



PEETER KÖÖBI

Arterial Tone in Chronic Renal Insufficiency



ACADEMIC DISSERTATION

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for public discussion in the main auditorium of Building B,
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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, which are referred to in the text by Roman numerals I-V:

- I** Kööbi P, Kalliovalkama J, Jolma P, Rysä J, Ruskoaho H, Vuolteenaho O, Kähönen M, Tikkanen I, Fan M, Ylitalo P and Pörsti I (2003): AT₁ receptor blockade improves vasorelaxation in experimental renal failure. *Hypertension*, 41(6):1364-1371.
- II** Kööbi P, Jolma P, Kalliovalkama J, Tikkanen I, Fan M, Kähönen M, Moilanen E and Pörsti I (2004): Effect of angiotensin II type 1 receptor blockade on conduit artery tone in subtotaly nephrectomized rats. *Nephron Physiology*, 96(3):91-98.
- III** Jolma P, Kööbi P, Kalliovalkama J, Saha H, Fan M, Jokihaara J, Moilanen E, Tikkanen I and Pörsti I (2003): Treatment of secondary hyperparathyroidism by high calcium diet is associated with enhanced resistance artery relaxation in experimental renal failure. *Nephrology Dialysis Transplantation*, 18(12):2560-2569.
- IV** Kööbi P, Vehmas TI, Jolma P, Kalliovalkama J, Fan M, Niemelä O, Saha H, Kähönen M, Ylitalo P, Rysä J, Ruskoaho H and Pörsti I: Advanced experimental renal failure and resistance artery tone: Effect of calcium-phosphorus balance. Submitted.
- V** Pörsti I, Fan M, Kööbi P, Jolma P, Kalliovalkama J, Vehmas TI, Hélin H, Holthöfer H, Mervaala E, Nyman T and Tikkanen I (2004): High calcium diet down-regulates kidney angiotensin-converting enzyme in experimental renal failure. *Kidney International*, 66(6):2155-2166.

ABBREVIATIONS

1,25D	1,25-dihydroxyvitamin D ₃
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AT ₁	Angiotensin II type 1
AT ₂	Angiotensin II type 2
BK _{Ca}	Large-conductance Ca ²⁺ -activated K ⁺ channels
BNP	B-type natriuretic peptide
BP	Blood pressure
[Ca ²⁺] _i	Intracellular free Ca ²⁺ concentration
cAMP	Cyclic adenosine 3',5'-monophosphate
cGMP	Cyclic guanosine 3',5'-monophosphate
COX	Cyclooxygenase
CRI	Chronic renal insufficiency
CTGF	Connective tissue growth factor
EDHF	Endothelium-derived hyperpolarizing factor
EET	Epoxyeicosatrienoic acid
ET	Endothelin
ET _A	Endothelin-1 type A
ET _B	Endothelin-1 type B
GFR	Glomerular filtration rate
G protein	Guanosine 5'-triphosphate-binding protein
IK _{Ca}	Intermediate-conductance Ca ²⁺ -activated K ⁺ channels
IP ₃	Inositol 1,4,5-trisphosphate
K/DOQI	Kidney Disease Outcomes Quality Initiative
K _{ATP}	ATP-sensitive K ⁺ channels
K _{Ca}	Ca ²⁺ -activated K ⁺ channels
K _{IR}	Inward rectifier K ⁺ channels
K _V	Voltage-dependent K ⁺ channels
L-NAME	N ^G -nitro-L-arginine methyl ester
MLCK	Myosin light chain kinase
NA	Noradrenaline

NO	Nitric oxide
NOS	Nitric oxide synthase
NT-proANP	N-terminal pro-atrial natriuretic peptide
NTX	5/6 nephrectomized
PG	Prostaglandin
PGI ₂	Prostacyclin
PRA	Plasma renin activity
PSS	Physiological salt solution
PTH	Parathyroid hormone
RAS	Renin-angiotensin system
SH	Secondary hyperparathyroidism
SK _{Ca}	Small-conductance Ca ²⁺ -activated K ⁺ channels
SR	Sarcoplasmic reticulum
SNP	Sodium nitroprusside
TGF-β	Transforming growth factor β
TXA ₂	Thromboxane A ₂
VSMC	Vascular smooth muscle cell

ABSTRACT

Chronic renal insufficiency is associated with high morbidity and mortality due to cardiovascular complications. Accumulating evidence shows that the physiological functions of vascular endothelium are disturbed already at the early stages of uremic disease, and impaired regulation of both renovascular and systemic arterial tone plays an important role in the progression of chronic kidney disease itself, as well as in the manifestation of cardiovascular complications that are currently the most frequent cause of death in patients with end-stage renal disease. However, the present knowledge about the underlying pathophysiological mechanisms is scarce, and the current lines in the treatment of renal patients are probably not optimal for the cardiovascular system. Therefore, the objective of this study was to examine the functional and morphological alterations of isolated resistance and conductance arteries in moderate and advanced experimental chronic renal insufficiency.

Angiotensin II type 1 receptor antagonists, a class of drugs that has been recently reported to slow down the progression of chronic kidney disease, are widely used to control arterial blood pressure in renal patients. However, the influence of these drugs on the regulation of vascular tone in uremia is unknown, and therefore the current study evaluated the effects of long-term losartan treatment on the uremic changes of small and large arteries in experimental renal insufficiency.

High calcium intake has been reported to reduce blood pressure and improve vasorelaxation in experimental hypertension, whereas in renal patients such diet is used to manage hyperphosphatemia and secondary hyperparathyroidism. Therefore, the effects of diet-induced changes in calcium-phosphate balance on resistance artery tone were studied in moderate and advanced chronic renal insufficiency. In addition, the influences of high calcium intake on the uremic changes of local renin-angiotensin system in the kidney, the degree of ectopic calcifications, and renal histology were studied in both moderate and advanced chronic renal insufficiency.

Male Sprague-Dawley rats were subjected to 5/6 nephrectomy or sham-operation at the age of 8 weeks. Four weeks later treatments with either losartan or high calcium diet were started and continued for 8 weeks (studies with moderate renal insufficiency). In order to mimic advanced chronic renal insufficiency, the rats were followed for 15 weeks after the subtotal nephrectomy, and thereafter the high calcium and high phosphate diets were applied for 12 weeks. The levels of arterial blood pressure of conscious animals were measured using the tail cuff method. The cardiac synthesis of natriuretic peptides was determined to verify the volume status in the study groups. Renal density of angiotensin II receptors, and renal and aortic angiotensin converting enzyme content, were measured using autoradiography. The *in vitro* responses of mesenteric resistance and conductance arteries were performed using myographs to measure the changes in arterial wall tension induced by pharmacological vasoconstrictors and vasodilators added to the organ bath containing physiological salt solution.

In moderate renal insufficiency, endothelium-mediated vasorelaxation to acetylcholine in both resistance and conductance arteries was impaired via K^+ channels, whereas nitric oxide-mediated component of arterial relaxation was preserved. The small arteries of uremic rats also featured eutrophic inward remodelling, as suggested by the increased wall-to-lumen ratio and unchanged cross-sectional area when compared with the arteries of sham-operated controls. Losartan treatment normalized both functional and morphological changes of the arteries in moderate renal insufficiency, without any effect on blood pressure, volume overload or kidney functional parameters. Furthermore, high calcium diet suppressed the elevated levels of parathyroid hormone and phosphate, the effect of which was associated with decreased renal tissue ACE content, reduced albuminuria, inhibited extraskelatal calcification, decreased glomerulosclerosis and tubulo-interstitial fibrosis, reduced volume overload, and improved K^+ channel-mediated relaxation of resistance arteries. Moreover, in advanced renal insufficiency, the resistance arteries featured impaired relaxation via both nitric oxide- and K^+ channel-mediated pathways. High calcium intake reduced the increased arterial blood pressure, retarded the progression of renal insufficiency, improved survival, and normalized the functional changes of resistance vessels. In contrast, the arteries of uremic rats on high phosphate diet showed virtually no response to acetylcholine.

Collectively, the results of the present study suggested that beyond the clinically evidenced benefits on arterial blood pressure and progression of renal scarring, AT_1 receptor antagonists confer distinct advantages on arterial tone and morphology in chronic kidney disease. Furthermore, the current study showed that calcium-phosphate balance is a significant modulator of resistance artery tone in chronic renal insufficiency. Finally, these experiments for the first time suggested a link between calcium metabolism and ACE expression in the kidney, which may play a role in the progression of renal damage.

INTRODUCTION

Patients suffering from chronic renal insufficiency (CRI), a progressive disease that finally leads to the end-stage renal failure, died earlier mainly due to the accumulation of uremic toxins and associated metabolic disturbances. However, in favour of the highly sophisticated dialysis techniques and largely distributed kidney transplantation, the mortality of renal patients is no more due to renal failure. These treatments are able to sustain a patient's life for years or even for decades, leading thus to the situation that the long-term complications of chronic renal disease have become actual. In a number of clinical studies, an abnormally high rate of mortality due to cardiovascular complications has been reported in this population. Nowadays, cardiovascular death accounts nearly 50% of all deaths in end-stage renal disease, the proportion of which is much higher than in general population (USRDS 1997). Virtually, the prognosis of the dialysis patients is determined by the morbidity due to accelerated atherosclerosis (Foley et al. 1994). Furthermore, occlusive accidents involving coronary, peripheral, or cerebrovascular arteries, account for at least half of cardiovascular deaths in end-stage renal disease (USRDS 1997). However, the cause of the enhanced incidence of atherosclerosis in patients with end-stage renal disease is still not well understood. Various risk factors are involved, including secondary hypertension, endothelial dysfunction, high rate of diabetes mellitus, increased plasma lipid concentrations, disturbed calcium-phosphorus metabolism, and inflammation.

The vascular endothelium plays a central role in the local regulation of arterial tone via the determination of contractile state of the underlying vascular smooth muscle, i.e. by releasing relaxing and contracting factors. Another important physiological role of the endothelium is the prevention of blood cell aggregation and thrombus formation, while the impairment of this function may contribute to the early development of atherosclerotic lesions (van Guldener et al. 1997). Endothelial dysfunction is a sensitive indicator of cardiovascular disease, predicts its prognosis, and is closely associated with the development of atherosclerosis (Galle et al. 2003). There is accumulating evidence that CRI is associated with endothelial dysfunction, which plays an important role in the progression and pathogenesis of chronic renal disease, and contributes to the development of secondary hypertension (Kang et al. 2002). However, studies concerning the detailed mechanisms of endothelial dysfunction in CRI are scarce.

The renin-angiotensin system (RAS) regulates systemic and renal vasomotor activity, maintains optimal salt balance and volume homeostasis, participating thereby in the normal regulation of arterial pressure (Brown et al. 1995, He et al. 1998). Tissue remodelling and growth in the kidney, and also in the cardiovascular system, is largely controlled by the RAS. However, the pathologic consequences in CRI can result from overactivity of this cascade. An activated RAS contributes to both systemic and glomerular capillary hypertension, which can induce hemodynamic injury to the vascular endothelium and glomerulus (Brewster and Perazella 2004). In addition, direct profibrotic and proinflammatory actions of angiotensin II (Ang II) and aldosterone may also

promote renal damage. The majority of the untoward effects associated with Ang II appear to be mediated through its binding to the angiotensin II type 1 (AT₁) receptor. AT₁ receptor antagonists and angiotensin converting enzyme (ACE) inhibitors are widely used as antihypertensive agents in CRI patients. Furthermore, there is growing evidence that these drugs can retard the progression of CRI, reduce glomerulosclerosis and interstitial fibrosis, and decrease proteinuria independently of their effect on arterial blood pressure (BP). Whether these drugs can provide benefits on disturbed regulation of arterial tone in CRI, is unknown.

Disturbed calcium-phosphorus balance and secondary hyperparathyroidism (SH) are ubiquitous complications of CRI, and may also contribute to the functional and morphological changes of the arteries (Rostand and Drüeke 1999, Tyralla and Amann 2003). SH can also promote the remodelling of the vascular wall in CRI (Amann et al. 2003a), and elevated levels of parathyroid hormone (PTH) and phosphate are known to be associated with increased risk of cardiovascular complications in renal patients (Marco et al. 2003). Furthermore, endothelial dysfunction in patients with primary hyperparathyroidism has been reported to normalize after parathyroidectomy (Nilsson et al. 1999). Calcium salts are used as phosphate binders in order to treat SH and hyperphosphatemia in CRI. Previously, in experimental hypertension high calcium diet has been shown to consistently reduce BP (Hatton and McCarron 1994, Mäkynen et al. 1996). High calcium intake can also decrease plasma renin activity (Wuorela et al. 1992), reduce Ang II binding sites in apical brush-border membrane of renal cortex (Levi and Henrich 1991), increase sodium excretion in spontaneously hypertensive rats (Pörsti et al. 1991), and enhance vasorelaxation in experimental hypertension (Jolma et al. 2000). However, the effects of elevated calcium intake and subsequent changes in calcium-phosphate balance on the regulation of arterial tone and morphology, and possible associated changes on disturbed systemic and tissue-level RAS are unknown.

The present study was designed to evaluate the reactivity and morphology of resistance and conductance arteries at different stages of experimental CRI. Furthermore, the effects of long-term AT₁ receptor antagonism and high calcium intake on arteries in moderate CRI were examined. In advanced experimental CRI, high calcium and high phosphate intake-induced metabolic alterations and their vascular effects were elucidated. Finally, the influence of calcium balance on tissue components of RAS in the kidneys was determined in moderate and advanced CRI.

REVIEW OF THE LITERATURE

1 Chronic renal insufficiency

1.1 Pathophysiology and progression of impaired renal function

CRI is featured by a persistently reduced glomerular filtration rate (GFR). Independently of the initial disease, CRI is characterized by progressive loss of functioning nephrons that leads to terminal renal failure, whereas the rate of this progress can vary substantially (Yu 2003). The most common reasons that initiate this progressive disease are diabetes, other diagnoses (including hypertension), glomerulonephritis, cystic kidney disease, amyloidosis and pyelonephritis (Finnish Registry for Kidney Diseases, report 2003, <http://www.musili.fi/smtr/english/Report2003.pdf>). The progression of renal damage with various aetiologies consists many of pathological mechanisms, including changes in renal hemodynamics (Hostetter et al. 1981b), progressive proteinuria (Williams and Coles 1994) and participation of RAS.

Early diabetic nephropathy is known to be associated with an elevated GFR (Nelson et al. 1999, Vora et al. 1992), while hypertensive patients have fewer but larger glomeruli, suggesting compensatory hyperfiltration (Keller et al. 2003). In rats subjected to subtotal nephrectomy, compensatory hyperfiltration of the spared nephrons contributes to maintain overall GFR. However, this adaptation also leads to glomerular hypertension, proteinuria, and progressive CRI (Hostetter et al. 1981a). It has been suggested that there is a critical renal mass below which hyperfiltration becomes detrimental, since only patients with greater than 50% loss of renal mass have been shown to have a long-term increased risk for proteinuria and renal insufficiency (Novick et al. 1991). After the loss of a critical number of nephrons, the remaining nephrons undergo compensatory functional and structural adaptations. During this process, the surviving nephrons lose the capacity to autoregulate glomerular flow and pressure and become vulnerable to the effects of systemic hypertension, which is readily accompanied by glomerular hypertension, hyperfiltration and hypertrophy (Hostetter et al. 1981a, Hostetter et al. 1981b). The increased glomerular capillary pressure causes the stretching of the capillary tuft and thus also stretches the adjacent mesangial cells, which induces mesangial cell proliferation and glomerulosclerosis at least partly by overexpression of cytokines such as platelet-derived growth factor (Kato et al. 1999) and monocyte chemoattractant protein 1 (Suda et al. 2001). The first stage of glomerulosclerosis is the damage of endothelial cells, which may be induced by immune, hemodynamic or metabolic insults. Consequently the damaged endothelium loses its anticoagulant, anti-inflammatory and antiproliferative properties and starts to express cell adhesion molecules (Johnson 1994). The above changes lead to the attraction of platelets and inflammatory cells such as neutrophils and monocytes to the glomerular capillaries. Infiltrating monocytes interact with mesangial cells and stimulate their proliferation (Johnson 1994).

Disturbed regulation of apoptosis in renal tissue plays a significant role in the progression of CRI. It has been suggested that the normal extracellular matrix, which down-regulates apoptosis, becomes replaced by an abnormal one and loses its antiapoptotic properties (Sugiyama et al. 1998). This results in a decreased population of the normal glomerular and tubular epithelial cells. Furthermore, the glomerular epithelial cells lose their ability to replicate in response to injury, leading to their stretching along denuded areas of glomerular basement membrane, which would favour proteinuria and increase traffic of inflammatory, mitogenic and fibrogenic mediators, such as transforming growth factor β (TGF- β). This growth factor is strongly regulated by the levels of Ang II, and plays an important role in renal fibrogenesis through the stimulation of extracellular matrix synthesis (Johnson 1994).

In addition to glomerulosclerosis, tubulo-interstitial fibrosis contributes to the pathophysiology of impaired renal function. The degree of tubulo-interstitial changes correlates with renal function even better than glomerulosclerosis (Schainuck et al. 1970). Similarly with glomerulosclerosis, tubulo-interstitial fibrosis also involves inflammation, interstitial fibroblast proliferation and excessive deposition of interstitial extracellular matrix, leading to fibrosis. Injured renal tubular cells play a major role in the pathogenesis of tubulo-interstitial fibrosis (Suzuki et al. 2001). In pathological situations these cells express cell adhesion molecules, release inflammatory mediators and autacoids as well as chemokines, growth factors and pro-sclerotic cytokines, such as connective tissue growth factor (CTGF). The expression of CTGF is also mediated by Ang II via TGF- β (Okada et al. 2004a, Okada et al. 2004b). Furthermore, it has been suggested that local intrarenal RAS, being an important determinant of tissue injury, inflammation and progression of renal disease, plays an important role in the development of tubulo-interstitial fibrosis. Therefore, the kidney-protective effect of pharmacological inhibition of RAS (Aman et al. 2001b, Gilbert et al. 1999, Ruiz-Ortega et al. 2002) could be explained at least in part via the diminished Ang II-induced synthesis of CTGF and other pro-sclerotic cytokines.

In pathophysiology of CRI, much attention has been paid to the importance of persistent proteinuria, an independent risk factor of renal disease progression (Klahr et al. 1994, Williams and Coles 1994). Proteinuria is a result of glomerular capillary hypertension and damage to the permeability barrier in the glomerulus. Proteins leaking across the glomerulus cause protein-overload on the proximal tubular cells, leading to increased activation of the intrarenal ACE (Largo et al. 1999), endothelin (ET)-1, and other cytokines favouring fibrosis, apoptosis, and infiltration of monocytes, thus further amplifying the process (Figure 1). Moreover, it has been suggested that proteinuria directly increases the translocation of growth factors such as TGF- β and hepatocyte growth factor from plasma into tubular fluid, where these factors interact with receptors located at the apical membrane of tubular cells and thus promote interstitial fibrosis (Wang and Hirschberg 2000, Wang et al. 2000b). Consistent with its important role in pathophysiology, proteinuria is a strong predictor of clinical progression of CRI. The rate of GFR decline is proportional to the severity of proteinuria (GISEN-group 1997).

Table 1. Stages of chronic kidney disease (K/DOQI-guidelines 2002).

Stage	Description	GFR (mL/min/1.73 m ²)
1	Kidney damage with normal or increased GFR	≥90
2	Kidney damage with mild decrease in GFR	60-89
3	Moderate decrease in GFR	30-59
4	Severe decrease in GFR	15-29
5	Kidney failure	<15 (or dialysis)

Chronic kidney disease is defined as either kidney damage or GFR <60 mL/min/1.73 m² for ≥3 months. Kidney damage is defined as pathological abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies (K/DOQI-guidelines 2002).

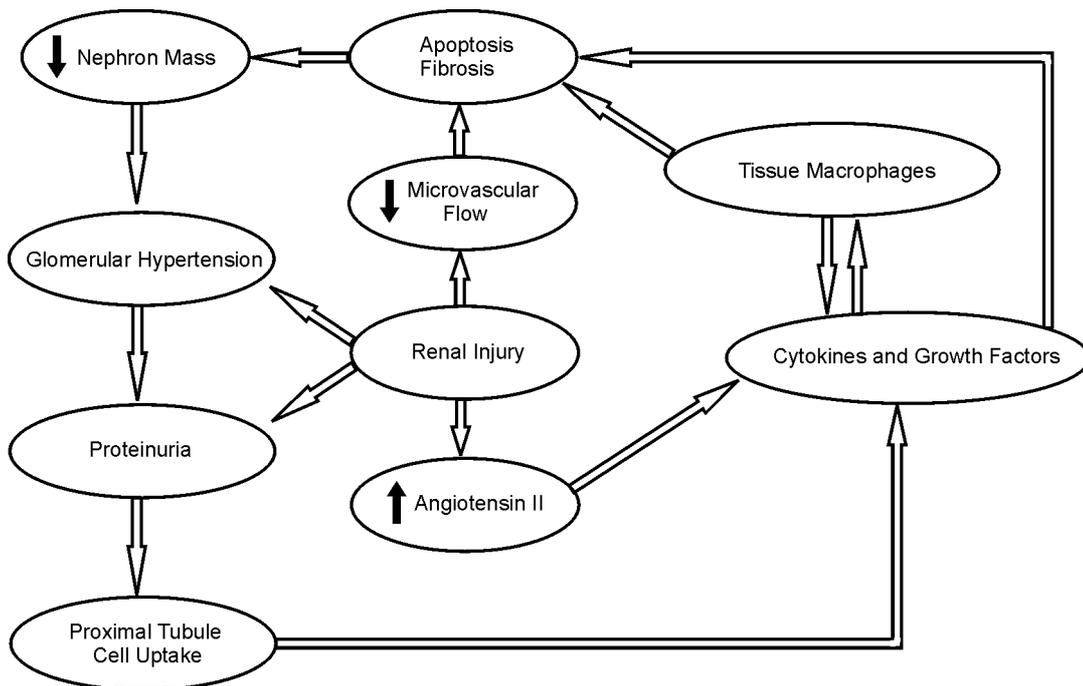


Figure 1. The figure shows the central mechanisms that contribute to the pathophysiology and progression of chronic renal insufficiency (modified from Yu 2003).

1.2 Vasoactive peptides - volume overload

Progressive CRI is characterized by an adaptive increase in the sodium excretion rate per nephron as the total glomerular filtration rate declines. This increase is caused, at least in part, by the effect of atrial natriuretic peptide (ANP) and other natriuretic peptides, the release of which is augmented in the setting of volume expansion in CRI (Charra and Chazot 2003). During the progressive decline of GFR towards end-stage renal disease, total renal sodium excretion eventually decreases, inducing thus extracellular volume expansion, hypertension, and oedema. Initially, the fractional sodium excretion increases in proportion to degree of renal dysfunction, in part by an effect of ANP (Charra and Chazot 2003). However, exogenous administration of natriuretic peptides in clinical chronic and acute renal disease does not consistently increase renal sodium excretion (Shemin and Dworkin 1997). Chronic volume overload in uremic patients increases filling pressures and venous return, thus imposing an increased workload on the left ventricle, which ultimately results in left ventricular dilatation and left ventricular hypertrophy as a result of increased load. Furthermore, in patients with CRI, the permanent fluid overload also contributes to the development of chronic heart failure, which is observed in 30% of individuals with CRI stage 5 (Harnett et al. 1995).

The synthesis of natriuretic peptides, ANP and B-type natriuretic peptide (BNP) is up-regulated in pressure and volume overload (de Bold et al. 1996, Magga et al. 1994, Nakao et al. 1992, Ruskoaho 1992). The major determinant of ANP and BNP secretion is myocyte stretch (de Bold et al. 1996, Kinnunen et al. 1993, Mäntymaa et al. 1993). Therefore, ANP and BNP are used as markers of volume overload. Plasma ANP levels strongly correlate with BP, and the secondary hypertension in CRI is largely attributed to positive sodium balance and volume overload (Vasavada and Agarwal 2003).

In hemodialysis patients plasma ANP is highly elevated and decreases during the dialysis session when fluid is removed. However, hemodialysis treatment does not completely correct the disturbed fluid and electrolyte metabolism, and plasma concentration of ANP does not decrease to levels observed in healthy controls (Franz et al. 2001). Furthermore, other factors besides uremia and chronic volume overload, such as cardiac dysfunction or hypertension may contribute to the elevated plasma concentrations of ANP. Thus, in patients with advanced CRI and other above-mentioned disorders, the results of ANP measurements should be interpreted cautiously.

Dialysis may have different effects on the elevated levels of natriuretic peptides: the concentrations of ANP have shown to be lowered more efficiently than the levels of BNP (Kohse et al. 1993), which, at least in part, can be explained by more effective elimination of ANP across the dialysers (Franz et al. 2001). BNP, that is mainly synthesized in heart ventricles and released in response to increased wall tension, is a highly useful tool in the evaluation of volume balance in CRI (Nakatani et al. 2002). In patients with advanced renal disease, BNP levels are increased, and this increase well correlates with pulmonary artery pressure, pulmonary artery wedge pressure, left ventricular end-diastolic pressure and left ventricular ejection fraction (Osajima et al. 2001). BNP is

thus a suitable marker of volume overload in patients with severe CRI. In some reports, the increase of BNP has been shown only in dialysis patients, suggesting differences of hemodynamic stress in predialysis renal insufficiency and during the dialysis treatment (Akiba et al. 1995). In hemodialysis patients, high plasma BNP concentrations have been shown to be associated also with left ventricular hypertrophy, cardiovascular diseases and diabetes mellitus (Naganuma et al. 2002). Thus, in addition to evaluating volume status in patients with CRI, plasma BNP concentration may be a useful variable for assessing the risk of cardiac death in patients with end stage renal disease.

Taken together, ANP and BNP involve both advantages and limitations in the evaluation of volume status in patients with impaired renal function, and therefore the simultaneous measurements of these markers may be more efficient than both separately, especially in dialysis patients (Akiba et al. 1995).

1.3 Calcium metabolism and secondary hyperparathyroidism

Disturbances of the metabolism of calcium and phosphate are characteristic of CRI (Drüeke 2001). When renal function is impaired, reduced phosphate excretion leads to the elevation of plasma phosphate, and this together with reduced 1,25-dihydroxyvitamin D₃ (1,25D) synthesis result in the development of SH (Llach 1995, Slatopolsky et al. 2001). In the parathyroid glands, increased synthesis of PTH is characterized by cellular hyperplasia (Mihai and Farndon 2000, Silver et al. 2002, Slatopolsky et al. 2001). Accumulating evidence suggests that high phosphate levels and SH play important roles in the pathophysiology of the cardiovascular complications of uremia (Amann et al. 2003a, Rostand and Drüeke 1999; Figure 2).

Already in mild CRI, decreased synthesis of 1,25D contributes to the characteristic lowering of plasma calcium levels (Figure 2). In the parathyroid cells, reduced synthesis of vitamin D receptors and Ca²⁺-sensing receptors have been found, the findings of which contribute to the development of hypocalcemia (Korkor 1987, Mihai and Farndon 2000). Via the Ca²⁺-sensing receptors, the extracellular Ca²⁺ concentration plays an important role in the regulation of plasma PTH levels (Drüeke 2001, Silver et al. 2002). Increased circulating levels of PTH are also detected already in patients with mild to moderate CRI. The synthesis and release of PTH are increased in response to low serum 1,25D, low ionised calcium, and high phosphate (Slatopolsky et al. 1999). The present view is that high plasma phosphate concentration, independent of the levels of Ca²⁺ and 1,25D, is an important stimulator of PTH secretion (Lopez-Hilker et al. 1990). In advanced CRI, phosphate retention markedly boosts the development of SH (Llach and Velasquez Forero 2001).

In parathyroid glands, 1,25D controls PTH gene transcription via the vitamin D receptor (Slatopolsky et al. 1999). In both clinical (Korkor 1987) and experimental uremia (Merke et al. 1987), the density of vitamin D receptors in the parathyroid glands is clearly reduced. During the progressive decline of renal function, the number of vitamin D receptors in the parathyroid glands is further decreased, which results in marked 1,25D resistance in the glands. The present knowledge

suggests that 1,25D is an important regulator of parathyroid cell growth, and that low levels of 1,25D contribute to the proliferation of parathyroid cells (Slatopolsky et al. 1999). Correspondingly, the administration of 1,25D can suppress parathyroid hyperplasia in uremia (Szabo et al. 1989).

PTH secretion in the parathyroid glands can rapidly react to hypocalcemia via a specific Ca^{2+} -sensing mechanism (calcium receptor) (Brown et al. 1993, Silver et al. 2002). Via this mechanism, extracellular calcium level can modify both PTH gene transcription and parathyroid cell proliferation (Okazaki et al. 1991, Silver et al. 2002). In uremia there may be an abnormality of the parathyroid glands, which results in disturbed calcium-regulated PTH secretion due to the insensitivity of the suppressive effect of calcium on PTH secretion (Brown et al. 1982). Additional factors that contribute to the development of SH and hypocalcaemia in CRI, are a frequently observed decrease in dietary calcium intake together with reduced intestinal calcium absorption due to low 1,25D levels (Drüeke 2001). Therefore, oral calcium salts are used early in CRI to treat the calcium deficiency and prevent the development of SH (Drüeke 2001, Fournier et al. 1996). Importantly, oral calcium salts bind phosphate in the intestine and thus reduce hyperphosphatemia, which is an important principle in the management of SH (Drüeke 2001).

High-dose oral calcium supplements may result in excessive intestinal absorption of calcium, increased circulating levels of phosphorus and calcium, and increased plasma $\text{Ca} \times \text{P}$ product. This may predispose to the deposition of calcium salts in soft tissues, i.e. extraskelatal calcification (Drüeke 2001). The use of high doses of calcium-based phosphate binders has been associated with cardiovascular calcification, in particular if mineral metabolism is not well controlled. In haemodialysis patients, excess calcium intake may adversely influence the balance of skeletal and extraskelatal calcification (Chertow et al. 2004). An association between the prescribed dose of oral calcium carbonate and arterial wall stiffness has also been published (Guérin et al. 2000). The risk of inducing extraskelatal calcifications may be further enhanced by parallel administration of vitamin D (Drüeke 2001).

In experimental CRI, however, the increased intake of calcium carbonate has actually reduced kidney calcification, the probable mechanism being the lowering of plasma phosphate (Cozzolino et al. 2002). Furthermore, most clinical studies concerning this complication have reported that high concurrent circulating levels of phosphate and calcium, or high $\text{Ca} \times \text{P}_i$ product, are risk factors for calcification, but have not given precise information about dietary calcium intake in these patients (Kimura et al. 1999, Raggi et al. 2002). Therefore, calcium-based phosphate binders may be associated with extensive ectopic calcification if hyperphosphatemia is not adequately controlled (Chertow et al. 2004). Nevertheless, not all of the clinical reports have shown that increased oral calcium load would be a risk factor for soft tissue calcification in dialysis patients (Moe et al. 2003). A recent analysis emphasized the importance of examining combinations of parameters of calcium-phosphate metabolism, and not single variables alone, when assessing cardiovascular risk in hemodialysis patients (Stevens et al. 2004). Without doubt, the control of the hyperphosphatemia and the increased $\text{Ca} \times \text{P}$ product are important measures in the treatment of SH in CRI (Locatelli et

al. 2002).

In the clinical setting, poor control of phosphorus is usually associated with higher intake of calcium salts, whereby it is difficult to differentiate the influences of hyperphosphatemia and high calcium intake in these patients. Hyperphosphatemia is without doubt toxic to the cardiovascular system (Stevens et al. 2004). Thus, high PTH and phosphate levels predispose to ectopic calcifications in CRI (Slatopolsky et al. 2001), rather than putative hypercalcemia induced by high calcium ingestion. Furthermore, excess of PTH is also associated with elevated BP, and it may directly influence the function of arterial smooth muscle (Rostand and Drüeke 1999). In experimental CRI, high dietary phosphate content and hyperphosphatemia have been reported to contribute to cardiac fibrosis and arterial wall thickening (Amann et al. 2003a). The elevated levels of PTH and phosphate have also been found to be markers of increased cardiovascular mortality in hemodialysis patients (Marco et al. 2003). Altogether, the disturbed calcium-phosphate balance appears to significantly contribute to the cardiovascular pathology in CRI (Slatopolsky et al. 2001).

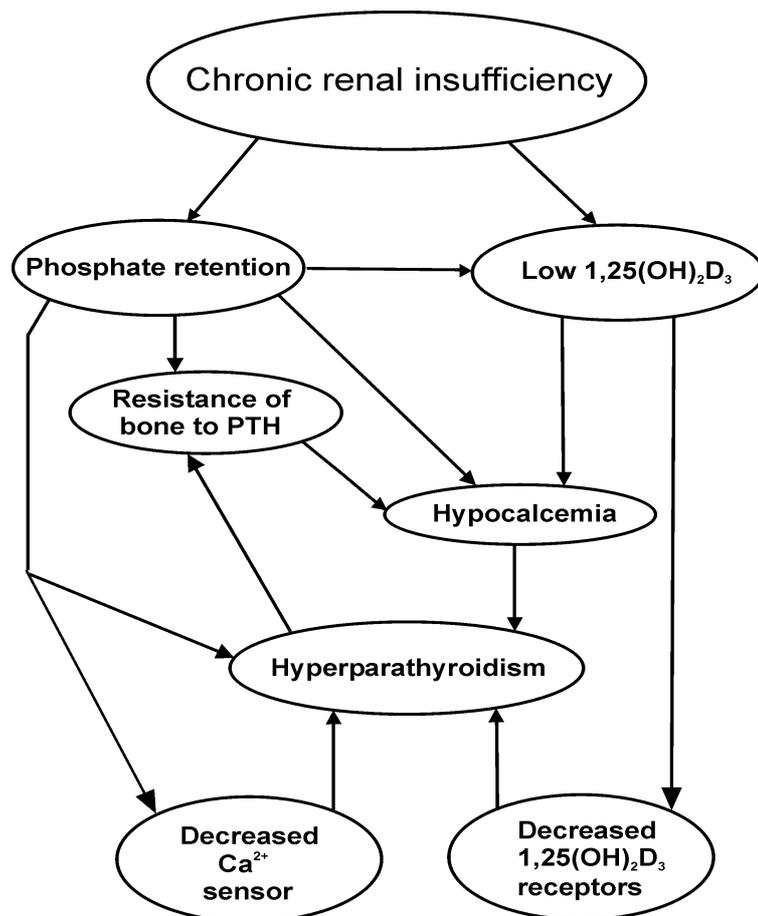


Figure 2. Schematic representation of the factors that participate in the pathogenesis of secondary hyperparathyroidism in chronic renal insufficiency (modified from Slatopolsky et al. 1999).

1.4 Cardiovascular complications

1.4.1 Secondary hypertension

Hypertension is a definite complication that emerges in most of patients with CRI. As the renal disease progresses, the prevalence of arterial hypertension increases, and prior to starting the dialysis, about 85–90% of the CRI patients suffer from elevated BP (Rodicio and Alcazar 2001). In these patients, hypertension is a strong predictor of left ventricular hypertrophy, cardiac dilatation, cardiac failure, and ischemic heart disease (Martinez-Maldonado 2001). Clinical studies with CRI patients suggest that independent risk factors in the development of hypertension are diabetes, advanced age, proteinuria and hypertriglyceridemia (Ridao et al. 2001). Numerous studies have established that chronic parenchymal renal disease or end-stage renal disease are the most common causes of secondary hypertension, accounting for about 5% of all patients with elevated arterial BP (Sinclair et al. 1987). For instance, in chronic glomerular disease, up to 80% of patients develop secondary hypertension, depending on the underlying diagnosis (Blythe 1985). Similarly, the tubulointerstitial disease is also associated with high incidence of hypertension (Hostetter et al. 1988). Moreover, secondary hypertension appears virtually in all patients with polycystic kidney disease by the time end stage renal disease is present (Chapman and Gabow 1997).

On the other hand, hypertension itself can lead to renal insufficiency, while secondary hypertension, as a complication of renal disease with other aetiology, contributes to the progression of kidney failure. Moreover, it is difficult to determine whether glomerular or interstitial damage is the result of hypertension or other causes, since the mechanism of injury of hypertension and other renal diseases may be similar (Sarnak and Levey 2000). As a consequence of arterial hypertension, CRI may appear by at least two different mechanisms: renal damage can develop via glomerular ischemia induced by the damage of preglomerular arteries and arterioles with progressive luminal narrowing and a subsequent fall in glomerular blood flow, while hypertensive renal damage can also be induced by direct transmission of the elevated systemic pressure to the glomeruli (Baldwin and Neugarten 1987).

Secondary hypertension may develop in CRI via several mechanisms, one of which is volume dependent increase of arterial BP leading to an increase in cardiac output. Definitely, it has been demonstrated that salt retention plays a fundamental role in this mechanism by increasing interchangeable sodium (Davies et al. 1973) and vascular wall sodium (Simon 1990), and by expanding the extracellular volume.

In several types of CRI, the presence of ischemic renal tissue leads to the activation of circulating RAS, and before the drugs capable of inhibiting the RAS became available, 10–15% of the dialysis patients required bilateral nephrectomy to control their high BP. For instance, in polycystic kidney disease and in renovascular hypertension, the elevation of arterial BP is mediated predominantly via increased arterial tone induced by circulating Ang II (Martinez-Maldonado

1991). On the other hand, high Ang II levels induce the excess production of aldosterone, which may contribute to the development of hypertension via sodium retention and subsequent increase in volume load (Lifton et al. 1992). Furthermore, aldosterone itself can contribute to the progression of cardiovascular and renal interstitial damage (Sun et al. 2000). However, in most types of CRI, the systemic RAS is not overactive and even lowered plasma renin activity (PRA) has been reported (Vasavada and Agarwal 2003). Despite this, increased activity of local RAS in kidney tissue may contribute to the progression of renal injury and to the disturbed regulation of arterial BP by paracrine/autocrine pathways (Martinez-Maldonado 2001).

Recent experimental and clinical data suggest that the increase in BP in CRI is to a significant part due to sympathetic overactivity, which is triggered by afferent signals emanating from the kidney, and results in resetting of sympathetic tone by stimulation of hypothalamic centres (Orth et al. 2001). Interestingly, increased traffic in the peripheral sympathetic nerves measured by microneurography is not detectable in such dialysis patients, who had been subjected to bilateral nephrectomy (Converse et al. 1992). This suggests that the diseased kidney may be a source of the sympathetic activation. The activation of systemic RAS has been suggested to play significant role in sympathetic overactivity in hypertensive patients with CRI, and high circulating Ang II levels can stimulate central sympathetic outflow by a direct effect on the vasomotor centre in the brain stem (Matsukawa et al. 1991). Furthermore, sympathetic hyperactivity can be reduced by ACE inhibition and AT₁ receptor antagonism in CRI patients (Klein et al. 2003). These findings support the view that Ang II is involved in the pathogenesis of the sympathetic hyperactivity in CRI. Beyond its increasing effect on BP, sympathetic overactivity has been suggested to accelerate the progression of renal insufficiency in experimental animals with CRI (Amann et al. 1996). Furthermore, a low dose of sympatholytic drug moxonidine that failed to lower BP, reduced microalbuminuria in patients with type I diabetes (Strojek et al. 2001). These findings, together with the fact that sympathetic overactivity increases the risk of cardiac arrhythmia in CRI (Orth et al. 2001), suggest that blockade of sympathetic nervous system may confer benefits to patients with CRI.

ET plays a significant role in the development of arterial hypertension in CRI (Shichiri et al. 1990). ET-1 contributes to the regulation of renal hemodynamics and glomerular filtration rate, and the modulation of sodium and water excretion. In the rat remnant kidney model of CRI, ET-1 production is increased in blood vessels and renal tissues (Lariviere et al. 1997). This change correlates with the rise in BP, the development of cardiovascular hypertrophy, and the degree of renal insufficiency and injury. In addition to its direct increasing effect on the peripheral vascular resistance, ET-1 may also contribute to hypertrophic remodelling of small arteries, and thus play a significant role in the development of end-organ damage and cardiovascular complications in CRI (Dao et al. 2001). Selective endothelin-1 type A (ET_A) receptor blockade slows down the progression of hypertension and vascular and renal damage, supporting a role for ET-1 in CRI progression (Brochu et al. 1999). The increase in ET-1 production can be associated with alterations

of the local production of other local mediators, including Ang II, TGF- β_1 and nitric oxide (NO) (Lariviere and Lebel 2003).

In humans with essential hypertension, atherosclerosis, and nephrosclerosis, plasma ET-1 levels are increased when compared to patients with uncomplicated essential hypertension. Similarly, plasma ET-1 concentrations are markedly increased in patients with end-stage renal disease undergoing dialysis, and this correlates with BP, suggesting that ET-1 may contribute to hypertension in these patients. Furthermore, in patients with CRI, the treatment of anemia using human recombinant erythropoietin has been suggested to increase arterial BP via the acceleration of endothelial dysfunction and increased vascular ET-1 production (Katoh et al. 1994). Thus, ET-1 pathway may be one of the important mechanisms in the development of secondary hypertension in patients with CRI.

1.4.2 Vascular calcification

Vascular calcification has been clearly defined as a risk factor for cardiovascular mortality in the general population, and it is highly prevalent in patients with impaired renal function (Davies and Hruska 2001). It has been shown that dialysis patients, even those younger than 30 years of age, have a high incidence of coronary artery calcification, and the progression of these lesions is relatively rapid (Goodman et al. 2000). Furthermore, vascular calcification in CRI patients is associated with a number of markers of increased mortality such as left ventricular hypertrophy (Davies and Hruska 2001), myocardial infarction, congestive heart failure, endocarditis, and valvular heart disease.

Arterial calcification in CRI is characterized by mineral deposition in the tunica media, in contrast to general population, where calcification of atheromatous plaques predominates. The hemodynamic disorders related to medial wall calcification are quite different from those due to atherosclerotic calcification (London et al. 2002). The diffuse deposition of mineral throughout the tunica media increases vascular stiffness and therefore reduces vascular compliance, the consequence of which is decreased capacity of the arterial circulation to dampen increases in arterial pressure with each ventricular systole. This causes an increase of systolic BP, pulse pressure and pulse wave velocity (London et al. 2002, London et al. 1990). These hemodynamic alterations lead to left ventricular hypertrophy, and may compromise coronary artery blood flow during diastole.

In CRI, alterations in phosphorus metabolism and concomitant SH play a significant role in the development of vascular calcification. Treatment of hyperphosphatemia and SH with high calcium intake is thought to exacerbate the passive deposition of mineral in soft-tissues, at least in dialysis patients (Goodman 2001). However, in experimental uremia the increased intake of calcium carbonate has been reported to reduce soft-tissue calcification in remnant kidney, probably via the effective lowering of plasma phosphate (Cozzolino et al. 2002). Therefore, in CRI the most

important risk factors for mineral deposition in soft tissues are probably the increased plasma levels of phosphate and PTH. Increased calcium intake could actually slow down the calcification process via the control of hyperphosphatemia, especially if enough residual renal function is preserved to maintain sufficient calcium excretion. Furthermore, many clinical studies have reported that high $\text{Ca} \times \text{Pi}$ product is an important risk factor for calcification, but have not given detailed information about dietary calcium intake of these patients (Kimura et al. 1999, Raggi et al. 2002). A thorough analysis of the role of dietary calcium intake as a risk factor for soft tissue calcification in CRI, with reliable information about patient compliance, should be performed in the future.

In addition to the calcium-phosphate-PTH balance in the regulation of soft tissue calcification, current evidence suggests that the pathophysiological mechanisms underlying arterial calcification involve disturbed active inhibition of the calcification process, rather than an increase of passive mineral precipitation. The calcification process is influenced by tissue-specific cellular mechanisms and by inhibitor proteins that are present in tissues and plasma (Schinke and Karsenty 2000, Schinke et al. 1998). Such inhibitors of mineral deposition prevent soft-tissue and vascular calcification under normal conditions *in vivo* (Schinke and Karsenty 2000).

The matrix GLA-protein contributes to the prevention of calcification in arterial tissue (Luo et al. 1997). Transgenic mice lacking matrix GLA-protein develop extensive calcification, the process of which involves cartilage formation in the large elastic arteries (El-Maadawy et al. 2003). Furthermore, the actions of this protein can be blocked by warfarin, since K-vitamin is an important co-factor in the γ -carboxylation and activation of the GLA-proteins (Jono et al. 2004, Schori and Stungis 2004). Sustained warfarin administration causes osteoporosis and arterial calcification in rats (Howe and Webster 2000, Price et al. 2000). Thus, in patients undergoing dialysis, the use of warfarin may be a risk factor for calcific uremic arteriolopathy, or so-called calciphylaxis (Mazhar et al. 2001). Based on these observations, matrix GLA-protein can be considered as an important physiological inhibitor of soft-tissue calcification in arterial and cardiac valve tissues.

There is compelling evidence that a variety of proteins normally involved in bone and mineral metabolism can be expressed under uremic conditions in arterial tissue (Shanahan et al. 1999, Shanahan et al. 2000). The bone-matrix protein osteopontin is such an additional regulator of vascular calcification (Speer et al. 2002, Steitz et al. 2002). The expression of osteopontin in arterial tissues has been demonstrated in CRI patients with calciphylaxis (Ahmed et al. 2001). Interestingly, increased phosphorus level and uremic serum can increase the expression of osteopontin in isolated vascular smooth muscle cells (VSMC) (Chen et al. 2002). Taken together, vascular calcification as a complication of CRI may be to a large part a consequence of disturbed calcium-phosphate balance, but deficient active inhibition of passive mineral precipitation into extraskelatal tissues may play an important role in the calcification process. Future studies should be targeted especially to determine the underlying mechanisms in more detail, and develop treatments capable of restoring and even enhancing the active inhibition of calcification.

1.4.3 Atherosclerosis

An abnormally high mortality from atherosclerotic cardiovascular accidents has long been reported in CRI patients on dialysis (Lindner et al. 1974). Cardiovascular death accounts nearly 50% of all deaths in end-stage renal disease, and this proportion is much higher when compared with general population (USRDS 1997). Furthermore, occlusive accidents involving coronary, peripheral, or cerebrovascular arteries account for at least half of cardiovascular deaths in end-stage renal disease (USRDS 1997). However, recent studies have demonstrated that thickening of arterial wall in patients with CRI is present already before starting hemodialysis treatment, and that advanced atherosclerosis in these patients is not due to the dialysis treatment, but to CRI and the associated metabolic abnormalities (Shoji et al. 2002).

In patients with CRI, specific risk factors for atherosclerosis include SH, increased sympathetic-nerve activity, elevated levels of oxidized low-density lipoproteins, and endothelial dysfunction (Luke 1998). In addition, the same risk factors that contribute to atherosclerosis in general population, such as arterial hypertension, smoking, hyperlipidemia, the insulin resistance syndrome, and hyperhomocysteinemia, play a significant role also in patients with CRI, in whom the risk is compounded due to the high prevalence of multiple risk factors (Luke 1998, Nishizawa et al. 2004).

Disturbed endothelial function plays a central role in the pathophysiology of atherosclerosis, whereas CRI has been well known to contribute to the endothelial dysfunction (Gris et al. 1994). Several studies have shown that impaired endothelial function is a prominent feature in patients with moderate CRI (Annuk et al. 2001), as well as in patients with advanced renal impairment and dialysis treatment (van Guldener et al. 1998, van Guldener et al. 1997). Damaged vascular endothelium loses its antithrombotic properties that normally inhibit the adhesion of blood cells to the vessel wall, thus initiating the development of atherosclerotic plaques.

Another important function of healthy endothelium is vasorelaxation. The impairment of both of these functions, and augmented excretion of vasoconstrictive agents such as ET-1 and thromboxane A₂ (TXA₂) by the endothelial cells have been reported in uremic conditions (Lariviere et al. 1997). It has also been identified that growth factors and chemotactic substances, which are produced by damaged or overstimulated endothelial cells, contribute to the structural changes of the arterial wall and to the progression of atherosclerotic lesions (Cotran and Pober 1990). Various factors associated with CRI, such as hypertension, increased oxidative stress, dyslipidaemia, hyperglycaemia, hyperhomocysteinemia, and retention of L-arginine inhibitors, all have been suggested to contribute the endothelial dysfunction in uremia. However, the observed correlation between creatinine clearance and endothelial function supports the view that renal insufficiency *per se* is the most important reason for the endothelial dysfunction and atherosclerosis in CRI (Annuk et al. 2001).

During the progression of atherosclerosis, intimal lesions impinge upon the arterial lumen and, in more advanced stages of the disease, can disrupt blood flow, leading to ischemia and necrosis in tissues. Ischemic events can also occur acutely in previously unobstructed vessels when atherosclerotic plaques rupture causing thrombus formation and arterial occlusion (Berliner et al. 1995). A high rate of the above complications has been reported in CRI patients, probably due to more extensive deposition of calcium to coronary plaques and increased media thickness when compared to general population (Schwarz et al. 2000). The calcification presumably occurs throughout the course of plaque development, being most pronounced in larger and more mature lesions (Goodman et al. 2004). An augmented calcification of the atherosclerotic plaques in CRI is contributed principally by the same disturbances that cause the calcification in tunica media of the vascular wall, and in other soft tissues. The most important of these factors are impaired calcium-phosphorus balance, SH, inflammation, dialysis treatment, dyslipidaemia, hypertension, diabetes, sodium and volume overload, participation of oxidized lipids, and elevated homocysteine levels (McCullough et al. 2004, Salusky and Goodman 2002). Accelerated calcification of atherosclerotic lesions in CRI is, at least in part, due to the enhanced local expression of proteins that are normally involved in bone and mineral metabolism, and to disturbances in the metabolism of proteins that normally inhibit the precipitation of calcium to the soft tissues (Goodman et al. 2004).

1.5 Renin-angiotensin system and chronic renal insufficiency

1.5.1 Tissue versus circulating renin-angiotensin system

The RAS has a substantial role in the maintenance of volume homeostasis and salt balance, and thereby participates in the normal regulation of arterial pressure in mammals (Brown et al. 1995, He et al. 1998). The conceptual theory that “renal substances” might circulate in the blood and elevate BP was proposed by Richard Bright already in 1836 on the basis of his observations in patients with renal failure. Renin was discovered in 1898 by Tigerstedt and Bergman, who showed the development of systemic hypertension by injecting an extract from rabbit renal cortex into normotensive animals. Renin is synthesized and released from the juxtaglomerular cells of the afferent arteriole in the kidney. This enzyme has high substrate specificity, and its only known substrate is angiotensinogen, which is primarily formed in the liver. Renin cleaves the N terminus of circulating angiotensinogen to angiotensin I (Ang I), which is then transformed to Ang II through the action of ACE, that is located mainly in the luminal surface of vascular endothelium (Riordan 1995; Figure 3). Circulating levels of angiotensinogen are in excess of 1000-fold greater than angiotensins I and II (Brasier and Li 1996). Thus, the activity of systemic RAS is primarily regulated by the PRA and the rate-limiting step in the RAS is Ang I generation, even though Ang I does not seem to have major or widely confirmed biologic functions.

The major biologically active peptide of RAS is Ang II (Nguyen et al. 2002), which has several well-known functions, including constriction of VSMCs via AT₁ receptors, direct suppression of renin release, and stimulation of aldosterone release (Dzau 2001). The AT₁ receptors are widely distributed and expressed by many cell types (Ardaillou 1999). Components of the RAS and Ang II receptors are found in the brain (Davisson et al. 2000) and in many peripheral tissues such as the heart (Dostal and Baker 1999) and kidney (Siragy 2000). Recent studies have shown that the RAS is involved in diverse physiological and pathological processes such as growth and remodelling (Tamura et al. 2000), inflammation (Schieffer et al. 2000), vascular hypertrophy, and thrombosis (Brown and Vaughan 2000).

The major biologic actions of Ang II in the kidney are mediated by two well-characterized receptors (Figure 3). These recently described subtypes have been labelled AT₁ and angiotensin II type 2 (AT₂) receptors. The AT₁ receptor is distributed in the vasculature, kidney, adrenal gland, heart, liver, and brain and mediates the hemodynamic actions, endocrine functions, and mitogenic effects of Ang II in the kidney. The AT₂ receptor is abundantly present in fetus, whereas in adults only in the adrenal medulla, uterus, ovary, vascular endothelium, and distinct brain areas, mediating for instance vasodilatory and antiproliferative effects. Most of the deleterious effects of Ang II are mediated by the AT₁ receptor. Ang II causes glomerular injury and interstitial proliferation in two kidney-one clip rats via increased TGF- β in the renal interstitium, leading to fibrosis and renal damage (Peters et al. 2000). Furthermore, this mechanism has also been observed in an experimental model of obstructive nephropathy (Pimentel et al. 1995), as well in stroke-prone hypertensive rats in which interstitial and glomerular damage are severe (Nakamura et al. 1996).

Local intrarenal RAS is considered to play an important role in the progression of kidney diseases (Ruiz-Ortega et al. 2002). Despite that systemic RAS is primarily not activated in many types of chronic renal disease (Gilbert et al. 1999), the interruption of RAS by either ACE inhibition or AT₁ receptor antagonism reduces renal injury in several types of experimental and human kidney disease (1997, Noble and Border 1997). This finding has led to the view that a local RAS in the kidney may be an important determinant in the pathophysiology and progression of chronic renal disease (Gilbert et al. 1999). The existence of tissue level RAS in the kidney has been suggested by the high concentration of Ang II in the glomerular filtrate and proximal tubular lumen relative to plasma (Seikaly et al. 1990). It has been identified that all components of the RAS are present in the kidneys and constitute a functioning renal RAS. The mRNA transcripts and protein have been demonstrated for multiple components of the RAS including renin, angiotensinogen, and ACE, thereby documenting their presence and production locally in the kidney (Dzau 2001). Furthermore, Ang II receptor subtypes AT₁ and AT₂ have been found in the afferent and efferent arterioles, glomeruli, mesangial cells, and proximal tubules (Siragy 2000). Recent studies have also provided compelling evidence that the local production of intrarenal RAS components can act independently of the Ang II production known to be a part of the general hormonal or endocrine circulation (Neal and Greene 2002).

Intrarenal Ang II has important functions in regulating urine flow, urinary sodium excretion and glomerular filtration rate, but can also contribute to the regulation of systemic arterial BP, since expression of human angiotensinogen in the kidneys of mice results in hypertension in the absence of changes in systemic Ang II (Sigmund 2001). The presence of Ang II in renal tissue depends on two mechanisms: local production, and uptake from circulation, which has been observed in heart, adrenal and kidney (van Kats et al. 1997, Zou et al. 1996a, Zou et al. 1996b). However, it has been shown that normally most of the Ang II in the kidney is not derived from the circulation, but is formed locally by conversion of locally produced Ang I (van Kats et al. 2001). This corresponds with results in experimental CRI, where the activity of systemic RAS was not activated, but the expression of renin and Ang II in kidney tissue of animals with CRI was increased (Gilbert et al. 1999). Moreover, local RAS may be important determinant in the progression of CRI, since ACE inhibition and AT₁ receptor antagonism can reduce renal injury under conditions, where the systemic RAS is not activated (Gilbert et al. 1999).

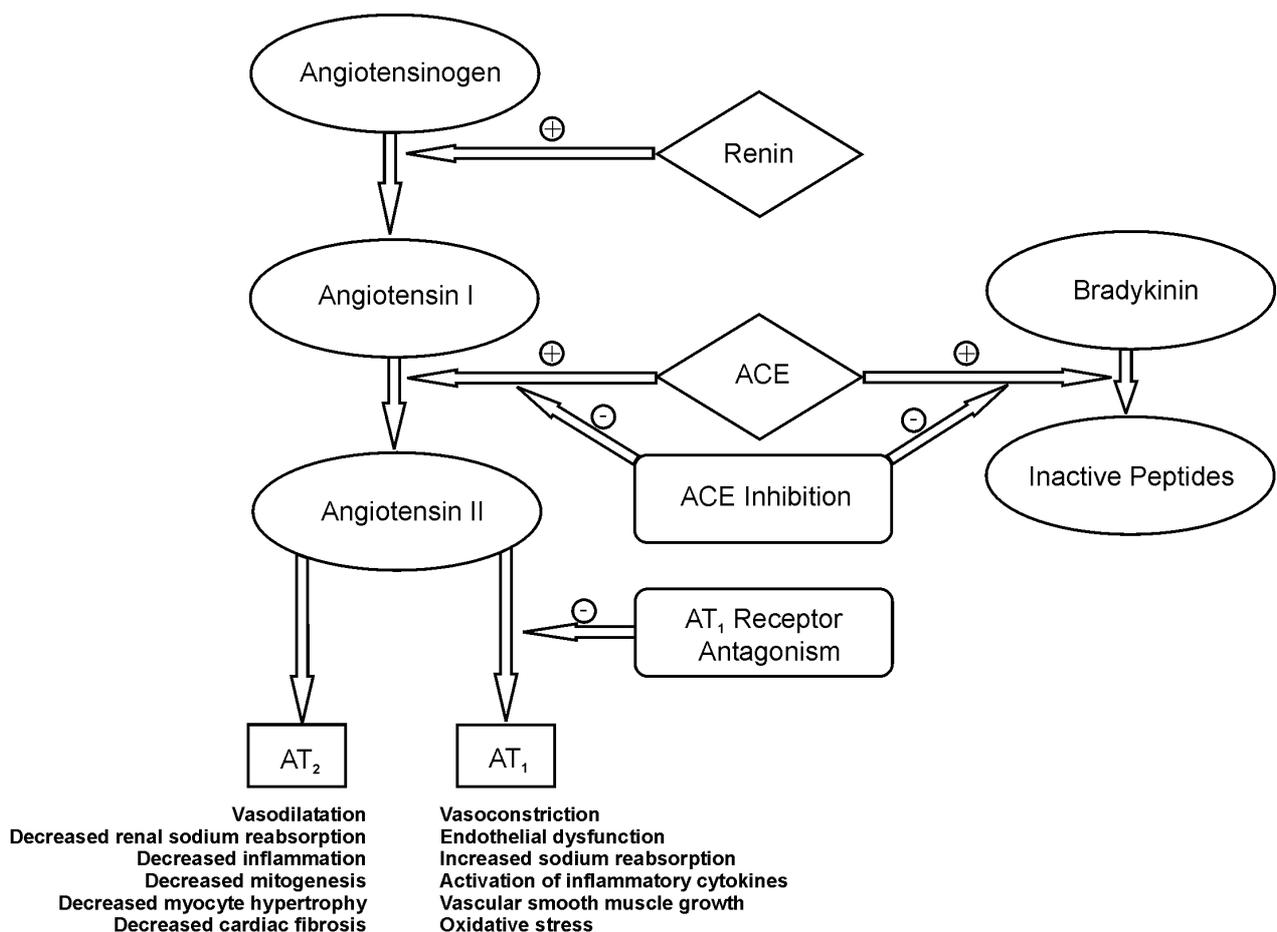


Figure 3. Schematic diagram depicts the renin-angiotensin system and its major effects via angiotensin II type 1 receptor (AT₁) and angiotensin II type 2 receptor (AT₂), and the mechanisms of angiotensin converting enzyme (ACE) inhibition and AT₁ antagonism (modified from Brewster and Perazella 2004).

1.5.2 Inhibition of the actions of the renin-angiotensin system

Interruption of the RAS by either ACE inhibition or AT₁ receptor antagonism reduces renal injury in several models of experimental and human kidney disease (Noble and Border 1997). There is convincing evidence that administration of RAS blocking agents promise better control of glomerular hypertension and long-term outcome of kidney preservation than diuretics, adrenoceptor blocking agents, beta-blockers, calcium channel blockers and other antihypertensive medications (Hallab et al. 1993, Zucchelli et al. 1992). Moreover, clinical studies have suggested that ACE inhibitors reduce proteinuria and retard the progression of renal disease to a greater degree than can be explained by their BP lowering effects alone (Kasiske et al. 1993, Maschio et al. 1996). Similarly, the renoprotective effect of AT₁ receptor antagonists has been suggested to be partially independent of the BP reduction caused by these drugs (Lewis et al. 2001, Parving et al. 2001).

The decreased Ang II generation induced by the use of ACE inhibitors dilates glomerular efferent arterioles, thereby reducing glomerular capillary pressure (Raij and Keane 1985). The subsequent fall in filtration pressure contributes to the antiproteinuric effect and to the long-term renoprotection (Anderson et al. 1985). Since proteinuria *per se* has been shown to play an important role in the final pathway of progressive renal function loss, the beneficial effects of RAS blocking agents in CRI could be largely attributed to the reduction of proteinuria (Remuzzi et al. 1997). Large quantities of protein in renal tubules may have a damaging effect on the tubular cells and interstitium. This view is supported by the evidence that reabsorption of filtered proteins may activate the proximal tubular epithelium and subsequent upregulation of inflammatory and vasoactive genes such as monocyte chemoattractant protein-1 and ETs (Remuzzi et al. 1997). These molecules are associated with a tubulointerstitial inflammatory reaction that in most forms of glomerulonephritis consistently proceeds to renal scarring. Altogether, both glomerulosclerosis and interstitial fibrosis can be ameliorated by ACE inhibitors and AT₁ receptor antagonists (Ots et al. 1998).

Experimental studies that have compared the effects of ACE inhibitors and AT₁ receptor antagonists have shown a striking similarity between these classes of drugs (Table 2). However, in contrast to ACE inhibitors, AT₁ receptor antagonists do not inhibit the breakdown of bradykinin (Hilgers and Mann 2002). On the other hand, AT₁ receptor antagonists increase Ang II concentrations, leading to the activation of AT₂ receptors and subsequent slight increase of bradykinin (Hilgers and Mann 2002). Kinins contribute significantly to the BP-lowering effects of ACE inhibitors in humans (Gainer et al. 1998). However, kinins also promote unwanted side effects of ACE inhibitors such as cough, angioneurotic oedema (Israili and Hall 1992), and anaphylactic reactions to dialysis membranes (Verresen et al. 1994). Moreover, the increased levels of Ang II during the AT₁ blockade and subsequent activation of AT₂ receptors may have some advantages when compared to the ACE inhibition. The strong evidence that AT₂ receptors counteract the vasoconstriction (Siragy et al. 1999) and proliferative actions (Stoll et al. 1995) support this view. It

has been shown that AT₂ receptor can exert an antifibrotic effect on the kidney during experimental obstructive nephropathy, in opposition to the profibrotic effects of Ang II operating through the AT₁ receptor (Morrissey and Klahr 1999), whereas the AT₂ knockout mice with ureteral obstruction develop accelerated fibrosis and collagen deposition in the renal interstitium (Ma et al. 1998).

Clinical trials have shown that despite the differences in mechanisms of action, both ACE inhibitors and AT₁ receptor antagonists appear to maintain relatively equal potency in providing nephroprotection (Barnett et al. 2004, Pitt et al. 2000). Also similar effects of enalapril and irbesartan on proteinuria and dextran sieving coefficients have been observed in patients with IgA nephropathy (Remuzzi et al. 1999), whereas in patients with type 2 diabetes lisinopril and candesartan lowered BP and albumin excretion to the same degree (Mogensen et al. 2000). However, in patients with CRI, AT₁ receptor antagonists increase serum potassium less than ACE inhibitors (Bakris et al. 2000) and are better tolerated also due to fewer side effects such as cough and angioneurotic oedema (Pitt et al. 2000, Remuzzi et al. 1999).

Table 2. The comparison of the effects of ACE inhibitors and AT₁ receptor antagonists in CRI.

Experimental model (reference)	Treatment	Blood pressure	Proteinuria	Glomerulo- sclerosis	Tubulointerstitial fibrosis
Diabetic nephropathy (Allen et al. 1997)		↑	↑	↑	-
	Valsartan	↓	↓	↓	-
	Ramipril	↓	↓	↓	-
Diabetic nephropathy (Remuzzi et al. 1998)		↑	↑	↑	-
	Valsartan	↓	↓	↓	-
	Benazepril	↓	↓	↓	-
Hypertensive nephrosclerosis (Nakaya et al. 1999)		↑	↑	↑	↑
	Irbesartan	↓	↓	↓	↓
	Enalapril	↓	↓	↓	↓
Nephrectomy (Noda et al. 1999)		-	↑	↑	↑
	Candesartan	-	↓	↓	↓
	Enalapril	-	↓	↓	↓

↑ and ↓ indicate a deterioration and reduction when compared with the corresponding control group, respectively.
- indicates “data not available”.

2 Local control of arterial tone

2.1 Endothelium-derived vasodilatory factors

The endothelial cell monolayer is a highly important regulator of vascular homeostasis. The balance between substances that cause either contraction or relaxation regulates the vasomotor tone of underlying arterial smooth muscle. Several vasoactive substances like NO, hyperpolarizing factor, cyclooxygenase (COX) metabolites and ET are continuously released by the endothelium, which thus controls blood flow and arterial tone according to the metabolic needs of tissues, in response to various humoral, neural, and mechanical stimuli (Behrendt and Ganz 2002). The endothelium also regulates several other functions like coagulation, lipid transport, immunological responses and even vascular structure (Haynes and Webb 1998).

2.1.1 Nitric oxide

Furchgott and Zawadzki first reported that the presence of intact endothelium in isolated rabbit aorta is an absolute requirement for the acetylcholine (ACh) -induced vasorelaxation (Furchgott and Zawadzki 1980). In contrast, in the absence of the endothelium, ACh only induced a modest contraction in the arteries *in vitro*, which could be explained by the direct contractile effect of ACh on smooth muscle (Furchgott 1996, Ignarro et al. 1987). It took several years until the potent vasodilating substance released from the endothelium by ACh could be identified as NO (Furchgott 1996, Ignarro et al. 1987).

The enzyme nitric oxide synthase (NOS) generates NO via the conversion of L-arginine to NO and L-citrulline (Palmer et al. 1988; Figure 4). The endothelial isoform of NOS (eNOS) is constitutively expressed, and in smooth muscle NO activates guanylate cyclase, increases the production of cyclic guanosine 3',5'-monophosphate (cGMP), reduces intracellular free calcium, and, subsequently, causes relaxation (Behrendt and Ganz 2002). In addition to vasodilatation, NO also inhibits platelet aggregation (de Graaf et al. 1992) and leukocyte adhesion (Gauthier et al. 1995, Kubes et al. 1991), and reduces smooth muscle proliferation (Cornwell et al. 1994). The present widely accepted view is that NO protects against vascular injury, inflammation, and thrombosis, which are all key events in the genesis and progression of atherosclerosis (Behrendt and Ganz 2002). In addition to the processes that can mechanically disrupt the endothelium like atherosclerosis, increased inactivation of NO caused by superoxide and other reactive oxygen species, are probably involved in reduced NO bioactivity (Ohara et al. 1993). The above findings have led to the development of a novel concept: several pathologic disease states can affect the endothelium and result in a situation where the NO-elicited vasodilator tone is reduced (endothelial dysfunction)

2.1.2 Prostacyclin

Prostacyclin (PGI₂) is the most significant prostanoid produced in the endothelium, although other PGs are also synthesized in the vascular wall (Busse et al. 1994). PGI₂ especially inhibits platelet aggregation, but also causes vasodilatation (Busse et al. 1994) and reduces VSMC proliferation (Weber et al. 1998). The enzyme phospholipase A₂ liberates arachidonic acid from cell membrane phospholipids, and thereafter COX converts arachidonic acid to PGG₂ and PGH₂. Then PGI₂ synthase converts PGH₂ to PGI₂ (Cohen 1995, Gryglewski 1995; Figure 4).

Like in the case of NO, the endothelial cells release PGI₂ in response to various stimuli like activation of cellular receptors, shear stress, hypoxia, and vasoconstriction (Gryglewski 1995, Lüscher and Noll 1995). In smooth muscle, PGI₂ binds to membrane receptors, activates adenylate cyclase and increases intracellular cyclic adenosine 3',5'-monophosphate (cAMP) (Busse et al. 1994). Subsequently, intracellular free Ca²⁺ concentration ([Ca²⁺]_i) is reduced, sensitivity of contractile proteins to Ca²⁺ is decreased, and cell membrane is hyperpolarized (Bülbring and Tomita 1987, Cohen and Vanhoutte 1995, Ushio-Fukai et al. 1993). PGI₂ is rather a local than a circulating hormone, since its circulating levels are very low (Vane and Botting 1993), and the blockade of COX has a negligible influence on BP (Ruoff 1998). PGs synthesis can be blocked by nonsteroidal anti-inflammatory drugs that inhibit COX-1, and by coxibs that inhibit COX-2 (FitzGerald and Patrono 2001, Vane and Botting 1993).

Experiments with 5/6 nephrectomized (NTX) rats suggest that PGI₂ and COX-2 are upregulated in renal cortex of CRI animals, perhaps stimulated by a decrease in renal blood flow, which upregulates PGI₂ synthesis to protect the kidney from ischemia in renal insufficiency. PGI₂ is known to dilate glomerular afferent and efferent arterioles and vasa recta in renal medulla, enhancing thus total renal blood flow without an increase in glomerular filtration pressure (Bolger et al. 1978, Yoshida et al. 1986). Therefore, PGI₂ has been suggested to protect kidney from ischemia and to delay the progression of CRI. Furthermore, treatment with the PGI₂ analogue beraprost has also been reported to diminish the vascular resistance of afferent and efferent arterioles in CRI patients, resulting in an increase of total renal blood flow without glomerular hyperfiltration and mitigating thus the progression rate of renal dysfunction (Fujita et al. 2001).

2.1.3 Endothelium-derived hyperpolarizing factor

In several types of arteries from different locations, endothelium-dependent relaxations cannot be completely blocked by COX and NOS inhibitors, and the remaining response is considered to be mediated via VSMC hyperpolarization (Bolton et al. 1984, Chen and Suzuki 1989, Félétou and Vanhoutte 1988, Huang et al. 1988, Taylor et al. 1988). Since the vasorelaxation and membrane hyperpolarization occur without detectable changes in intracellular of cyclic nucleotide contents (Cowan and Cohen 1991, Mombouli et al. 1992, Taylor et al. 1988), this effect has been attributed

to a so called endothelium-derived hyperpolarizing factor (EDHF) that has not been yet fully chemically characterized (McGuire et al. 2001). In general, the contribution of EDHF-mediated responses to endothelium-dependent relaxation is more pronounced in small resistance-caliber arteries when compared with larger conduit-size vessels (Shimokawa et al. 1996). However, in the coronary and renal circulation EDHF appears to play a significant role also in conduit-size arteries (Félétou and Vanhoutte 1999, Quilley et al. 1997).

Like the NO-mediated responses, also the relaxations to EDHF are characterized by an increase in endothelial cell $[Ca^{2+}]_i$ (Johns et al. 1988, Lückhoff et al. 1988; Figure 4). Therefore, for EDHF-mediated responses to be observed, an increase in endothelial $[Ca^{2+}]_i$ is required (Nilius and Droogmans 2001). A multitude of agonists and substances that increase endothelial $[Ca^{2+}]_i$ can result in augmented EDHF action, regardless of the mechanism of the increase in $[Ca^{2+}]_i$ (Fukao et al. 1995, Illiano et al. 1992).

When smooth muscle is hyperpolarized, Ca^{2+} entry via voltage-dependent Ca^{2+} channels is reduced and the turnover of intracellular phosphatidylinositides is decreased, and subsequently, $[Ca^{2+}]_i$ is reduced, whereby relaxation follows (Nelson et al. 1990). The finding that the endothelium-dependent hyperpolarization of smooth muscle cells increases K^+ conductance, strongly suggests that EDHF is a K^+ channel opener (Chen and Suzuki 1989, Chen et al. 1988, Taylor et al. 1988). The EDHF-mediated response can be abolished by Ca^{2+} -activated K^+ channel (K_{Ca}) inhibition, and in a number of studies this has been accomplished by the combination of apamin [inhibitor of small-conductance K_{Ca} (SK_{Ca})] and charybdotoxin [a nonselective inhibitor of large-conductance K_{Ca} (BK_{Ca}) and intermediate-conductance K_{Ca} (IK_{Ca}) channels, and also of some voltage-dependent K^+ -channels] (Corriu et al. 1996, Plane and Garland 1996, Zygmunt and Hogestatt 1996). However, in addition to blockade of K_{Ca} in smooth muscle, the combination of apamin and charybdotoxin appears to inhibit two types of K_{Ca} (IK_{Ca} and SK_{Ca}) also in the endothelial cells. Therefore, this combination may prevent endothelial cell hyperpolarization in addition to blocking K^+ channels in smooth muscle cells. This methodological shortcoming has been overcome by the application of another K^+ channel inhibitor iberiotoxin that appears to selectively block BK_{Ca} in VSMCs (Burnham et al. 2002, Cai et al. 1998, Coleman et al. 2001, Doughty et al. 1999, Edwards et al. 1998, Edwards et al. 1999b, Edwards et al. 2000, Marchenko and Sage 1996).

Several candidates for EDHF have been suggested. In many studies epoxyeicosatrienoic acids (EETs), that are arachidonic acid-derived products of cytochrome P450 epoxygenases, have been proposed to be EDHF(s) (Archer et al. 2003, Bolz et al. 2000, Campbell et al. 1996, Coats et al. 2001, Cohen and Vanhoutte 1995, Fisslthaler et al. 1999, Garland et al. 1995, Vanhoutte and Mombouli 1996). The EETs can stimulate K_{Ca} in smooth muscle and induce vasorelaxation, and they have also been shown to be produced in the endothelium and to be released in response endothelial cell stimulation (Quilley et al. 1997). The stimulation of the endothelium is characterized by cellular hyperpolarization, and the EETs (and other products of cytochrome P450)

may play an important role in the hyperpolarization of endothelial cell, which is a requirement of the EDHF-mediated hyperpolarization of VSMCs: the EETs may modulate endothelial Ca^{2+} influx in response to Ca^{2+} -store depletion (Hoebel et al. 1997), and they may also increase the sensitivity of endothelial K^+ channels to Ca^{2+} (Baron et al. 1997, Li and Campbell 1997).

The K^+ ion has also been proposed as EDHF, since increased myoendothelial K^+ concentration can hyperpolarize VSMCs via activation of inward rectifier K^+ channels (K_{IR}) and Na^+ - K^+ -ATPase (Nelson and Quayle 1995, Prior et al. 1998). However, the putative role of K^+ ion as an EDHF has been a subject for much debate (Doughty et al. 2000, Lacy et al. 2000). Interestingly, the gap junctions between the endothelium and smooth muscle may play a role in EDHF-mediated responses: the spreading of endothelial hyperpolarization electrotonically via the gap junctions to smooth muscle may play a role in the EDHF response of rat hepatic and mesenteric arteries, and especially of guinea-pig internal carotid artery (Edwards et al. 1999a). The number of the myoendothelial gap junctions increases when arterial size decreases (Sandow and Hill 2000). This finding fits remarkably well with the conception that the contribution of EDHF to the control of vascular tone increases when arterial size decreases (Shimokawa et al. 1996).

The NO may inhibit the production and action of EDHF (Bauersachs et al. 1996, McCulloch et al. 1997). Thus, in pathophysiological conditions with reduced endothelium-derived NO bioactivity, the significance of the EDHF-mediated vasorelaxation could be increased (Bauersachs et al. 1996). However, the majority of studies have published results in contradiction with the above view: impaired endothelium-mediated hyperpolarization has repeatedly been reported in experimental hypertension (Fujii et al. 1992, Mäkynen et al. 1996, Sunano et al. 1999, Van de Voorde et al. 1992) and also in experimental CRI (Kalliovalkama et al. 1999a). Taken together, the present evidence suggests that there are multiple EDHFs, and that the chemical mediator and the significance of the EDHF response varies from one vascular bed to another (Campbell and Harder 1999, Edwards and Weston 1998).

2.2 Endothelium-derived contractile factors

The endothelium can also synthesize and release powerful contractile mediators like vasoconstrictor prostanoids, ET-1, superoxide and Ang II. Like in the case of relaxing factors, the production of the contractile factors is induced by a multitude of stimuli: vasoconstriction, hypoxia, pulsatile stretch, pressure, and a large number of local and circulating hormonally active compounds (Lüscher et al. 1992, Rubanyi 1993, Schiffrin 2001). Interestingly, in the case of ET, hypoxia and low shear stress can induce ET production, whereas high shear stress and endothelial formation of cGMP and cAMP decrease ET synthesis (Kuchan and Frangos 1993, Lüscher and Noll 1995, Lüscher et al. 1993b).

TXA_2 and the intermediates PGG_2 and PGH_2 of the PG of the endoperoxide synthesis are contractile factors produced by COX. In target cells these vasoconstrictor prostanoids act via the same TXA_2 receptor (Cohen 1995), and their synthesis can be blocked by COX inhibitors (Auch-

Schwelk and Vanhoutte 1991). The vasoconstrictor products of COX may limit the endothelium-dependent vasodilatations, but COX products do not appear to play a major role in the endothelial dysfunction of hypertensive patients (Campia et al. 2002). It has been reported that TXA₂ is probably involved in the pathogenesis of hypertension and progression of renal dysfunction in experimental CRI, since the levels of TXA₂ concentrations were increased in blood vessels, renal cortex and in urine of NTX rats. In addition, TXA₂ synthase inhibitor and receptor antagonist ridogrel lowered systolic BP, reduced proteinuria and blunted the increase of serum creatinine, and resulted in a marked fall in vascular, renal and urine TXA₂ concentrations (Lariviere et al. 2004).

Endothelium-derived ET-1 is a remarkably potent vasoconstrictor. Its vasoconstrictor action is mediated through ET_A and type B (ET_B) receptors in smooth muscle, while the endothelial ET_B receptors simultaneously oppose ET_A- and ET_B-mediated vasoconstriction by vasodilator autacoid formation (Lüscher et al. 1993a, Nava and Lüscher 1995, Taddei et al. 2000). In smooth muscle, ETs activate phosphatidylinositol metabolism, mobilize intracellular Ca²⁺ stores, increase calcium entry via Ca²⁺ channels and protein kinase C (Black et al. 2003, Schiffrin 1995a). All 3 forms of ET (ET-1, ET-2, ET-3) are converted from preproendothelins to proendothelin, which is then converted by the ET converting enzymes to ET (Haynes and Webb 1998). The endothelial can probably only produce ET-1 and not the other forms of ET (Agapitov and Haynes 2002, Lüscher et al. 1992).

ET-1-induced vasoconstrictor may be increased in essential hypertension (Taddei et al. 2001), and the actions of ET-1 in the kidney, the nervous system and other hormone systems could participate genesis and in the pathophysiology of hypertension (Goddard and Webb 2000). Theoretically, ET antagonists carry significant potential to become essentials tool in the treatment of different forms of cardiovascular disease (Schiffrin 1995b), but at the moment the only clinical application of ET antagonists (bosentan) is the treatment of primary pulmonary hypertension. ET-1 is produced by, and binds to, most renal cell types. In the kidney ET-1 exerts many biological effects including constriction of most renal vessels, mesangial cell contraction, inhibition of sodium and water reabsorption by the nephron, enhancement of glomerular cell proliferation, and stimulation of extracellular matrix accumulation (Kohan 1997). Several actions mediated by ET may play a role in the progression of renal damage in CRI: blockage of inducible nitric oxide synthase transcription via the ET_A receptor; increased expression of the collagen 1 gene, vascular remodelling, mediation of proteinuria, macrophage chemotaxis, and stimulation of interstitial fibroblast proliferation and extracellular matrix synthesis (Yu 2003). Finally, it must be emphasized that a marked heterogeneity of the endothelium-derived vasoconstrictor responses exists among species, strains, and different vascular beds (Lüscher et al. 1992, Rubanyi 1993, Schiffrin 2001).

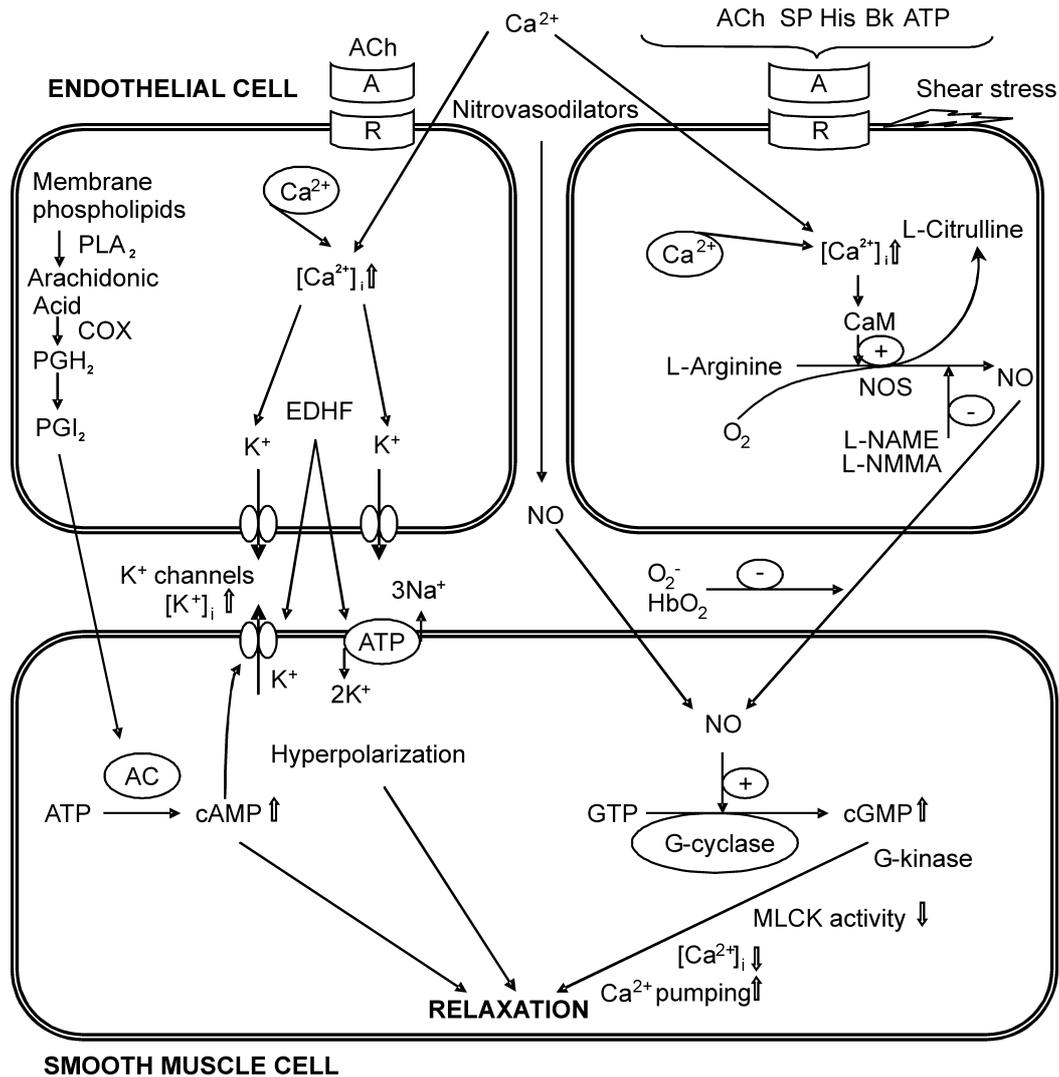


Figure 4. Mechanisms of endothelium-dependent arterial relaxation. Abbreviations: A, agent; AC, adenylate cyclase; ACh, Acetylcholine; ATP, adenosine 5'-triphosphate; Bk, Bradykinin; $[Ca^{2+}]_i$, intracellular free calcium concentration; $[K^+]_i$, free potassium concentration; cAMP, cyclic adenosine 3',5'-monophosphate; cGMP, cyclic guanosine 3',5'-monophosphate; CaM, calmodulin; Ca^{2+} pump, Ca^{2+} - Mg^{2+} ATPase; EDHF, endothelium-derived hyperpolarizing factor; G-cyclase, guanylyl cyclase; GTP, guanosine 5'-triphosphate; Hb, hemoglobin; His, histidine; L-NAME, NG-nitro-L-arginine; L-NMMA, NG-monomethyl-L-arginine; MLCK, myosin light chain kinase; NO, nitric oxide; NOS, nitric oxide synthase; PGH_2 , prostaglandin H_2 ; PGI_2 , prostacyclin; R, receptor; SP substance P. -, inhibition; +, stimulation; \uparrow , increase; \downarrow , decrease (modified from Busse et al. 2002, Busse et al. 1994, Jaggar et al. 1998, Nelson and Quayle 1995, Standen and Quayle 1998).

2.3 Vascular smooth muscle

2.3.1 Contraction and cellular calcium regulation

The contractile activity of smooth muscle depends on the level of free intracellular Ca^{2+} available to the contractile apparatus. Vasoconstrictor agents bind to cell surface receptors, which activate G proteins such as phospholipase C (metabolises phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol) and adenylate cyclase (metabolises ATP to produce cAMP) (Abdel-Latif 1986, Nishizuka 1995). Subsequently, IP_3 releases Ca^{2+} from intracellular stores by binding to IP_3 -receptors in SR (Nahorski et al. 1994). Vasoconstrictors can also depolarize arterial smooth muscle and activate voltage-dependent Ca^{2+} channels in the plasma membrane, which increases Ca^{2+} influx to the cell. Thus, $[\text{Ca}^{2+}]_i$ increases both via Ca^{2+} entry and Ca^{2+} release from intracellular stores (Allen and Walsh 1994). Intracellular Ca^{2+} stores can also be mobilised by Ca^{2+} -induced Ca^{2+} release: the influx of a small amount of Ca^{2+} releases Ca^{2+} from SR via so-called ryanodine receptors (Allen and Walsh 1994, Horowitz et al. 1996, Marks 1992). This mechanism can amplify the IP_3 -induced Ca^{2+} release (Finch et al. 1991, Nahorski et al. 1994).

In smooth muscle cytoplasm, Ca^{2+} forms a complex with calmodulin, thus taking out myosin light chain kinase (MLCK) autoinhibition, with a subsequent myosin light chain phosphorylation and myosin ATPase activation (Walsh 1994, Winder et al. 1998). Phosphorylated myosin binds to actin filaments, which produces the contraction of smooth muscle (Walsh 1994). When $[\text{Ca}^{2+}]_i$ is reduced, MLCK is inactivated, myosin light chain phosphatase dephosphorylates myosin, and relaxation follows (Allen and Walsh 1994, Cirillo 1992, Rembold 1992, Stull et al. 1991).

The influx of Ca^{2+} across the plasmalemma takes place via voltage-gated and receptor-operated Ca^{2+} channels, or via ion exchangers (Horowitz et al. 1996). Voltage-gated Ca^{2+} channels are divided into two subgroups: the T-type channel is activated by small depolarizations and is rapidly inactivated, while the L-type channel requires strong depolarizations and is slowly inactivated (Spedding and Paoletti 1992). The extrusion of Ca^{2+} from vascular smooth muscle takes place via a plasmalemmal ATP-driven Ca^{2+} pump or $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Allen and Walsh 1994, Horowitz et al. 1996). Ca^{2+} -ATPase predominantly accounts for cellular Ca^{2+} efflux, while the $\text{Na}^+/\text{Ca}^{2+}$ exchange is an antiporter, which can exchange one Ca^{2+} ion with the influx of three Na^+ ions (Cirillo 1992). In VSMCs the SR plays a predominant role in the storage of Ca^{2+} , and thus Ca^{2+} is both sequestered and released by SR following agonist-induced activation of receptors in the plasmalemma (DeLong and Blasie 1993, Martonosi et al. 1990, Minneman 1988). An ATP-driven Ca^{2+} pump in the membrane of SR actively transports Ca^{2+} ions from the cytosol into the SR (Allen and Walsh 1994, Horowitz et al. 1996, van Breemen and Saida 1989).

The contractile status of smooth muscle does not entirely depend on $[\text{Ca}^{2+}]_i$: mechanisms that alter the Ca^{2+} sensitivity of contractile machinery, in collaboration with the regulatory mechanisms for cellular Ca^{2+} metabolism, play an important role in the control of vascular tone (Somlyo et al.

1999, Takuwa 1996, Walsh 1994). Thus, contractile force can increase when the responsiveness of the contractile machinery, or sensitivity of the myofilaments, to $[Ca^{2+}]_i$ is enhanced (Andrea and Walsh 1992, Ruegg 1999). The Ca^{2+} sensitivity can change in response to alterations in calmodulin concentrations, myosin light chain phosphorylation (e.g. by the small G protein Rho A and Rho-associated kinase), and changes in myosin phosphatase activity (Hori and Karaki 1998, Winder et al. 1998). However, the general rule is that for smooth muscle contraction to take place, $[Ca^{2+}]_i$ must be elevated and Ca^{2+} entry increased, or storage of Ca^{2+} to cellular stores or the extrusion of Ca^{2+} must be decreased. Whether abnormalities in these mechanisms exist in VSMCs of hypertensive subjects remains unresolved (Gonzalez and Suki 1995).

2.3.2 $Na^+-K^+-ATPase$

The $Na^+-K^+-ATPase$ (sodium pump), is a plasma membrane enzyme that plays a pivotal role in VSMC homeostasis by upholding Na^+ and K^+ gradients between the intra- and extracellular space that are important for the maintenance of cell volume and tone. By the use of the energy from the hydrolysis of one molecule of ATP, it transports three Na^+ out in exchange for two K^+ that are taken in, and generates thus a hyperpolarizing current (Blanco and Mercer 1998, Kaplan 2002). Cardiac glycosides such as digoxin and ouabain inhibit vascular $Na^+-K^+-ATPase$ (Blaustein 1993, O'Donnell and Owen 1994). In patients with CRI, the concentrations of ouabain-like factors that inhibit $Na^+-K^+-ATPase$ may be elevated (Kelly et al. 1986). Labile sodium pump inhibitor identified from peritoneal dialysate of volume-expanded hypertensive patients with CRI has been reported to induce a qualitatively similar contraction to that aggravated by ouabain in rabbit aorta, suggesting that accumulation of one or more endogenous sodium pump inhibitors may contribute to uremic changes of vascular tone (Krep et al. 1996). The ouabain-like substances present in uremic sera have also been reported to increase the force of contraction and impair recovery of relaxation and $[Ca^{2+}]_i$ in cardiomyocytes (Periyasamy et al. 2001). Whether such uremic substances can induce inhibition of vascular $Na^+-K^+-ATPase$ sufficient to raise peripheral vascular resistance *in vivo* remains to be studied.

The functional changes of $Na^+-K^+-ATPase$ could affect nucleic acid synthesis and subsequently alter the proliferation of rat VSMCs, which could contribute to vascular remodelling also in CRI (Orlov et al. 2001). Recently, it has been reported that the inhibition of $Na^+-K^+-ATPase$ can activate proliferation of cultured human VSMCs (Abramowitz et al. 2003), ensuring the need of further studies concerning the role of endogenous $Na^+-K^+-ATPase$ inhibitors in uremic vasculopathy.

2.3.3 K⁺ channels

K⁺ channels have a substantial contribution to the regulation of VSMC membrane potential. Under physiological conditions, K⁺ concentration in the VSMC is approximately 25-fold higher than in the extracellular fluid. An increase in membrane permeability to K⁺ induces an outward current that opposes the depolarization and cellular excitability, and leads to a loss of positive charge and hyperpolarization of cell membrane (Jackson 1998, Quast et al. 1994). The known types of K⁺ channels in vascular smooth muscle are K_{Ca}, K_{IR}, ATP-sensitive K⁺ channels (K_{ATP}), and voltage-activated K⁺ channels (K_V) (Jackson 1998, Jackson 2000, Jackson and Blair 1998, Jackson et al. 1997). All these channels contribute to the local control of arterial tone by determining the membrane potential, which in turn controls [Ca²⁺]_i and thus contraction of smooth muscle (Standen and Quayle 1998).

Pharmacological agents such as cromakalim, levcromakalim, diazoxide and pinacidil can dilate arteries via the opening of K_{ATP} (Jackson 2000, Quast et al. 1994). Vice versa, the sulphonylureas glibenclamide and glimepiride can block these channels and thus cause arterial contractions (Ravel et al. 2003). Physiologically the K_{ATP} close if intracellular ATP concentration increases, whereas an elevation in cytosolic adenosine 5'-diphosphate or intracellular acidification raises the open state probability of these channels (Ishizaka et al. 1999, Nelson and Brayden 1993). In experimental CRI, an impaired vasorelaxation to cromakalim has been reported, suggesting that uremia can result in reduced action of K_{ATP} in vascular smooth muscle (Kalliovalkama et al. 1999a).

K_{Ca} channels in VSMCs are opened by the potent vasodilators EETs, which are produced by the vascular endothelium and hyperpolarize VSMCs (Sacerdoti et al. 2003). Therefore, it has been proposed that one or more EETs may serve as EDHF. Furthermore, the increases of [Ca²⁺]_i as well as membrane depolarization can also activate K_{Ca} (Carl et al. 1996, Nelson and Quayle 1995). The most important subtype of K_{Ca}, BK_{Ca}, largely contributes to the regulation of arterial tone by adjusting the resting membrane potential, whereas the selective inhibitor of BK_{Ca} iberiotoxin can cause membrane depolarization and contraction of smooth muscle (Amberg et al. 2003). The normal function of BK_{Ca} could provide protective limitation against increased vascular tone, since these channels have been reported to prevent vasospasm via a negative feedback mechanism (Amberg et al. 2003, Jackson 2000). Beside EETs, BK_{Ca} can be opened by carbon monoxide (Wang and Wu 1997) and vasodilators that act through the cGMP and cAMP cascades (Nelson and Quayle 1995, Paterno et al. 1996). Metabolic disturbances associated with CRI may affect the function of K_{Ca} channels. For instance, increased PTH levels due to SH have been suggested to increase the synthesis of K_{Ca} blocker 20-hydroxyeicosatetraenoic acid in renal tubular cells (Roman 2002). Moreover, in tracheal smooth muscle, the activation of type 1 PTH receptors can lead to increased intracellular cAMP and subsequent activation of BK_{Ca}, and thus initiation of the relaxation of smooth muscle (Shenberger et al. 1997). The expression of type 1 PTH receptors has been reported

to be down-regulated in uraemia (Disthabanchong et al. 2004), which could thus suppress the activity of K^+ channels in CRI.

K_V are another class of K^+ channels described widely in VSMCs (Nelson and Quayle 1995). These channels are activated by membrane depolarization and they participate in the regulation of resting membrane potential (Jackson 1998, Jackson et al. 1997, Nelson and Quayle 1995). K_V blockade by 4-aminopyridine increases the amplitude and duration of spontaneous contractions of VSMCs (Cogolludo et al. 2001). K_V play critical role in controlling pulmonary arterial tone under physiological and pathological conditions, whereas the inhibition of these channels can induce significant vasoconstriction in rat pulmonary circulation via depolarization and activation of L-type Ca^{2+} channels (Cogolludo et al. 2003). A recent *in vitro* study suggests that in rat conductance arteries K_V are involved in the control of oscillatory activity and thus contribute to the regulation of vascular excitability and contractility (Tammaro et al. 2004).

The phenomenon that modest elevation of K^+ concentration (for instance by the application of 6 to 16 mM KCl) induces relaxation in precontracted small arteries has been attributed to the action of K_{IR} (Knot et al. 1996). In isolated arteries, micromolar concentrations of Ba^{2+} can be used to block K_{IR} to distinguish K_{IR} -induced vasodilatation from responses mediated via the activation of other K^+ channels (Quayle et al. 1997). K_{IR} are the least characterized from four known classes of K^+ channels due to their restricted expression in resistance vessels, whereas in most conduit vessels K_{IR} are absent. The physiological importance and density of K_{IR} increase with decreasing vessel calibre. K_{IR} have been established in coronary and cerebral small arteries (Knot et al. 1996), but it has also been shown that K_{IR} are a major determinant of resting membrane potential and tone in renal afferent arterioles (Chilton and Loutzenhiser 2001). The existence of K_{IR} in renal microvessels provides a putative explanation to the earlier findings that mild elevation of plasma K^+ concentration leads to an increase of renal blood flow and GFR (Budtz-Olsen et al. 1975, Lin and Young 1988). Thus, the action of K_{IR} could be notable factor that contributes to the regulation of renal hemodynamics. Whether uremic conditions can influence K_{IR} , would be of interest, but remains to be studied.

Finally, a link between Ca^{2+} metabolism and the control of arterial tone via K^+ channels is the extracellular Ca^{2+} receptor located in the perivascular sensory nerves: the activation of this receptor by increased extracellular Ca^{2+} concentration can induce vasorelaxation via the release of a hyperpolarizing mediator that acts on K^+ channels in the underlying smooth muscle (Bukoski 1998, Ishioka and Bukoski 1999).

3 Arterial tone and structure in chronic renal insufficiency

Epidemiological studies have shown that damage of large conduit arteries is a major factor that contributes to morbidity and mortality in patients with end-stage renal disease (USRDS 1998). The arterial system of CRI patients undergoes remodelling that is characterized by dilatation and intima-

media hypertrophy of conduit arteries, and isolated wall hypertrophy of peripheral muscular-type conduit arteries. These changes are caused by vasculopathic and atherogenic factors related to uremia such as homocysteine, asymmetric dimethylarginine (ADMA) and other endogenous NO synthase inhibitors, oxidative stress, inflammation, and accumulation of oxidized high-density lipoproteins (Annuk et al. 2003, Morris et al. 2001, Stenvinkel et al. 1999). Elevated plasma levels of ET-1 may also contribute to the cardiovascular remodelling in CRI patients (Demuth et al. 1998).

Several studies have shown that regulation of arterial tone is disturbed in patients with CRI as well as in experimental models of uremia. Endothelium-dependent vasodilatation has been shown to be impaired in hemodialysis patients (van Guldener et al. 1997), and in uremic rats (Kalliovalkama et al. 1999a). Endothelial dysfunction may predispose the patient to accelerated atherosclerosis and may be involved in the pathogenesis of secondary hypertension in CRI. Moreover, in a recent study the impaired endothelial function was associated with all-cause mortality of patients with advanced CRI, independently of the presence of end-organ damage such as left ventricular hypertrophy or arteriosclerosis (London et al. 2004). There is evidence that endothelial dysfunction can be improved by kidney transplantation in end-stage renal failure patients (Passauer et al. 2003). Endothelial dysfunction may also contribute to the development and progression of renal insufficiency, and because endothelial dysfunction is detected at an early phase in the process of renal injury, it appears to be an attractive target for therapy (Rabelink and Koomans 1997).

As suggested by many recent reports, the elevation of endogenous inhibitors of NO synthesis could play an important role in the development and progression of CRI and its cardiovascular complications. ADMA, an endogenous NO synthesis inhibitor that is present also in normal human plasma, has been found to accumulate in CRI (Kielstein et al. 1999, Vallance et al. 1992). This may contribute to the changes in arterial function and subsequent secondary hypertension in CRI (Kielstein et al. 1999, MacAllister et al. 1996, Vallance et al. 1992). However, a lack of relationship between plasma ADMA and creatinine levels has been reported in CRI patients with low total NO production (Schmidt and Baylis 2000). Interestingly, the vascular expression of NO synthase has been reported to be unaltered or even augmented in experimental CRI, probably in order to oppose the elevation of systemic BP (Aiello et al. 1997, Vaziri et al. 1998b).

3.1 Conductance arteries

Clinical studies have depicted that BP values in CRI patients are most frequently characterized by increased systolic BP with normal or even low diastolic BP, or high pulse pressure (London et al. 1998). This hemodynamic pattern is a consequence of vascular remodelling, characterized by dilation and increased wall thickness and stiffness of conduit arteries (London et al. 1996). In CRI, the remodelling of conductance vessels includes medial calcifications, increased extracellular matrix content and hyperplasia of smooth muscle (Amann et al. 1997, Hafner et al. 1995). It is of

note that end-stage renal disease frequently causes coronary artery calcification in adult patients already at a very young age (Goodman et al. 2000).

The intima-media thickness of carotid arteries and aortic stiffness are increased in nondiabetic CRI patients already before they start hemodialysis, suggesting that the remodelling and increased stiffness of large arteries are not due to the hemodialysis treatment, but rather to renal insufficiency *per se* or due to the associated secondary metabolic abnormalities (Shinohara et al. 2004, Shoji et al. 2002). In patients with advanced CRI, arterial remodelling and increased arterial stiffness measured from aortic pulse wave velocity are strong independent predictors of cardiovascular mortality (Blacher et al. 1999). It has been shown that long-term antihypertensive treatment, which effectively lowered BP, also significantly decreased aortic pulse wave velocity and wave reflections in large part of patients, and improved their survival (Guérin et al. 2001). In CRI patients whose pulse wave velocity decreased in parallel with BP, survival was much better than in those patients whose pulse wave velocity did not respond to BP decrease (Guérin et al. 2001).

As already stated above, in addition to the structural changes, growing evidence suggests that CRI is associated with impaired endothelial vasomotor function (Annuk et al. 2003, Rabelink and Koomans 1997). Even in patients with mild renal insufficiency and negligible signs of atherosclerosis, endothelial dysfunction of large arteries has been reported, suggesting that CRI *per se* may directly promote the development vascular disease (Thambyrajah et al. 2000). In the brachial arteries of hemodialysis patients, the impaired vasodilatation has been attributed to reduced NO-mediated endothelium-dependent vasodilatation (Joannides et al. 1997). Besides the reduced endothelium-dependent relaxation via NO, the EDHF-mediated vasodilatation was found to be depressed in the arteries of hypertensive NTX rats (Kimura and Nishio 1999). In normotensive rats with moderate renal insufficiency, the NO-mediated vasodilatation was actually preserved, whereas endothelium-mediated vasorelaxation was impaired via mechanism that involved K^+ channels in arterial smooth muscle (Kalliovalkama et al. 1999a).

3.2 Resistance arteries

Despite the fact that the morphological changes of large arteries in CRI have been well described, the knowledge regarding the remodelling of resistance arteries in the uremic conditions is scarce. Substantial increase in wall thickness of small intramyocardial arteries has been reported in rats with CRI (Nabokov et al. 1999), while high dietary phosphorus intake and the subsequent hyperphosphatemia have been shown to aggravate arteriolar wall thickening (Amann et al. 2003a). The increase in intimal and medial thickness of small mesenteric arteries has been abrogated by ET_A receptor antagonism, suggesting an important role of ET-1 in mechanisms of small artery remodelling in uremia (Amann et al. 2001a).

In CRI patients, advanced morphological changes of small arteries feature calcification in the tunica media and proliferation in the intima (Goodman 2001). Clinical manifestation of small artery

calcification in patients with end-stage renal disease is characterized by painful skin lesions, tissue ischemia, and necrosis of the skin and subcutaneous tissue, and known as calcific uremic arteriopathy or “calciophylaxis” (Gipstein et al. 1976). The concentrations of calcium and phosphate are physiologically perched at levels within the solubility product. In advanced renal insufficiency, when the serum calcium-phosphate product exceeds $60 \text{ mg}^2/\text{dl}^2$ ($4.8 \text{ mmol}^2/\text{l}^2$), widespread tissue deposition of amorphous calcium phosphate can occur (Goodman 2001). In many CRI patients with calciophylaxis, marked clinical improvement of skin lesions after parathyroidectomy has been reported, suggesting that severe SH is an important pathogenetic factor in that vascular complication (Hafner et al. 1995). Interestingly, it has been reported that treatment with the bisphosphonate pamidronate resulted in rapid improvement of clinical condition in one patient suffering from calciophylaxis (Monney et al. 2004). In concert, earlier experimental studies have shown that bisphosphonates may have beneficial effects on the development of experimental calciophylaxis (Miller et al. 1984, Price et al. 2002). However, the pathogenesis of calciophylaxis remains poorly understood and its treatment largely empirical being mainly based on the correction of the disturbances in calcium–phosphate metabolism.

The functional disturbances of resistance arteries in CRI have not been studied as thoroughly as those in larger arteries, and several results concerning this topic are inconsistent. Nevertheless, endothelial dysfunction of small arteries may contribute to the development of secondary hypertension in CRI (Morris et al. 2001). However, disturbed regulation of resistance artery tone may also exist in renal patients as a consequence of secondary hypertension or other cardiovascular complications. In a recent study, reduced dilatation using non-invasive laser Doppler flowmetry was shown in arterioles of end-stage renal failure patients without cardiovascular disease and diabetes (Stewart et al. 2004). In these patients, the post-occlusion reactive hyperaemia and thermal hyperaemic responses were impaired, and also a reduction in the number of functional arterioles was observed (Stewart et al. 2004). Impaired endothelium-dependent relaxation has also been reported in isolated subcutaneous resistance vessels from normotensive patients with polycystic kidney disease, without changes in morphological variables (Wang et al. 2000a). Similarly, reduced vasodilatation to ACh, but normal vasodilatation to sodium nitroprusside (SNP) has been reported in uremic human subcutaneous resistance arteries (Morris et al. 2001), suggesting endothelial dysfunction and unaltered responsiveness of arterial smooth muscle to exogenous NO in small vessels. However, these studies failed to determine which component(s) of the endothelium-mediated relaxation contribute to the observed changes. The involvement of reduced NO synthesis in the endothelial cells was speculated but not addressed (Morris et al. 2001), whereas the relative roles of EDHF and PGI_2 in vasorelaxation were not studied in the above reports.

In contrast to the studies above, it has also been reported that endothelium-dependent and -independent vasorelaxation responses of skin microvessels of normotensive CRI patients were not different from those observed in healthy controls (Cupisti et al. 2000). Moreover, in experimental

animals with moderate CRI, the endothelium-mediated relaxations of resistance arteries were not different from the responses of corresponding healthy controls (Thuraisingham and Raine 1999).

AIMS OF THE PRESENT STUDY

The objective of the present series of investigations was to examine the control of arterial tone at different stages of experimental chronic kidney disease. In moderate CRI, the effect of long-term AT₁ receptor antagonism on resistance and conductance artery tone was studied. Furthermore, the changes in the regulation of resistance artery tone induced by modifications of the calcium-phosphate balance were examined in moderate and advanced CRI. Finally, the effects of high calcium intake on the expression of renal ACE and ectopic calcification in renal and aortic tissues were elucidated in experimental CRI.

The detailed aims were:

1. To examine the influence of AT₁ receptor antagonism by long-term losartan treatment on resistance artery tone and morphology and aortic ACE expression in moderate experimental CRI.
2. To study the effect of 8-week losartan treatment on the regulation of conductance artery tone in a rat model of moderate renal insufficiency.
3. To investigate the effect of the treatment of SH by high calcium intake on the tone of resistance arteries in experimental animals with moderate CRI.
4. To study the influences of diet-induced changes in calcium-phosphate balance on the regulation of small artery tone in NTX rats with advanced CRI.
5. To determine the influence of high calcium intake on renal components of RAS and kidney morphology in moderate and advanced CRI.

MATERIALS AND METHODS

1 Experimental animals

Normotensive male Sprague-Dawley rats were obtained from the colony of the Medical School at the University of Tampere. The rats were housed two to a cage in a standard animal laboratory room (temperature +22°C, a controlled environmental 12 h light-dark cycle). The studies were approved by the Animal Experimentation Committee of the University of Tampere, and by the Provincial Government of Western Finland, Department of Social Affairs and Health.

2 Diets and drug treatments

All animals in studies I and II received standard laboratory food pellets containing 0.9% calcium, 0.8 % phosphorus, 0.27 % sodium, 0.2 % magnesium, 0.6 % potassium, 1500 IU/kg vitamin D, and 12550 kJ/kg energy AnalyCen, Lindköping, Sweden). In studies III, IV and V the control chow contained 0.3% calcium and 0.5% phosphorus, whereas the high calcium diet contained 3.0% calcium and 0.5% phosphorus (modified chow also manufactured by AnalyCen, Sweden). In study IV the high phosphorus diet contained 0.3% calcium and 1.5% phosphorus. Extra calcium was supplied as the carbonate salt (III, IV, V), and extra phosphorus was provided as the phosphate salt (IV), otherwise the chows were identical.

All rats were freely provided with tap water, while the losartan-treated animals in studies I and II received losartan (20 mg/kg/day) in their drinking fluid. The daily-prepared solutions were kept in lightproof bottles.

3 Blood pressure measurements

The systolic BPs of conscious rats restrained in plastic holders were measured indirectly by the tail cuff method at +28°C. All measurements were performed with an IITC Inc. Model 129 Blood Pressure Meter (Woodland Hills, California, USA) equipped with a photoelectric pulse detector. The BP of each rat was obtained by averaging three reliable recordings.

4 Anaesthesia, 5/6-nephrectomy and sham-operation.

At the age of 8 weeks, the 5/6-nephrectomy or sham-operation were performed under ketamine/diazepam anaesthesia. The anaesthetics were given intraperitoneally, 75 mg/kg and 2.5 mg/kg, respectively. In the nephrectomized groups, surgical resections of the upper and lower poles were performed, comprising about 2/3 of the left kidney, followed by contralateral nephrectomy (Ylitalo et al. 1976). In the sham group both kidneys were decapsulated. Antibiotics (metronidazole

60 mg/kg, cefuroxim 225 mg/kg) were given postoperatively, and pain was relieved with buprenorphine (0.2 mg/kg, 3 times daily, 3 days).

5 Urine collection and measurement of fluid intake

Urine was collected for 24 h individually in metabolic cages where the animals had free access to food and water. Urine volumes were measured and samples stored at -20°C. The consumption of drinking fluid was measured by weighing the bottles after a 24 h period.

6 Blood and tissue samples

The rats were anaesthetised by the intraperitoneal administration of urethane (1.3 g/kg) and the carotid arteries were cannulated. Blood samples were drawn into chilled tubes containing EDTA and glass capillaries on ice containing heparin as anticoagulant, after which the tubes with samples were centrifuged, and the plasma stored at -70°C until analysis. After exsanguination, the thoracic and abdominal cavities of the animals were opened, the hearts and the kidneys removed and weighed. The tissue samples were frozen in liquid nitrogen and stored at -70°C until analyses.

7 Biochemical determinations

7.1. Plasma renin activity, electrolytes, urea nitrogen, phosphate, creatinine, proteins, PTH, 1,25(OH)₂D₃, 25OH-D₃, ionised calcium, haemoglobin, urine albumin and calcium

PRA (V) was determined by radioimmunoassay (Ang I RIA kit, Diasorin S.p.A., Italy) according to the manufacturer's instructions. Plasma sodium and potassium concentrations were measured by potentiometric direct dry chemistry, urea nitrogen by colorimetric enzymatic dry chemistry, and phosphate (III, IV, V) by colorimetric end-point dry chemistry (Vitros 950 analyzer, Johnson & Johnson Clinical Diagnostics, Rochester, New York, USA). Creatinine was determined by the kinetic colorimetric assay according to Jaffe, and plasma proteins (III, IV, V) were measured by colorimetric measurement according to Biuret (Cobas Integra analyzer, F. Hoffman-La Roche Ltd, Diagnostics Division, Basel, Switzerland). Creatinine clearance was derived using the standard formula: creatinine clearance = (urine creatinine X urine volume) / plasma creatinine. PTH (II, III, IV, V) levels were measured by an immunoradiometric assay specific for intact rat PTH (Immutopics, San Clemente, California, USA), and vitamin D (III, V) by radioassay designed for the quantitative determination of 1,25(OH)₂D₃ (competitive protein-binding assay, Nichols Institute Diagnostics, San Juan Capistrano, California, USA). In study IV, plasma 1,25(OH)₂D₃ and 25-hydroxyvitamin D₃ (25OH-D₃) were determined by the use of commercial kits (IDS Ltd). Ionised calcium (I, II, III, IV, V) was measured by an ion selective electrode (Ciba Corning 634 Ca²⁺/pH

Analyzer, Ciba Corning Diagnostics, Sudbury, UK). Haemoglobin was determined by photometric analysis using Technicon cyanide free haemoglobin reagent (Technicon H*2™, Technicon Instruments Corporation, Tarrytown, New York, USA). Urine albumin (V) was determined by nephelometry (Behring Nephelometer 100 analyzer, Behringwerke, Marburg, Germany) using rabbit anti-rat albumin antibodies (Cappel, Cochranville, Philadelphia, Pennsylvania, USA) as reported earlier (Luimula et al. 2000). Urine calcium (IV, V) was measured by flame photometry (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany).

7.2. Isolation and analysis of cytoplasmic RNA (I, IV)

Total RNA was isolated from ventricular samples by the guanidine thiocyanate-CsCl method (Chirgwin et al. 1979). For Northern blot analysis, 20 µg sample of RNA from ventricular tissue was transferred to MAGNA nylon membrane (Osmonics Inc.). PCR-amplified probes corresponding to bases 76-509 of rat BNP (GeneEMBL access number M25297), an oligonucleotide probe complementary of rat ribosomal 18S RNA (M11188) (Majalahti-Palviainen et al. 2000) and a full-length rat ANP cDNA probe Car-55 (provided by Dr. Peter L. Davies, Queen's University, Kingston, Canada) were labelled with [³²P]-dCTP with Quick Prime Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). The membranes were hybridised overnight at +42°C in 5 x SSC (saline sodium citrate, 1 x SSC = 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate, pH 7), 0.5% sodium dodecyl sulphate, 5 x Denhardt's solution, 50% formamide and 100 µg/ml sheared herring sperm DNA. After hybridization, the membrane was washed in 0.1 x SSC, 0.1% sodium dodecyl sulphate three times for 20 min at +60°C and exposed to Phosphor Screen (Eastman Kodak) at room temperature. Radioactivity was measured with PhosphorImager SI equipment using ImageQuant software (Molecular Dynamics). The signals of ANP and BNP mRNA were normalized to 18S in each sample to correct for potential differences in loading and/or transfer.

7.3. Radioimmunoassay of BNP and NT-proANP (I, IV)

Tissue and plasma samples were extracted by Sep-Pak C₁₈ cartridges (Ruskoaho et al. 1989). Eluates were lyophilised and redissolved in radioimmunoassay buffer. Plasma immunoreactive N-terminal pro-atrial natriuretic peptide (NT-proANP; I) was determined by radioimmunoassay without prior extraction (Vuolteenaho et al. 1992). For the BNP radioimmunoassay (I, IV), the ventricular guanidine thiocyanate extracts were diluted 50-fold. The extracted samples were incubated in duplicate with the specific rabbit BNP (Kinnunen et al. 1993). Synthetic rat BNP₅₁₋₉₅ was incubated as standard. The BNP and NT-proANP tracers were prepared by chloramine-T iodination of synthetic rat [Tyr₀]-BNP₅₁₋₉₅ followed by reverse phase HPLC purification. After incubation for 48 h at +4°C, the ¹²⁵I-labelled rat [Tyr₀]-BNP₅₁₋₉₅ with normal rabbit serum were

added. After incubation for another 24 h at +4°C the immunocomplexes were precipitated with sheep antiserum directed against rabbit gammaglobulin in the presence of 8% polyethylene glycol 6000, pH 7, followed by centrifugation at 3000 g for 30 min. The plasma samples were incubated with the rabbit NT-proANP antiserum overnight at +4°C and the bound and free fractions were separated with double antibody in the presence of polyethylene glycol. The sensitivities for BNP and NT-proANP determinations were 0.5 fmol/tube and 0.75 fmol/tube, respectively. The 50% displacements of the respective standard curves were at 4.3 fmol/tube for BNP and at 10 fmol/tube for NT-proANP. The intra- and interassay variations of BNP was less than 10%. Serial dilutions of tissue extracts showed parallelism with the standards. The BNP antiserum did not recognise ANP or C-type natriuretic peptide.

7.4 In vitro autoradiography of aortic ACE (I), kidney ACE (V) and renal Ang II receptors (II, V)

Frozen aortic and kidney sections (20 µm thick) were cut on a cryostat at -17 °C, mounted onto Super Frost[®] Plus slides, dried in a dessicator under reduced pressure at +4 °C overnight and stored at -80 °C with silica gel until further processing (Bäcklund et al. 2001). Quantitative *in vitro* autoradiography of ACE and Ang II receptors was performed on 20 µm aortic or kidney tissue sections with the radioligands [125I]-MK351A and [125I]-Sar1,Ile8-Ang II, respectively (Bäcklund et al. 2001, Zhuo et al. 1999). The density of AT₁ receptors was determined in the presence of the AT₂ antagonist PD 123,313 (10 µM), while the density of AT₂ receptors was measured in the presence of the AT₁ antagonist losartan (10 µM). The optical densities were quantified by AIDA computer image analyzing system (AIDA 2D densitometry) coupled to the FUJIFILM BAS-5000 phosphoimager (Tamro, Finland). Specific binding was calculated as total binding minus non-specific binding.

7.5 Western blotting of renal ACE (V)

Frozen tissues (100 mg) were lysed in 1 ml of sodium dodecyl sulphate buffer containing 10 mM Tris HCl, pH 7.4, 2% sodium dodecyl sulphate, and protein inhibitors (Complete[™] Mini EDTA-free, Roche Diagnostics, Mannheim, Germany). The homogenate was stored on ice before removal of nuclear and other debris by centrifugation (10.000 g, 15 min). Protein concentrations in the extracts were determined by Bio-Rad Protein Assay system (Bio-Rad Laboratories Inc., Richmond, CA, USA). Aliquots of homogenate containing 50 µg of protein in loading buffer (10% glycerol, 2% sodium dodecyl sulphate, 60 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue, and 100 mM dithiothreitol) were boiled for 5 minutes before electrophoresis on 12% sodium dodecyl sulphate-polyacrylamide gels. The proteins in the gel were electrophoretically transferred to Immun-Blot PVDF membrane (Bio-Rad Laboratories Inc.) in 25 mM Tris-HCl, pH 8.0, 192 mM glycine, and 20% methanol at 50 Volts overnight. After washing in H₂O and TBS-T (20 mM Tris-HCl, pH 7.6,

136 mM NaCl, 0.3% Tween-20), membranes were blocked in 5% non-fat milk powder in TBS-T (room temperature, 1 hour), and incubated for 3 hours with goat polyclonal antibody against rat ACE (Santa Cruz Biotechnology Inc., California, USA) diluted 1:200 in 5% milk in TBS-T buffer. After extensive washing with 2.5% milk/TBS-T buffer, membranes were incubated with 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG for 1 hour (Sigma-Aldrich Co., St. Louis, MO, USA). Antibody binding was detected by chemiluminescence (WB Chemiluminescent Reagent plus, NEN Inc., Boston, MA, USA), and the autoradiograph was analyzed with Image Gauge 3.3 software (Fuji Photo Film Co., Japan).

8 Mesenteric arterial responses *in vitro*

8.1. Arterial preparations and organ bath solutions

The superior mesenteric arteries (II) were carefully cleaned of adherent connective tissue, excised, and placed on a Petri dish containing physiological salt solution (PSS; pH=7.4) of the following composition (mM): NaCl 119.0, NaHCO₃ 25.0, glucose 11.1, KCl 4.7, CaCl₂ 1.6, KH₂PO₄ 1.2 and MgSO₄ 1.2, and aerated with 95 % O₂ and 5 % CO₂. Standard sections of the mesenteric artery (3 mm in length) were cut, beginning 3 mm distally from the mesenteric artery-aorta junction. The endothelium was either left intact or removed by gently rubbing it with a jagged injection needle (Arvola et al. 1992). The rings were placed between stainless steel hooks (diameter 0.3 mm) and mounted in an organ bath chamber (volume 20 ml) in PSS described above. The small second (IV) or third order (I, III) branches from the mesenteric arterial bed were carefully excised under a dissecting microscope (Nikon SMZ-2T, Nikon Inc., Japan) and mounted over two 40 µm wires in a small organ bath chamber (volume 5 ml) containing PSS. The endothelium was left intact or removed by perfusing air through the vascular lumen. The preparations were aerated with 95% O₂ and 5 % CO₂ at +37°C, and rinsed with fresh solutions at least every 20 min, during which time the pH in the baths remained stable. In solutions containing high concentrations of K⁺ (20-125mM), NaCl was replaced with KCl on an equimolar basis.

8.2. Arterial contractile and relaxation responses

In study II the arterial rings were initially equilibrated for 1 h at +37°C with a resting preload of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FT 03 transducer and Model 7 E Polygraph; Grass Instrument Co., Quincy, MA, USA). The presence of the functional endothelium in vascular preparations was confirmed by a clear relaxation response to 1 µM ACh in NA-precontracted arterial rings, and the absence of endothelium by the lack of this response. If any relaxation was observed in the endothelium-denuded rings, the endothelium was further rubbed. In studies I, III and IV a Mulvany

multimyograph Model 610A (J.P. Trading, Aarhus, Denmark) was employed for experiments with vascular preparations. In this system the isometric micromyographs consist of two jaws, one of which is connected to a length displacement device and the other to a force transducer linked to a computer with Myodaq software (J.P. Trading, Aarhus, Denmark). The small arterial rings were placed over two thin wires, each of which was attached to one of the myograph jaws. Normalisation of the vascular preparations was then performed so that the internal diameter of the vessel was set at 90 % of that obtained when exposed to an intraluminal pressure of 100 mmHg in the relaxed state (Mulvany and Halpern 1977). The presence of intact endothelium in the vascular preparations was confirmed by a clear relaxation to 1 μ M ACh in NA-precontracted rings, and the absence of endothelium by the complete lack of this response.

Agonist-induced contractions. The cumulative contractions of the endothelium-intact preparations to NA (I – IV) and Ang II (I) were studied. In study II, the contractions elicited by ET-1 were investigated in the endothelium-intact preparations in the presence and absence of 0.1 mmol/L N^G-nitro-L-arginine methyl ester (L-NAME) and 3 μ mol/L diclofenac.

Depolarization-induced contractions. The concentration-response curves of the endothelium-denuded rings to KCl were determined.

Endothelium-dependent relaxations to ACh. Mesenteric arterial relaxations were studied in response to ACh in rings precontracted with NA (1 μ M in II; 5 μ M in I, III and IV). The ACh-induced relaxations after NA-precontraction were also elicited in the presence of 0.1 mM L-NAME (I - IV), L-NAME and 3 μ M diclofenac (I - IV), L-NAME, diclofenac and apamin (50 nM) plus charybdotoxin (0.1 μ M) (I, II, III), and L-NAME, diclofenac and apamin (50 nM) plus iberiotoxin (0.1 μ M) (IV). The responses to ACh were further studied in the presence of 1 mM L-arginine (II); and in the presence of 1 mM L-arginine plus 0.1 μ M SQ-30741 (II). Furthermore, the relaxations to ACh were investigated in rings precontracted with KCl (50 mM) (I).

Endothelium-independent relaxations to SNP, isoprenaline, levcromakalim and EET. The relaxation responses of NA-precontracted endothelium-denuded rings to SNP were examined (I - IV). The vasorelaxations elicited by isoprenaline (I, II, III) and levcromakalim (I, II, III) were studied in endothelium-denuded rings precontracted with NA. In studies III and IV, the relaxation responses to EET were examined after precontraction with NA.

9 Morphological studies

9.1 Morphology of mesenteric resistance arteries

The morphology of small vascular rings from the second (IV) or third order (I, III) branches of the rat superior mesenteric arterial bed was examined with a pressure myograph (Living Systems Instrumentation Inc., Burlington, Vermont, USA). Second (IV) or third order (I, III) branches from the rat superior mesenteric arterial bed (2-6 cm prior to the ileocecal junction) were carefully

excised. A segment (3 mm in length) of the artery was isolated under a dissection microscope (Nikon SMZ-2T, Nikon Inc., Japan) and transferred to the myograph chamber containing 8 ml of PSS aerated with 95% O₂ and 5% CO₂. The proximal end of the vessel was cannulated with a micropipette and flushed to remove the remaining blood before the cannulation of the distal end. Then arteries were deactivated by perfusing extraluminally with PSS containing 30 mmol/l EDTA. Thereafter the intraluminal pressure was slowly raised to 140 mm Hg with a servocontrolled pump (Pressure servo control, Living Systems Instrumentation Inc., Burlington, Vermont, U.S.A.) and the axial length of the arterial segment was adjusted by carefully moving the cannula until the artery was unbuckled and the vascular walls were parallel. After intravascular pressure was established, the arterial segments were checked for leaks, which were identified by a reduction of the intraluminal pressure. The arterial segments were then equilibrated for 40 min in 60 mm Hg. Thereafter the intravascular pressure was increased to 100 mm Hg by the use of the servocontrolled pump and the arteries were allowed to equilibrate for 1 minute. Wall thickness and lumen diameter were then recorded by the use of a video monitoring system (Video dimension analyzer, Living Systems Instrumentation Inc., Burlington, Vermont, USA).

9.2 Morphological analyses of the kidneys and aorta (V)

Five- μ m sagittal kidney sections were stained either with hematoxylin-eosin and periodic acid-Schiff (PAS) or von Kossa stain, and processed for light microscopic evaluation. An expert who was blinded to the treatment of the rats quantified kidney tissue histology. Five- μ m transversal sections of thoracic aorta were stained with von Kossa stain.

Glomerulosclerosis (hematoxylin-eosin and PAS stain): a score for each animal was derived by examining 100 systematically sampled glomeruli at a magnification of X 400. The severity of scarring was expressed at the following arbitrary scale: 0=normal, 1=mesangial expansion or thickening of basement membrane, 2=mild or moderate segmental glomerular hyalinosis/sclerosis involving < 50% of the tuft, 3=diffuse glomerular hyalinosis/sclerosis involving > 50% of the tuft, 4=diffuse glomerulosclerosis, total tuft obliteration and collapse. The index for each rat was expressed as the mean score of the calculated 100 glomeruli (Schwarz et al. 1998).

Tubulointerstitial damage (hematoxylin-eosin and PAS stain): a scoring system was applied (from 0 to 4), in which tubular atrophy, dilation, casts, interstitial inflammation, and fibrosis were assessed in 10 kidney fields at a magnification of X 100: 0=normal, 1=lesions in < 25% of the area, 2=lesions in 25-50% of the area, 3=lesions in > 50% of the area, 4=lesions involving the entire area (Schwarz et al. 1998).

Calcification (von Kossa stain): All foci of calcification per entire kidney section were counted, and the number of calcifications was related to sample area (cm²). Calcifications were also measured from aortic sections at X 200 magnification. The total area of each aortic section, and area of calcification, was measured by a computerized interactive system (Scion Image Beta 4.02,

Frederick, Maryland, USA). The index of calcification for each rat was expressed as percentage of the calcified area related to the total area of the aortic cross-section.

10 Immunohistochemistry of CTGF (V)

Five- μ m-thick kidney samples were processed as described previously (Inkinen et al. 2003). The samples were incubated in blocking serum, and primary polyclonal antibody against mouse CTGF that cross-reacts with rat CTGF (ab6992, 1:400, Abcam, Cambridge, UK) was applied for 60 min at RT. Then the slides were incubated for 30 min with biotinylated secondary antibody (anti-rabbit IgG, Vector Laboratories, Burlingame, USA), and for 30 min with peroxidase labelled biotin-avidin-complex using a commercial Elite ABC kit (Vector Laboratories, California, USA). The colour reaction was developed by incubation for 15 min in a 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Finally, the sections were counterstained with Mayer's hemalum and mounted. Negative controls were treated with blocking serum with and without non-specific IgG instead of the primary antibody. Positive CTGF label in tissue was scored from 0 to 3 using light microscope (Inkinen et al. 2003).

11 Compounds

The following drugs and chemicals were used: ACh chloride, Ang II, apamin, charybdotoxin, EET, ET-1, iberiotoxin, isoprenaline hydrochloride, NA bitartrate, L-NAME hydrochloride, (Sigma Chemical Co., St. Louis, Missouri, USA), levcromakalim (SmithKline Beecham AB, West Sussex, U.K.), ketamine (Parke-Davis Scandinavia AB, Solna, Sweden), cefuroxim, diazepam (Orion Pharma Ltd., Espoo, Finland), metronidazole (B. Braun AG, Melsungen, Germany), buprenorphine (Reckitt & Colman, Hull, U.K.), SNP (Fluka Chemie AG, Buchs SG, Switzerland), diclofenac (Voltaren[®] injection solution, Ciba-Geigy, Basle, Switzerland) and losartan potassium (Merck Pharmaceutical Company, Wilmington, DE, USA). The stock solutions of the compounds used in the *in vitro* studies were made by dissolving the compounds in distilled water, with the exception of levcromakalim (in 50 % ethanol), and EET (in 99% ethanol). Drinking fluids containing losartan were made by dissolving the compound in tap water. All solutions were freshly prepared before use and protected from light. The chemicals used in the preparation of PSS were of highest grade available and obtained from E. Merck AG (Darmstadt, Germany).

12 Analyses of results

The statistical analysis was performed using one-way and two-way analyses of variance (ANOVA) supported by Bonferroni test or Least Significant Difference test when carrying out pairwise comparisons between the study groups. If variable distribution was skewed, the Kruskal-Wallis and

Mann-Whitney U-tests were applied, and *p* values were corrected with the Bonferroni equation (IV-V). ANOVA for repeated measurements was applied for data consisting of repeated observations at successive time points. Maximal wall tensions for vascular contractions were expressed in mN/mm. The EC₅₀ of contractions was calculated as percentage of maximal response, and presented as the negative logarithm (pD₂). The relaxations were presented as percentage of pre-existing contraction. Spearman's correlation coefficient was used in the correlation analyses. All results were expressed as mean ± SEM. The data were analysed with BMDP Statistical Software version PC90 (Los Angeles, California, USA) and SPSS 9.0 (SPSS Inc., Chicago, IL, USA).

Table 3. Summary of the experimental design of the studies on arterial reactivity and morphology, cardiac natriuretic peptides, lipid profile, aortic ACE and renal AT₁ receptors.

Study	Follow-up after NTX Treatment Vessel	E+ relaxations (precontraction)	E- relaxations (precontraction)	Contractions	Morphology	Other
I	12 weeks Losartan Small mesenteric artery	ACh (NA) + L-NAME + diclofenac + apamin and charybdotoxin ACh (KCl)	Isoprenaline (NA) Levcromakalim (NA) Sodium nitroprusside (NA)	NA KCl Ang II	+	Aortic ACE Vasoactive peptides
II	12 weeks Losartan Main mesenteric artery	ACh (NA) + L-NAME + diclofenac + apamin and charybdotoxin ACh (NA) + L-arginine + SQ-30741	Isoprenaline (NA) Levcromakalim (NA) Sodium nitroprusside (NA)	NA KCl ET-1	-	Renal AT ₁ receptors
III	12 weeks High calcium intake Small mesenteric artery	ACh (NA) + L-NAME + diclofenac + apamin and charybdotoxin	Isoprenaline (NA) Levcromakalim (NA) Sodium nitroprusside (NA) EET (NA)	NA KCl ET-1	+	
IV	27 weeks High calcium intake High phosphorus intake Small mesenteric artery	ACh (NA) + L-NAME + diclofenac + apamin and iberiotoxin	Sodium nitroprusside (NA) EET (NA)	NA KCl	+	Vasoactive peptides
V	12 weeks and 27 weeks High calcium intake Aorta	-	-	-	Aortic calci- fication	Kidney calcification, glomerulo- sclerosis and tubulointerstitial fibrosis Renal ACE and Ang II receptors

ACh, acetylcholine; AT₁, angiotensin II type 1; E+, endothelium-dependent; E-, endothelium-independent; EET, 11,12-epoxyeicosatrienoic acid; ET-1, endothelin-1; L-NAME, N^G-nitro-L-arginine methyl ester; NA, noradrenaline

RESULTS

1 Blood pressure, resistance artery morphology, renal and aortic histology, heart weight, total renal mass, drinking fluid and urine volumes, and rat survival in the study groups

Blood pressure. In studies I-III, during the 12-week follow-up after the operations, arterial BP in CRI rats was not changed when compared with sham-operated animals. However, in study III, when analysed by two-way ANOVA, a small but significant increase in BP was uncovered in rats with renal insufficiency when compared with sham-operated controls (NTX and NTX-Ca groups were pooled and compared with sham rats pooled with sham-Ca group). In study V, mild elevation of systolic BP was found in both CRI groups 12 weeks after NTX. In these CRI rats, which were followed for 24 (I) or 27 weeks after NTX (IV, V), the arterial BP was clearly increased from the rat age of 24 weeks (I) or from 23 weeks (IV, V), when compared with age-matched sham-operated animals. Calcium supplementation clearly decreased the elevated BP (IV, V). However, 8-week losartan treatment had no effect on BPs in CRI or sham-operated rats (I-II).

Morphology of the resistance arteries. CRI resulted in eutrophic inward remodelling of second (IV) or third order (I, III) small mesenteric arteries: wall to lumen ratio was increased, without change in wall cross-sectional area. Losartan treatment completely normalized the remodelling in small artery (I), while high calcium intake had no effect on the changed arterial morphology in CRI rats (III, IV).

Renal and aortic histology. 12-weeks after the NTX, the indices of glomerulosclerosis and interstitial damage (arbitrary scale from 0 to 4), and number of calcifications (deposits/cm²) in kidney tissue, were higher in the NTX group than the Calcium-NTX group (V). No differences in tissue histology were detected between the sham and Calcium-sham groups.

After the 27-week follow-up, the indices of glomerulosclerosis, tubulointerstitial damage, and kidney tissue calcification were clearly increased in the NTX group. All of these indices were lower in the Ca-NTX than the NTX group. High calcium diet also significantly reduced calcifications in the thoracic aorta (V).

Heart weight and total renal mass. The heart-to-body weight ratios were comparable in CRI and sham-operated rats measured 12 weeks after the operations (I, II, III, V), while in CRI rats followed for 24 weeks (I) or 27 weeks (IV, V) the heart-to-body weight ratios were increased when compared with their controls. Calcium supplementation was without significant effect on the relative heart weights (III, IV, V). 8-week losartan treatment did not influence the relative heart weights in CRI rats, but decreased it in sham-operated rats (I, II). 12 weeks after the operations, the total renal tissue mass in the nephrectomized animals was approximately 70% of the weight of the two kidneys of sham-operated controls (I, II, III, V). In studies IV and V, 27 week after the operations, the remnant kidneys of NTX groups appeared macroscopically swollen whereby the renal tissue/body weight ratio was similar to sham, whereas the calcium-fed group showed lower

renal tissue weights.

Drinking fluid and urine volumes. CRI increased the drinking fluid intake and urine output. Losartan treatment (I, II) and high Ca intake (III, IV, V) had no significant effect on fluid consumption or urine excretion, while high phosphorus diet increased significantly both urine excretion and fluid intake in rats with advanced CRI (IV).

Rat survival in the study groups. In study V after the 27-week follow-up, only 7 of the initial 14 rats survived in the NTX group, whereas survival was significantly improved in the Calcium-NTX group.

2 Plasma sodium, potassium, ionized calcium, 1,25(OH)₂D₃, 25OH-D₃, pH, urea, creatinine, PTH, phosphate, proteins, haemoglobin, lipids, urine albumin and calcium excretion.

The plasma PTH, phosphate, creatinine and urea nitrogen values were increased (I-IV), while plasma 1,25(OH)₂D₃ (III, IV, V), 25OH-D₃ (IV), ionized calcium, haemoglobin, and creatinine clearance were decreased in rats with CRI when compared with sham rats. The high calcium intake suppressed the plasma PTH and phosphate levels and moderately elevated the ionized calcium in rats with CRI (III, IV, V). Furthermore, in study IV the increase of plasma creatinine during the follow-up was lower in calcium-treated CRI group when compared with untreated CRI rats. In study III, the plasma 1,25(OH)₂D₃ levels in calcium-treated CRI rats did not differ from those in sham rats. In study V (12-week follow-up), plasma levels of 1,25(OH)₂D₃ in individual study groups did not differ (NTX group vs. sham group $p=0.056$), but analyses by two-way ANOVA showed that plasma 1,25(OH)₂D₃ was lower in the two NTX groups than the sham groups. In rats with advanced CRI (IV), high calcium and high phosphate diets did not influence the levels of 1,25(OH)₂D₃ and 25OH-D₃.

The plasma Na⁺ and K⁺ were similar in all groups in studies I-III and in study V in the groups that were followed for 12 weeks after NTX. However, in studies IV and V (27-week studies), the plasma K⁺ concentrations were higher in rats with advanced CRI, while in high calcium treatment decreased the plasma K⁺ levels. The plasma sodium levels were not affected by CRI in study IV, but high calcium intake decreased and high phosphate diet increased the plasma sodium levels. In study III the levels of plasma proteins were decreased in both untreated and calcium-treated rats with CRI. However, in the 27-week study (V) the plasma proteins were significantly higher in calcium-NTX when compared with untreated NTX rats, but were still lower in the calcium-NTX group than in the age-matched sham rats.

Plasma pH levels were comparable in all study groups in rats with moderate CRI (I-III), while in more advanced CRI (IV, V) the pH levels were decreased in NTX rats vs. sham, and normalized after high calcium diet. High density lipoprotein (HDL) levels did not differ between the study groups (IV), while plasma cholesterol was increased and HDL/total cholesterol ratio decreased in all NTX groups. Plasma triglycerides were higher in calcium-NTX rats than in other groups (IV).

The 24-hour urine calcium excretion was increased in all NTX rats with advanced CRI when compared with sham rats, and was approximately 7-fold higher in the calcium-NTX than in NTX group (IV, V). The level of daily urinary albumin excretion was increased in NTX rats with moderate CRI, but was lowered by 8-week high calcium intake (V). Finally, 8-week losartan treatment had no effect on any of the measured laboratory variables (I, II).

3 Cardiac synthesis and the levels of vasoactive peptides in plasma and cardiac ventricles

3.1. Ventricular levels of ANP and BNP mRNA

Ventricular ANP mRNA and BNP mRNA (I, IV) contents were higher in CRI rats than in the sham-operated controls. Losartan treatment had no effect on ANP and BNP mRNA in moderate CRI (I), while high calcium intake normalized the elevated ventricular ANP and BNP mRNA levels in advanced CRI (IV). Furthermore, high phosphate intake did not affect the ventricular ANP and BNP mRNA content in CRI rats (IV).

3.2. Plasma NT-proANP levels and ventricular BNP levels

Plasma concentration of NT-proANP (I) and ventricular BNP levels (I, IV) were increased in rats with CRI when compared with sham rats. In losartan-treated rats with moderate CRI, the NT-proANP and BNP levels remained comparable to those measured in untreated CRI animals (I). In advanced CRI, the increased ventricular BNP levels were effectively suppressed by high calcium intake, while elevated phosphate intake had no effect on ventricular BNP concentration in CRI rats (IV).

4 Plasma renin activity, aortic and renal ACE, renal Ang II receptors and CTGF

PRA was decreased in NTX rats with moderate and advanced CRI, while high calcium intake did not have any effect on PRA (V). The content of aortic ACE in rats with moderate CRI did not significantly differ from the sham group (I). Losartan treatment reduced aortic ACE in sham-operated rats, but not in CRI rats (I). 12 weeks after NTX the aortic ACE content directly correlated with the level of plasma urea and inversely correlated with creatinine clearance (I). In rats with more advanced CRI (followed for 24 weeks after renal ablation), a 1.5-fold increase in aortic ACE was observed (I).

In rats followed for 12 weeks after NTX, the renal tissue ACE content was approximately 40% lower in the calcium-sham and calcium-NTX groups than in the sham and NTX groups (V; analysed by the use of quantitative *in vitro* autoradiography). The distribution of ACE was different between NTX and sham rats: highest ACE signal was detected in a circular fashion in the inner

cortex and outer medulla in the sham group, whereas ACE was more widely distributed throughout the remnant kidney in the NTX group. The outcome of ACE protein determination by Western blotting well paralleled with the results of autoradiography: renal ACE was lower in the calcium-NTX group when compared with the NTX and sham rats. However, ACE did not differ between NTX and sham groups. 27 weeks after NTX the kidney tissue ACE content determined by autoradiography was still lower in the calcium-treated CRI rats when compared with the sham and NTX groups. The outcome of Western blotting confirmed that also in the 27-week study renal ACE protein in calcium-fed NTX rats was significantly reduced when compared with the NTX control, while ACE protein was increased in the NTX group when compared with sham-operated animals.

12 weeks after NTX the AT₁ receptor density in kidney cortex was slightly reduced in NTX rats (II, V) and calcium-fed sham group (V) when compared with untreated sham-operated animals. No significant differences in cortical AT₁ receptor density were detected between the NTX and calcium-NTX groups. AT₁ density in renal medulla (II, V), and AT₂ density in cortex and medulla (V), were similar in the study groups. Furthermore, the 12-week study (rat age 20 weeks) showed that the average proportion of AT₂ of all AT receptors in the kidney varied 0.65-2.23%, with no significant differences between the groups (V). AT₁ receptor density was higher in the renal medulla than cortex, while there were no significant differences in AT₁ receptor medulla-to-cortex ratios between study groups (II). Moreover, after 8-weeks of losartan treatment the renal cortical and medullary AT₁ receptor binding (autoradiography) was clearly reduced in both CRI and sham-operated rats, suggesting an effective AT₁ blockade (II). In the 27-week study (V), the AT₁ density in renal cortex was lower in calcium-NTX when compared with the NTX group. No differences in renal cortical AT₁ density between the NTX and sham groups, and renal medullary AT₁ density between any of the study groups, were observed. At the rat age of 35 weeks, the proportion of cortical and medullary AT₂ was less than 0.34% of all AT receptors, with no significant differences between the study groups.

27 weeks after NTX, kidney CTGF score was markedly increased in rats with advanced CRI when compared with sham-operated controls, whereas the high calcium intake significantly lowered the CTGF score in the NTX rats.

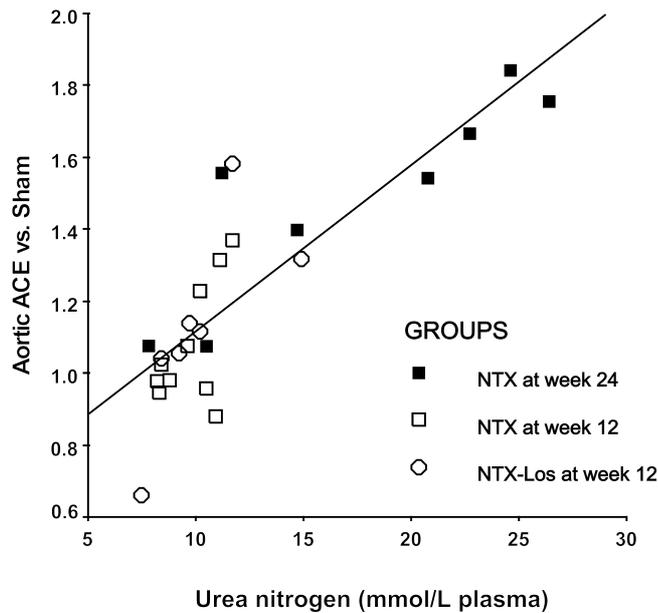


Figure 5. Correlations between aortic ACE and plasma urea nitrogen in 5/6 nephrectomized (NTX) rats 12 and 24 weeks after the operations, and losartan-treated 5/6 nephrectomized (NTX-Los) rats 12 weeks after the operations.

5 Control of arterial tone *in vitro* in CRI

5.1 Arterial tone in moderate and advanced CRI

5.1.1 Arterial contractile responses

The small arterial rings of the CRI and sham-operated rats showed comparable contractile sensitivities (i.e. pD_2 values) and maximal wall tensions to NA and KCl, evaluated both 12 weeks (I, III) and 27 weeks (IV) after nephrectomy. Furthermore, in study I the contractile sensitivities and maximal wall tensions in mesenteric resistance arteries to Ang II were comparable between CRI and sham rats 12 weeks after the operations. However, in the same study, the maximal wall tensions induced by Ang II were clearly increased in CRI rats followed for 24 weeks, when compared with age-matched sham-operated controls, while contractile sensitivity remained comparable between the groups (I).

In the main branch of the superior mesenteric artery, the contractile sensitivities and maximal wall tensions to NA and KCl were also unchanged in rats with moderate CRI when compared with sham-operated controls (II). Furthermore, in the conductance artery, maximal wall tensions and contractile sensitivities to ET-1 in the absence and presence of L-NAME plus diclofenac were comparable in the sham-operated and CRI rats (II).

5.1.2 Arterial relaxation responses

Endothelium-independent relaxations. The relaxations of endothelium-denuded NA-precontracted small mesenteric arterial rings (I, III) and main mesenteric arterial rings (II) to SNP and isoprenaline were comparable in CRI and sham rats after 12-weeks follow-up. However, in these rats with moderate CRI, the relaxations to levromakalim (I, III) and EET (III) were clearly impaired in mesenteric resistance arteries. In the main branch of the same arterial bed, the responses to levromakalim were also impaired in CRI rats when compared with sham group after 12-week follow-up (II). Furthermore, in rats with more advanced renal insufficiency, the cumulative relaxations of mesenteric resistance arteries to EET were clearly reduced when compared with those detected in sham-operated controls, while the responses to SNP did not differ between the groups (IV).

Endothelium-dependent relaxations. The relaxations induced by ACh in endothelium-intact NA-precontracted *resistance arterial rings* were reduced in rats with moderate CRI when compared with the sham group (I, III). Also in study IV at the moderate stage of CRI (11 weeks after NTX, prior to the dietary interventions), the relaxations to ACh were impaired in mesenteric resistance arteries of CRI rats when compared to sham. The NOS inhibition with L-NAME moderately diminished the relaxations to ACh, but the remaining response was still reduced in CRI rats when compared with sham (I, III). COX inhibition with diclofenac did not significantly influence the relaxations to ACh in CRI or sham-operated study groups. The inhibition of K_{Ca} with charybdotoxin and apamin markedly reduced the responses to ACh, and the reduction in relaxation was clearly less marked in CRI rats, while the remaining response was similar between the study groups (I, III). Furthermore, in mesenteric resistance vessels the relaxations to ACh were comparable between CRI and sham groups when precontraction was induced using 50 mmol/L KCl (I).

In the *main branch of the mesenteric artery*, relaxations to ACh were impaired in rats with moderate CRI when compared with sham rats in the absence and presence of L-NAME and diclofenac (II). As indicated by the analyses of area under each ACh response curve (AUC), the reduction in relaxation induced by L-NAME was similar between CRI and sham groups. However, the change by the NOS inhibition in ACh pD_2 and maximal relaxation was slightly but significantly higher in CRI rats when compared with the sham group (II). The change in AUC induced by K_{Ca} blockade with charybdotoxin and apamin was clearly lower in rats with CRI than in sham rats (II). Moreover, the addition of NO substrate L-arginine improved the maximal response to ACh in CRI rats, but not in sham group, while sensitivity to ACh was not affected by L-arginine in the conductance artery of either study group (II). Furthermore, the addition of TXA₂ receptor antagonist SQ-30741 had no effect on the responses to ACh in CRI or sham groups (II).

In advanced CRI, the relaxations to ACh in endothelium-intact NA-precontracted *small mesenteric arterial rings* were distinctively impaired in CRI rats when compared with the sham

group (IV). The addition of L-NAME to the organ bath reduced the relaxation to ACh in both groups, while the reduction in AUC was significantly lower in CRI rats than in sham-operated animals (IV). In contrast, the addition of COX inhibitor diclofenac did not significantly affect the microvessel relaxations to ACh in either study group. However, the K_{Ca} blockade with iberiotoxin plus apamin clearly reduced the remaining response to ACh in both CRI and sham rats, and as indicated by the AUC analyses, the effect of K_{Ca} inhibition was less marked in CRI rats when compared with sham.

5.2 Influence of long-term AT_1 blockade on arterial tone in moderate CRI

5.2.1 Arterial contractile responses

In the small arterial rings the contractile sensitivities and maximal wall tensions to NA and KCl were not affected by 8-week AT_1 blockade with losartan in either CRI or sham-operated rats (I). In the main branch of superior mesenteric artery, the contractile sensitivities to NA and KCl were also similar in untreated and losartan-treated CRI and sham rats (II). However, losartan treatment decreased the maximal wall tensions to NA in the sham group, but not in rats with CRI (II). The maximal wall tensions in main mesenteric artery induced by KCl were comparable in all study groups (II).

In the main branch of the superior mesenteric artery, losartan treatment had no effect on the contractile sensitivities to ET-1 in either CRI or sham animals, but decreased the ET-1-induced maximal wall tensions similarly in both uremic and sham-operated rats (II).

5.2.2 Arterial relaxation responses

Endothelium-independent relaxations. The relaxations of endothelium-denuded NA-precontracted small mesenteric arterial rings (I) and main mesenteric arterial rings (II) to SNP and isoprenaline were similar in CRI and sham rats with or without the long-term losartan treatment. However, losartan improved the impaired relaxations to levcromakalim detected in CRI rats in mesenteric resistance arteries (I) and also in the main branch of the same arterial bed (II), since the responses in both small and large arterial rings from losartan-treated CRI rats did not differ from those detected in sham-operated controls (I, II). Losartan treatment had no effect on relaxations to levcromakalim in sham-operated rats (I, II)

Endothelium-dependent relaxations. The reduced relaxations to ACh in resistance vessels from CRI rats were completely normalized in losartan-treated CRI rats (I). The NOS inhibition with L-NAME moderately and similarly diminished the relaxations to ACh in all groups (I). COX inhibition with diclofenac did not significantly influence the relaxation to ACh in any of the study groups, whereas the inhibition of K_{Ca} with charybdotoxin and apamin markedly reduced the

relaxation to ACh, so that the remaining response was similar between all of the study groups (I). The reduction in relaxation induced by K_{Ca} blockade was clearly higher in losartan-treated CRI rats than in untreated uremic animals (I). Furthermore, in mesenteric resistance vessels the relaxations to ACh were comparable in losartan-treated and untreated study groups when precontraction was induced using 50 mmol/L KCl (I).

In the main branch of the mesenteric artery, the impaired relaxations to ACh in rats with moderate CRI were also normalized after long-term AT_1 blockade (II). AUC analyses of the ACh response showed that the reduction in relaxation induced by L-NAME was equal in the study groups, while change in AUC by the K_{Ca} inhibitors charybdotoxin and apamin was clearly higher in losartan-treated CRI rats, when compared with the uremic controls (II). Furthermore, following the addition of L-arginine the maximal response to ACh was improved in CRI rats, but not in losartan-treated CRI rats and sham groups. However, the sensitivity to ACh was not affected by L-arginine in any of the study groups. The addition of TXA_2 receptor antagonist SQ-30741 had no effect on the responses to ACh in any of the study groups (II).

5.3 Influence of changes in calcium-phosphate balance on arterial tone in moderate and advanced CRI

5.3.1 Arterial contractile responses

Vasoconstrictor responses. High calcium intake did not have any influence on contractile responses of resistance arteries in moderate CRI, since the vessels of NTX and sham-operated rats exhibited similar contractile sensitivity and maximal wall tensions to NA, KCl and ET-1 on both high calcium or control diet (III). In advanced CRI, the contractile sensitivities to NA and KCl also remained unchanged in CRI rats on high calcium and high phosphorus diets when compared with CRI rats on control diet and sham-operated controls (IV). Maximal wall tensions to NA were similar in all study groups, while maximal wall tensions to KCl were higher in calcium-treated CRI rats than sham-operated and CRI controls. The high phosphorus intake had no influence on maximal wall tensions induced by KCl (IV).

5.3.2 Arterial relaxation responses

Endothelium-independent relaxations. In the 12-week study (III), the relaxations of endothelium-denuded NA-precontracted small mesenteric arterial rings to SNP and isoprenaline were similar in CRI and sham rats on high calcium diet, when compared with those on control diet. However, high calcium intake completely normalized the impairments in relaxations to EET and levromakalim in CRI rats (III). In the 27-week study the responses to EET, which were clearly impaired in NTX rats on control diet, were completely normalized by high calcium intake (IV). High phosphorus intake

did not influence the EET-induced relaxations in advanced CRI (IV). Interestingly, the responses to SNP were slightly but significantly enhanced in CRI rats after high phosphate diet, when compared to the responses detected in sham and calcium-treated CRI rats (IV).

Endothelium-dependent relaxations. The reduced relaxations to ACh in endothelium-intact resistance vessels of NTX rats were completely normalized in rats with moderate CRI on high calcium diet (III). The NOS inhibition with L-NAME moderately and similarly diminished the relaxations to ACh in all groups (III). COX inhibition with diclofenac did not significantly influence the relaxation to ACh in any of the study groups, whereas the inhibition of K_{Ca} with charybdotoxin and apamin markedly reduced the relaxation to ACh, so that the remaining response was similar between all the study groups (III). The reduction in relaxation induced by K_{Ca} blockade was clearly higher in calcium-treated CRI rats than in NTX animals on control chow (III).

In advanced CRI, the impaired relaxations to ACh were also normalized following high calcium intake, while high phosphate intake further deteriorated the ACh-induced relaxations (IV). AUC analyses of the ACh response showed that the reduction in relaxation induced by L-NAME was clearly higher in CRI rats on high calcium diet when compared with those on control or high phosphorus diet (IV). The addition of the COX inhibitor diclofenac did not affect the responses to ACh in any of the study groups. However, the change in AUC of the ACh response induced by the K_{Ca} inhibitors iberiotoxin and apamin was higher in calcium-treated CRI rats when compared with untreated CRI groups (IV). The addition of iberiotoxin and apamin did not have any influence on the relaxations to ACh in CRI rats on high phosphorus intake.

Table 4. Summary of the alterations in arterial relaxations in rats with moderate and advanced chronic renal insufficiency compared with sham-operated controls, and the effects of AT₁ blockade, high calcium intake and high phosphorus intake.

Variable	Moderate CRI					Advanced CRI		
	Conductance artery +losartan		Resistance artery +losartan +Ca ²⁺ diet			Resistance artery +Ca ²⁺ diet +Pi diet		
E+ relaxations (precontraction)								
Acetylcholine (NA)	↓	↑	↓	↑	↑	↓	↑	↓
Change by NOS inhibition	↔	↔	↔	↔	↔	↓	↑	↔
Change by COX inhibition		↔	↔	↔	↔	↔	↔	↔
Change by K _{Ca} inhibition	↓	↑	↓	↑	↑	↓	↔	↔
Change by L-arginine	↑	↓						
Change by SQ-30741	↔	↔						
Acetylcholine (KCl)			↔	↔				
E- relaxations (precontraction)								
Sodium nitroprusside (NA)	↔	↔	↔	↔	↔	↔	↔	↑
Isoprenaline (NA)	↔	↔	↔	↔	↔			
EET (NA)			↓		↓	↓	↑	↔
Levcromakalim (NA)	↓	↑	↓	↑	↑			

COX, cyclooxygenase; CRI, chronic renal insufficiency; E+, endothelium-dependent; E-, endothelium-independent; EET, 11,12-epoxyeicosatrienoic acid; K_{Ca}, calcium-activated potassium channels; NA, noradrenaline; NOS nitric oxide synthase. ↑, ↓ and ↔ indicate an increase, reduction and no change when compared with the corresponding control group, respectively.

DISCUSSION

The present investigation examined alterations in conductance and resistance artery tone at different stages of experimental CRI. In moderate CRI, the effects of long-term AT₁ receptor antagonism on uraemia-induced changes in large and small artery function and morphology were evaluated. The influence of changes in calcium-phosphorus balance on vascular reactivity was studied in moderate and advanced CRI. Moreover, the changes in renal RAS components, and the effects of high calcium intake on local RAS in the remnant kidney, were studied in rat models of moderate and advanced CRI.

1 Experimental model of the study

The experimental model employed in the current study was subtotal (5/6) renal ablation by the resection of upper and lower poles of the left kidney, following by contralateral nephrectomy (Ylitalo et al. 1976) in Sprague-Dawley rat. The excision method is known as a low-renin model of CRI, which results in slow but progressive increase in BP over a period of weeks to months (Cowley et al. 1994, Koletsky and Goodsitt 1960). An alternative experimental method, renal mass reduction by the ligation of renal arterial branches, causes severe and immediate hypertension due to renal ischemia with high intrarenal and circulating renin concentrations, and leads to permanent overactivity of systemic RAS due to decreased BP applied to juxtaglomerular receptors near the ligated arterial branches (Kleinknecht et al. 1995). Because of the differences in the development and progression of hypertension between the two methods, results of studies in one model cannot be readily extrapolated to the other. In our study, the rats with both moderate and advanced CRI showed clearly decreased PRA, suggesting that the renal ablation method we used resulted in CRI with low activity of systemic RAS. These results correspond with previous findings in Sprague-Dawley rats with reduced renal mass (Amann et al. 2001b). Since in most types of clinical chronic renal disease the systemic RAS is not activated, the excision model of renal ablation is eligible for studying the vascular functional disturbances and changes in tissue level RAS in different stages of renal impairment.

The excision model of experimental CRI mimics adequately the different stages of clinical CRI, and the progression of renal impairment depends on the duration of follow-up: in studies I-III and V, 12 weeks after the operations, NTX rats showed characteristic findings of moderate renal insufficiency: plasma creatinine and urea nitrogen were increased by 1.7-fold and 1.6-fold, respectively. Furthermore, plasma PTH was elevated, creatinine clearance was decreased, and the permanent volume overload was documented by the clear increase of natriuretic peptides. As expected, the arterial BP of NTX rats remained similar, or was only slightly elevated, to those measured in sham rats during the 12-week follow-up after renal ablation. Therefore, the earlier

stage of the model of CRI we used was appropriate to evaluate vascular changes induced by renal insufficiency *per se*.

In studies IV and V, in order to mimic the advanced clinical CRI, the rats were followed also for 27 weeks after NTX, and the laboratory findings at the end of these studies suggested that CRI could be considered as advanced: plasma creatinine and urea nitrogen were increased approximately by 2.6-fold and 4.4-fold, respectively, phosphate by 2.2-fold, PTH by 11.8-fold, when compared with sham. The 2-fold increases in urine output and fluid intake suggested that urine-concentrating capability was clearly decreased 27 weeks after the NTX. However, these NTX rats still showed relatively good residual renal function and therefore the results from rats with advanced CRI cannot be extrapolated to the terminal stage of clinical kidney disease where dialysis is required.

2 Cardiovascular remodelling and morphology, aortic and kidney calcification, changes in blood pressure and volume load in moderate and advanced chronic renal insufficiency

Cardiac hypertrophy is the primary chronic compensatory mechanism to increased haemodynamic overload in hypertensive patients. In CRI, the positive sodium balance and increased extracellular volume lead to systemic hypertension and vascular and cardiac remodelling (De Francisco and Pinera 2004). The structural alterations of myocardium occur early during the course of renal insufficiency, and left ventricular hypertrophy is present in 75% of subjects at the start of dialysis (London et al. 1987). The primary cause of cardiac hypertrophy in renal patients is combined volume and pressure overload (De Francisco and Pinera 2004).

In our study, the NTX rats with advanced CRI showed increased heart to body weight ratios, elevated BPs and increased volume load, as suggested by elevated levels of natriuretic peptides. However, in moderate CRI the volume overload was already detected in the absence of significant increases of BP and heart to body weight ratio. Thus, in this low-renin model of CRI the volume overload seems to be present already at the normotensive stage of chronic renal impairment, while cardiac hypertrophy was only observed following the increase in systolic BP. Interestingly, despite losartan treatment did not influence BP in sham-operated rats, it still lowered heart to body weight ratio in this group. Such therapy has previously reduced heart weights in normal Wistar rats (Kalliovalkama et al. 1999b), which could possibly be explained by the accumulation of Ang II following AT₁ receptor antagonism, leading to increased stimulation of unopposed AT₂ receptors, which in turn may exert antigrowth effects on rat cardiomyocytes (van Kesteren et al. 1997). The finding that losartan did not reduce heart weights in CRI rats may be attributed to the prevailing volume overload in these animals.

Patients with CRI have been characterized by abnormal elastic properties of large arteries, reflected as decreased distensibility and compliance (Barenbrock et al. 1994, London et al. 1996). The increased stiffness of the conduit arteries has even been seen in the absence of structural changes (Mourad et al. 1997). We found that the small arteries of rats with CRI investigated either

12 weeks or 27 weeks following NTX featured increased wall to lumen ratio, which was not affected by the diet-induced changes in calcium-phosphate balance, but was completely normalized by 8-week AT₁ receptor blockade by losartan. Since the cross-sectional area of arterial wall was not increased, the observed change in vascular morphology in CRI rats is compatible with eutrophic inward remodelling (Mulvany 1999). The vascular wall to lumen ratio exhibits the ability of the vessel to contract against intravascular pressure, while the cross-sectional area indicates the amount of material within the vascular wall, and provides information of vascular growth (Mulvany 1999). Therefore, the present results indicated that calcium intake in both moderate and advanced CRI enhanced vasorelaxation, although the structure of the resistance vessels was not corrected. However, 8-week AT₁ receptor blockade normalized both structure and arterial relaxation of mesenteric resistance arteries, despite the absence of any effects on BP and increased volume load in moderate CRI.

Although high calcium intake did not have any influence on the altered morphology of resistance arteries, the increase in aortic and renal tissue calcifications was prevented by elevated calcium ingestion in NTX rats, probably because of the effective suppression of plasma phosphate and Ca x Pi product. The reduced mortality in the CRI rats on the high calcium diet argues against the view that increased calcium intake would be toxic to the animals in this model of CRI. Furthermore, since the samples of the most severely affected rats with advanced CRI were lost due to the reduced survival in the untreated NTX group, the present evaluation probably underestimated the extent of tissue damage and decline of renal function in this group. Thus, the present results suggest that especially hyperphosphatemia, but not mild hypercalcemia alone, is detrimental to the kidneys and vasculature in CRI.

3 Aortic ACE and the renal AT₁ receptor binding following AT₁ receptor blockade

The RAS is a major regulator of sodium metabolism, renal function, arterial tone and BP. Expression of human angiotensinogen in the kidneys of mice results in hypertension in the absence of changes in systemic Ang II (Sigmund 2001), and it has been thought that local RAS may contribute to many pathophysiological mechanisms related to target organs also in CRI. Thus, local intrarenal RAS is considered to play an important role in the progression of kidney diseases (Ruiz-Ortega et al. 2002). Studies concerning the pathophysiological changes of local RAS in CRI are scarce. Some reports have suggested the activation of tissue RAS in adrenals (Endemann et al. 2004), heart (Amann et al. 2003b) and kidney (Gilbert et al. 1999) in the absence of overactivation in systemic RAS.

As expected, the PRA in NTX rats was clearly decreased, whereas the changes in calcium-phosphate-PTH balance induced by high calcium diet had no effect on PRA. These findings showed that the activity of systemic RAS was decreased in this model of CRI independently of the status of the calcium-phosphate balance. However, 24 weeks after NTX the aortic content of ACE was

increased by 1.5-fold when compared with age-matched controls. Thus, despite low activity of systemic RAS, the vascular tissue RAS may be overactive in advanced experimental CRI. Earlier, in moderate CRI, the content of aortic ACE in NTX rats did not significantly differ from control rats. Interestingly, 8-week losartan treatment reduced aortic ACE content only in sham-operated animals, the effect of which was not observed in NTX rats with moderate CRI. Therefore, our findings suggest that the regulation of tissue RAS in aorta is influenced by uremic circumstances already at moderate stage of CRI. This anomaly could also contribute to the changes in vascular function and morphology observed in the present study.

In study I we for the first time showed a strong correlation between the degree of renal insufficiency and aortic ACE content: 12 weeks after the operations aortic ACE content directly correlated with the level of plasma urea nitrogen and inversely correlated with creatinine clearance. Furthermore, when the NTX rats with more advanced CRI were included to the correlation analyses, the Spearman's correlation between urea nitrogen level and aortic ACE content was even more significant than in analyses concerning only the rats followed for 12-weeks (unpublished data; $r = 0.829$, $p = 0.000$; Figure 5 in results section). Moreover, no clustering of the data points from the different time points of measurements was observed. Therefore, it seems that in the present animal model the increase of aortic ACE content is probably not predicted by the duration of renal insufficiency, but rather by the degree of CRI.

Effective AT₁ receptor blockade by losartan was verified by the use of autoradiography. Both NTX and sham-operated losartan-treated rats showed clearly decreased renal AT₁ receptor binding density, which suggests that AT₁ receptors were successfully occupied by the active metabolite of losartan. Furthermore, the AT₁ receptor density in the renal cortex was also decreased in the untreated NTX rats when compared with the untreated sham-operated rats, while AT₁ receptor expression in renal medulla remained unchanged between the groups. This result agrees with previous finding in rats with gentamicin-induced renal insufficiency, where the density of Ang II receptors in glomeruli has been found to be decreased (Esquerro et al. 1995). Putative explanation to this finding could be the negative feedback to AT₁ receptor expression by overactive local RAS in the kidney tissue.

4 Renal components of RAS, CTGF score, and histological changes in remnant kidneys

This study showed that despite low activity of systemic RAS, the local ACE content in renal tissue was clearly increased in advanced stage of CRI. Treatment of SH by high calcium intake reduced ACE content in rat remnant kidney, and also reduced albuminuria, favourably influenced kidney morphology and diminished soft tissue calcification and improved survival. These findings suggest a link between calcium metabolism and ACE expression in kidney tissue that could also be important in the progression of renal damage.

In the present study, renal ACE content measured by the use of autoradiography and Western

blotting did not significantly differ between rats with moderate CRI and sham-operated animals. However, the distribution of ACE was different between NTX and sham rats: highest ACE signal was detected in a circular fashion in the inner cortex and outer medulla in animals with intact kidneys, whereas ACE was more widely distributed throughout the remnant kidney in the NTX group. In CRI rats followed for 27 weeks, the renal ACE expression (autoradiography) did not significantly differ from their controls, but there was a trend of an increase, and the ACE protein content by Western blotting showed a clear upregulation of renal ACE in rats with advanced CRI. Furthermore, the elevated renal ACE content was associated with a marked increase in CTGF score in the kidneys of NTX rats. Previously, pathological expressions of the RAS components renin and Ang II have been reported in rat remnant kidneys, with an associated over-expression of TGF- β_1 (Gilbert et al. 1999). The prosclerotic effects of TGF- β_1 have been recently shown to be mediated predominantly by CTGF in rat remnant kidney (Okada et al. 2004a). The aforementioned altered tissue level expression of renin and Ang II would lead to increased local Ang II action even without concurrent changes in renal ACE content. This provides a plausible explanation to the reduced cortical AT₁ receptor density in CRI rats, which could serve as a compensatory mechanism to counteract the increased activity of RAS in the remnant kidney.

The regulation of ACE in tissues is not well understood. Intense proteinuria up-regulates ACE in the kidney, which may contribute to the progression of renal disease (Largo et al. 1999). Balloon injury of arteries induces ACE expression after endothelial disruption, the mechanism of which plays a possible role in neointima formation (Fernandez-Alfonso et al. 1997). In cultured endothelial cells ACE expression or activity is induced by platelet activating factor (Kawaguchi et al. 1990), ET-1 (Kawaguchi et al. 1991), dexamethasone (Dasarathy et al. 1992), ANP (Saijonmaa and Fyhrquist 1998), and vascular endothelial growth factor (Saijonmaa et al. 2001). TNF- α decreases the levels of ACE protein in endothelial cells (Papapetropoulos et al. 1996), and down-regulates ACE in differentiating macrophages (Viinikainen et al. 2002). Estrogen has also been suggested to reduce the gene expression of ACE in rat kidneys (Gallagher et al. 1999). Interestingly, AT₂ receptor activation has been reported to decrease ACE activity, which may partially underlie AT₂'s attenuation of AT₁-mediated actions (Hunley et al. 2000). In the present study an inverse correlation between renal contents of ACE and AT₂ receptors was observed, supporting a link between the regulations of these two components of RAS in remnant kidney tissue.

The present study for the first time demonstrated that high calcium diet reduces kidney ACE content in CRI rats, with a simultaneous decrease in albuminuria and a beneficial influence on kidney morphology. Local intrarenal RAS is known to be an important determinant of tissue injury, inflammation and progression of renal disease, while inhibition of the actions of RAS can reduce proteinuria and preserve renal function in kidney diseases (Amann et al. 2001b, Brenner et al. 2001, Gilbert et al. 1999, Ruiz-Ortega et al. 2002). It is of note that in experimental CRI, disturbed calcium balance and hyperphosphatemia have been linked with increased tissue fibrosis and thickening of arterial wall (Amann et al. 2003a), the findings of which thus resemble the structural

changes that are associated with long-term activation of RAS at the tissue level (Gilbert et al. 1999, Ruiz-Ortega et al. 2002).

The present results indicate that alterations in the calcium-phosphate balance contribute to the regulation of ACE in the kidney, since we found that reduced renal tissue ACE content was associated with reduced plasma levels of PTH and phosphate, elevated plasma levels of calcium, and increased urinary calcium excretion. In addition to its other physiological roles, extracellular calcium ion also functions like a hormone, since it regulates cellular processes through specific calcium receptor-mediated mechanisms (Brown 1999). In the nephron high calcium delivery stimulates tubular calcium-sensing receptors, the mechanism of which plays a role in the enhanced natriuresis and diuresis induced by high calcium ingestion or hypercalcemia (Brown 1999). In addition, elevated level of extracellular calcium can increase the synthesis of proteins that inhibit soft tissue calcification, and this increase is mediated via a mechanism that is functionally related to the calcium-sensing receptor (Farzaneh-Far et al. 2000). Some of the proteins that regulate calcification belong to the TGF- β superfamily of cytokines (Lund et al. 2002). Because of the well-known increasing effect of Ang II on TGF- β_1 synthesis (Gilbert et al. 1999, Ruiz-Ortega et al. 2002), a hypothesis could be presented whereby high renal calcium delivery could increase the synthesis of calcification-regulation proteins via the stimulation of calcium-sensing receptors, which, in turn, could influence the expression of renal components of RAS.

Rats with CRI showed higher albumin excretion that well paralleled the moderate increases in glomerulosclerosis and interstitial damage, while high calcium intake was associated with lower albumin excretion and beneficial influence on kidney morphology in the NTX rats. These findings may be due to the observed changes in calcium metabolism. Another putative explanation would be down-regulation of local components of RAS in the kidney, since renal tissue ACE content, determined by two distinct methods, was significantly reduced after increased calcium ingestion in NTX rats. No changes in renal AT₁ or AT₂ receptor densities were observed in NTX rats after the high calcium diet, but a significant decrease in cortical AT₁ receptor density was detected in the calcium-supplemented sham-operated rats. Subsequently, the net effect of lower ACE without simultaneous changes, or a slight concurrent decrease, in cortical AT₁ receptor density, could result in reduced Ang II action at the tissue level, and provide an explanation to the observed beneficial results on morphology and albumin excretion.

5 Resistance artery tone at different stages of experimental chronic renal insufficiency

This study showed that resistance artery relaxation via K⁺ channels was impaired in moderate experimental CRI, while the vasorelaxation mediated by the NO pathway was preserved. Furthermore, in advanced CRI the impaired endothelium-dependent dilatation of resistance vessels featured deficiency of both NO- and K⁺ channel-mediated vasorelaxation.

The endothelium-dependent relaxations induced by ACh in NA-precontracted resistance arterial rings were reduced in rats with moderate CRI, the finding of which corresponds to previous studies in microvessels from patients (Morris et al. 2001, Wang et al. 2000a) and laboratory animals (Bagi et al. 2003) with renal disease. The vasodilatation induced by ACh is mediated by NO, PGI₂, and endothelium-derived hyperpolarization, and the relative roles of these components were addressed by NOS inhibition, COX inhibition, and K⁺ channel blockade, respectively. The NOS inhibition using L-NAME moderately diminished the relaxations to ACh similarly in both NTX and sham rats, suggesting, that the NO-mediated component of the relaxation in small mesenteric arteries was similar between the groups. The sensitivity of arterial smooth muscle to cGMP, as examined by the relaxation to the NO-donor SNP, was also similar in the NTX and sham groups after the 12-week follow-up. Furthermore, COX inhibition with diclofenac did not have significant influence on the relaxation to ACh in the study groups. This shows that COX-derived compounds did not significantly contribute to the endothelium-dependent relaxation in resistance arteries of these rats, and suggests also that CRI did not have detectable effect on that pathway.

The major mechanism that contributes to the vasodilatation in resistance arteries is via the activation of K⁺ channels (Coats et al. 2001). In agreement with this, the inhibition of K_{Ca} with charybdotoxin and apamin markedly reduced the relaxation to ACh, whereas the reduction in the relaxation was clearly less marked in CRI rats than in sham group, and the remaining response was similar between the study groups (Cohen and Vanhoutte 1995). This finding suggests an attenuated vasorelaxation via K⁺ channel-mediated hyperpolarization in rats with moderate CRI. The impaired endothelium-derived hyperpolarization could result from reduced sensitivity to, or decreased endothelial release of, hyperpolarizing factors (Roman 2002). Therefore, endothelium-independent relaxations to EET (K_{Ca} agonist) and levcromakalim (K_{ATP} opener) were performed. Interestingly, both of these responses were clearly reduced in the rats with moderate CRI, showing that the impaired arterial relaxation could be attributed to reduced vasodilatation via K⁺ channels in vascular smooth muscle. It is of note that since the defective K⁺ channel-mediated resistance artery relaxation was detected already at normotensive stage of CRI, the observed changes were presumably caused by renal insufficiency *per se*. Previously, PTH has been found to increase the synthesis of the K_{Ca} blocker 20-hydroxyeicosatetraenoic acid in renal tubular cells (Roman 2002). Thus, SH observed in NTX rats could contribute to the defective K⁺ channel mediated relaxation in vascular smooth muscle.

When compared with findings observed in moderate CRI, the advanced stage of renal disease featured clearly further disturbed regulation of resistance artery tone: mesenteric resistance vessels of NTX rats followed for 27 weeks showed poor K_{Ca} channel mediated relaxation, and clearly deteriorated endothelium-dependent relaxation via NO pathway was also detected. In study IV, the relative roles of NO, PGI₂ and endothelium-derived hyperpolarization were evaluated by the AUC changes of the relaxation responses to ACh induced by NOS inhibition (L-NAME), COX inhibition (diclofenac) and K_{Ca} blockade (charybdotoxin and apamin), respectively (Gschwend et al. 2002).

NOS inhibition reduced the relaxations to ACh, and AUC analyses showed that contribution of NO to the ACh response was lower in NTX rats than in sham group. Thus, NO-mediated endothelium-dependent relaxation was impaired in rats with advanced CRI. Several putative mechanisms can explain impaired vasorelaxation via endothelium-derived NO in uraemia. Increased concentrations of endogenous NOS inhibitors, like asymmetric dimethylarginine, may reduce NO synthesis (Fliser et al. 2003). Endothelial cells may feature deficiency of NO precursor L-arginine, the transport of which into the endothelium is inhibited by increased urea nitrogen (Wagner et al. 2002). NOS activity could also be affected by SH, since parathyroidectomy has been reported to improve NO production in NTX rats (Vaziri et al. 1998a).

As in moderate CRI, the sensitivity of arterial smooth muscle to cGMP, as examined using the NO-donor SNP, was similar in the NTX and sham rats also at advanced stage of CRI. In contrast, the endothelium-independent relaxations induced by the K_{Ca} agonist EET (Plane et al. 1997) were impaired in NTX rats after both 12 and 27 weeks of follow-up. Therefore, vasorelaxation via K_{Ca} in smooth muscle was decreased in earlier as well as in more advanced stages of CRI. High levels of PTH can increase the synthesis of the K_{Ca} blocker 20-hydroxyeicosatetraenoic acid in renal tubular cells (Roman 2002) and therefore suppress K^+ channel activity. Moreover, the activation of type 1 PTH receptors has a relaxing effect on tracheal smooth muscle via the stimulation of cAMP and activation of BK_{Ca} (Shenberger et al. 1997). PTH can also act as a vasodilator via type 1 PTH receptors, whereas expression of these receptors is down-regulated in uraemia (Disthabanchong et al. 2004), the mechanism of which may contribute to the attenuated vasodilatation. For instance, downregulation of type 1 PTH receptor mRNA has been reported in human osteoblasts in end-stage renal failure (Picton et al. 2000). Furthermore, the expression of these receptors has been reported to be decreased in renal blood vessels of spontaneously hypertensive rats, which may partially explain the elevated renovascular resistance in these animals (Massfelder et al. 2002). The putative CRI-induced downregulation of type 1 PTH receptors in vascular smooth muscle, and its possible relationship to attenuated K^+ channel mediated vasorelaxation, remains to be studied.

Despite the low activity of systemic RAS in NTX rats, possible changes of RAS in vascular wall may contribute to the changes in vascular function. Therefore, we evaluated the contractile sensitivities and maximal wall tensions in mesenteric resistance arteries in response to Ang II. These vasoconstrictor responses were not altered in rats with moderate CRI 12 (weeks after the operations). However, in the same study, the maximal wall tensions induced by Ang II were clearly increased in the NTX rats that were followed for 24 weeks and featured more advanced renal insufficiency. These findings together with the observed increase of aortic ACE content suggest that CRI seems to have an enhancing influence on vascular local RAS, which may contribute to the disturbed regulation of arterial tone in CRI.

The sensitivities and maximal wall tensions to NA and KCl were not altered in both moderate and advanced stage of CRI. These results agree with a previous clinical study, where forearm vasoconstriction to NA was not altered in end-stage renal insufficiency patients (Passauer et al.

2003). Since no contractile changes were observed in the responses to NA and KCl, the differences observed in vasorelaxation could not result from alterations in the responses to the precontractile agents used in this study.

6 Conductance artery tone in moderate chronic renal insufficiency

Impaired endothelial function of large arteries has been reported in clinical and experimental uremia (Kalliovalkama et al. 1999a, Morris et al. 2000, Thambyrajah et al. 2000). The Results of the present study showed that at moderate stage of experimental CRI the mesenteric conduit arteries featured deficient vasorelaxation via K^+ channels, whereas NO-mediated component of endothelium-dependent relaxation was preserved. Furthermore, vasoconstriction to ET-1 was not altered in these animals with normal arterial BP.

Cardiovascular complications in renal patients are predominantly related to the damage of conduit-size arteries, and reduced compliance of large arteries is recognized as an independent predictor of cardiovascular mortality in CRI (London 2000). Possible explanations to the reduced vascular compliance in CRI, in addition to atherosclerosis, are increased extracellular matrix content, hyperplasia of smooth muscle, and calcification in the media of blood vessels (Amann et al. 1997, Hafner et al. 1995). On the other hand, deficient endothelial vasodilator function may also contribute to the large vessel pathophysiology in CRI (Rabelink and Koomans 1997), since endothelium-dependent vasodilatation is impaired in the forearm circulation of hemodialysis patients (van Guldener et al. 1997) and in conduit-size arteries of NTX rats (Kalliovalkama et al. 1999a).

In concert with previous studies, we found that endothelium-dependent relaxation to ACh was impaired in mesenteric conduit artery of rats with moderate CRI. The AUC analyses showed that the contribution of NO to the total ACh response was comparable in NTX and sham rats. Nevertheless, the reductions induced by NOS inhibition in the sensitivity (pD_2 value) and maximal relaxation to ACh were more pronounced in the NTX group when compared to sham group. Thus, the NO-mediated component of endothelium-dependent relaxation was probably preserved, and its relative contribution to the entire endothelium-mediated relaxation was even augmented in rats with CRI when compared with sham rats. The addition of diclofenac did not cause significant change in AUC of the responses to ACh in either study group. Similarly, the responses to ACh remained unchanged in the presence of TXA_2 and PGH_2 receptor blocker SQ-30741. These results suggest that COX-derived compounds were not playing a significant role in the modulation of the endothelium-dependent responses, whereas CRI does not seem to have any effect on COX pathway in conduit artery. In contrast, the contribution of K_{Ca} to the total ACh response, as evaluated by the use of K_{Ca} blockers charybdotoxin and apamin, was clearly decreased in NTX rats when compared with sham (Cohen and Vanhoutte 1995, Plane et al. 1997). Thus, the endothelium-dependent vasodilatation in mesenteric conduit artery of NTX rats was reduced via a

mechanism, which involved activation of K^+ channels and hyperpolarization of arterial smooth muscle.

The sensitivity of conductance artery smooth muscle to cGMP or cAMP was not altered in rats with moderate CRI, since the relaxations to the NO-donor SNP and β -adrenoceptor agonist isoprenaline were similar in the study groups. In contrast, the endothelium-independent relaxations to K_{ATP} opener levcromakalim were impaired in NTX group, suggesting that the sensitivity of smooth muscle to hyperpolarizing stimuli was decreased in the mesenteric conductance artery of rats with moderate CRI (Roman 2002).

Dorsal hand vein responsiveness to local infusion of ET-1 has shown to be reduced in hypertensive renal insufficiency patients, but not in those with normal BP (Hand et al. 2001). A putative explanation to this alteration is the downregulation of vascular receptors because of increased exposure to ET-1, which is elevated in CRI (Lariviere et al. 1997). This agrees with the view that changes in vascular reactivity to ET-1 may be inversely correlated with the increase of arterial BP. Our results support this, since arterial BP remained unchanged during the 12-week follow-up, and accordingly, the arterial contractions to ET-1 in conduit artery were similar between NTX and sham group.

7 The effects of AT_1 receptor blockade on arterial tone in resistance and conductance arteries in moderate chronic renal insufficiency

There is compelling evidence that the inhibition of RAS with ACE inhibitors or AT_1 receptor antagonists can slow the progression of renal insufficiency in many chronic nephropathies (Praga 2002). However, the information about the effects of such therapy on the regulation of arterial tone and morphology has been scarce. Therefore, in this study we examined the effects of long-term losartan treatment in normotensive rats with moderate CRI. Furthermore, the aim of this study was also to evaluate these effects especially in a model of CRI, in which systemic RAS is not overactive, giving thus valuable information concerning the changes induced by both CRI and RAS blockade on the tissue level RAS in the arteries and kidney.

The results of the present study showed that AT_1 receptor antagonism by 8-week losartan treatment normalized the deficient vasorelaxation in both resistance and conductance mesenteric arteries in moderate CRI without any effect on BP, volume overload, SH and residual kidney function. Furthermore, losartan treatment also normalized the eutrophic inward remodelling detected in mesenteric resistance arteries of NTX rats. Clinical studies suggest that in addition to lowering arterial BP, AT_1 receptor antagonists can also retard the progression of diabetic nephropathy (Brenner et al. 2001), and inhibit the development of glomerulosclerosis and interstitial fibrosis in the kidney (Noda et al. 1999). It is noteworthy that these benefits cannot be solely explained by BP reduction (Brenner et al. 2001, Lewis et al. 2001). Our results suggest that AT_1 receptor blockade can also ameliorate the functional and structural disturbances of uremic arteries.

In mesenteric resistance vessels of losartan-NTX rats, the endothelium-dependent relaxations to ACh were improved when compared with untreated NTX group, and were similar to those of sham rats. Furthermore, there was no difference between the sham and losartan-NTX groups after the addition of NOS inhibitor L-NAME, COX inhibitor diclofenac, and K_{Ca} inhibitors charybdotoxin and apamin. Therefore, the contributions to the ACh response by NO, PGI_2 and K^+ channels, respectively, were similar in losartan-treated NTX rats and sham group. These results suggest that the impaired endothelium-dependent relaxation via K^+ channels was normalized by losartan treatment in small vessels of rats with moderate CRI. Similar effect was observed in the mesenteric conduit-size artery of the uremic animals: the AUC analyses showed equal responses to ACh in losartan-NTX and sham groups, and relative roles of NO, PGI_2 and K^+ channels were also similar in these groups. Thus, losartan treatment normalized EDHF-mediated deficient arterial relaxation of both resistance and conductance vessels in rats with CRI. Furthermore, the defective endothelium-independent relaxation via K_{ATP} induced by levcromakalim in NTX rats was also improved in losartan-treated rats with CRI when compared with untreated NTX group on both levels of mesenteric arterial bed. Therefore, losartan therapy improved the decreased sensitivity of arterial smooth muscle to hyperpolarizing stimuli.

As mentioned above, SH related to CRI may be one cause of deficient K^+ channel function, since PTH has been found to increase the synthesis of the K_{Ca} blocker 20-hydroxyeicosatetraenoic acid in renal tubular cells (Roman 2002). However, since losartan treatment had no effect on elevated PTH levels in NTX rats, the improved vasorelaxation in losartan-treated CRI rats could not be explained by this mechanism. Rather, since the vascular local RAS was overactive in this model of CRI, the vascular effects by the losartan treatment may be the result of the blockade of tissue level RAS in the arteries. This view is supported by the finding that losartan dramatically reduced the maximal wall tensions induced by Ang II in resistance arteries. Moreover, recent studies have suggested that AT_1 receptor blockade and ACE inhibition enhance K^+ efflux from cardiac cells, which has been found to be impaired due to RAS overactivity (Shimoni 2001, Shimoni and Liu 2003). However, the precise link between local RAS and hyperpolarization of vascular smooth muscle remains to be studied.

Plasma and urinary levels of ET-1 are elevated in patients with CRI as well as in experimental models of uraemia (Lariviere et al. 1997). This elevation is related to enhanced ET-1 production in vascular and renal tissues (Brochu et al. 1999) and is modulated, at least in part, by Ang II through the AT_1 receptor (Lariviere et al. 1998). In our study, losartan treatment reduced maximal wall tensions induced by ET-1 similarly in both NTX and sham rats, while contractile sensitivities remained unchanged. The same phenomenon has been observed in aortic rings of spontaneously hypertensive rats contracted by ET-1 after pre-incubation with losartan (Maeso et al. 1997). Moreover, since the changes in maximal wall tensions after the AT_1 receptor blockade in NTX and sham rats were similar, the link between Ang II and ET-1 is probably not modulated by CRI in this experimental model. Furthermore, losartan has been reported to reduce increased ET-1 production

in glomeruli and blood vessels of NTX rats (Lariviere et al. 1998). Thus, the beneficial effects of losartan in CRI could be, at least partially, attributable to the attenuation of Ang II-induced ET-1 production.

8 Influence of changes in calcium-phosphate balance on resistance artery tone in moderate and advanced experimental renal insufficiency

The present results showed that 8-week treatment of SH by high calcium intake improved resistance artery relaxation in moderate experimental renal insufficiency. Furthermore, high calcium diet applied for 12 weeks normalized the deficient vasorelaxation in advanced CRI. As expected, high phosphorus intake had a detrimental influence on SH, the effect of which was associated with exceedingly poor endothelium-dependent vasodilatation. In addition to lowering of PTH, high calcium intake also reduced BP and alleviated the decline of residual renal function in advanced CRI. These effects may partially explain the improved relaxation in small arteries. However, since in moderate CRI the enhanced vasorelaxation was observed in the absence of changes in renal function or BP, these results suggest that calcium-phosphate-PTH balance *per se* is an important regulator of resistance artery tone in CRI.

The small mesenteric arteries of rats with moderate CRI showed attenuated endothelium-dependent relaxation to ACh when compared with sham rats. This impairment was observed in the presence and absence of L-NAME and diclofenac, but was not detected during K_{Ca} blockade. In calcium-fed CRI rats, the relaxations to ACh were similar to those observed in sham-operated animals. Furthermore, the reduction in relaxation induced by the addition of the K_{Ca} blockers charybdotoxin and apamin was clearly higher in calcium-treated CRI rats when compared with CRI rats on normal diet. Thus, high calcium intake improved the endothelium-dependent relaxation via a mechanism that involved the activation of K^+ channels in arterial smooth muscle (Roman 2002).

Deficient endothelium-dependent relaxation via K^+ channels in CRI could result from decreased endothelial release of EDHF, or reduced sensitivity of smooth muscle to EDHF. Therefore, we evaluated the relaxation properties of the arteries in response to the K_{Ca} agonist EET and K_{ATP} agonist levcromakalim. These results showed that endothelium-independent relaxations via both of these subtypes of K^+ channels were impaired in rats with moderate CRI, whereas high calcium intake normalized this aberration. Since this effect was not associated with changes in BP or levels of urea and creatinine, but was related to the reduced plasma levels of PTH and phosphate, our results suggest that alterations in calcium-phosphate balance contribute to the impaired vasodilatation in CRI, while correction of disturbed calcium-phosphorus metabolism confers benefits on the regulation of arterial tone in uremia.

In study IV on advanced experimental CRI, deficient endothelium-dependent relaxation in NTX rats already observed prior to the modification of dietary calcium or phosphate intakes. Despite that the high calcium intake was started at a relatively late stage of CRI, it reversed the

functional impairments detected in small vessels of CRI rats. The relative roles of the components that contribute to endothelium-mediated relaxation were analyzed by the evaluation of the AUC changes induced by NOS inhibition (L-NAME), COX inhibition (diclofenac) and K^+ channel blockade (iberiotoxin+apamin) in the series of consecutive ACh responses. The contribution of NO to the ACh response was augmented in calcium-treated NTX rats when compared with NTX and phosphate-NTX rats. Thus, the defective NO-mediated endothelium-dependent relaxation in advanced CRI was improved by calcium intake.

Several putative mechanisms may explain the impaired vasorelaxation via endothelium-derived NO in CRI. For instance, increased concentrations of endogenous NOS inhibitor asymmetric dimethylarginine may reduce NO synthesis (Fliser et al. 2003). The impairment of endothelial NO synthesis in CRI could be caused by deficiency of the NO precursor L-arginine, since elevated urea nitrogen may inhibit the transport of L-arginine into endothelial cells (Wagner et al. 2002). However, it has been suggested that additional factors related to CRI are necessary to impair NO production, since the administration of urea did not cause any changes in renal hemodynamics or NO deficiency in rats with normal renal function (Xiao et al. 2001). For instance, NOS activity could be affected independently by hypertension, SH, volume expansion, anemia, inflammatory disorders or immune activation. Parathyroidectomy has also been suggested to improve reduced NO production in NTX rats (Vaziri et al. 1998a). This finding corresponds to our results, since increased calcium intake reduced plasma PTH levels and normalized resistance artery relaxation via NO.

High calcium intake also normalized the impaired K^+ channel-mediated component of ACh-induced relaxation in advanced CRI, since the contribution of K_{Ca} to the endothelium-dependent ACh response in calcium-NTX rats and sham rats was similar, but was clearly decreased in NTX and phosphorus-NTX groups. It is of note that in phosphorus-fed rats with advanced CRI, the use of K_{Ca} blockade had virtually no effect on the ACh response, whereby these rats with very high PTH levels featured very poor vasorelaxation via K^+ channels. This finding supports the view that SH may contribute to the deficient K^+ channel function in CRI.

Corresponding to the results in moderate CRI, the sensitivity of arterial smooth muscle to cGMP was unaltered in advanced CRI, since the endothelium-independent relaxations to SNP were comparable in NTX, calcium-NTX and sham rats. Interestingly, the phosphorus-NTX rats showed enhanced response to SNP when compared with sham. This finding could be explained by a compensatory increase in smooth muscle sensitivity to NO due to reduced bioavailability of endothelial NO. It seems probable that endothelial NO release in phosphorus-NTX rats was impaired to a greater extent than suggested by the AUC analyses, since this assessment did not take into consideration that smooth muscle sensitivity to NO was increased in the phosphate-NTX rats. In contrast to the response induced by SNP, the endothelium-independent relaxations to the K_{Ca} agonist EET were impaired in NTX and phosphorus-NTX rats, whereas this response did not differ between sham and calcium-NTX rats (Plane et al. 1997). Therefore, vasorelaxation via K_{Ca} in

smooth muscle was decreased in CRI, and normalized by high calcium intake. Since PTH may increase the synthesis of the K_{Ca} blocker 20-hydroxyeicosatetraenoic acid (Roman 2002), it would be of interest to examine whether modified calcium-phosphorus balance influences the synthesis of 20-hydroxyeicosatetraenoic acid in CRI.

Collectively these experiments showed that modification of calcium-phosphate balance influences the regulation of resistance artery tone in moderate and advanced experimental CRI. Treatment of SH by high calcium intake improved vasorelaxation via a mechanism that involved K^+ channels at both stages of CRI, and also via NO-mediated pathway in advanced CRI. This effect was associated with beneficial influences on BP, volume overload, decline of residual renal function, and survival in advanced CRI, whereas in moderate CRI the vascular effects of calcium intake were observed without any effect on BP and kidney function. In contrast, high phosphorus intake had a detrimental influence on both SH and vasodilatation in resistance arteries, the effects of which were independent of changes in BP or volume overload.

SUMMARY AND CONCLUSIONS

The present study was designed to examine the alterations in local control of arterial tone at different stages of chronic kidney disease using the low-renin model 5/6 nephrectomized rat. Furthermore, the effects of AT₁ receptor blockade, and high calcium and high phosphate intake on arterial reactivity were studied in experimental renal insufficiency. The influence of high calcium ingestion on ACE expression in the remnant kidney was evaluated.

The major findings and conclusions are:

1. The attenuated arterial relaxation in moderate experimental CRI, that was not yet associated with elevated blood pressure, was explained by reduced vasodilatation via K⁺ channels, whereas the NO-mediated component of endothelium-mediated relaxation appeared to be preserved. In both small and large arteries, the effects of NOS inhibition on relaxations to ACh were similar, while the inhibition of K⁺ channels reduced the relaxations to ACh less effectively in CRI rats than in sham-operated controls.
2. At advanced stage of CRI, when arterial blood pressure was also increased, the impaired relaxation of resistance arteries was due to diminished vasodilatation via both K⁺ channels and the NO pathway: the reductions in relaxation induced by K⁺ channel inhibitors, as well as by NOS inhibition, were clearly less marked in rats with advanced CRI when compared with sham-operated rats.
3. The resistance arteries in moderate and advanced experimental CRI featured eutrophic inward remodelling: wall to lumen ratio was increased when compared with sham-operated controls, lumen diameter was decreased, while wall thickness and cross-sectional area were similar between the study groups.
4. Long-term AT₁ receptor antagonism with losartan normalized the observed impairments of resistance and conductance artery function, and corrected the morphology of resistance arteries, in the absence of effects on blood pressure, SH, volume overload or functional kidney variables in this model of moderate CRI. Thus, long-term AT₁ receptor antagonism conferred benefits to vascular function and morphology in CRI that could not be explained by favourable influences on BP or proteinuria.
5. Local RAS in vascular tissue was enhanced in experimental uremia: ACE expression in the aorta was clearly increased in advanced CRI, while aortic ACE content directly

correlated with the level of plasma urea and inversely correlated with the calculated creatinine clearance. Furthermore, the maximal wall tensions induced by Ang II were increased in resistance vessels of rats with advanced CRI when compared with sham rats. The observed enhancement of local vascular RAS may significantly contribute to the vascular pathophysiology in CRI.

6. Calcium-phosphate balance seems to be an important modulator of resistance artery tone in CRI: high calcium suppressed the elevated levels of PTH and phosphate, the effect of which was associated with augmented endothelium-mediated relaxation of resistance arteries in moderate CRI. Furthermore, in advanced CRI, the high calcium diet reduced elevated blood pressure, retarded the progression of CRI, decreased volume overload, and restored the diminished NO- and K⁺ channel-mediated relaxations of small mesenteric arteries. In contrast, high phosphate intake had a clear detrimental effect on vascular relaxation.
7. The findings of the present study suggest a link between calcium metabolism and ACE expression in kidney tissue that could be important in the progression of renal damage: high calcium intake down-regulated ACE expression in remnant kidney, an effect that was accompanied by reduced albuminuria, favourable influence on kidney morphology, and improved survival of rats with advanced CRI.

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