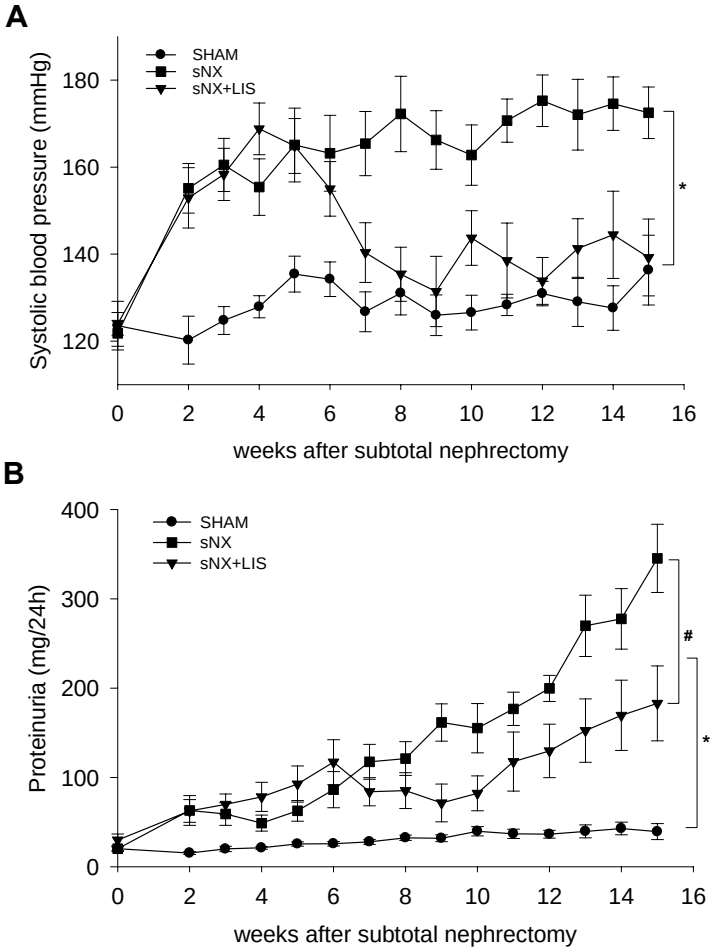

Figure 1. *The development of* ***A)*** *systolic blood pressure (mmHg) and* ***B)*** *proteinuria (mg/24h) in time after the subtotal nephrectomy. Note that after the development of the disease, the rats were stratified to different treatment groups at week 6. SHAM operated rats, sNX- subtotally nephrectomized and sNX+LIS- subtotally nephrectomized treated with lisinopril 2.5 mg/kg, * $p<0.05$ compared with SHAM; $^{\#}$ $p<0.05$ compared with sNX*

Renal disease progressed further in sNX animals, whereas the progression was slowed down by ACE-i. Upon sacrifice, 15 weeks after the operation, sNX rats displayed elevated SBP *(Figure 1A)*, severe proteinuria *(Figure 1B)*, double plasma creatinine levels, higher water intake, urine output, kidney and left ventricle mass (*Table 1, termination*) as compared to age-matched SHAM rats. ACE-i treatment substantially reduced proteinuria compared with sNX group, although the levels were still higher than in SHAM animals. Plasma creatinine levels and left ventricle mass tended to be lower in the sNX+LIS group, which was however not statistically significant. Increased SBP in the sNX group was restored by ACE-i to the levels measured in SHAMs *(Table 1, termination)* and had no effect on wet kidney weight, water intake and urine output.

Vascular experiments

Myogenic constriction

As shown in *Figure 2A*, passive diameters of small mesenteric arteries did not differ among the experimental groups over the whole pressure range, suggesting no apparent changes in maximal relaxant ability of the investigated arteries. Similarly, no differences were observed in the receptor-mediated contraction to the thromboxane A_2 analogue U46619 (data not shown). Small mesenteric arteries developed a substantial myogenic tone dependent on the intraluminal pressure applied to the vessel. Active diameters were significantly higher in the sNX group over the pressure range of 100-160 mmHg when compared with SHAM (*Figure 2A*). As a consequence, myogenic tone was blunted over this pressure range *(Figure 3A).* Threshold for the development of active myogenic constriction, indicated by negative myogenic index, was shifted in sNX rats to 80-100 mmHg pressure step as compared to 60-80 mmHg in SHAM rats (*Figure 2B*). Furthermore, the arteries of sNX rats displayed less pronounced negative values of myogenic index at pressure steps 60-80, 80-100 and 100-120 mmHg when compared with SHAM animals, indicating less steep slope of active pressure-diameter curve in this pressure range *(Figure 2B).* Maximal myogenic tone ($p=0.001$, *Table 2*) and peak myogenic index ($p=0.004$, *Table 2*) were also impaired in the vessels isolated from sNX rats. Interestingly, chronic treatment with lisinopril reversed active responses to pressure (*Figure 2A*). Consequently, myogenic tone was restored over the whole pressure range (*Figure 3A*) and maximal myogenic tone was comparable to the SHAM values ($p=0.03$ compared to sNX, *Table 2).* Additionally, the myogenic index was reversed to more negative values in sNX+LIS group at pressure steps 60-80, 80-100 mmHg *(Figure 2B).*

EDHF-mediated vasodilation

A single dose of acetylcholine induced significantly lower endothelium-dependent relaxation in the sNX group (100±3, 87±2 and 101±5 % of precontraction level for SHAM, sNX and sNX+LIS, respectively), suggesting an impairment in endothelium-mediated responses. EDHF-mediated relaxation of small mesenteric arteries was significantly diminished after sNX as compared to SHAM arteries (*Figure 3B*). The acetylcholine curve

performed under blockade of cyclooxygenase and nitric oxide synthase showed a reduction in both maximal response E_{max} (p= 0.009, *Table 2*) and pD_2 values (p= 0.006, *Table 2*) in sNX rats when compared to SHAM.

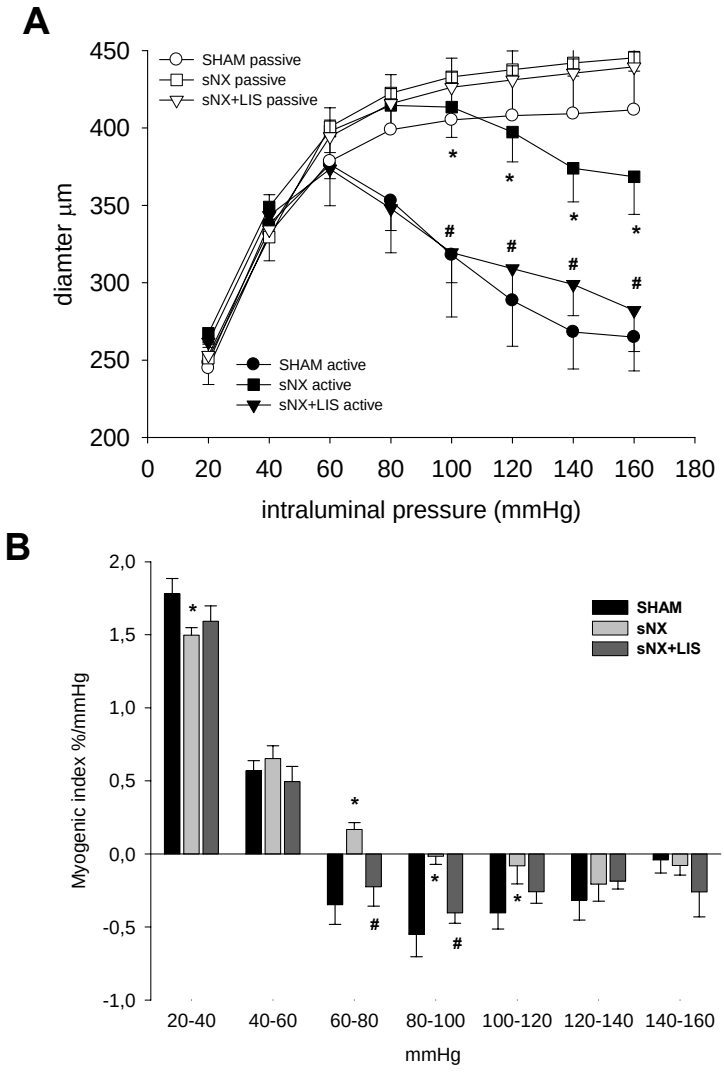

Figure 2. A) *Diameters of small mesenteric arteries in response to stepwise increase of intraluminal pressure in the presence (black symbols, active tone) or absence (white symbols, passive tone) of extracellular calcium and* ***B)*** *calculated myogenic index (in % of active diameter/mmHg) developed in response to stepwise increase of intraluminal pressure studied in SHAM operated, subtotally nephrectomized (sNX) and subtotally nephrectomized rats treated with lisinopril 2.5 mg/kg (sNX+LIS) 15 weeks after the operation. * p<0.05 compared with SHAM; # p<0.05 compared with sNX*

ACE-i treatment effectively restored blunted EDHF-dependent dilation observed in vessels from sNx rats *(Figure 3B)*. Rats from sNX+LIS displayed increased E_{max} (p= 0.04) and pD_2 (p= 0.04) values of EDHF-acetylcholine curve when compared to sNX animals (*Table 2*).

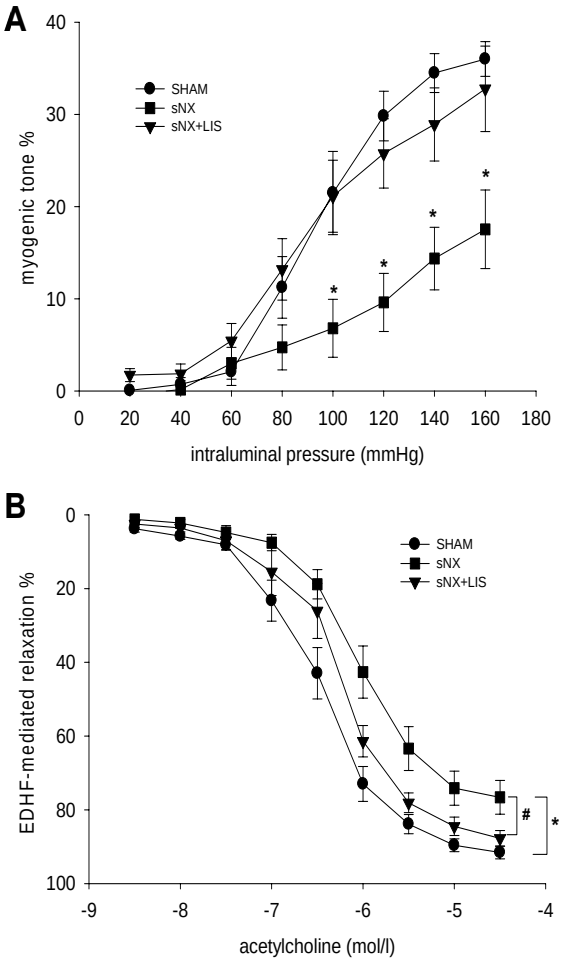

Figure 3. *Impaired* ***A)*** *myogenic tone (in % of passive diameter) and* ***B)*** *EDHF-dependent vasodilation (as acetylcholine-induced relaxation in % of precontraction) in small mesenteric arteries of subtotally nephrectomized (sNX) rats as compared to SHAM-operated, and subtotally nephrectomized rats treated with lisinopril 2.5 mg/kg (sNX+LIS).* ** $p<0.05$ compared with SHAM; $^{\#}$ $p<0.05$ compared with sNX*

Correlation analysis

To characterize the role of blood pressure changes in the development of renal damage after sNX and in the renoprotective effects of ACE-i, we analyzed relationship between the clinical parameters. SBP correlated with both proteinuria and plasma creatinine (r= 0.64, p=

0.02 and r= 0.66, p= 0.02, respectively) in sNX+LIS group, but not in sNX group (r= 0.18, p= NS and r= 0.12, p= NS, respectively).
EDHF-dependent relaxation positively correlated with acetylcholine-induced response, suggesting that EDHF represents principal endothelial vasodilatory mechanism in small mesenteric arteries (r= 0.61, p= 0.002, all groups included). To test our hypothesis on the relation between myogenic constriction and EDHF-dependent relaxation we performed correlation analysis in sNX animals. However, no relation was observed between these two parameters in small mesenteric arteries used in this study (r= 0.06, p= NS). When investigating whether beneficial effects of ACEi on both vascular parameters in sNX+LIS group might be interrelated,, we did not find any relation between EDHF and myogenic tone either (r= 0.25, p= NS). Similarly, no correlation was found between maximal total acetylcholine-induced response and myogenic tone (r= 0.11, p= NS and r= 0.20, p= NS for sNX and sNX+LIS group respectively), suggesting no role for endothelium-mediated mechanisms in myogenic tone changes.

Table 2. *Comparison of vascular reactivity in small mesenteric arteries of SHAM operated, subtotally nephrectomized (sNX) and subtotally nephrectomized rats treated with lisinopril 2.5 mg/kg (sNX+LIS) 15 weeks after the operation.*

	SHAM	**sNX**	**sNX+LIS**
Myogenic tone			
Maximal myogenic tone (% of passive diameter)	37±2	18±4*	33±5#
Peak myogenic index (% of active diameter/mmHg)	-0.80±0.08	-0.40±0.12*	-0.68±0.15
EDHF vasodilation			
E_{max} (% of precontraction)	92±2	77±5*	88±2#
pD_2	6.5±0.1	5.9±0.1*	6.3±0.1#

EDHF- endothelium-dependent hyperpolarizing factor, E_{max}- maximal relaxation, pD_2- negative logarithm of the acetylcholine concentration causing half maximal responses. * $p<0.05$ compared with SHAM; # $p<0.05$ compared with sNX

To address the role of renal failure and hypertension in the development or ACE-i mediated reversal of systemic vascular changes, additional correlation analyses were performed in sNX and sNX+LIS animals, respectively. Interestingly, in sNX rats maximal myogenic tone and maximal EDHF-dependent dilation of small mesenteric arteries inversely correlated or tended to correlate with proteinuria as a marker of renal damage, (*Figure 4A*, *B*; r= -0.60, p= 0.04 and r= -0.52, p= 0.08 for maximal myogenic tone and maximal EDHF dilation, respectively), but not with SBP (r= 0.06, p= NS and r= -0.02, p= NS, for maximal

myogenic tone, and maximal EDHF dilation, respectively). When the treated group was analyzed, an inverse correlation of borderline significance was found between myogenic tone and proteinuria (*Figure 4A*; r= -0.53, p= 0.06), but not between EDHF dilation and proteinuria (*Figure 4B*, r= -0.04, p= NS). No significant correlations were observed between blood pressure and vascular parameters in sNX+LIS group.

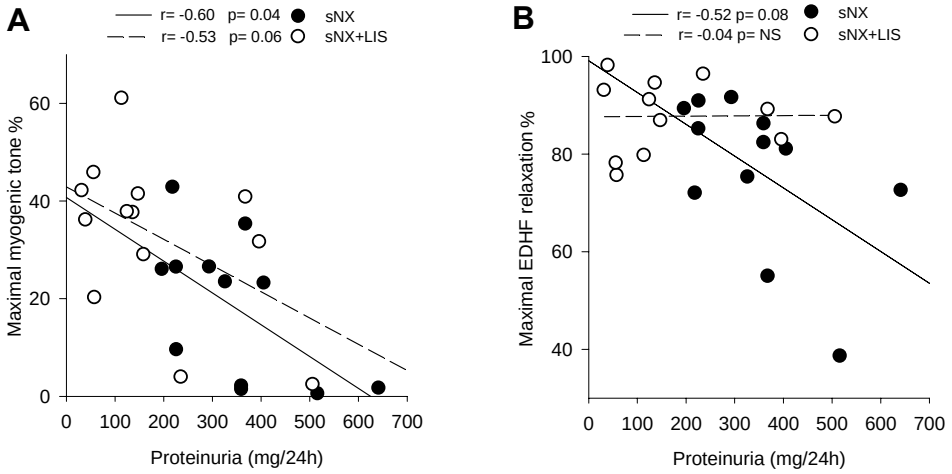

Figure 4. *Correlation between individual values of proteinuria (mg/24h) and* ***A)*** *maximal myogenic tone (% of passive diameter) and* ***B)*** *maximal EDHF-mediated relaxation (maximal relaxation in % of precontraction) of small mesenteric arteries in subtotally nephrectomized rats (sNX) and subtotally nephrectomized rats treated with lisinopril 2.5 mg/kg (sNX+LIS).*

Discussion

Chronic renal failure (CRF) is associated with altered arterial tone of the systemic vasculature, which may contribute to elevated blood pressure and accelerated rate of cardiovascular events observed in renal patients. In the current study, we demonstrate that two major vasoregulatory mechanisms of small mesenteric arteries, namely myogenic constriction and EDHF-mediated vasodilation, are impaired in a rat model of subtotal nephrectomy (sNX) with hypertension. Both vascular alterations in this experimental model might be reversed by renoprotective therapy with an ACE inhibitor.

Rats with sNX employed in the current study displayed characteristic features of CRF, as evidenced by severe proteinuria and almost doubled plasma creatinine levels, increased fluid intake, elevated urine production and increased renal mass, probably due to compensatory renal growth[20]. CRF was accompanied by an increase in blood pressure, a

complication frequently occurring in patients with end-stage renal disease and the ultimate risk factor for cardiovascular events[5]. The severity of hypertension did not correlate with the markers of renal damage, suggesting extrarenal factors may be involved in the increase of systemic blood pressure. Since hypertension in CRF is associated with increased peripheral resistance[21], systemic vascular changes might well contribute to CRF-associated elevations in blood pressure. Furthermore, a significant body of evidence suggests that myogenic response of small peripheral arteries is enhanced in SHR rats[15-17] implying that enhanced intrinsic reactivity of resistance arteries to increased pressure might be involved in elevated peripheral resistance in hypertension. However, as suggested by our current data this does not seem to be the case in CRF-associated hypertension. In contrast to our hypothesis, in sNX we observed a reduction in myogenic response of small mesenteric arteries, characterized by the impaired myogenic tone in the pressure range of 100-160 mmHg and altered myogenic index resulting in a shifted threshold for the development of active myogenic constriction to higher pressures. To our knowledge, the only other study investigating myogenic response of small resistance arteries in CRF did not find any difference between myogenic reactivity of small cremaster arteries in sNX and SHAM-operated rats[22]. Differences in anatomic localization, vascular diameter and function may account for the observed discrepancy with our data. Collectively however, both studies suggest that changes in systemic myogenic response do not contribute to the development of CRF-associated hypertension. Consequently, mechanisms other than excessive local myogenic reactivity, such as volume overload, overactivation of renin-angiotensin or sympathetic system, are probably responsible for increased peripheral resistance in CRF. The observed reduction of myogenic tone might represent a compensatory mechanism counteracting the increase in peripheral resistance in CRF. However, we found the level of myogenic tone not to be related with severity of hypertension, but rather with the severity of renal disease, suggesting that myogenic tone is specifically modulated by the state of renal insufficiency. Furthermore, the reduction of systemic myogenic tone was completely reversed after chronic treatment with lisinopril. Since ACE-i therapy resulted in a complete reversal of elevated systolic blood pressure and prevented the progression of renal damage, all antihypertensive, renoprotective and/or specific local effects of ACE-i (e.g. resulting from local ACE inhibition) might play a role in myogenic tone reversal. A positive correlation between blood pressure and severity of renal damage in treated animals suggests that antihypertensive and renoprotective effects of ACEi might be related. However, it is likely that kidney-related mechanisms modulate myogenic reactivity after ACE-i treatment, since the level myogenic tone was associated with renal damage rather than with blood pressure.

In addition to a reduction in smooth-muscle mediated myogenic mechanisms we also found an alteration in the major endothelium-dependent vasodilatory mechanism of small mesenteric artery, e.g. EDHF. Our data are in agreement with studies showing impaired EDHF-mediated relaxation in large conduit arteries of experimental animals with CRF[12,23]. However it is difficult to assess to which extent compromised EDHF relaxation of

resistance arteries contributes to CRF-associated hypertension, since impaired EDHF relaxation was also demonstrated in CRF-rats without hypertension, suggesting that CRF *per se* is associated with disturbed EDHF-mediated relaxation[24]. Our finding of a correlation between EDHF-mediated relaxation and markers of renal failure, but not hypertension, may support this view. Moreover, EDHF is differently modulated in various forms of hypertension ranging from altered[25,26] to elevated EDHF relaxations in some animal models[27-30]. In latter cases, EDHF might compensate for reduced nitric oxide bioavailability. Therefore the role of altered EDHF relaxation in CRF-related hypertension will have to be defined further.

The mechanisms responsible for concomitant decrease of myogenic constriction and EDHF-mediated dilation of systemic arteries in CRF cannot be directly inferred from this study. In contrast to our hypothesis we could not confirm a direct antagonistic relation between these two parameters. Similarly, ACEi-induced changes in endothelium (EDHF)- and myogenic- mediated mechanisms do not seem to be correlated either, suggesting that endothelial and smooth-muscle mediated mechanisms are altered independently in CRF. Thus, rather than a result of endothelial dysfunction, myogenic alterations might be a consequence of inappropriate chronic stimulation of sympathetic[31], endothelin[32] or renin-angiotensin[33] systems occurring in CRF. Myogenic tone is strongly potentiated by vasoconstricting agents, such as noradrenaline[34], endothelin-1[16], and angiotensin-II[35,36] and the chronic overactivation might result in its adaptive downregulation. Involvement of renin-angiotensin system is also supported by the finding of complete reversal of systemic myogenic tone after chronic treatment with ACEi. Interestingly, the angiotensin AT_1 receptor is involved in changes of myogenic reactivity observed in mesenteric arteries after experimental heart failure[35]. Yet, specific molecular mechanisms responsible for myogenic alterations will still have to be worked out. Although we cannot fully exclude the possibility of a generalized contractile defect on the level of smooth muscle cells, we found no differences in reactivity to the thromboxane A_2 agonist and no change in general contractile ability was reported previously in this model and vascular bed[24]. Therefore, signaling mechanisms relatively specific for pressure-induced vasoconstriction are more likely to be involved, including altered activity or mechanosensitive, voltage- or calcium-regulated ion channels[14]. One may speculate on the role of smooth muscle membrane Ca^{2+}-regulated potassium large conductance channels (BK_{Ca}), closure of which is implicated in myogenic-induced depolarization[14,37], whereas opening is involved in endothelium-induced hyperpolarization[8,38]. Reduced availability of BK_{Ca} channel might possibly explain the concomitant defect in myogenic tone and EDHF-mediated dilation in CRF. In line with renin-angiotensin system overactivity hypothesis, angiotensin II has been shown to inhibit vascular smooth muscle K_{Ca} channels[39]. However, further studies are needed to explore the mechanisms underlying vascular changes in CRF.

In conclusion, the present study revealed impaired myogenic constriction and EDHF-dependent dilation of small mesenteric arteries in a rat model of CRF with hypertension. The alterations in systemic vasoactive mechanisms do not seem to participate in the

development of CRF-associated hypertension, being rather directly related to the severity of renal impairment. Furthermore, both systemic vascular changes might be reversed by the renoprotective treatment with an ACE inhibitor.

References

1. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 2004; 351:1296-1305
2. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Hypertension 2003; 42:1050-1065
3. Foley RN, Parfrey PS, Harnett JD, Kent GM, Murray DC, Barre PE. The prognostic importance of left ventricular geometry in uremic cardiomyopathy. J Am Soc Nephrol 1995; 5:2024-2031
4. Klassen PS, Lowrie EG, Reddan DN, DeLong ER, Coladonato JA, Szczech LA, Lazarus JM, Owen WF, Jr. Association between pulse pressure and mortality in patients undergoing maintenance hemodialysis. JAMA 2002; 287:1548-1555
5. Flack JM, Peters R, Shafi T, Alrefai H, Nasser SA, Crook E. Prevention of hypertension and its complications: theoretical basis and guidelines for treatment. J Am Soc Nephrol 2003; 14:S92-S98
6. Benchetrit S, Green J, Katz D, Bernheim J, Rathaus M. Early endothelial dysfunction following renal mass reduction in rats. Eur J Clin Invest 2003; 33:26-33
7. Hasdan G, Benchetrit S, Rashid G, Green J, Bernheim J, Rathaus M. Endothelial dysfunction and hypertension in 5/6 nephrectomized rats are mediated by vascular superoxide. Kidney Int 2002; 61:586-590
8. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. Trends Pharmacol Sci 2002; 23:374-380
9. Gschwend S, Pinto-Sietsma SJ, Buikema H, Pinto YM, van Gilst WH, Schulz A, de Zeeuw D, Kreutz R. Impaired coronary endothelial function in a rat model of spontaneous albuminuria. Kidney Int 2002; 62:181-191
10. Lagaud GJ, Skarsgard PL, Laher I, van Breemen C. Heterogeneity of endothelium-dependent vasodilation in pressurized cerebral and small mesenteric resistance arteries of the rat. J Pharmacol Exp Ther 1999; 290:832-839
11. Ochodnicky P, Henning RH, Provoost AP, de Zeeuw D, van Dokkum RP. Heterogeneity of systemic and renal endothelial dysfunction in a rat model of hypertension-associated renal damage [Abstract]. J Am Soc Nephrol 2005; 16:185A-
12. Kohler R, Eichler I, Schonfelder H, Grgic I, Heinau P, Si H, Hoyer J. Impaired EDHF-mediated vasodilation and function of endothelial Ca-activated K channels in uremic rats. Kidney Int 2005; 67:2280-2287

13. Kimura K, Nishio I. Impaired endothelium-dependent relaxation in mesenteric arteries of reduced renal mass hypertensive rats. Scand J Clin Lab Invest 1999; 59:199-204
14. Davis MJ, Hill MA. Signaling mechanisms underlying the vascular myogenic response. Physiol Rev 1999; 79:387-423
15. Falcone JC, Granger HJ, Meininger GA. Enhanced myogenic activation in skeletal muscle arterioles from spontaneously hypertensive rats. Am J Physiol 1993; 265:H1847-H1855
16. Huang A, Koller A. Endothelin and prostaglandin H2 enhance arteriolar myogenic tone in hypertension. Hypertension 1997; 30:1210-1215
17. Shibuya J, Ohyanagi M, Iwasaki T. Enhanced myogenic response in resistance small arteries from spontaneously hypertensive rats: relationship to the voltage-dependent calcium channel. Am J Hypertens 1998; 11:767-773
18. Gschwend S, Henning RH, de Zeeuw D, Buikema H. Coronary myogenic constriction antagonizes EDHF-mediated dilation: role of KCa channels. Hypertension 2003; 41:912-918
19. Hughes JM, Bund SJ. Arterial myogenic properties of the spontaneously hypertensive rat. Exp Physiol 2002; 87:527-534
20. Hostetter TH. Progression of renal disease and renal hypertrophy. Annu Rev Physiol 1995; 57:263-278
21. Kim KE, Onesti G, Swartz CD. Hemodynamics of hypertension in uremia. Kidney Int Suppl 1975; 155-162
22. New DI, Chesser AM, Raftery MJ, Yaqoob MM. The myogenic response in uremic hypertension. Kidney Int 2003; 63:642-646
23. Kalliovalkama J, Jolma P, Tolvanen JP, Kahonen M, Hutri-Kahonen N, Saha H, Tuorila S, Moilanen E, Porsti I. Potassium channel-mediated vasorelaxation is impaired in experimental renal failure. Am J Physiol 1999; 277:H1622-H1629
24. Koobi P, Kalliovalkama J, Jolma P, Rysa J, Ruskoaho H, Vuolteenaho O, Kahonen M, Tikkanen I, Fan M, Ylitalo P, Porsti I. AT1 receptor blockade improves vasorelaxation in experimental renal failure. Hypertension 2003; 41:1364-1371
25. Fujii K, Tominaga M, Ohmori S, Kobayashi K, Koga T, Takata Y, Fujishima M. Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. Circ Res 1992; 70:660-669
26. Wu X, Makynen H, Kahonen M, Arvola P, Porsti I. Mesenteric arterial function in vitro in three models of experimental hypertension. J Hypertens 1996; 14:365-372
27. Sofola OA, Knill A, Hainsworth R, Drinkhill M. Change in endothelial function in mesenteric arteries of Sprague-Dawley rats fed a high salt diet. J Physiol 2002; 543:255-260
28. Huang A, Sun D, Smith CJ, Connetta JA, Shesely EG, Koller A, Kaley G. In eNOS knockout mice skeletal muscle arteriolar dilation to acetylcholine is mediated by EDHF. Am J Physiol Heart Circ Physiol 2000; 278:H762-H768

29. Corriu C, Feletou M, Puybasset L, Bea ML, Berdeaux A, Vanhoutte PM. Endothelium-dependent hyperpolarization in isolated arteries taken from animals treated with NO-synthase inhibitors. J Cardiovasc Pharmacol 1998; 32:944-950
30. Kalliovalkama J, Jolma P, Tolvanen JP, Kahonen M, Hutri-Kahonen N, Wu X, Holm P, Porsti I. Arterial function in nitric oxide-deficient hypertension: influence of long-term angiotensin II receptor antagonism. Cardiovasc Res 1999; 42:773-782
31. Rump LC, Amann K, Orth S, Ritz E. Sympathetic overactivity in renal disease: a window to understand progression and cardiovascular complications of uraemia? Nephrol Dial Transplant 2000; 15:1735-1738
32. Goddard J, Johnston NR, Hand MF, Cumming AD, Rabelink TJ, Rankin AJ, Webb DJ. Endothelin-A receptor antagonism reduces blood pressure and increases renal blood flow in hypertensive patients with chronic renal failure: a comparison of selective and combined endothelin receptor blockade. Circulation 2004; 109:1186-1193
33. Gretz N, Waldherr R, Strauch M. The remnant kidney model. In: Gretz, N. and Strauch, M. (editors). Experimental and genetic models of chronic renal failure. Basel: Karger; 1993. pp. 1-23.
34. Meininger GA, Faber JE. Adrenergic facilitation of myogenic response in skeletal muscle arterioles. Am J Physiol 1991; 260:H1424-H1432
35. Gschwend S, Henning RH, Pinto YM, de Zeeuw D, van Gilst WH, Buikema H. Myogenic constriction is increased in mesenteric resistance arteries from rats with chronic heart failure: instantaneous counteraction by acute AT1 receptor blockade. Br J Pharmacol 2003; 139:1317-1325
36. Nurkiewicz TR, Boegehold MA. Reinforcement of arteriolar myogenic activity by endogenous ANG II: susceptibility to dietary salt. Am J Physiol Heart Circ Physiol 2000; 279:H269-H278
37. Wesselman JP, Schubert R, VanBavel ED, Nilsson H, Mulvany MJ. KCa-channel blockade prevents sustained pressure-induced depolarization in rat mesenteric small arteries. Am J Physiol 1997; 272:H2241-H2249
38. Brayden JE, Nelson MT. Regulation of arterial tone by activation of calcium-dependent potassium channels. Science 1992; 256:532-535
39. Minami K, Hirata Y, Tokumura A, Nakaya Y, Fukuzawa K. Protein kinase C-independent inhibition of the Ca(2+)-activated K+ channel by angiotensin II and endothelin-1. Biochem Pharmacol 1995; 49:1051-1056

Chapter 7

Endothelial dysfunction in chronic kidney disease: determinant of susceptibility to end-organ damage and therapeutic response

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Abstract

Endothelial dysfunction (ED) seems to be a crucial mediator of increased cardiovascular risk observed among patients with chronic kidney disease (CKD). Importantly, systemic ED does not only occur in patients with severe renal failure, but also in individuals with earlier stages of CKD. Close association between microalbuminuria and systemic ED renders renal vascular function an important marker for the severity of cardiovascular damage. Furthermore, alterations of the renal endothelium might be actively involved in the progression of renal end-organ damage. Recently, experimental evidence showed that interindividual variability in endothelial function of healthy rats predicts the susceptibility to renal damage and the efficacy of renoprotective treatment. Therefore, a specific manipulation of renal and systemic ED might provide benefit in various stages of CKD. Thus, ED may represent an ideal therapeutic target not only for treatment, but also for primary prevention of renal disease.

Introduction

Chronic kidney disease (CKD) represents a major worldwide health care and economic problem. Apart from the enormous number of patients with chronic renal failure requiring treatment by dialysis of transplantation, there is also a constantly raising number of individuals suffering from earlier stages of CKD. In the United States, a total of 8 million individuals are estimated to have a GFR of less than 60 ml/min/1.73 m^2 and another 12 million had evidence of microalbuminuria[1]. CKD is associated with increased prevalence of cardiovascular disease and in fact, patients with CKD have even higher chance to experience a cardiovascular event than to progress to renal failure[2]. Early detection of individuals at risk for renal impairment might therefore prevent both renal failure and cardiovascular-related burden in these patients.

Endothelial injury is currently recognized as a common denominator of vascular damage in various conditions associated with increased cardiovascular risk, including CKD. Several important aspects of endothelial dysfunction (ED) in CKD have recently received a great deal of attention. Firstly, the occurrence of systemic ED in various stages of CKD has been proposed as an explanation for the accelerated rate of cardiovascular events associated with impaired renal function. Moreover, specific intrarenal ED could play an active role in the development and progression of renal damage itself. Finally, recent data suggest that the renal endothelium might be involved in the individual susceptibility to renal damage and might govern the sensitivity to renoprotective treatment. In this chapter, we discuss these aspects of ED in CKD (*Figure 1*) and propose that the endothelium represents a specific target for early detection and prevention of both renal and cardiovascular damage progression in patients with CKD.

Endothelial dysfunction, general considerations

Based on vascular research carried out over the last 25 years, the endothelium, inner lining of the vasculature, is now recognized as the principal regulator of vascular function (recently reviewed by[3,4]). Endothelial dysfunction (ED), defined as alterations in the normal properties of the endothelium that are inappropriate for preservation of organ function[5], is characterized by loss of protective endothelial characteristics in favour of deleterious mechanisms. ED is now recognized as a crucial event in the initiation and progression of atherosclerosis and a common denominator for conditions associated with elevated cardiovascular risk, including hypertension, diabetes, dyslipidemia, obesity or smoking[6]. In all these states, ED is characterized by altered production and/or decreased bioavailability of nitric oxide (NO), the most extensively studied endothelial mediator, and excessive production of reactive oxygen species.

Several estimates of ED are available in clinics, most of them are however difficult to interpret. Plasma levels of endothelial markers, such as vWF, PAI-1, or soluble adhesive and chemoattractant molecules are frequently used to estimate the state of systemic ED.

Unfortunately, they do not provide any information on endothelial function of clinically relevant vascular beds (e.g. coronary or renal). Localization of ED might be of paramount importance when investigating the role of the endothelium in the progression of specific end-organ damage as the structural and functional properties of the endothelium differ among vascular beds (*e.g.* renal versus systemic resistance arteries). Specificity of endothelial function measurements might be achieved by assessment of vascular tone in a defined vascular bed (*Table 1*).

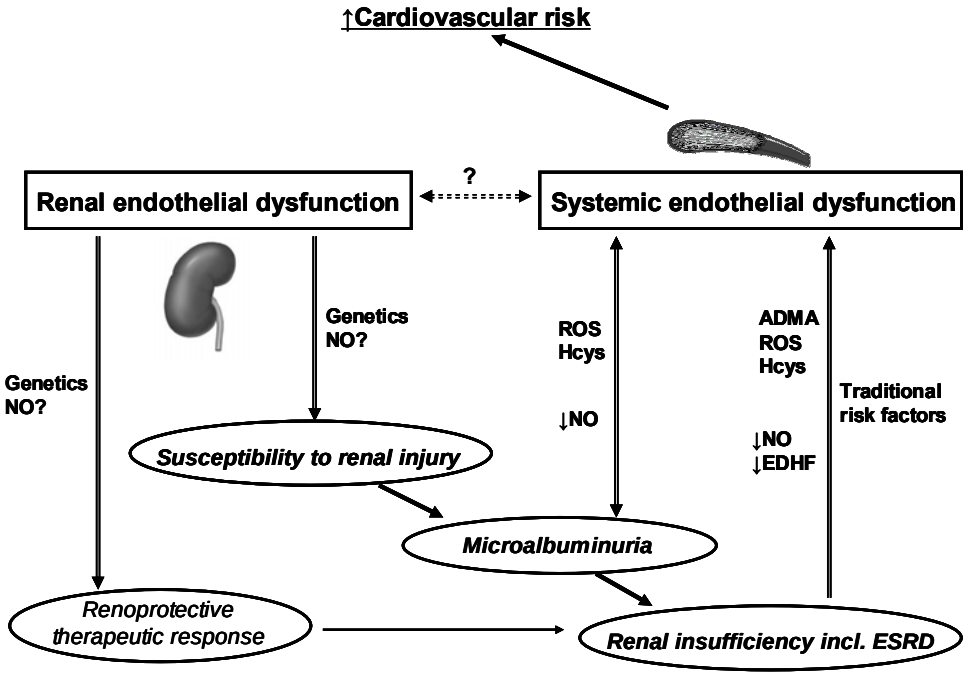

Figure 1. The role of renal and systemic endothelial function in the progression of the end-organ damage in various stages of chronic kidney disease.

Renal endothelial dysfunction might be involved in the susceptibility of the individual to renal injury, the progression of renal end-organ damage to the stage of renal insufficiency and may possibly co-determine the variability in renoprotective therapeutic response. Microalbuminuria and ESRD are also associated with systemic endothelial dysfunction, which is critically involved in the development of cardiovascular end-organ damage. The exact relations between renal and systemic endothelial function are not completely understood. Arrows depict the mutual relations. The most important mechanisms of endothelial dysfunction are also denoted. NO-nitric oxide, EDHF-endothelium-derived hyperpolarizing factor, ADMA- asymmetric dimethylarginine, HCys- homocysteine, ROS-reactive oxygen species.

Table 1. *Methods available for assessment of endothelial function in defined vascular beds in humans.*

Vascular bed	Method	Comment
Systemic	*Flow-mediated dilation (FMD)*	Non invasive measurements of diameter changes in conduit brachial artery by Doppler ultrasound after postischemic induction of flow, mainly NO-dependent
	Forearm blood flow (FBF)	Invasive measurement of flow in forearm microcirculation by strain gauge venous pletysmography, local infusion of endothelium-activating substances (acetylcholine)
	Microcirculation	Non-invasive laser digital Doppler flow measurements in skin microcirculation, endothelium-activating substances (acetylcholine) applied locally by iontophoresis
	Isolated vessels	Subcutaneous arteries from skin biopsies
Coronary	*Coronary angiography*	Functional angiogram after intracoronary infusion of acetylcholine
Renal	*Renal blood flow changes afterNO-modulating agents*	Measurements of renal hemodynamics after L-NAME or L-arginine infusion

Chronic renal failure is associated with systemic endothelial dysfunction

It has long been recognized that the last stage of CKD, end-stage renal disease, is invariably associated with systemic ED[7], a finding that has been interpreted as an important event in the development of renal insufficiency-related cardiovascular complications. This may not be that surprising since renal patients usually have several of the conventional cardiovascular risk factors such as hypertension, dyslipidemia or diabetes, all of which are associated with ED. However, several data suggest that the occurrence of these events can not be solely explained by traditional risk factors and that additional 'kidney-specific' mechanisms may contribute to the CKD-related ED.

Many authors report elevated plasma markers of endothelial injury, such as vWF, fibrinogen or thrombomodulin in patients on maintenance dialysis[8,9]. Others have confirmed localized impaired endothelium-dependent vasodilation in peripheral vascular beds of uremic patients *in vivo*[7,10,11], as well as in isolated vascular preparations[12]. Interestingly, peripheral vasodilatory ED was not only found in dialyzed subjects, but also in patients with moderate renal failure even when adjusted for hypertension, blood glucose and serum cholesterol[13]. In this study, the severity of systemic ED was proportionally

related to the degree of renal dysfunction. A study by *Kari et al.* demonstrated the presence of ED in uremic children without hypertension and lipid alterations, suggesting a renal failure specific effect[14]. In experimental animal studies, 5/6 nephrectomy resulting in chronic renal failure within several weeks is also associated with ED in both resistance[15] and conduit arteries[16] even without the presence of concomitant hypertension. Therefore, it is now widely accepted that malfunction of the kidneys elicits profound deleterious effects on systemic vascular endothelium.

The majority of the studies investigating the mechanisms of ED in chronic renal failure has focused on the systemic NO deficiency as the principal event leading to ED. Functional evidence of impaired stimulated NO bioactivity in forearm vasculature of hemodialysis patients was provided by *Passauer et al.*[11,17]. Mechanisms responsible for altered NO activity have not been completely elucidated yet, however some non-traditional risk factors relatively specific for uremia are being extensively discussed. Firstly, chronic renal failure is believed to be a state of excessive oxidative stress, as demonstrated in numerous experimental and clinical studies[18,19]. Reactive oxygen species result in premature inactivation of NO and its decreased bioavailability. The involvement of increased oxidative stress in systemic ED is further supported by a direct relation between endothelium-dependent vasodilation and plasma markers of oxidative stress[20] and beneficial effect of antioxidant treatment on ED in patients with chronic renal failure[21,22]. Hyperhomocysteinemia might be a specific condition responsible for excessive generation of reactive oxygen species in chronic renal failure as it is found in the majority of renal patients[23] and has been shown to adversely affect NO production[24] and endothelial function[25]. Finally, in dialyzed patients elevated plasma levels of endogenous competitive inhibitors of constitutive nitric oxide synthase (eNOS), such as asymmetric dimethylarginine (ADMA), are found[26]. It has been proposed that this substance may *in vivo* reach the plasma levels relevant for significant inhibition of NO production[27]. Thus, while prevailing evidence shows that chronic renal failure is a state of systemic NO deficiency, the exact role of 'kidney-specific' mechanisms has yet to be characterized.

Although not yet systematically addressed, several data suggest that mechanisms other than reduced NO bioavailability might be responsible for ED in patients with chronic renal failure. Additionally to NO, prostaglandins and endothelium-derived hyperpolarizing factor (EDHF) are released from the endothelium upon stimulation with acetylcholine. Most of the authors did not observe any contribution of prostaglandins to ED in experimental models of chronic renal failure, arguing against the important role of these mediators in renal insufficiency-related systemic ED. However, one human study reported that inhibition of cyclooxygenase by diclofenac acutely improves endothelium-dependent dilation in the forearm of the patients with mild to moderate renal insufficiency[28]. The authors speculate that beneficial effect of diclofenac might be attributed to inhibition of prostanoid endothelium-derived contracting factor (EDCF) or decreased production of cyclooxygenase-derived free radicals. In addition to nitric oxide and prostaglandins, yet another mediator, termed EDHF, is responsible for acetylcholine-induced endothelial

relaxation. We[29] and others[15,16] demonstrated reduced EDHF-dependent dilation in small resistance and conduit arteries of 5/6 nephrectomized rats. Under normal physiological conditions EDHF is responsible for the majority of the acetylcholine-induced relaxation in small arteries, where it prevails over NO-dependent mechanisms[30]. However, the role of EDHF in ED is not clearly defined since its identity remains illusive. Several mediators, such as cytochrome P450 metabolites of arachidonic acid, potassium ions, hydrogen peroxide or gap junctions have been proposed to account for EDHF-mediated dilation in various vascular beds[31]. It is however recognized that all these mechanisms involve the opening endothelial Ca^{2+}-sensitive potassium channels with small (SK_{Ca}) and intermediate (IK_{Ca}) conductance. *Kohler et al.* showed altered expression of these channels in vascular endothelium of 5/6 nephrectomized rats[32]. However, the only study addressing EDHF-mediated vasodilation in uremic humans failed to show any changes in forearm non-NO/prostaglandin vasodilation in hemodyalized patients[11]. Additional studies need to be performed to answer the question whether impaired EDHF-dependent ED, as observed in animal models, may occur also in humans and to which extent it may participate in the development of renal insufficiency-related cardiovascular complications.

Although it is now established that chronic renal failure represents a state of systemic NO and possibly EDHF-mediated ED, the role of ED in the excessive occurrence of cardiovascular risk of renal patients will have to be further clarified. *Pannier et al.* reported a direct relation between forearm post-ischemic endothelial vasodilation and markers of end-organ cardiovascular damage including intima-media thickness of common carotid artery and left ventricular hypertrophy[33] in subjects on hemodialysis. Several plasma markers of ED or molecules potentially involved in ED, including ADMA, hyperhomocysteine and PAI-1[34-37], all have been shown to be related with future cardiovascular morbidity and mortality in longitudinal studies in hemodialysis patients. So far, the only evidence available for such prognostic value of specific vasomotor endothelial dysfunction has been reported by *London et al.*[38]. They have shown that flow-mediated vasodilation was related with all-cause mortality in patients with end-stage renal disease with 60 months median follow-up. Thus, the current data support the view of systemic ED being a cardiovascular risk factor in patients with chronic renal failure.

Systemic endothelial dysfunction and microalbuminuria

Although most of the clinical data regarding systemic ED in CKD has been collected in uremic patients on dialysis, ED is often present in milder stages of renal insufficiency[13,39]. Moreover, the severity of ED might be related to the degree of renal insufficiency in renal patients[22], further suggesting a crucial link between kidney and systemic vascular damage. Interestingly, systemic ED can be already detected in subjects with minor increases in urinary protein excretion, which may be a marker of initial renal injury. A minor increase in protein (albumin) excretion (microalbuminuria) might be interpreted as the reflection of impaired glomerular endothelial function. Therefore it has been hypothesized (Steno hypothesis) that microalbuminuria reflects the specific renal feature of generalized vascular

dysfunction[40]. The view of the kidney as an organ reflecting general vascular damage has stemmed from numerous studies showing the predictive value of microalbuminuria for cardiovascular mortality in diabetic and high risk non-diabetic individuals[41,42]. Recently, the PREVEND study reported predictive value of microalbuminuria for all-cause mortality in the general population with low cardiovascular risk[43], suggesting that minor renal injury might be an early indicator of systemic vascular damage.

ED estimated from elevated plasma levels of endothelial markers[44,45] or measured as endothelium-dependent dilation in systemic vascular beds[46] is found in diabetic or hypertensive patients with microalbuminuria compared to normoalbuminemic counterparts. Furthermore, a direct inverse relationship has been found between systemic acetylcholine-induced dilation and microalbuminuria in diabetic patients[47]. Finally, microalbuminuria is associated with increased permeability to albumin in peripheral vessels in both diabetics[48] and clinically healthy subjects[49]. Altered endothelium-dependent vasodilation could be also found in clinically healthy subjects with microalbuminuria[50] as well as in subjects with asymptomatic proteinuria[51], *e.g.* in populations with minimal presence of other cardiovascular risk factors. At present, it remains unclear whether minor renal damage itself adds a new risk to the systemic vasculature or whether microalbuminuria represents just a reflection of a more severe, generalized cardiovascular phenotype. In support of the latter hypothesis, some authors suggest that generalized ED precedes the development of microalbuminuria, as the plasma levels of endothelial markers such as vWF are strongly related to *de novo* development of microalbuminuria in the follow-up of diabetic or healthy cohorts[52,53].

Only a small number of studies addressed the mechanisms responsible for selective contribution of increased urinary protein (albumin) excretion to systemic ED. *Elliot et al.* has found blunted vasoconstriction in response to the NOS inhibitor L-NAME in forearm vasculature of insulin-dependent diabetic patients with microalbuminuria as compared to normoalbuminuria, suggesting loss of NO-dependent vasodilation[54]. In another study, *Stehouwer et al.* demonstrated that the presence of microalbuminuria was associated with impaired flow (NO)-mediated systemic vasodilation in elderly individuals with and without diabetes[55]. Mechanisms responsible for potential NO deficiency in microalbuminuric patients might involve excessive oxidative stress. *Giner et al*.[56] found the elevated levels of markers of peripheral oxidative stress in hypertensive patients with microalbuminuria compared to normoalbuminurics. Similar results were reported from diabetic patients[57]. Recently, higher plasma levels of homocysteine were related with the occurrence of microalbuminuria[58,59] in diabetic populations, rendering homocysteinemia a potential mechanism responsible for increased oxidative stress and ED in microalbuminuria. Interestingly, animal data suggest that systems other than NO might be affected by minor renal impairment. EDHF–mediated dilation is reduced in coronary arteries of Munich Wistar Fromter (MWF) rat model of spontaneous albuminuria[60]. In yet another model of spontaneous renal disease, the Fawn-Hooded rat, slightly elevated urinary protein excretion at a young age has already been associated with a selective EDHF defect in systemic

resistance arteries[61]. However, since in such animal models it is difficult to dissociate between the effect of renal impairment itself and concomitant hypertension, the altered EDHF-mediated relaxation in systemic vasculature related to minor renal injury will need to be further confirmed.

Several studies investigated whether markers of systemic ED are related to cardiovascular risk in microalbuminuric patients. Plasma markers related to ED, such as levels of VCAM-1[62] and homocysteine[63], predicted mortality in diabetic patients with microalbuminuria. However, no such prognostic value has been reported for endothelium-dependent vasodilation. Nevertheless, the relation between putative renal and systemic ED seems to be established and may help to explain the increased occurrence of cardiovascular complications in patients with minor renal injury.

Renal endothelial dysfunction and the progression of renal damage

It is generally agreed that ED might be crucially involved in the development of systemic end-organ damage associated with renal dysfunction. However, ED might have profound consequences for the progression of damage in the kidney itself. Proteinuria or microalbuminuria may be the reflection of elevated glomerular permeability, in which endothelium might be involved. It suggests that at least some aspects of endothelial dysfunction might be present in very early stages of renal disease progression. Microalbuminuria predicts the rate of progression of renal function loss in diabetic patients and general population[64,65], raising the possibility that renal ED is involved in the progression of renal disease. Interestingly, the conditions associated with increased risk of renal impairment, including hypertension, diabetes or aging are all characterized by impaired renal endothelial function both in humans[66-68] and experimental animals[69-71]. Endothelial injury might play a prominent role in hemodynamic adaptations of the kidney to renal injury, which are responsible for the progressive nature of kidney damage. According to the most prevailing concept, remnant nephrons, e.g. those surviving the initial damaging event, adapt to the increased hemodynamic load by allowing higher intraglomerular pressure and hyperfiltration to compensate for reduced filtration. Glomerular hyperfiltration seems to represent the key event in the development of glomerulosclerosis, thus creating a vicious circle of additional nephron loss. Endothelium-derived NO has been long defined as a key regulator of glomerular pressure and renal perfusion[72]. Administration of the NO synthase inhibitor L-NAME leads to severe glomerular hypertension, glomerulosclerosis, interstitial fibrosis and eventually renal failure in rats[73-76]. On the other hand, administration of NO precursor L-arginine attenuates proteinuria and glomerular hypertension in experimental chronic renal failure induced by 5/6 nephrectomy[77]. Interestingly, direct renal hemodynamic NO dysfunction has been scarcely addressed in chronic renal failure. Recently, *Okuda et al.* reported impaired endothelium-mediated vasodilation of afferent and efferent arterioles in subtotally nephrectomized dogs, which was attributed to the increased levels of endogenous NO synthase inhibitor ADMA due to lower activity of its degrading enzyme dimethylarginine

dimethylaminohydrolase (DDAH). These data suggest that increased ADMA levels might represent the important mechanism of renal ED. However, reduction in other than hemodynamic properties of NO, including inhibition of mesangial growth and extracellular matrix production, limitation of leucocyte influx and adhesion, may importantly contribute to the progression of renal disease. *Kang et al.* highlighted the role of proliferative properties of glomerular microvascular endothelium in the progression of renal disease[78]. They showed that L-NAME treatment lead to the loss of microvascular endothelium and loss of the endothelial proliferative response in 5/6 nephrectomized rats[79]. Overall, although not precisely characterized, renal ED might crucially contribute to the development and progression renal end-organ damage.

Renal endothelial function and variation in the susceptibility to renal damage

Several authors have tried to linked endothelial damage in the kidney to progression of renal damage, recognizing renal ED as an early event in the renal pathophysiology. This has led to the hypothesis that renal endothelium is not only involved in the progression, but also in the susceptibility to renal damage. Interindividual differences in susceptibility to the development of renal disease are long recognized from clinical studies. The systemic factors including hypertension or diabetes represent the ultimate risk for renal damage, however the majority of patients with these disorders would never develop nephropathy. Additional evidence of the predisposition to renal disease is represented by clustering of renal disease with a specific racial and ethnic background[80,81]. Comparably, specific animal strains spontaneously developing renal disease have been described suggesting a role of genetic components in susceptibility to CKD[82]. Although the genetic background of CKD is a subject of intensive research, the involved genes have not been identified yet[83,84].

The role of the endothelium and /or NO in the predisposition to renal disease has been recently proposed by some investigators. An inbred strain of the Wistar-Furth rat is largely resistant to the development of renal damage after 5/6 nephrectomy[85] or after puromycin aminonucleoside injection[86] as compared to the widely used Sprague-Dawley strain. Low level of NOS inhibition with no impact on disease progression in the Sprague Dawley rat, converted Wistar Furth rat into a model of rapidly progressive renal disease[87]. Similar data were presented by using C57B16 mouse, a strain highly resistant to the development of nephrectomy-induced renal damage[88]. In our laboratory, we addressed the role of endothelial function in the susceptibility to end-organ damage using different approaches. Not only the various inbred strains differ in their sensitivity to organ damage, but also the animals of the same outbred strain such as the Wistar rat. When renal damage is induced by a well-defined uniform damaging procedure, such as 5/6 nephrectomy or standard injection of adriamycin, individual animals develop renal disease of highly variable severity. Some healthy rats seem to be more susceptible to end-organ damage than others. To investigate the factors responsible for this variability, we measured endothelium-mediated vasodilation in small renal arteries of these healthy animals. Following these measurements renal damage was induced. Interestingly, nitric oxide-mediated vasodilatory ability of renal

arteries measured in healthy kidney inversely predicted the subsequent development of proteinuria and focal glomerulosclerosis after 5/6 nephrectomy[89] and adriamycin nephrosis[90]. The rats with more pronounced endothelial nitric oxide-mediated relaxation seem to be protected against end-organ damage. This is in agreement with the protective role of nitric oxide against the development of renal damage. However, many questions remain unanswered. We measured vasomotor endothelial function, but it is unclear whether the renal endothelium provides direct hemodynamic protection against renal damage, or whether it is just a reflection of other protective properties of nitric oxide. Support for the latter explanation comes from the fact that the predictive value of NO-mediated dilation is found in models with various etiology of renal disease, e.g. 5/6 nephrectomy with hemodynamic-mediated injury and adriamycin nephrosis with nephrotoxic agent-induced damage. Interestingly, recent data suggest that the predictive value of endothelial vasomotor function is found in yet another rat model, in which renal damage is induced by combined unilateral nephrectomy and myocardial infarction[91]. The second important question is whether this predictive ability of renal endothelium may have a genetic basis. Polymorphisms of endothelial nitric oxide synthase have been associated with end-stage renal disease in several studies[92,93]. Clearly, additional experiments need to be performed, however the current evidence point out that interindividual differences in endothelial function might account for the variability in the susceptibility to renal injury.

Endothelial function and variability in renoprotective therapeutic response

Recognizing the important role of ED in the development of both systemic and renal end-organ damage, the endothelium might represent a valid therapeutic target. A wide spectrum of treatments has been shown to improve ED in several conditions. However, it is very difficult to assess to which extent this improvement contributes to the prevention or regression of end-organ damage, since these beneficial agents often possess antihypertensive, antilipidemic or hypoglycemic properties thus additionally targeting traditional cardiovascular risk factors.

The beneficial effect of several interventions has been tested in patients with chronic renal failure. Interestingly, restoration of renal function, *e.g*. by transplantation, is associated with reversal of systemic ED[94]. Dialysis might also acutely improve systemic ED, which could be attributed to removal of elevated ADMA[95] or homocysteine levels[96]. In contrast, other studies highlight dialysis-induced oxidative stress, which may eventually lead to impaired endothelial function[97]. Decreasing oxidative stress by antioxidant treatment might improve endothelial function in some vascular beds[21]. Attempts for NO bioavailability restoration either by L-arginine supplementations or by decreasing homocysteine levels with folic acid administration have led to disappointing results[98-100]. Recently, the beneficial effect of cyclooxygenase inhibition has been reported[28]. Additionally, in experimental settings, selective restoration of EDHF-mediated dilation was achieved by chronic angiotensin AT_1 receptor blocker losartan[15]. As previously stated, it is not known whether observed beneficial endothelial effects would translate to the reduction of cardiovascular risk. In this

respect, it is important to mention the results of the recent studies in hemodialysis patients, in which treatment with acetylcysteine[101] and vitamin E[102] were associated with a lower incidence of cardiovascular events, suggesting that targeting oxidative stress associated with endothelial dysfunction might prove a useful therapeutic strategy in chronic renal failure.

ED in the peripheral vasculature is also an emerging treatment target in patients with minute renal injury manifested as microalbuminuria. Several studies have been performed in diabetic patients, mainly investigating the effect of drugs interfering with the renin-angiotensin system, such as ACE inhibitors (ACEi) and angiotensin AT_1 receptor blockers (ARB). Although ACEi might improve some aspects of endothelial function in diabetic microalbuminurics, this is not always associated with reduction in microalbuminuria[103-105]. Furthermore, reduction in microalbuminuria by ARB was not accompanied by improvement of endothelium-dependent vasodilation[106]. Therefore, it seems that improvement of microalbuminuria is not always related to the improved systemic ED, at least in diabetic patients. The exact relation between renal and systemic ED will have to be defined, since lowering of microalbuminuria efficiently prevents cardiovascular events in both high[107] and low risk populations[108].

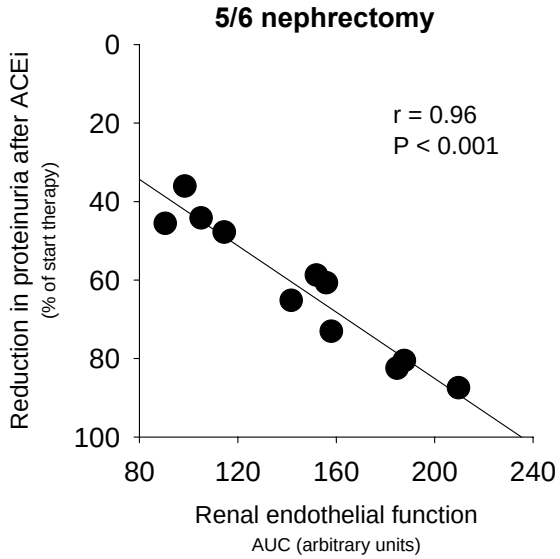

Figure 2. Renal endothelial function of healthy rat predicts the antiproteinuric effect of ACE inhibition.

Endothelium-dependent relaxation measured in small renal arteries of healthy animals (in arbitrary units of Area Under dose-response Curve to Acetylcholine- AUC) predicts the reduction in proteinuria after 10 weeks therapy with lisinopril (2.5mg/kg) preceded by 6 weeks of chronic renal disease induced by 5/6 nephrectomy.

Importantly, specific lowering of microalbuminuria translates in the reduction of renal events in several populations. *Parving et al.* showed that lowering of albuminuria with ARB irbesartan is dose-dependently associated with reduced progression to diabetic nephropathy in hypertensive type II diabetics independent of blood pressure control[109]. This may also suggest that reversal of ED might play a role in the inhibition of renal disease progression. Many of the agents known to improve ED, including ACEi, ARB, vasopeptidase inhibitors and probably statins, have been shown to inhibit the progression of renal disease. Furthermore, all these agents have profound effects on renal hemodynamics thus potentially interfering with endothelium-dependent control of renal function.

Among the renoprotective treatments, blockade of renin-angiotensin system seem to provide superior protection as compared to other drugs. In patients, ACEi effectively reduce proteinuria, glomerulosclerosis and decline of renal function. The antiproteinuric effect of ACEi is a valid index of therapeutic efficacy since it predicts long-term effect on renal disease[110]. Comparably to the rate of renal disease progression, the response to ACE inhibition is highly variable among individuals, suggesting that some patients are resistant while others are more sensitive to renoprotection by ACEi[111]. Several factors have been implicated in this variability of therapeutic response, including variability in severity of systemic complications (*e.g.* hypercholesterolemia, hypertension, obesity, insulin resistance), volume status and/or genetic background. Given the potential role of endothelium in the therapeutic effects of ACEi, we studied whether variability in renal endothelial function represent a factor involved in variability of ACEi antiproteinuric response. In the experimental setting described above, we measured vasomotor endothelial function in healthy Wistar rats. The animals were subsequently subjected to 5/6 nephrectomy and developed severe renal damage within 6 weeks. From this time point on, the rats were treated by ACEi lisinopril during additional 10 weeks. Antiproteinuric response to ACEi was highly variable in these rats and was not related to the level of blood pressure or volume status. However, ACEi antiproteinuric response was strongly predicted by baseline endothelial function *(Figure 2)*. The rats with more pronounced endothelial vasodilation were found to benefit less from ACEi[112]. Therefore, in contrast to the predictive value of endothelial function for the progression of renal damage, better renal endothelial function was associated with less beneficial outcome. This suggests that individual variability in antiproteinuric response might be independent of the severity of renal damage and that specific endothelial effects of ACEi might be involved in their renoprotective effects. Indeed, ACEi lowers the level of oxidative stress thereby preventing reduction in NO bioavailability, and retards the degradation of bradykinin, an NO-releasing autacoid. In fact, angiotensin II-induced release of NO is an important physiologic mechanism to counteract the excessive renal vasoconstriction[113]. The present results only allow speculation on the role of NO in predictive value of endothelial function for antiproteinuric effects of ACEi. It might be hypothesized that individuals with well-preserved endothelium (eventually NO) seem to be less dependent on activity of angiotensin II after the induction of renal damage and thus resistant to the beneficial effects

of ACEi. Alternatively, the favorable effects of ACEi might be directly related to their NO-releasing ability. Individuals with saturated mechanisms of NO production might be less sensitive for additional ACEi-induced NO release. Clearly, more data on this subject are required, however at present it seems possible that variability in endothelial function among healthy individuals might provide prognostic information on the ACEi therapeutic response.

Conclusions

Endothelial dysfunction seems to be a crucial mediator of increased cardiovascular risk observed among patients with CKD. Importantly, systemic ED does not only occur in patients with severe renal failure, but also in the individuals with earlier stages of renal impairment. Close association between microalbuminuria and systemic ED renders renal vascular function an important marker for the severity of cardiovascular damage. Furthermore, changes in renal endothelium might be actively involved in the progression of renal end-organ damage. Recently, variability in endothelial function of healthy individuals emerged to predict the susceptibility to renal damage and the efficacy of renoprotective treatment (*Figure 1*). Therefore, a specific manipulation of renal and systemic ED might provide benefit in various stages of CKD. ED thus represents an ideal therapeutic target not only for treatment, but also for primary prevention of renal disease.

References

1. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 2003; 139:137-147
2. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation 2003; 108:2154-2169
3. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 2004; 15:1983-1992
4. Goligorsky MS. Endothelial cell dysfunction: can't live with it, how to live without it. Am J Physiol Renal Physiol 2005; 288:F871-F880
5. Stehouwer CD. Endothelial dysfunction in diabetic nephropathy: state of the art and potential significance for non-diabetic renal disease. Nephrol Dial Transplant 2004; 19:778-781
6. Brunner H, Cockcroft JR, Deanfield J, Donald A, Ferrannini E, Halcox J, Kiowski W, Luscher TF, Mancia G, Natali A, Oliver JJ, Pessina AC, Rizzoni D, Rossi GP, Salvetti A, Spieker LE, Taddei S, Webb DJ. Endothelial function and dysfunction. Part II: Association with cardiovascular risk factors and diseases. A statement by the Working Group on Endothelins and Endothelial Factors of the European Society of Hypertension. J Hypertens 2005; 23:233-246
7. Morris ST, Jardine AG. The vascular endothelium in chronic renal failure. J Nephrol 2000; 13:96-105
8. Gris JC, Branger B, Vecina F, al Sabadani B, Fourcade J, Schved JF. Increased cardiovascular risk factors and features of endothelial activation and dysfunction in dialyzed uremic patients. Kidney Int 1994; 46:807-813
9. Nakayama M, Yamada K, Yamamoto Y, Yokoyama K, Nakano H, Kubo H, Shigematsu T, Kawaguchi Y, Sakai O. Vascular endothelial dysfunction in patients on regular dialysis treatment. Clin Nephrol 1994; 42:117-120
10. Cupisti A, Rossi M, Placidi S, Caprioli R, Morelli E, Vagheggini G, Barsotti G. Responses of the skin microcirculation to acetylcholine and to sodium nitroprusside in chronic uremic patients. Int J Clin Lab Res 2000; 30:157-162
11. Passauer J, Bussemaker E, Range U, Plug M, Gross P. Evidence in vivo showing increase of baseline nitric oxide generation and impairment of endothelium-dependent vasodilation in normotensive patients on chronic hemodialysis. J Am Soc Nephrol 2000; 11:1726-1734

12. Morris ST, McMurray JJ, Spiers A, Jardine AG. Impaired endothelial function in isolated human uremic resistance arteries. Kidney Int 2001; 60:1077-1082
13. Annuk M, Lind L, Linde T, Fellstrom B. Impaired endothelium-dependent vasodilatation in renal failure in humans. Nephrol Dial Transplant 2001; 16:302-306
14. Kari JA, Donald AE, Vallance DT, Bruckdorfer KR, Leone A, Mullen MJ, Bunce T, Dorado B, Deanfield JE, Rees L. Physiology and biochemistry of endothelial function in children with chronic renal failure. Kidney Int 1997; 52:468-472
15. Koobi P, Kalliovalkama J, Jolma P, Rysa J, Ruskoaho H, Vuolteenaho O, Kahonen M, Tikkanen I, Fan M, Ylitalo P, Porsti I. AT1 receptor blockade improves vasorelaxation in experimental renal failure. Hypertension 2003; 41:1364-1371
16. Koobi P, Jolma P, Kalliovalkama J, Tikkanen I, Fan M, Kahonen M, Moilanen E, Porsti I. Effect of angiotensin II type 1 receptor blockade on conduit artery tone in subtotally nephrectomized rats. Nephron Physiol 2004; 96:91-98
17. Passauer J, Pistrosch F, Bussemaker E, Lassig G, Herbrig K, Gross P. Reduced agonist-induced endothelium-dependent vasodilation in uremia is attributable to an impairment of vascular nitric oxide. J Am Soc Nephrol 2005; 16:959-965
18. Ferraro B, Galli F, Frei B, Kingdon E, Canestrari F, Rice-Evans C, Buoncristiani U, Davenport A, Moore KP. Peroxynitrite-induced oxidation of plasma lipids is enhanced in stable hemodialysis patients. Kidney Int 2003; 63:2207-2213
19. Hasdan G, Benchetrit S, Rashid G, Green J, Bernheim J, Rathaus M. Endothelial dysfunction and hypertension in 5/6 nephrectomized rats are mediated by vascular superoxide. Kidney Int 2002; 61:586-590
20. Annuk M, Zilmer M, Lind L, Linde T, Fellstrom B. Oxidative stress and endothelial function in chronic renal failure. J Am Soc Nephrol 2001; 12:2747-2752
21. Cross JM, Donald AE, Nuttall SL, Deanfield JE, Woolfson RG, Macallister RJ. Vitamin C improves resistance but not conduit artery endothelial function in patients with chronic renal failure. Kidney Int 2003; 63:1433-1442
22. Ghiadoni L, Cupisti A, Huang Y, Mattei P, Cardinal H, Favilla S, Rindi P, Barsotti G, Taddei S, Salvetti A. Endothelial dysfunction and oxidative stress in chronic renal failure. J Nephrol 2004; 17:512-519
23. Bostom AG, Culleton BF. Hyperhomocysteinemia in chronic renal disease. J Am Soc Nephrol 1999; 10:891-900
24. Zhang X, Li H, Jin H, Ebin Z, Brodsky S, Goligorsky MS. Effects of homocysteine on endothelial nitric oxide production. Am J Physiol Renal Physiol 2000; 279:F671-F678
25. Virdis A, Ghiadoni L, Cardinal H, Favilla S, Duranti P, Birindelli R, Magagna A, Bernini G, Salvetti G, Taddei S, Salvetti A. Mechanisms responsible for endothelial dysfunction induced by fasting hyperhomocystinemia in normotensive subjects and patients with essential hypertension. J Am Coll Cardiol 2001; 38:1106-1115
26. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 1992; 339:572-575

27. Schmidt RJ, Baylis C. Total nitric oxide production is low in patients with chronic renal disease. Kidney Int 2000; 58:1261-1266
28. Annuk M, Fellstrom B, Lind L. Cyclooxygenase inhibition improves endothelium-dependent vasodilatation in patients with chronic renal failure. Nephrol Dial Transplant 2002; 17:2159-2163
29. Ochodnicky P, Vettoretti S, Henning RH, Kluppel CA, de Zeeuw D, van Dokkum RP. Concomitant decrease of systemic arterial myogenic tone and EDHF-mediated dilation in subtotally nephrectomized rat [Abstract]. J Am Soc Nephrol 2005; 16:185A-
30. Feletou M, Vanhoutte PM. EDHF: new therapeutic targets? Pharmacol Res 2004; 49:565-580
31. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. Trends Pharmacol Sci 2002; 23:374-380
32. Kohler R, Eichler I, Schonfelder H, Grgic I, Heinau P, Si H, Hoyer J. Impaired EDHF-mediated vasodilation and function of endothelial Ca-activated K channels in uremic rats. Kidney Int 2005; 67:2280-2287
33. Pannier B, Guerin AP, Marchais SJ, Metivier F, Safar ME, London GM. Postischemic vasodilation, endothelial activation, and cardiovascular remodeling in end-stage renal disease. Kidney Int 2000; 57:1091-1099
34. Mallamaci F, Zoccali C, Tripepi G, Fermo I, Benedetto FA, Cataliotti A, Bellanuova I, Malatino LS, Soldarini A. Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. Kidney Int 2002; 61:609-614
35. Mallamaci F, Tripepi G, Cutrupi S, Malatino LS, Zoccali C. Prognostic value of combined use of biomarkers of inflammation, endothelial dysfunction, and myocardiopathy in patients with ESRD. Kidney Int 2005; 67:2330-2337
36. Segarra A, Chacon P, Martinez-Eyarre C, Argelaguer X, Vila J, Ruiz P, Fort J, Bartolome J, Camps J, Moliner E, Pelegri A, Marco F, Olmos A, Piera L. Circulating levels of plasminogen activator inhibitor type-1, tissue plasminogen activator, and thrombomodulin in hemodialysis patients: biochemical correlations and role as independent predictors of coronary artery stenosis. J Am Soc Nephrol 2001; 12:1255-1263
37. Zoccali C. Endothelial damage, asymmetric dimethylarginine and cardiovascular risk in end-stage renal disease. Blood Purif 2002; 20:469-472
38. London GM, Pannier B, Agharazii M, Guerin AP, Verbeke FH, Marchais SJ. Forearm reactive hyperemia and mortality in end-stage renal disease. Kidney Int 2004; 65:700-704
39. Thambyrajah J, Landray MJ, McGlynn FJ, Jones HJ, Wheeler DC, Townend JN. Abnormalities of endothelial function in patients with predialysis renal failure. Heart 2000; 83:205-209
40. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage. The Steno hypothesis. Diabetologia 1989; 32:219-226

41. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. The New England Journal of Medicine 1984; 310:356-360
42. Yudkin JS. Microalbuminuria as predictor of vascular disease in non-diabetic subjects. Islington Diabetes Survey. The Lancet 1988; 2:530-533
43. Hillege HL, Fidler V, Diercks GF, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, Gans RO, Janssen WM, Grobbee DE, de Jong PE. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. Circulation 2002; 106:1777-1782
44. Clausen P, Jacobsen P, Rossing K, Jensen JS, Parving HH, Feldt-Rasmussen B. Plasma concentrations of VCAM-1 and ICAM-1 are elevated in patients with Type 1 diabetes mellitus with microalbuminuria and overt nephropathy. Diabet Med 2000; 17:644-649
45. Perticone F, Maio R, Tripepi G, Zoccali C. Endothelial dysfunction and mild renal insufficiency in essential hypertension. Circulation 2004; 110:821-825
46. Dogra G, Rich L, Stanton K, Watts GF. Endothelium-dependent and independent vasodilation studies at normoglycaemia in type I diabetes mellitus with and without microalbuminuria. Diabetologia 2001; 44:593-601
47. Papaioannou GI, Seip RL, Grey NJ, Katten D, Taylor A, Inzucchi SE, Young LH, Chyun DA, Davey JA, Wackers FJ, Iskandrian AE, Ratner RE, Robinson EC, Carolan S, Engel S, Heller GV. Brachial artery reactivity in asymptomatic patients with type 2 diabetes mellitus and microalbuminuria (from the Detection of Ischemia in Asymptomatic Diabetics-brachial artery reactivity study). Am J Cardiol 2004; 94:294-299
48. Feldt-Rasmussen B. Increased transcapillary escape rate of albumin in type 1 (insulin-dependent) diabetic patients with microalbuminuria. Diabetologia 1986; 29:282-286
49. Jensen JS, Borch-Johnsen K, Jensen G, Feldt-Rasmussen B. Microalbuminuria reflects a generalized transvascular albumin leakiness in clinically healthy subjects. Clin Sci (Lond) 1995; 88:629-633
50. Clausen P, Jensen JS, Jensen G, Borch-Johnsen K, Feldt-Rasmussen B. Elevated urinary albumin excretion is associated with impaired arterial dilatory capacity in clinically healthy subjects. Circulation 2001; 103:1869-1874
51. Paisley KE, Beaman M, Tooke JE, Mohamed-Ali V, Lowe GD, Shore AC. Endothelial dysfunction and inflammation in asymptomatic proteinuria. Kidney Int 2003; 63:624-633
52. Clausen P, Feldt-Rasmussen B, Jensen G, Jensen JS. Endothelial haemostatic factors are associated with progression of urinary albumin excretion in clinically healthy subjects: a 4-year prospective study. Clin Sci (Lond) 1999; 97:37-43
53. Stehouwer CD, Nauta JJ, Zeldenrust GC, Hackeng WH, Donker AJ, den Ottolander GJ. Urinary albumin excretion, cardiovascular disease, and endothelial dysfunction in non-insulin-dependent diabetes mellitus. Lancet 1992; 340:319-323

54. Elliott TG, Cockcroft JR, Groop PH, Viberti GC, Ritter JM. Inhibition of nitric oxide synthesis in forearm vasculature of insulin-dependent diabetic patients: blunted vasoconstriction in patients with microalbuminuria. Clin Sci (Lond) 1993; 85:687-693
55. Stehouwer CD, Henry RM, Dekker JM, Nijpels G, Heine RJ, Bouter LM. Microalbuminuria is associated with impaired brachial artery, flow-mediated vasodilation in elderly individuals without and with diabetes: further evidence for a link between microalbuminuria and endothelial dysfunction--the Hoorn Study. Kidney Int Suppl 2004; S42-S44
56. Giner V, Tormos C, Chaves FJ, Saez G, Redon J. Microalbuminuria and oxidative stress in essential hypertension. J Intern Med 2004; 255:588-594
57. Ozdemir G, Ozden M, Maral H, Kuskay S, Cetinalp P, Tarkun I. Malondialdehyde, glutathione, glutathione peroxidase and homocysteine levels in type 2 diabetic patients with and without microalbuminuria. Ann Clin Biochem 2005; 42:99-104
58. de Luis DA, Fernandez N, Arranz ML, Aller R, Izaola O, Romero E. Total homocysteine levels relation with chronic complications of diabetes, body composition, and other cardiovascular risk factors in a population of patients with diabetes mellitus type 2. J Diabetes Complications 2005; 19:42-46
59. Hoogeveen EK, Kostense PJ, Jager A, Heine RJ, Jakobs C, Bouter LM, Donker AJ, Stehouwer CD. Serum homocysteine level and protein intake are related to risk of microalbuminuria: the Hoorn Study. Kidney Int 1998; 54:203-209
60. Gschwend S, Pinto-Sietsma SJ, Buikema H, Pinto YM, van Gilst WH, Schulz A, de Zeeuw D, Kreutz R. Impaired coronary endothelial function in a rat model of spontaneous albuminuria. Kidney Int 2002; 62:181-191
61. Ochodnicky P, Henning RH, Provoost AP, de Zeeuw D, van Dokkum RP. Heterogeneity of systemic and renal endothelial dysfunction in a rat model of hypertension-associated renal damage [Abstract]. J Am Soc Nephrol 2005; 16:185A-
62. Jager A, van Hinsbergh VW, Kostense PJ, Emeis JJ, Nijpels G, Dekker JM, Heine RJ, Bouter LM, Stehouwer CD. Increased levels of soluble vascular cell adhesion molecule 1 are associated with risk of cardiovascular mortality in type 2 diabetes: the Hoorn study. Diabetes 2000; 49:485-491
63. Stehouwer CD, Gall MA, Hougaard P, Jakobs C, Parving HH. Plasma homocysteine concentration predicts mortality in non-insulin-dependent diabetic patients with and without albuminuria. Kidney Int 1999; 55:308-314
64. Verhave JC, Gansevoort RT, Hillege HL, Bakker SJ, de Zeeuw D, de Jong PE. An elevated urinary albumin excretion predicts de novo development of renal function impairment in the general population. Kidney Int Suppl 2004; S18-S21
65. Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet 1982; 1:1430-1432
66. Dalla Vestra M, Sacerdoti D, Bombonato G, Fioretto P, Finucci G, Saller A, Sfriso A, Bruseghin M, Sambataro M, Velussi M, Baggio B, Nosadini R, Crepaldi G. Nitric

oxide modulation of renal and cardiac hemodynamics in type 2 diabetes. Eur J Endocrinol 2002; 146:687-694
67. Higashi Y, Oshima T, Ozono R, Matsuura H, Kajiyama G. Aging and severity of hypertension attenuate endothelium-dependent renal vascular relaxation in humans. Hypertension 1997; 30:252-258
68. van den Meiracker AH, van der Linde NA, Broere A, Derkx FH, Boomsma F. Effects of L-arginine and L-NAME on the renal function in hypertensive and normotensive subjects. Nephron 2002; 91:444-451
69. Hayashi K, Matsuda H, Nagahama T, Fujiwara K, Ozawa Y, Kubota E, Honda M, Tokuyama H, Saruta T. Impaired nitric oxide-independent dilation of renal afferent arterioles in spontaneously hypertensive rats. Hypertens Res 1999; 22:31-37
70. Hayashi K, Kanda T, Homma K, Tokuyama H, Okubo K, Takamatsu I, Tatematsu S, Kumagai H, Saruta T. Altered renal microvascular response in Zucker obese rats. Metabolism 2002; 51:1553-1561
71. Long DA, Newaz MA, Prabhakar SS, Price KL, Truong LD, Feng L, Mu W, Oyekan AO, Johnson RJ. Loss of nitric oxide and endothelial-derived hyperpolarizing factor-mediated responses in aging. Kidney Int 2005; 68:2154-2163
72. Baylis C, Qiu C. Importance of nitric oxide in the control of renal hemodynamics. Kidney Int 1996; 49:1727-1731
73. Zatz R, Baylis C. Chronic nitric oxide inhibition model six years on. Hypertension 1998; 32:958-964
74. Fujihara CK, Michellazzo SM, de Nucci G, Zatz R. Sodium excess aggravates hypertension and renal parenchymal injury in rats with chronic NO inhibition. Am J Physiol 1994; 266:F697-F705
75. Kashiwagi M, Shinozaki M, Hirakata H, Tamaki K, Hirano T, Tokumoto M, Goto H, Okuda S, Fujishima M. Locally activated renin-angiotensin system associated with TGF-beta1 as a major factor for renal injury induced by chronic inhibition of nitric oxide synthase in rats. J Am Soc Nephrol 2000; 11:616-624
76. Baylis C, Mitruka B, Deng A. Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage. J Clin Invest 1992; 90:278-281
77. Reyes AA, Purkerson ML, Karl I, Klahr S. Dietary supplementation with L-arginine ameliorates the progression of renal disease in rats with subtotal nephrectomy. Am J Kidney Dis 1992; 20:168-176
78. Kang DH, Kanellis J, Hugo C, Truong L, Anderson S, Kerjaschki D, Schreiner GF, Johnson RJ. Role of the microvascular endothelium in progressive renal disease. J Am Soc Nephrol 2002; 13:806-816
79. Kang DH, Nakagawa T, Feng L, Johnson RJ. Nitric oxide modulates vascular disease in the remnant kidney model. Am J Pathol 2002; 161:239-248
80. Feldman HI, Klag MJ, Chiapella AP, Whelton PK. End-stage renal disease in US minority groups. Am J Kidney Dis 1992; 19:397-410

81. Hayden PS, Iyengar SK, Schelling JR, Sedor JR. Kidney disease, genotype and the pathogenesis of vasculopathy. Curr Opin Nephrol Hypertens 2003; 12:71-78
82. Provoost AP. Spontaneous glomerulosclerosis: insights from the fawn-hooded rat. Kidney Int Suppl 1994; 45:S2-S5
83. Bowden DW. Genetics of kidney disease. Kidney Int Suppl 2003; S8-12
84. Korstanje R, DiPetrillo K. Unraveling the genetics of chronic kidney disease using animal models. Am J Physiol Renal Physiol 2004; 287:F347-F352
85. Fitzgibbon WR, Greene EL, Grewal JS, Hutchison FN, Self SE, Latten SY, Ullian ME. Resistance to remnant nephropathy in the Wistar-Furth rat. J Am Soc Nephrol 1999; 10:814-821
86. Erdely A, Freshour G, Smith C, Engels K, Olson JL, Baylis C. Protection against puromycin aminonucleoside-induced chronic renal disease in the Wistar-Furth rat. Am J Physiol Renal Physiol 2004; 287:F81-F89
87. Erdely A, Wagner L, Muller V, Szabo A, Baylis C. Protection of wistar furth rats from chronic renal disease is associated with maintained renal nitric oxide synthase. J Am Soc Nephrol 2003; 14:2526-2533
88. Muller V, Engels K, Baylis C. Chronic inhibition of nitric oxide synthase (NOS) renders the C57BL6 mouse susceptible to the development of chronic renal disease. [Abstract]. J Am Soc Nephrol 2003; 14:625A-
89. Gschwend S, Buikema H, Navis G, Henning RH, de Zeeuw D, van Dokkum RP. Endothelial dilatory function predicts individual susceptibility to renal damage in the 5/6 nephrectomized rat. J Am Soc Nephrol 2002; 13:2909-2915
90. Ochodnicky P, Henning RH, Buikema H, de Zeeuw D, van Dokkum RP. Renal endothelial function predicts the individual susceptibility to adriamycin-induced renal damage [Abstract]. J Am Soc Nephrol 2005; 16:800A-
91. Ochodnicky P, de Zeeuw D, Henning RH, Kluppel CA, van Dokkum RP. Endothelial function predicts the development of renal damage after combined nephrectomy and myocardial infarction. J Am Soc Nephrol 2006; 17 Suppl 2:S49-S52
92. Nagase S, Suzuki H, Wang Y, Kikuchi S, Hirayama A, Ueda A, Takada K, Oteki T, Obara M, Aoyagi K, Koyama A. Association of ecNOS gene polymorphisms with end stage renal diseases. Mol Cell Biochem 2003; 244:113-118
93. Noiri E, Satoh H, Taguchi J, Brodsky SV, Nakao A, Ogawa Y, Nishijima S, Yokomizo T, Tokunaga K, Fujita T. Association of eNOS Glu298Asp polymorphism with end-stage renal disease. Hypertension 2002; 40:535-540
94. Passauer J, Bussemaker E, Lassig G, Gross P. Kidney transplantation improves endothelium-dependent vasodilation in patients with endstage renal disease. Transplantation 2003; 75:1907-1910
95. Kielstein JT, Boger RH, Bode-Boger SM, Schaffer J, Barbey M, Koch KM, Frolich JC. Asymmetric dimethylarginine plasma concentrations differ in patients with end-stage renal disease: relationship to treatment method and atherosclerotic disease. J Am Soc Nephrol 1999; 10:594-600

96. McGregor DO, Buttimore AL, Lynn KL, Yandle T, Nicholls MG. Effects of long and short hemodialysis on endothelial function: a short-term study. Kidney Int 2003; 63:709-715
97. Miyazaki H, Matsuoka H, Itabe H, Usui M, Ueda S, Okuda S, Imaizumi T. Hemodialysis impairs endothelial function via oxidative stress: effects of vitamin E-coated dialyzer. Circulation 2000; 101:1002-1006
98. Bennett-Richards KJ, Kattenhorn M, Donald AE, Oakley GR, Varghese Z, Bruckdorfer KR, Deanfield JE, Rees L. Oral L-arginine does not improve endothelial dysfunction in children with chronic renal failure. Kidney Int 2002; 62:1372-1378
99. Chan NN, Chan WB, Chan JC. Reduction of total homocysteine levels by oral folic acid fails to improve endothelial function in children with chronic renal failure. Circulation 2003; 107:e6-e7
100. Cross JM, Donald AE, Kharbanda R, Deanfield JE, Woolfson RG, Macallister RJ. Acute administration of L-arginine does not improve arterial endothelial function in chronic renal failure. Kidney Int 2001; 60:2318-2323
101. Tepel M, van der GM, Statz M, Jankowski J, Zidek W. The antioxidant acetylcysteine reduces cardiovascular events in patients with end-stage renal failure: a randomized, controlled trial. Circulation 2003; 107:992-995
102. Boaz M, Smetana S, Weinstein T, Matas Z, Gafter U, Iaina A, Knecht A, Weissgarten Y, Brunner D, Fainaru M, Green MS. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. Lancet 2000; 356:1213-1218
103. Arcaro G, Zenere BM, Saggiani F, Zenti MG, Monauni T, Lechi A, Muggeo M, Bonadonna RC. ACE inhibitors improve endothelial function in type 1 diabetic patients with normal arterial pressure and microalbuminuria. Diabetes Care 1999; 22:1536-1542
104. Gasic S, Wagner OF, Fasching P, Ludwig C, Veitl M, Kapiotis S, Jilma B. Fosinopril decreases levels of soluble vascular cell adhesion molecule-1 in borderline hypertensive type II diabetic patients with microalbuminuria. Am J Hypertens 1999; 12:217-222
105. Schalkwijk CG, Smulders RA, Lambert J, Donker AJ, Stehouwer CD. ACE-inhibition modulates some endothelial functions in healthy subjects and in normotensive type 1 diabetic patients. Eur J Clin Invest 2000; 30:853-860
106. Tan KC, Chow WS, Ai VH, Lam KS. Effects of angiotensin II receptor antagonist on endothelial vasomotor function and urinary albumin excretion in type 2 diabetic patients with microalbuminuria. Diabetes Metab Res Rev 2002; 18:71-76
107. Ibsen H, Olsen MH, Wachtell K, Borch-Johnsen K, Lindholm LH, Mogensen CE, Dahlof B, Devereux RB, de Faire U, Fyhrquist F, Julius S, Kjeldsen SE, Lederballe-Pedersen O, Nieminen MS, Omvik P, Oparil S, Wan Y. Reduction in albuminuria translates to reduction in cardiovascular events in hypertensive patients: losartan

intervention for endpoint reduction in hypertension study. Hypertension 2005; 45:198-202

108. Asselbergs FW, Diercks GF, Hillege HL, van Boven AJ, Janssen WM, Voors AA, de Zeeuw D, de Jong PE, van Veldhuisen DJ, van Gilst WH. Effects of fosinopril and pravastatin on cardiovascular events in subjects with microalbuminuria. Circulation 2004; 110:2809-2816
109. Parving HH, Lehnert H, Brochner-Mortensen J, Gomis R, Andersen S, Arner P. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. N Engl J Med 2001; 345:870-878
110. Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, Marcantoni C, de Jong PE, de Zeeuw D, Shahinfar S, Ruggenenti P, Remuzzi G, Levey AS. Proteinuria as a modifiable risk factor for the progression of non-diabetic renal disease. Kidney Int 2001; 60:1131-1140
111. Laverman GD, de Zeeuw D, Navis G. Between-patient differences in the renal response to renin-angiotensin system intervention: clue to optimising renoprotective therapy? J Renin Angiotensin Aldosterone Syst 2002; 3:205-213
112. van Dokkum RP, Vettoretti S, Ochodnicky P, Kluppel CA, Henning RH, de Zeeuw D. Endothelial function of small renal arteries of healthy rat predicts the antiproteinuric response to ACE-inhibitor (ACE-i) therapy following 5/6 nephrectomy (Nx). [Abstract]. J Am Soc Nephrol 2005; 16:164A-
113. Patzak A, Lai E, Persson PB, Persson AE. Angiotensin II-nitric oxide interaction in glomerular arterioles. Clin Exp Pharmacol Physiol 2005; 32:410-414

Chapter 8

Summary and conclusions

Samenvatting

Zhrnutie

Summary

Chronic kidney disease (CKD) is currently viewed as a generalized vasculopathic state associated with multiple alterations in various vascular beds. Moreover, the kidney can be regarded as an organ, where vascular changes are immediately and sensitively detected by protein (albumin) excretion, already in early stages of vascular disease. The vascular changes have been often considered a consequence of renal disease. However, the fact that several forms of vascular dysfunction occur early in the pathophysiology of CKD has brought the possibility that vascular changes may actually preceed and maybe even determine the progression of renal end-organ damage. This thesis provides data on the role of intrarenal and systemic vascular reactivity in early renal pathology in animal models of experimental and spontaneous renal disease. **Chapter 2** introduces the concept, in which microalbuminuria, an early marker of CKD, is associated with both renal and systemic vascular dysfunction. Furthermore, we suggest that therapies targeted to improve early endothelial dysfunction may provide benefit in the primary prevention of CKD and related cardiovascular alterations.

Endothelial function predicts the susceptibility to renal end-organ damage

Early vascular changes observed in the individuals with microalbuminuria suggest the role of vascular alterations in the development of CKD. Development of renal damage varies among individuals despite similar levels of blood pressure and it has been proposed that genetic factors might be responsible for increased susceptibility to CKD[1,2]. Previously, it has been shown that variability in endothelial function among healthy rats of outbred Wistar rat strain predicts the susceptibility of the individual animals to renal injury after 5/6 nephrectomy (5/6Nx)[3]. In **chapter 3** we demonstrated the predictive property of intrarenal endothelial function in yet another model of experimental renal damage induced by combined unilateral nephrectomy (UnX) and myocardial infarction (MI). We found that individuals with pronounced endothelium-dependent vasodilation of small renal arteries developed lower proteinuria and glomerulosclerosis after UnX+MI. This correlation suggests the presence of an intrarenal vascular factor determining the susceptibility to renal damage induced by cardiac injury. Together with observations from the 5/6 nephrectomy model, it is suggested that hemodynamic alterations mediated by endothelial reactivity may play a role in the development of experimental end-organ damage irrespective of how this damage is inflicted. The present findings implicate that modulation of endothelial function might provide protection against the development of end-organ damage in patients after MI. In **chapter 4** we tested the concept of the predictive value of endothelial function in an experimental model with a different etiology of renal damage, *i.e.*, nephropathy induced by the nephrotoxic drug adriamycin. In contrast to previous models, the rats with prominent endothelial relaxation seem to be more prone to the development of renal damage after injection of adriamycin. These contrasting findings suggest that predictive value of renal

vascular function is critically dependent on the type of renal injury and etiology of progressive renal damage. However, we additionally found a positive significant correlation between the resulting proteinuria and the level of renal blood flow measured before the induction of injury. This might indicate that in this model, the endothelium-mediated hemodynamic status of the kidney determines the extent of renal damage through regulation of drug delivery to the kidney. Yet, in agreement with hemodynamic models of 5/6Nx and UnX+MI, individuals with pronounced nitric oxide (NO)-mediated vasodilation also seem to be protected against the toxic renal damage, regardless of the total endothelium-mediated vasodilation. Therefore, NO seems to play a protective role in various models of renal injury. Thus, assessment of NO-mediated relaxation may provide more consistent information on the susceptibility to renal end-organ damage than total endothelial response. This is in line with the protective role of NO against renal damage, described by several other authors[4,5]. Consequently, drugs aiming at increasing renal NO bioavailability might prove effective in prevention of renal end-organ damage, induced either by nephron number reduction, nephrotoxic drug or myocardial infarction.

Endothelial and myogenic dysfunction precede end-organ damage in spontaneous renal disease

Spontaneous animal models prone to the development of renal damage provide a valuable tool to define the genetic background for susceptibility to renal injury[2]. In line with the hypothesis of the predictive value of endothelial function for the development of renal damage, in **chapter 5** we describe endothelial dysfunction, preceding the development of proteinuria in the Fawn Hooded hypertensive (FHH) rats, a model of spontaneous hypertension-associated renal disease[6], when compared to FHL rats, a related strain resistant to hypertension and renal impairment. In this case, however, rather than NO, excessive production of cyclooxygenase-derived constrictive prostanoids was responsible for the impaired renal endothelial vasodilation prior to the development of renal injury. This suggests a specific mechanism of vascular alteration, possibly related to minor elevations of systemic blood pressure in this inbred strain. Furthermore, in the same arteries prior to the development of renal damage, we observed impaired myogenic reactivity, an additional vasoactive mechanism probably involved in the altered renal hemodynamics, hyperfiltration and renal injury, as previously described in other models of spontaneous renal disease[7-9]. Interestingly, both endothelial and VSMC-mediated changes were specific for the renal vasculature and were not observed in systemic resistance or large conduit arteries, arguing against an *a priori* relationship between renal and systemic vascular dysfunction in CKD. Yet, another endothelial mediator, EDHF, seems to be impaired in the early course of renal disease in small systemic arteries. This suggests that renal injury might be associated with early renal as well as systemic vascular changes.

Myogenic response and EDHF are impaired in systemic vasculature in experimental CKD

Whereas renal vascular changes may determine the severity of renal end-organ damage, impaired systemic reactivity might participate in CKD-related complications, such as hypertension. In **chapter 5** we found impaired systemic vascular reactivity in a model of spontaneous renal disease prior to the development of end-organ damage. In **chapter 6** we explored the systemic vascular reactivity in the experimental model of 5/6 nephrectomy. Two principal local vasomotor mechanisms in small systemic resistance arteries, VSMC-mediated myogenic constriction and endothelium-mediated EDHF-dependent relaxation were concomitantly altered in this setting, arguing against the hypothesis of their mutual inverse relationship[10]. In this case, myogenic reactivity represents rather a beneficial mechanism counteracting elevated peripheral resistance, whereas loss of EDHF might actively participate in the development of CKD-related cardiovascular problems. Importantly, renoprotective therapeutic intervention in the renin-angiotensin system reversed both systemic vascular changes providing a useful therapeutic strategy to prevent CKD-related cardiovascular alterations.

Conclusions and future perspectives

The association of endothelial dysfunction with cardiovascular disease has represented one of the major issues of cardiovascular medicine in recent 20 years[11-14]. Currently, it becomes more and more clear that vascular changes may be involved in earlier stages of chronic disease development and may even determine the susceptibility to organ damage. Based on our results, measurements of endothelial function may provide a useful prognostic tool to identify individuals prone to end-organ impairment. A similar concept has been proposed for endothelial function in cardiac and systemic arteries in predicting cardiovascular outcomes, however, the majority of studies included high risk individual[15-17] in contrast to our healthy experimental animals. In kidney, our experimental results will have to be confirmed by human studies. Furthermore, a specific test of nitric oxide-mediated vasodilation should be preferred to total endothelium-mediated vasodilation, as the other endothelial mediators may not properly reflect the sensitivity to renal injury. Although it cannot be concluded from our experiments whether the observed variability in endothelial function is genetically determined, several other authors suggest that the decrease expression of nitric oxide synthase (NOS) is associated with susceptibility to renal damage[18,19]. Moreover, clinical studies showed an association between polymorphisms of endothelial NOS and CKD in various patient populations[20,21]. Additionally, endothelial function might be a determinant of renoprotective therapeutic response as suggested in *chapter 7*. Dietary or pharmacologic strategies, such as plant-derived antioxidants or drugs interfering with the renin-angiotensin system, known to interfere with endothelial function, may prove beneficial in primary preventive strategies against CKD and related cardiovascular events. In addition to the endothelium, VSMC-mediated myogenic response may provide a novel therapeutic target for prevention of early hyperfiltration and

subsequent renal pathology. Further characterization of the heterogeneous mechanisms underlying arterial reactivity in various vascular beds will help to further define the intricate relation between renal and systemic vasculature and the role of vascular tone regulation in the development of chronic end-organ damage.

References

1. Bowden DW. Genetics of kidney disease. Kidney Int Suppl 2003; S8-12
2. Korstanje R, DiPetrillo K. Unraveling the genetics of chronic kidney disease using animal models. Am J Physiol Renal Physiol 2004; 287:F347-F352
3. Gschwend S, Buikema H, Navis G, Henning RH, de Zeeuw D, van Dokkum RP. Endothelial dilatory function predicts individual susceptibility to renal damage in the 5/6 nephrectomized rat. J Am Soc Nephrol 2002; 13:2909-2915
4. Baylis C, Mitruka B, Deng A. Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage. J Clin Invest 1992; 90:278-281
5. Kang DH, Nakagawa T, Feng L, Johnson RJ. Nitric oxide modulates vascular disease in the remnant kidney model. Am J Pathol 2002; 161:239-248
6. Provoost AP. Spontaneous glomerulosclerosis: insights from the fawn-hooded rat. Kidney Int Suppl 1994; 45:S2-S5
7. Hayashi K, Kanda T, Homma K, Tokuyama H, Okubo K, Takamatsu I, Tatematsu S, Kumagai H, Saruta T. Altered renal microvascular response in Zucker obese rats. Metabolism 2002; 51:1553-1561
8. Palmer BF. Impaired renal autoregulation: implications for the genesis of hypertension and hypertension-induced renal injury. Am J Med Sci 2001; 321:388-400
9. Takenaka T, Forster H, De Micheli A, Epstein M. Impaired myogenic responsiveness of renal microvessels in Dahl salt- sensitive rats. Circ Res 1992; 71:471-480
10. Gschwend S, Henning RH, de Zeeuw D, Buikema H. Coronary myogenic constriction antagonizes EDHF-mediated dilation: role of KCa channels. Hypertension 2003; 41:912-918
11. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 2004; 15:1983-1992
12. Goligorsky MS. Endothelial cell dysfunction: can't live with it, how to live without it. Am J Physiol Renal Physiol 2005; 288:F871-F880
13. Luscher TF. Imbalance of endothelium-derived relaxing and contracting factors. A new concept in hypertension? Am J Hypertens 1990; 3:317-330
14. Vanhoutte PM. Endothelium and control of vascular function. State of the Art lecture. Hypertension 1989; 13:658-667
15. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 106:653-658
16. London GM, Pannier B, Agharazii M, Guerin AP, Verbeke FH, Marchais SJ. Forearm reactive hyperemia and mortality in end-stage renal disease. Kidney Int 2004; 65:700-704

17. Neunteufl T, Heher S, Katzenschlager R, Wolfl G, Kostner K, Maurer G, Weidinger F. Late prognostic value of flow-mediated dilation in the brachial artery of patients with chest pain. Am J Cardiol 2000; 86:207-210
18. Erdely A, Wagner L, Muller V, Szabo A, Baylis C. Protection of wistar furth rats from chronic renal disease is associated with maintained renal nitric oxide synthase. J Am Soc Nephrol 2003; 14:2526-2533
19. Erdely A, Freshour G, Baylis C. Resistance to renal damage by chronic nitric oxide synthase inhibition in the Wistar-Furth rat. Am J Physiol Regul Integr Comp Physiol 2006; 290:R66-R72
20. Nagase S, Suzuki H, Wang Y, Kikuchi S, Hirayama A, Ueda A, Takada K, Oteki T, Obara M, Aoyagi K, Koyama A. Association of ecNOS gene polymorphisms with end stage renal diseases. Mol Cell Biochem 2003; 244:113-118
21. Noiri E, Satoh H, Taguchi J, Brodsky SV, Nakao A, Ogawa Y, Nishijima S, Yokomizo T, Tokunaga K, Fujita T. Association of eNOS Glu298Asp polymorphism with end-stage renal disease. Hypertension 2002; 40:535-540

Samenvatting

Patienten met een chronische nierziekte overlijden in meer gevallen aan hart- of vaataandoeningen dan aan de nieraandoening zelf. Een mogelijke verklaring voor dit fenomeen is de aanwezigheid van gegeneraliseerde vaatschade als gevolg van het falen van de nieren. De relatie tussen vaatschade en nierziekte lijkt echter tweeledig. Recent onderzoek suggereert dat de vaatdysfunctie mogelijk het beloop van de progressie van nierschade mede kan bepalen. Deze hypothese is gebaseerd op de bevinding dat patiënten met reeds aanwezige vaatfunctiestoornissen een gering verhoogde hoeveelheid eiwit in de urine (microalbuminurie) hadden, maar nog geen nierfunctieverlies vertoonden. Microalbuminurie, het eerste teken van een nieraandoening, ontstaat als het gevolg van lekkage in de bloedvatwand van de nier en wordt beschouwd als de weerspiegeling van gegeneraliseerde vaatschade, ook in het systemische vaatbed. Dit zou betekenen dat de nier een orgaan is waarin de mate van vaatdysfunctie relatief eenvoudig en in een vroeg stadium geconstateerd zou kunnen worden. Onderliggende mechanismen van vaatstoornissen bij nieraandoeningen en hun relatie met eind-orgaan schade zijn echter niet voldoende onderzocht.

Vaatdysfunctie wordt gekenmerkt door veranderingen in de lokale regulatie van de vaattonus. De tonus van de kleine weerstands arteriën wordt bepaald door de werking van de gladde spiercel en het endotheel, de cellaag die de binnenbekleding van de vaten vormt. Het endotheel speelt een belangrijke rol bij de regulatie van de vaattonus, onder andere door het afgeven van vaatverwijdende mediatoren. De functie van het endotheel wordt getest door middel van acetylcholine-gemedieerde dilatatie. Vermindering van vaatdilatatie wordt endotheeldysfunctie genoemd. Het weerspiegelt niet alleen het gebrek aan vaatverwijdend vermogen van het endotheel, maar ook de achteruitgang in andere fysiologische eigenschappen ervan. Endotheelschade wordt tegenwoordig gezien als vroege stap in het ontwikkelen van atherosclerose en is daarmee een risico-indicator voor hart- en vaatziekten. Endotheel afhankelijke dilatatie wordt gemedieerd door het afgeven van verschillende biologisch actieve mediatoren, met name stikstof oxide (NO), prostaglandines en een zogenoemde endothelium-derived hyperpolarizing factor (EDHF). De bijdrage van deze stoffen aan de endotheelafhankelijke relaxatie verschilt tussen de specifieke vaatbedden, zoals b.v. nier-, hart- en perifere vaten. Daardoor variëren ook de mechanismen van de endotheeldysfunctie in de verschillende vaatbedden en hun rol in het ontwikkelen van eindorgaanschade. Het is daarom van groot belang de relaties tussen de specifieke organen en vaatdisfunctie te begrijpen in ziektes waarbij sprake is van gegeneraliseerde vaatschade, zoals een chronische nierziekte. Naast het endotheel draagt de gladde spiercel van de vaatwand bij aan de regulatie van de vaattonus. De gladde spiercel reageert op verhogingen in de bloeddruk met contractie, ook wel myogene respons genoemd. Dit mechanisme in de nierarteriën beschermt de nier tegen het schadelijke effect van een verhoogde bloeddruk. Verder draagt de myogene response in het systemisch vaatbed bij aan

de regulatie van de perifere weerstand en bepaald hierdoor mede de systemisch bloeddruk. Over de rol van gladde spiercel afwijkingen in hart- en vaatziektes is nog verassend weinig bekend.
Dit proefschrift bestudeert in detail de rol van de vaatfunctie, zowel het endotheel als de myogene respons, in de ontwikkeling van eindorgaanschade in spontane en experimentele (geïnduceerde) diermodellen van nierschade. **Hoofdstuk 2** introduceert het klinisch concept van de relatie tussen microalbuminurie en systemische endotheeldysfunctie. Dit hoofdstuk geeft een samenvatting van zowel experimentele als klinische data, die laten zien dat endotheeldysfunctie vooraf gaat aan en voorspellend is voor het ontstaan van microalbuminurie, chronische nierziekte en geassocieerde hart- en vaatziekten. Mogelijk kan behandeling gericht op het verbeteren van endotheeldysfunctie worden toegepast als primaire preventie ervan.

Endotheelfunctie voorspelt de ontwikkeling van chronische, experimentele nierschade
Individuen vertonen aanzienlijke variatie in hun gevoeligheid voor het ontwikkelen van chronische nierziekte. Deze variatie blijkt gedeeltelijk onafhankelijk te zijn van de klassieke (systemische) risicofactoren zoals hoge bloeddruk, diabetes of verstoringen van vetspiegels in het bloed. Dit suggereert dat onbekende, intrarenale factoren medebepalend zijn voor het ontwikkelen van nierschade. Aangezien endotheeldysfunctie al vroeg in de pathogenese van eindorgaanschade een rol lijkt te spelen was de initiële hypothese dat het functioneren van de niervaten de gevoeligheid voor nierschade bepaald. In **hoofdstuk 3** wordt aangetoond dat de endotheelfunctie gemeten als acetylcholine-gemedieerde dilatatie van kleine nierarteriën in gezonde Wistar ratten, voorspellend is voor de mate van nierschade na het aanbrengen van een nefrectomie gecombineerd met een hartinfarct. Deze data illustreren dat de variatie in niervaatfunctie, die onder normale omstandigheden aanwezig is, de mate van de nierschade die als gevolg van de verminderde functie van de andere organen ontstaat, mede kan bepalen. Daarnaast zou de modulatie van de intrarenale endotheelfunctie beschermend kunnen werken tegen de achteruitgang in nierfunctie in patiënten met een hartinfarct. Het concept van de voorspellende waarde van endotheelfunctie voor het ontwikkelen van nierschade wordt in **hoofdstuk 4** getest in een ander experimenteel model van nierschade, namelijk adriamycine-geïnduceerde nefropatie. Na het meten van de vaatfunctie werd de nierschade aangebracht door het eenmalig inspuiten van het nefrotoxische middel adriamycine. Het resultaat was dat de ratten met een goede basale, NO-afhankelijk relaxatie beschermd waren tegen de adriamycine-geïnduceerde nierschade. Aan de andere kant, dieren met een uitgesproken goede acetylcholine-geinduceërd totale endotheelrelaxatie (die gemedieërd is door het gecombineerde effect van NO, prostaglandines en EDHF) ontwikkelden meer nierschade. Deze data demonstreren dat de voorspellende waarde van de endotheelfunctie sterk afhankelijk is van de bijdrage van de onderliggende mediatoren. De beschermende rol van NO komt overeen met data in de literatuur die een beschermend effect van NO bij nierziekten laten zien. De omgekeerde relatie tussen de totale endotheelrelaxatie en de

nierschade bij adriamycine nefrose zou verklaard kunnen worden door de rol van het endotheel in de regulatie van de nierdoorbloeding tijdens het inspuiten van adriamycine. In het tweede deel van deze studie werd aangetoond dat ratten met een uitgesproken goede nierdoorbloeding, gemeten net voor het inspuiten van adriamycine, geneigd zijn meer nierschade te ontwikkelen. Dit suggereert dat de aanvoer van het nefrotoxische middel in de nier medebepalend is voor de ontwikkeling cq. inductie van nierschade in dit diermodel. Deze bevindingen kunnen tot een nieuwe risicoinschatting en behandelingsstrategie bij patienten met nierziekten leiden. Het meten van specifieke NO-gemedieërde relaxatie zou een betere prognostisch parameter kunnen zijn dan de totale, acetylcholine-gemedieerde dilatatie. Daarnaast zouden geneesmiddelen gericht op het verbeteren van de NO activiteit het onstaan en/of de progressie van chronisch nierziekten kunnen voorkomen.

Endotheel- en myogene dysfunctie gaan vooraf aan eindorgaan schade in een spontaan nierziekte model

Naast de modellen waarin de nierschade wordt aangebracht door een experimentele ingreep, bestaan er ook rattenstammen met een specifieke genetische achtergrond waardoor de nierschade zich spontaan ontwikkelt. Gezien de relatie met de humane situatie is het belangrijk te bestuderen of de voorspellende waarde gezien in de experimentele modellen bevestigd kan worden in de zich spontaan ontwikkelende nierziekten. In **hoofdstuk 5** wordt de endotheel- en myogene dysfunctie van de kleine nierarteriën bestudeerd die vooraf gaat aan het ontwikkelen van nierschade in de zgn. Fawn-Hooded Hypertensieve (FHH) rat. Deze rat ontwikkelt reeds op jonge leeftijd spontaan hypertensie-geassocieërde nierschade, in tegenstelling tot de FHL stam, waarin de nierfunctie nagenoeg onaangetast blijft. Inderdaad werd een verminderde endotheelafhankelijke dilatatie van de nierarteriën gevonden in jonge FHH ratten, maar niet in jonge FHL ratten. Dit als gevolg van overmatige productie van vaatvernauwende prostaglandines en vrije zuurstof radicalen. Tegelijkertijd bleek ook de myogene respons van de niervaten in de FHH stam sterk verminderd. Daardoor is de beschermende werking van myogene contractie tegen de verhoogde bloeddruk (hypertensie) aangetast. Dit zou kunnen leiden tot intraglomerulaire hypertensie en het ontwikkelen van nierschade in de FHH rat. Zowel afwijkingen in het endotheel en de gladde spiercel bleken selectief veranderd in de niervaten, maar niet aanwezig in de systemische vasculatuur. Deze waarneming pleit tegen een directe relatie in vaatdysfunctie tussen niervaten en systemische vaten in de zich ontwikkelende nierziekte. In kleine mesenteriale arterieën, die de perifere weerstandsvaten vertegenwoordigen, was de EDHF-afhankelijke endotheel relaxatie echter selectief verminderd, waarschijnlijk door het ontwikkelen van de hypertensie in de FHH stam. In het algemeen lijkt de endotheliale- en myogene functie echter selectief aangetast in de kleine nierarterieën, ook in dit diermodel van spontaan nierziekte voorafgaand aan het ontstaan van eind orgaanschade.

Endotheel- en myogene dysfunctie in perifere weerstandsvaten als gevolg van experimenteel nierfunctieverlies is te herstellen met een ACE-remmer

Terwijl de voorspellende waarde van de vaatfunctie voor eindorgaanschade een relatief nieuw concept representeert, is het schadelijke effect van de nierfunctieverlies op de perifere vaatfunctie al langer bekend. De verhoogde bloeddruk, systemische hypertensie, vertegenwoordigd de meest voorkomende geassocieerde aandoening bij chronische nierziekten. Het is echter niet bekend of de lokale vaatdysfunctie betrokken is bij het ontstaan van hypertensie bij nierpatiënten. In **hoofdstuk 6** wordt de vaatfunctie van mesenteriale vaten bestudeerd in een diermodel van chronisch nierziekte geïnduceerd door het verwijderen van 5/6 deel van het nierweefsel (nefrectomie). In de kleine weerstandsvaten, zoals mesenteriale arteriën, zijn gladde spiercel-gemedieerde myogene constrictie en endotheel-gemedieerde EDHF-afhankelijke relaxatie de belangrijkste lokale mechanismen betrokken bij de regulatie van de vaattonus. In onze studie was experimenteel nierfalen geassocieerd met gelijktijdige reductie van myogene constrictie en EDHF-afhankelijke dilatatie. Verminderde endotheel-afhankelijke relaxatie zou kunnen bijdragen aan de nierfalen-gerelateerde verhoging in de systemische bloeddruk, terwijl de reductie in myogene constrictie lijkt te dienen als compensatie mechanisme tegen de toegenomene perifere weerstand. In het tweede gedeelte van de studie werd aangetoond dat de behandeling met de ACE-remmer lisinopril beide vaatveranderingen hersteld. De interventie in het renine-angiotensine systeem is derhalve een mogelijk effectieve therapeutische strategie voor het moduleren van de systemische vaatafwijkingen en het verminderen van het toegenomen cardiovasculaire risico.

Conclusies en toekomstperspectief

De rol van endotheeldysfunctie in het ontwikkelen van cardiovasculaire eindorgaanschade behoorde in de afgelopen decennia tot een van de meest onderzocht onderwerpen in de vaatbiologie. Nog steeds lijken de mechanismen en de relatie tussen deze twee fenomenen niet definitief vastgesteld. Het onderzoek gepresenteerd in dit proefschrift ondersteunt de hypothese dat vaatziekte vooraf gaat aan het ontwikkelen van eindorgaanschade en de ontwikkeling ervan mede bepaald. Vaatdysfunctie zou derhalve vroeg en selectief in de nier gedetecteerd kunnen worden. Deze bevindingen bieden enkele klinisch relevante perpectieven.

Ten eerste zou het meten van de endotheelfuctie toegepast kunnen worden als een parameter voor individuele risicoinschatting in vroege stadia van nierziekten of zelfs in gezonde individuen. Om dit concept tot een klinische toepassing te brengen moeten de data geverifieerd worden in humane studies. Daarnaast blijft de discussie met betrekking tot de meest universeel toepasbare methode voor het meten van endotheelfunctie in de mens nog steeds open. Uit de dierexperimenten beschreven in dit proefschrift blijkt ook dat specifiek NO-afhankelijke endotheel dilatatie een nog beter voorspellende parameter zou kunnen zijn dan de totale, acetylcholine-geïnduceerde vasodilatatie. NO blijkt dus de meest belangrijke beschermende mediator tegen het ontstaan van nierschade te zijn.

Ten tweede representeert het verbeteren van endotheelfunctie en dan met name de NO activiteit in de nier een behandelingstrategie, die het beloop en de uitkomst van aandoeningen in nierpatienten gunstig zou kunnen beïnvloeden. Misschien zou hierdoor het ontwikkelen van nierziekten zelfs wel kunnen worden voorkomen. Enkele van de meest klinisch succesvolle nierbeschermende interventies, met name gericht op het renine-angiotensine systeem, interfereereert ook met de endotheelfunctie. Met betrekking tot de renoprotectieve effecten van ACE-remmers blijkt uit **hoofdstuk 7** dat de endotheel afhankelijke dilatatie de sterkte van het renoprotectieve effect van ACE-remmers voorspelt. Daarmee lijkt de relatie tussen de endotheelfunctie en nierbeschermend effect van ACE remmers aangetoond.

Tenslotte speelt naast het endotheel ook de reactiviteit van de gladde spiercel een belangrijke rol in het ontstaan van nierschade. De myogene respons zou medebepalend kunnen zijn voor de gevoeligheid voor het ontwikkelen van nierschade. Nieuwe farmacologische strategieën gericht op het verbeteren van myogene functie moeten nog ontwikkeld worden. Gebaseerd op het betere begrip van de heterogene mechanismen die een rol spelen bij de vaatdysfunctie in diverse vaatbedden kunnen zo betere behandelingen van eindorgaanschade in chronische nierziekte en gerelateerde hart- en vaataandoeningen gerealiseerd worden.

Zhrnutie a závery

Chronická choroba obličiek (chronic kidney disease- CKD) predstavuje v sučasnosti významný medicínsky a ekonomický problém. Najvýraznejšou manifestáciou CKD je chronické obličkové zlyhanie pri výraznom znížení funkcie obličiek, ktoré vyžaduje liečbu hemodialýzou alebo transplantáciou. Avšak mnohonásobne väčší počet chorých má známky obličkového poškodenia (zvýšené vylučovanie proteínov- mikroalbuminúria, proteinúria, prípadne poškodenie potvrdené histologickým vyšeterením bioptickej vzorky) bez výraznejšieho zníženia filtračnej schopnosti obličiek. Títo pacienti už v skorých štádiách CKD majú enormne zvýšené riziko kardiovaskulárnych ochorení a existuje u nich oveľa väčšia pravdepodobnosť srdcovo-cievnej príhody ako samotnej progresie obličkového ochorenia do štádia obličkového zlyhania. Výskyt CKD je teda významným kardiovaskulárnym rizikovým faktorom a je spojený s prítomnosťou generalizovanej vaskulopatie. Donedávna sa biomedicínsky výskum väčšinou orientoval na odhalenie faktorov, vďaka ktorým zníženie obličkovej funkcie vedie k poškodeniu periférnych ciev. V posledných rokoch sa však ukazuje, že vzťah medzi vaskulopatiou a nefropatiou je obojstranný a cievna dysfunkcia môže predchádzať vzniku chronickej choroby obličiek a dokonca i hrať významnú úlohu pri rozvoji orgánového poškodenia.

Táto práca popisuje niekoľko experimentálnych štúdií skúmajúcich vzťah medzi vaskulárnou dysfunkciou a rozvojom chronického obličkového poškodenia a s ním spojených kardiovaskulárnych problémov.

Kapitolu 2 tvorí súhrn prác publikovaných vo vedeckej literatúre, ktoré sumarizujú vzťah medzi mikroalbuminúriou a endotelovou dysfunkciou. Mikroalbuminúria, čiže zvýšené vylučovanie albumínu v moči (30-300 mg za 24 hodín) je veľmi skorým indikátorom obličkového poškodenia. Výskyt mikroalbuminúrie je relatívne častý v populácii hypertonikov (20-30%), diabetikov (20-40%) a dokonca i v bežnej populácií (5-8%). Je veľmi zaujímavé, že prítomnosť mikroalbuminúrie je spojená s výskytom kardiovaskulárnych ťažkostí v prierezových štúdiách. V longitudinálnych klinických štúdiách hodnota albuminúrie dokonca predpovedá výskyt kardiovaskulárnej morbidity a mortality. Tento významný vzťah sa vysvetľuje hypotézou, že zvýšená permeabilita pre albumín v obličke je znakom generalizovaného poškodenia cievneho endotelu vo viacerých cievnych riečištiach. Niektoré práce naznačujú, že endotelová dysfunkcia je prítomná dokonca skôr ako sa objaví mikroalbuminúria. Oblička by tak mohla reprezentovať orgán, v ktorého funkcii sa odráža stav funkcie cievneho endotelu. Samotná endotelová dysfunkcia môže byť teda určujúcim faktorom pri rozvoji obličkového a srdcovocievneho poškodenia. Použitie farmakologických postupov zameraných na zlepšenie funkcie endotelu, ako napríklad liečiv interferujúcich s renín-angiotenzínovým systémom, predstavuje potenciálnu terapeutickú stratégiu pre primárnu prevenciu mikroalbuminúrie, a teda aj predchádzanie CKD a s ňou súvisiaceho kardiovaskulárneho rizika.

Individuálna variabilita endotelovej funkcie predpovedá rozsah experimentálneho obličkového poškodenia

Vyššie uvedené klinické výsledky naznačujú, že endotelová funkcia môže hrať významnú úlohu pri vzniku a rozvoji CKD. U individuálnych pacientov, či experimentálnych zvierat sa sklon k obličkovému poškodeniu líši čiastočne nezávisle od prítomnosti a závažnosti systémových rizikových faktorov ako sú hypertenzia či diabetes mellitus. Experimentálne i klinické štúdie naznačujú, že tento sklon je spoluurčovaný geneticky a určití jednotlivci môžu byť predisponovaní ku vzniku CKD. Avšak gény či faktory zodpovedné za túto predispozíciu dosiaľ nie sú známe. V **kapitole 3** uvádzame výsledky experimentálnej štúdie, v ktorej sme testovali hypotézu, či individuálna variabilita endotelovej reaktivity môže byť faktorom určujúcim predispozíciu k obličkovému poškodeniu. Endotelová funkcia bola meraná acetylcholínovým vazodilatačným testom na malých preglomerulárnych artériiách izolovaných z obličiek získaných pri unilaterálnej nefrektómii zdravých potkanov kmeňa Wistar. Následne bol u týchto potkanov indukovaný experimentálny infarkt myokardu podviazaním ľavej koronárnej artérie. Ten v spojení s unilaterálnou nefrektómiou v priebehu 16 týždňov vyvolal štrukturálne a funkčné poškodenie zostávajúcej obličky. Úroveň endotelovej reaktivity meraná u zdravých potkanov predpovedala závažnosť obličkového poškodenia, ktoré vzniklo po indukcii kombinovanej nefrektómie a infarktu myokardu. Potkany s výraznou od endotelu závislou dilatačnou schopnosťou obličkových artérií vykazovali nižšiu úroveň proteinúrie a glomerulárnej sklerózy, markerov obličkového orgánového poškodenia. Tieto dáta naznačujú existenciu intrarenálneho faktora, ktorý ovplyvňuje endotelovú funkciu obličkových ciev a spoluurčuje predispozíciu individuálnych zvierat k rozvoju obličkového poškodenia indukovaného dysfunkciou vzdialeného orgánu. Modulácia endotelovej funkcie u pacientov s infarktom myokardu môže poskytnúť ochranu pred obličkovým poškodením a z neho vyplývajúcim zvýšením kardiovaskulárneho rizika.

V **kapitole 4** sme horeuvedený koncept endotelovej funkcie ako prediktívneho parametra pre rozvoj obličkového poškodenia testovali u ďalšieho experimentálneho modelu s odlišnou etiológiou chronickej choroby obličiek- nefropatie indukovanej jednorázovou injekciou nefrotoxickej látky adriamycínu. Okrem toho sme sa pokúsili tento koncept rozpracovať sledovaním mechanizmov, ktoré potenciálne môžu hrať úlohu pri regulácii relaxácie závislej od endotelu. Aplikácia acetylcholínu vyvolá uvoľnenie troch hlavných skupín mediátorov- oxidu dusného (NO), zmesi vazokonstrikčných a vazodilatačných prostaglandínov a dosiaľ bližšie necharakterizovaného faktora, označovaného EDHF (hyperpolarizačný faktor odvodený od endotelu). Aj u zvierat s adriamycínovou nefropatiou predpovedala ich od endotelu závislá relaxačná schopnosť závažnosť obličkového poškodenia. Zvieratá s výraznou špecifickou reaktivitou závislou od NO boli chránené pred rozvojom adriamycínom indukovanej nefropatie. Tento výsledok je v súlade s mnohými publikáciami preukazujúcimi protektívne vlastnosti NO pri orgánovom poškodení. Na rozdiel od NO, u potkanov s markantnou celkovou od endotelu závislou relaxačnou schopnosťou (určenou spoločným efektom NO, prostaglandínov a EDHF) sa rozvinula

závažnejšia nefropatia. Znamená to, že prediktívna hodnota endotelovej funkcie je silne závislá od sledovaného vazoaktívneho mediátora a experimentálneho modelu. Opačný vzťah medzi celkovou endotelovou relaxáciou a renálnym výsledkom u modelu adriamycínovej nefropatie je možné vysvetliť úlohou endotelu v regulácií krvného prietoku obličiek počas podania adriamycínovej injekcie. V druhej časti našej štúdie sme ukázali, že u zvierat s vysokým krvným prietokom obličiek, meraným tesne pred podaním nefrotoxickej injekcie, sa rozvinulo závažnejšie obličkové poškodenie. Tento výsledok podporuje vysvetlenie, že akútny prítok nefrotoxickej látky do obličky počas injekcie je tak isto spoluurčujúcim faktorom renálneho poškodenia u tohto experimentálneho modelu. Uvedené dáta môžu viesť k novým diagnostickým a terapeutickým možnostiam u pacientov s CKD. Meranie špecifickej od NO závislej endotelovej relaxácie u zdravých osôb môže byť lepším diagnostickým indikátorom predispozície jednotlivých pacientov k rozvoju CKD ako meranie celkovej od endotelu závislej reaktivity. Liečivá, ktoré zasahujú do tvorby a biologickej aktivity NO sa môžu uplatniť v primárnej prevencii obličkového poškodenia.

Endotelová a myogénna cievna dysfunkcia predchádza rozvoju orgánového poškodenia u spontánne sa rozvíjajuceho obličkového ochorenia

Okrem experimentálnych modelov, u ktorých sa obličkové poškodenie vyvolá experimentálnym zásahom existujú aj „inbredné“ kmene potkanov so špecifickým genetickým pozadím, u ktorých sa spontánne v mladom veku rozvinie obličkové zlyhanie. Zaujímavou otázkou zostáva, či vaskulárna dysfunkcia hrá úlohu aj pri rozvoji tohto spontánneho genetického orgánového poškodenia. Preto sme v **kapitole 5** tejto práce hodnotili reaktivitu malých obličkových artérií u kmeňa hnedoškvrnného hypertenzného potkana (Fawn-hooded hypertensive rat- FHH), u ktorého sa v mladom veku spontánne rozvinie hypertenzné obličkové poškodenie. To je charakterizované proteinúriou a glomeruloskleróozou, ktoré môžu vyústiť až do obličkového zlyhávania. Na rozdiel od FHH potkana, geneticky príbuzný hnedoškvrnný potkan s nízkym tlakom (Fawn-hooded low pressure rat- FHL) je rezistentný voči tomuto obličkovému orgánovému poškodeniu. V súlade s našou hypotézou sme pozorovali endotelovú dysfunkciu malých preglomerulárnych artérií izolovaných z obličiek 7 týždňového FHH potkana, teda ešte pred vznikom štrukturálneho a funkčného obličkového poškodenia, v porovnaní s rovnako starými FHL potkanmi. Nadmerná produkcia kontraktilných prostaglandínov a voľných kyslíkových radikálov boli zodpovedné za pozorované endotelové poškodenie u FHH potkanov. Okrem endotelu závisí tonus malých artérií aj od reaktivity cievneho hladkého svalu. Hladký sval reaguje na zvýšenie intravaskulárneho tlaku kontrakciou a následným zvýšením napätia cievnej steny. Táto odpoveď sa nazýva myogénna a je významným fyziologickým mechanizmom ochraňujúcim orgány pred nadmerným zvýšením prietoku krvi pri zvýšení systémového tlaku. V obličke chráni myogénny mechanizmus glomerulus pred vznikom intraglomerulárnej hypertenzie, ktorá sa považuje za spúšťací faktor pri vzniku chronického obličkového poškodenia. Preto sme v našej štúdii pri charakterizácii

vaskulárneho poškodenia predchádzajúceho rozvoju CKD porovnávali aj myogénnu reaktivitu obličkových artérií FHH a FHL potkana. FHH potkany vykazovali výrazne nižšiu myogénnu reaktivitu ako FHL, čo môže hrať spolu s endotelovou dysfunkciou významnú úlohu pri vzniku spontánnej CKD u tohto kmeňa. Je zaujímavé, že endotelové aj hladkosvalové mechanizmy boli selektívne poškodené v obličkových cievach, a nie v periférnych rezistentných artériách izolovaných z mezetéria, či vo veľkých elastických cievach (aorta) FHL a FHH potkanov. Tieto dáta teda nepotvrdzujú priamy vzťah medzi vaskulárnym poškodením v obličkách a na periférii, ktorý sa používa na vysvetlenie zvýšeného kardiovaskulárneho rizika u CKD. Preto si presné mechanizmy generalizovanej vaskulárnej dysfunkcie pri spontánnej CKD vyžadujú ďalšie podrobné štúdium. Selektívne poškodenie endotelových a myogénnych mechanizmov v obličkových cievach predchádza rozvoju spontánneho orgánového poškodenia pri CKD.

Endotelová a myogénna dysfunkcia periférnych rezistentných ciev pri experimentálnom obličkovom zlyhaní je reverzibilná po použití ACE inhibítorov

V predchádzajúcich kapitolách sme sa sústredili na výskum cievnych zmien predchádzajúcich vzniku a rozvoju orgánového poškodenia pri CKD. Avšak ani mechanizmy vaskulárnych zmien, ktoré vznikajú ako dôsledok CKD nie sú dosiaľ uspokojivo vysvetlené. V **kapitole 6** tejto práce sme preto sledovali zmeny od endotelu a hladkého svalu závislých mechanizmov periférnych rezistentných artérií pri vzniku arteriálnej hypertenzie v dôsledku experimentálnej CKD. Skúmali sme dva najvýznamnejšie lokálne mechanizmy regulujúce tonus periférnych artérií: od EDHF závislú endotelovú relaxáciu a hladkým svalom sprostredkovanú myogénnu kontraktilitu malých mezenteriálnych artérií izolovaných z potkanov, u ktorých bolo obličkové zlyhávanie a následná hypertenzia vyvolané odobratím jednej obličky a podviazaním dvoch vetiev obličkovej artérie na kontralaterálnej strane. To malo za následok infarkt 2/3 zostávajúceho funkčného parenchýmu u druhej obličky (5/6 nefrektómia). 15 týždňov po indukcii obličkového zlyhania sme v periférnych rezistentných cievach pozorovali súčasné poškodenie od EDHF závislej endotelovej relaxácie a myogénnej kontraktility. Zatiaľčo znížená relaxačná schopnosť periférnych ciev môže prispievať k rozvoju hypertenzie po indukcii CKD, zmenšená kontrakčná reaktivita je pravdepodobne kompenzačným mechanizmom namiereným proti zvýšenej periférnej rezistencii. V druhej časti našej štúdie sme ukázali, že obe tieto vaskulárne zmeny sú modifikovateľné chronickou terapiou ACE inhibítorom lisinoprilom. Terapeutický zásah do renín-angiotenzínovej kaskády teda predstavuje účinnú stratégiu liečby systémového vaskulárneho poškodenia a predchádzania kardiovaskulárnych komplikácií spojených s rozvojom chronického obličkového zlyhania.

Závery a perspektívy

Úloha endotelovej dysfunkcie pri rozvoji srdcovocievnych ochorení patrí k najviac skúmaným otázkam kardiovaskulárnej medicíny v posledných desaťročiach. Avšak mechanizmy zodpovedné za tento proces a jeho presné miesto v patofyziológii orgánového

poškodenia nie sú dosiaľ dostatočne charakterizované. Experimentálne výsledky prezentované v tejto práci podporujú hypotézu, že vaskulárna dysfunkcia predchádza a spoluurčuje rozsah orgánového poškodenia pri chronickom ochorení obličiek. Oblička je zároveň orgánom, ktorý umožňuje skorú detekciu cievneho poškodenia a kardiovaskulárneho rizika (napr. meraním mikroalbuminúrie). Tieto závery ponúkajú niekoľko klinicky relevantných perspektív.

V prvom rade, meranie od endotelu závislej reaktivity sa môže stať potenciálnym diagnostickým parametrom umožňujúcim zhodnotenie renálneho, prípadne kardiovaskulárneho rizika v skorom štádiu ochorenia, možno dokonca i u zdravých osôb. Tento koncept si však vyžaduje potvrdenie platnosti v klinickej praxi za pomoci humánnych štúdií. Zároveň ostáva otvorenou otázka, aký endotelový test je najlepším indikátorom orgánového rizika. Dáta v predkladanej práci naznačujú, že meranie špecifickej od NO závislej endotelovej reaktivity môže poskytnúť lepšiu prediktívnu informáciu ako celková acetylcholínom indukovaná endotelová relaxácia. NO teda pravdepodobne reprezentuje najvýznamnejší protektívny mechanizmus proti rozvoju obličkového poškodenia.

Po druhé, zlepšenie endotelovej dysfunkcie môže tvoriť perspektívnu terapeutickú stratégiu pri chronickom poškodení obličiek. Potenciálne by dokonca takýto prístup mohol zabrániť vzniku obličkového ochorenia, a teda umožniť primárnu prevenciu CKD. Je zaujímavé, že niektoré v súčasnosti najúspešnejšie renoprotektívne intervencie, napr. zásah do renín-angiotenzínovej kaskády, majú preukázaný aj protektívny účinok na endotel. **Kapitola 7** tejto práce poskytuje súhrn, ktorý sumarizuje účinnosť endotelprotektívnych stratégií pri liečbe CKD. Zároveň v súvislosti s renoprotektívnym účinkom ACE inhibítorov uvádza originálne experimentálne dáta dokazujúce, že od endotelu závislá reaktivita malých obličkových ciev predpovedá variabilitu v renoprotektívnej účinnosti ACE inhibítorov a tak priamo ukazuje vzťah medzi endotel modulujúcim a renoprotektívnym účinkom týchto liečiv.

Na koniec táto práca ukazuje, že okrem endotelu, aj reaktivita buniek hladkej svalovny ciev môže hrať významnú úlohu pri vzniku obličkového poškodenia. Úroveň myogénnej reaktivity preglomerulárnych artérií môže spoluurčovať predispozíciu ku CKD. Nové farmakologické stratégie namierené na zlepšenie renálnej myogénnej dysfunkcie sú ešte v štádiu základného výskumu. Lepšie pochopenie heterogénnych mechanizmov zodpovedných za vaskulárnu dysfunkciu v renálnych, periférnych, či koronárnych riečištiach môže viesť k zlepšeniu terapie a primárnej prevencie orgánového poškodenia pri CKD a s ňou súvisiacich srdcovocievnych problémov.

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