Hyperuricemia and the renin-angiotensin-aldosterone system in experimental renal insufficiency

MASTER'S THESIS
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Acknowledgements

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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet – Veren korkea virtsahappopitoisuus, hyperurikemia, on tyypillistä pitkälle edenneessä munuaisten vajaatoiminnassa, mutta hyperurikemia voi altistaa natriumin erityksen vähentymiselle sekä korkealle verenpaineelle myös normaalin munuaistoiminnan vallitessa. Hyperurikemian merkityksestä sydän- ja verisuonitautien synnyssä kuitenkin kiistellään. Tämän tutkimuksen tavoitteena oli selvittää kokeellisen hyperurikemian vaikutuksia reniini-angiotensiini-aldosteronijärjestelmän komponentteihin munuaisissa ja verenkierrossa.

Tutkimusmenetelmät – 48 Sprague Dawley –rottaa jaettiin kahteen ryhmään, joille suoritettiin 5/6 nefrektomia (NX) tai munuaiskapselin poisto (Sham). Kolme viikkoa leikkausten jälkeen 12 rottaa kummastakin ryhmästä siirrettiin 2.0% oksonihappoa sisältävälle ruokavaliolle (Oxo). Ruokavalioiden kokonaiskesto oli 9 viikkoa. Verenpaine mitattiin epäsuoralla häntämansettimittauksella, minkä lisäksi rotista otettiin veri-, virtsa- ja munuaiskudosnäytteet. Munuaisten angiotensiinikonvertaasientsyymejä (ACE ja ACE2) ja angiotensiini II -reseptoreja (AT_{1R} ja AT_{2R}) tutkittiin kvantitiivisella käänteis-PCR:lla sekä autoradiografialla. Plasman reniiniaktiivisuutta (PRA) ja aldosteronipitoisuutta mitattiin radioimmunoassay-menetelmällä.

Tutkimustulokset – Oksonihappodieetti lisäsi selvästi plasman virtsahappopitoisuutta, mutta verenpaine nousi ainoastaan NX+Oxo –ryhmässä. Kreatiniinipuhdistuma väheni 60% molemmissa vajaatoimintaryhmissä ja 25% Sham+Oxo -ryhmässä. NX-ryhmän PRA oli 90% matalampi kuin Sham-ryhmällä. NX+Oxo ja Sham+Oxo –ryhmien PRA ja aldosteroni olivat selvästi korkeampia kuin vastaavien verrokkiryhmien arvot. Hyperurikemia lisäsi virtsan kalium / natrium -suhdetta yli 60% molemmissa hyperurikemiaryhmissä. Munuaisen reniini-angiotensiinijärjestelmän komponenteista (ACE, ACE2, AT_{1R} ja AT_{2R}) ei löytynyt tälle vaikutukselle selittävää tekijää, joten suolatasapainon muutos selittynee järjestelmän kiertävissä komponenteissa havaituilla muutoksilla.

Johtopäätökset – Tulosten perusteella hyperurikemian haitallisia vaikutuksia verenkiertoelimistöön voidaan selittää PRA:n, aldosteronin ja näistä seuraavan virtsan kalium / natrium -suhteen nousulla.

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ABSTRACT

Background and Aims – Hyperuricemia is associated with renal insufficiency and may predispose to Na^+ retention and hypertension. Whether hyperuricemia plays a causal role in the pathogenesis of cardiovascular disease remains controversial. The aim of the study was to examine the effects of hyperuricemia on circulating and renal components of the renin-angiotensin-aldosterone system in experimental renal insufficiency.

Methods – Three weeks after 5/6 nephrectomy (NX) or sham-operation, rats were put on 2.0% oxonic acid diet (Oxo) for 9 weeks. Blood pressure (BP) was monitored using tail-cuff, and blood, urine, and kidney samples were taken, as appropriate. Kidney angiotensin-converting enzymes (ACE, ACE2), and angiotensin II receptors (AT_{1R}, AT_{2R}) were examined using real-time RT-PCR and autoradiography, while plasma renin activity (PRA) and aldosterone were determined using radioimmunoassay.

Results – Oxo increased plasma uric acid as expected, while BP was elevated only in hyperuricemic NX rats. Creatinine clearance was reduced by 60% in both NX groups, and by 25% in hyperuricemic Sham rats. The NX group showed over 90% suppression of PRA, whereas Sham+Oxo group showed over 1.2-fold and NX+Oxo group over 2.3-fold increases in both PRA and plasma aldosterone. Hyperuricemia increased K^+ and decreased Na^+ excretion in Sham and NX rats, leading to over 1.6-fold increase in urine K^+ to Na^+ ratio. No changes in kidney ACE, ACE2, AT_{1R} or AT_{2R} were detected that could explain the hyperuricemia-induced alteration in Na^+-K^+ balance.

Conclusions – As oxonic acid diet increased PRA, plasma aldosterone, and urine K^+ to Na^+ ratio, these changes may play a significant role in the harmful cardiovascular actions of hyperuricemia.

CONTENTS

ABBREVIATIONS

ACE	angiotensin-converting enzyme
ACE2	angiotensin-converting enzyme 2
ACEI	angiotensin-converting enzyme inhibitor
Ang	angiotensin
Ang I	angiotensin I
Ang II	angiotensin II
ANOVA	analysis of variance
AT_{1aR}	angiotensin II receptor type 1 subtype A
AT_{1bR}	angiotensin II receptor type 1 subtype B
AT_{1R}	angiotensin II receptor type 1
AT_{2R}	angiotensin II receptor type 2
AT_{R}	angiotensin II receptors
$AT_{R}B$	angiotensin II receptor type 1 blocker
ATP	adenosine triphosphate
BP	blood pressure
CKD	chronic kidney disease
COX-2	cyclooxygenase-2
CRF	chronic renal failure
CRI	chronic renal insufficiency
c-Src	cellular Src tyrosine kinase
CVD	cardiovascular disease
EDTA	ethylene diamine tetraacetic acid
ERK1/2	extracellular signal-regulated kinases 1 and 2
ESRD	end-stage renal disease
FGF	fibroblast growth factor
GFR	glomerular filtration rate
K/DOQI	Kidney Disease Outcome Quality Initiative

MAP	mitogen-activated protein (kinase)
MDRD	Modification of Diet in Renal Disease study
NAD^+	nicotinamide adenine dinucleotide
NX	5/6 nephrectomy
NX+Oxo	5/6 nephrectomy with 2.0% oxonic acid diet
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pK_a	acid dissociation constant
PMSF	phenylmethylsulfonyl fluoride
PRA	plasma renin activity
RAAS	renin-angiotensin-aldosterone system
RAS	renin-angiotensin system
RIA	radioimmunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
Sham	sham operation
Sham+Oxo	sham operation with 2.0% oxonic acid diet
TGF-β	transforming growth factor β
UA	uric acid, urate
URAT1	uric acid transporter 1
VSMC	vascular smooth muscle cell

1. INTRODUCTION

Chronic kidney disease (CKD) and cardiovascular disease (CVD) are becoming increasingly prevalent in our modern society bringing along a plethora of related disorders. These disorders include hyperparathyroidism, insulin resistance, glucose intolerance, metabolic syndrome, and hyperuricemia, while new associations are discovered constantly (Pörsti et al., 2004; Nakagawa et al., 2008; Rosamond et al., 2008). Treatment of these diseases is highly expensive and will cause a substantial economic burden for future populations (Salonen et al., 2007). Therefore focusing research effort on the causes and disease prevention rather than simple treatment of symptoms is the key step forward.

The pathological mechanisms behind kidney disease and associated disorders are heavily tied to changes in kidney morphology and the renin-angiotensin-aldosterone system (RAAS) (Johnson et al., 2008a). Hypertension – the most common form of CVD – is present in virtually every CKD patient, while it may both result from, and contribute to CKD. Progression of CKD is accelerated by hypertension, which makes control of blood pressure (BP) one of the main elements in CKD treatment (Fogo, 2007). Inhibitors of the RAAS are the most typically used drugs with hypertensive CKD patients, while additional renal replacement therapies are given to end-stage renal disease (ESRD) patients with severely lowered glomerular filtration capacity (Lameire et al., 2005; Khwaja et al., 2007).

Hyperuricemia is a disorder caused by elevated serum uric acid (UA), generated during purine catabolism by the enzyme xanthine oxidoreductase. It is best known for its importance in gout, which is a disease recognized for centuries and currently the most common inflammatory arthritis in the Western world (Terkeltaub, 2006). Gout is caused by deposition of urate crystals in the joints in which they induce chronic inflammation and tissue damage. Hyperuricemia is also known to be present without crystal formation in many CKD and CVD patients, although its putative role as an independent risk factor for these diseases is still controverial (Johnson et al., 2003). Recent evidence has suggested that elevated UA concentration can, in fact, cause salt-sensitive hypertension and renal microvascular disease, thereby contributing to the progression of CKD (Sanchez-Lozada et al., 2005; Johnson et al., 2008a).

The present study is based on previously reported harmful effects of hyperuricemia and continues the line of experimental work performed at the University of Tampere using the 5/6 nephrectomy (NX) rat model (Jolma et al., 2003; Kööbi et al., 2003; Pörsti et al., 2004; Kööbi et al., 2006). The NX model has been extensively used to study events involved in loss of renal function and CKD progression (Fogo, 2007) proving its validity in these experiments. Hyperuricemia, on the other hand, was produced in this experiment using 2.0% oxonic acid diet, which inhibits the catabolism of UA in rats (Mazzali et al., 2001). The oxonic acid model of hyperuricemia has been shown to induce preglomerular arteriolar disease leading to tubular ischemia, interstitial infiltration of lymphocytes and macrophages, oxidant generation, and local vasoconstriction. These changes are associated with decreased sodium filtration and increased sodium reabsorption eventually resulting in salt sensitivity (Johnson et al., 2002).

The detrimental effects of UA may be mediated via enhanced renin release, and in the oxonic acid model the renal vasculopathy and hypertension are preventable by the blockade of the renin-angiotensin system (RAS) (Mazzali et al., 2001; Mazzali et al., 2002; Nakagawa et al., 2003). However, information about the circulating plasma renin activity (PRA) and aldosterone concentration in the oxonic acid model is lacking. The present study was, therefore, undertaken to examine the effects of oxonic acid-induced hyperuricemia on components of RAAS in circulation and in renal tissue, with a special interest in putative changes of plasma aldosterone concentration.

Manuscript presenting the results of this study titled “Oxonic acid-induced hyperuricemia elevates plasma aldosterone in experimental renal insufficiency” has been accepted for publication in the *Journal of Hypertension* on the 2nd of April 2008.

2. REVIEW OF THE LITERATURE

2.1. CHRONIC RENAL INSUFFICIENCY AND CHRONIC KIDNEY DISEASE

The increasing global prevalence of CKD, chronic renal insufficiency (CRI), and ESRD with the associated skyrocketing cost has profound public health and economic implications (Khwaja et al., 2007). This has made slowing the progression of kidney diseases a major health-care priority. Recent reports suggest that up to 10% of the population in Europe (Lameire et al., 2005) and almost 17% of the population in the USA (Rosamond et al., 2008) are affected by CKD, while the global pandemic is further fuelled by ageing population, as well as the rise in the numbers of those affected by diabetes, obesity, and hypertension (Khwaja et al., 2007).

In February 2002, the Kidney Disease Outcome Quality Initiative (K/DOQI) of the National Kidney Foundation published clinical practice guidelines for CKD (2002) that were based on a systematic literature review (Johnson et al., 2004). According to the K/DOQI guidelines, CKD is defined as kidney damage or glomerular filtration rate (GFR) of <60 ml/min/1.73 m^2 for 3 months or more, irrespective of cause (Levey et al., 2005). Kidney damage in many kidney diseases can be ascertained by the presence of albuminuria, defined as albumin-to-creatinine ratio >30 mg/g in two of three spot urine specimens. GFR value can be obtained by measuring plasma creatinine or the amount of creatinine excreted in 24-hour urine sample, and subsequently using estimating equations such as the Modification of Diet in Renal Disease (MDRD) Study equation (Klahr, 1989) or the Cockcroft-Gault formula (Cockcroft & Gault, 1976) to determine the correct value.

The severity of kidney disease is classified according to the level of GFR into five stages in which lower stage number represents a less severe form of the disease (2002; Levey et al., 2005) (Table 2.1). The first stage exhibits kidney damage with normal GFR of ≥90 ml/min/1.73 m^2, while the second stage is defined with a GFR of 89-60 ml/min/1.73 m^2. The related terms used to describe these two stages are albuminuria (excess albumin in urine), proteinuria (excess protein in urine) or hematuria (blood in urine) depending on the symptoms and manifestations of the renal disorder. Kidney disease can be termed as early-CRI when GFR drops under 60 ml/min/1.73 m^2 marking the third stage of kidney disease. The fourth stage is late-CRI or pre-ESRD and features a GFR between 29-15 ml/min/1.73

m^2, whereas the fifth and the last stage - ESRD or kidney failure - presents itself with GFR under 15 ml/min/1.73 m^2 (Levey et al., 2005).

Table 2.1 *Classification of chronic kidney disease*

Stage:	Description:	GFR* (ml/min/1.73 $m^{2)}$	Related terms:
1	Kidney damage with normal GFR	≥90	Albuminuria, proteinuria, hematuria
2	Kidney damage with mildly lowered GFR	89-60	Albuminuria, proteinuria, hematuria
3	Moderately lowered GFR	59-30	Early chronic renal insufficiency
4	Severely lowered GFR	29-15	Late chronic renal insufficiency
5	Kidney failure	<15	Renal failure, uremia, end-state renal disease

* glomerular filtration rate Table adapted from Levey et. al (Levey et al., 2005)

Progressing CKD exhibits a histological appearance of glomerulosclerosis, vascular sclerosis and tubulointerstitial fibrosis, suggesting a common final pathway of injury (Fogo, 2007). The response to the initial glomerular and tubulointerstitial cell injury in kidney disease invariably involves changes in cell number (cell proliferation, apoptosis, and necrosis) and cell size (hypertrophy) (Khwaja et al., 2007). These events typically precede the accumulation of extracellular matrix, and therefore the balance between these differing processes determines whether the response to the kidney damage results in resolution of glomerular / tubulointerstitial injury and healing, or in progressive fibrosis and scarring. Numerous growth factors appear to modulate progression of glomerular and tubulointerstitial scarring (Bohle et al., 1992; Khwaja et al., 2007). These factors and their roles may differ at the various stages of injury. Previous studies in pathophysiological settings have implicated changes in PDGF, TGF-β, Ang II, basic FGF, endothelin-1, and various chemokines among others, in progressive renal scarring (Fogo, 2007). Thus, it may be possible to slow down the fibrotic process in CKD through pharmacological manipulation of these factors. The particular importance of tubulointerstitial damage in

progressive CKD was highlighted in an extensive systematic morphometric analysis of over 1700 renal biopsies, which showed that interstitial inflammation and fibrosis were significantly associated with a more rapid decline of kidney function (Bohle et al., 1992). So far, however, there are no clinical therapies specifically targeting kidney remodeling and the ensuing fibrotic process itself (Khwaja et al., 2007).

The RAAS has been the focus of investigation of progression in CKD because of the efficacy of inhibition of its components in CKD. Angiotensin-converting enzyme (ACE) inhibitors (ACEIs) decrease glomerular capillary pressure by preferential dilation of the efferent arteriole (Foreman & Chan, 1988), likely mediated by both inhibition of angiotensin II (Ang II) and also by the effect of ACEIs in augmenting bradykinin, which is degraded by ACE (Fogo, 2007). Indeed, angiotensin II type 1 receptor blockers (AT_RBs), which do not have this activity to increase bradykinin, do not preferentially dilate the efferent arteriole or decrease glomerular pressure to the extent of that seen with ACEIs in most experimental studies. However, both ACEIs and AT_RBs have shown superior efficacy in slowing progressive CKD in experimental models and in human CKD when compared with other BP-lowering agents. (MacKinnon et al., 2006; Fogo, 2007).

As CKD progresses, new disorders and symptoms add up. Chronic renal failure (CRF), the last stage of CKD, is associated with increased cardiovascular morbidity and mortality, while the underlying causes include anemia, acidosis, hypertension, volume overload, and accumulation of uremic toxins (Luke, 1998; Rostand & Drueke, 1999; Pörsti et al., 2004). Changes in calcium-phosphate balance, especially hyperphosphatemia, are also closely associated with the decline of kidney function, ectopic calcification, and reduced survival in renal patients. Disturbed calciumphosphorus balance is a major cause for cardiovascular complications during impaired kidney function (Luke, 1998; Rostand & Drueke, 1999; Pörsti et al., 2004), while phosphate retention, hypocalcemia, and reduced vitamin D levels lead to the development of secondary hyperparathyroidism in CRI (Rostand & Drueke, 1999). Elevated plasma phosphate and parathyroid hormone can be suppressed by dietary phosphate restriction, but oral phosphate binders, most commonly calcium salts, are usually required to manage hyperphosphatemia and hyperparathyroidism during CKD (Rostand & Drueke, 1999; Pörsti et al., 2004).

Treatment of CKD is currently divided in therapies that aim to treat the causes of CKD and therapies to maintain kidney function (dialysis – renal replacement) (Levey et al., 2007). The main treatment after diagnosis is aggressive control of hypertension with a particular focus on RAAS inhibitors. Systemic hypertension often accompanies renal disease and may both result from, and contribute to, CKD. Specifically, progression of CKD is accelerated by hypertension, and therefore control of BP is the key element in the treatment of CKD (Fogo, 2007).

2.2. CARDIOVASCULAR DISEASE AND HYPERTENSION

CVD and hypertension are epidemic in the modern society. CVD is the number one cause of death in the United States, claiming nearly 1 million lives yearly and accounting for 36% of all-cause mortality and more deaths than the next 7 leading causes combined (Rosamond et al., 2008). Nearly 2400 Americans die of CVD each day - an average of 1 death every 37 seconds. The most common form of CVD is hypertension, which is present in approximately 70 million people in the United States and the prevalence of which increases dramatically with age, affecting the majority of the population over the age of 60 (Watanabe et al., 2002; Rosamond et al., 2008). The prevalence of hypertension in the USA has increased markedly in the past 100 years, from a frequency of 6 to 11% in the population in the early 1900s to over 30% today. This increase correlates with the epidemic increase in obesity, metabolic syndrome, type II diabetes, and ESRD, raising the likelihood that these conditions are pathogenetically related and intricately linked to environmental and especially dietary changes that have occurred in the world population during the 100 year span (Johnson et al., 2005c). Hypertension markedly increases the risk for myocardial infarction, stroke, congestive heart failure, and peripheral vascular disease, while successful early treatment reduces these complications and can reduce the number of cardiovascular deaths as well as improve overall quality of life (Watanabe et al., 2002; Rosamond et al., 2008).

The increased prevalence of hypertension seems to be a recent event in human history, and there is evidence showing a correlation between hypertension and the changes in diet. Previous reports have suggested that a key nutritional factor that may account for the increased prevalence of hypertension in modern societies is the dietary sodium intake

(Watanabe et al., 2002). This hypothesis has received credibility from studies showing that primitive societies, whose populations ingest a very small amount of sodium, have a complete absence of hypertension (Oliver et al., 1975; Johnson et al., 2008a). The sodium content of early hunter-gatherers of the Paleolithic Period was also extremely low and has been estimated to be only 690 mg/d (equivalent to 30 mEq Na^+ or 1.9 g NaCl). In contrast, the sodium intake in the current Western diets average 4000 mg/d (170 mEq Na^+ or approximately 10 g NaCl). Although individuals with normal kidneys might be able to excrete the increased sodium content without altering systemic BP, there is evidence that individuals who develop essential hypertension have a relative defect in their ability to excrete sodium. It has thus been speculated that the sudden increase in sodium content in the diet of industrialized nations will unearth individuals with this physiological renal defect and thereby increase the overall prevalence of hypertension (Watanabe et al., 2002).

Hypertension has been defined as systolic BP of >140mmHg or diastolic BP of >90mmHg, or both, measured in a relaxed, sitting position. For ambulatory BP monitoring, hypertension is usually defined when BPs are >140/90mmHg for more than 25% of the readings for any given 24 h period (Feig & Johnson, 2003). The cut-off of 140/90mmHg was selected in the early 1900s based on the fact that only 5–10% of the US population had BPs in that range (Johnson et al., 2008a). In addition, it was recognized from the start that BPs in the hypertensive range were almost inevitably accompanied with arteriolosclerosis as well as kidneys that were grossly contracted and granular in appearance, with glomerular, and more commonly tubular, changes on microscopic examination. This suggests that hypertension should not simply be defined by an elevation in BP but rather should be considered a syndrome in which microvascular disease and renal involvement are key components (Johnson et al., 2008a). Afferent arteriolopathy and concomitant microvascular disease narrows glomerular afferent arteriolar lumens via hypercellularity and thickening, thereby inducing glomerular hypertension (Johnson et al., 2003)

Further support for a key role for the kidney in the pathogenesis of hypertension has come from transplant studies in experimental models of hypertension (Dahl & Heine, 1975; Rettig et al., 1990) and in humans (Curtis et al., 1983). Dahl & Heine (Dahl & Heine, 1975) demonstrated that the transplantation of a kidney from a rat with salt-sensitive hypertension to a normotensive rat will transfer the salt sensitivity. Curtis et al. (Curtis et

al., 1983) also showed in humans with hypertension-induced ESRD that the transplantation of a kidney from a normotensive donor could cure the hypertension. This latter finding, as well as more recent studies (Kvist & Mulvany, 2003), demonstrated that systemic vascular disease cannot be the cause of hypertension. The observation of the primary importance of the kidney in essential hypertension does not negate the role of non-renal mechanisms in the process. For example, the elevation in peripheral vascular resistance is mediated by vasoconstriction that is dependent on Ang II receptors and other mediator systems (Crowley et al., 2005). Persistent activation of the sympathetic nervous system may also cause chronic hypertension (Johnson et al., 2008a).

It has been observed that salt restriction causes some individuals with essential hypertension to have a greater fall in BP than others, and this has led to the concept of further classifying essential hypertension as salt-sensitive or salt-resistant. Intricately associated with the vascular lesion in the kidney is an infiltration into the interstitium of inflammatory cells consisting of T-cells and macrophages, many of which are producing oxidants and Ang II (Johnson et al., 2008a). Because renal microvascular disease is non-uniform, the rise in systemic BP results in some nephrons being overperfused whereas others are underperfused. Peritubular capillaries may also be damaged. The net effect is that ischemia is not completely relieved, and this leads to continued stimulation of sodium reabsorptive mechanisms by renin-dependent and renin-independent mechanisms (Sealey et al., 1988). As a consequence, the pressure natriuresis curve is shifted to the right and flattened, and the hypertension is salt-sensitive (Johnson et al., 2008a).

2.3. THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

The critical role of the circulating RAAS in the regulation of arterial pressure and sodium homeostasis has been recognized for many years (Zhuo et al., 1999; Navar & Nishiyama, 2001; Bernstein, 2006; Paul et al., 2006). The RAAS is the best known regulator of BP and determinant of target-organ damage from hypertension. It also controls fluid and electrolyte balance through coordinated effects on the heart, blood vessels, and kidneys (Remuzzi et al., 2005). Ang II is the most powerful biologically active product of the RAAS, although there are other bioactive Ang peptides, including Ang III, Ang IV, and Ang 1-7. Ang II directly constricts vascular smooth muscle cells, enhances myocardial

contractility, stimulates aldosterone production, stimulates release of catecholamines from the adrenal medulla and sympathetic nerve endings, increases sympathetic nervous system activity, and stimulates thirst and salt appetite (Kobori et al., 2007b). Ang II also regulates sodium transport by epithelial cells in the intestine and kidney. There has also been a growing appreciation of the organ-specific roles exerted by Ang II acting as a paracrine factor (Paul et al., 2006). In addition to its physiological roles, locally produced Ang II induces inflammation, cell growth, mitogenesis, apoptosis, cell migration, and differentiation, regulates the gene expression of bioactive substances, and activates multiple intracellular signalling pathways; all of which might contribute to tissue injury (Navar, 1997; Kobori et al., 2007a).

2.3.1. *The classic circulating renin-angiotensin system*

Ang II is produced systemically via the classic RAS. An aspartyl protease, renin, in the plasma is released primarily from the juxtaglomerular cells on the afferent arterioles of the kidney (Hackenthal et al., 1990). Although circulating active renin and prorenin are released mainly from the kidney, other tissues also secrete prorenin into the circulation, and prorenin can be converted to renin by limited proteolysis such as that with trypsin activation in the circulation (Sealey et al., 1986). Angiotensinogen is primarily formed and constitutively secreted by hepatic cells into the circulation, thus allowing systemic formation of Ang II throughout the circulation (Brasier & Li, 1996). On release into the circulation, renin cleaves angiotensinogen at the N terminus to form the decapeptide Ang I (Navar, 1997). The circulating concentrations of angiotensinogen are abundant, being more than 1000 times greater than the plasma Ang I and Ang II concentrations (Navar & Nishiyama, 2001). Although some species variation exists, changes in renin activity determine the rate of Ang I formation in the plasma from the huge stores of circulating angiotensinogen, effectively making it the rate-limiting step of the RAS (Ichihara et al., 2004; Paul et al., 2006).

Renin is synthesized and stored in substantial quantities in the granules of juxtaglomerular cells and is released in response to various stimuli (Schweda & Kurtz, 2004; Paul et al., 2006). Thus, large changes in plasma renin levels can occur rapidly, leading to changes in Ang I generation. Ang I is easily converted to Ang II, due not only to the circulating ACE, but also to the widespread presence of ACE on endothelial cells of many vascular beds

including the lung (Navar, 1997; Ichihara et al., 2004; Paul et al., 2006). Although other pathways for Ang II formation have been identified in certain tissues, the circulating levels of Ang II reflect primarily the consequences of the renin and ACE enzymatic cascade on angiotensinogen (Johnston, 1994; Kobori et al., 2007a). The resultant increases in plasma Ang II exert powerful actions throughout the body through activation of Ang II receptors (AT_R) (Paul et al., 2006) (Figure 2.1). Several angiotensinases and peptidases are then able to metabolize Ang II further (Kobori et al., 2007a). It is recognized that several of the smaller peptides, including Ang III, Ang IV, and Ang 1-7, have biological activity, but their plasma levels are much lower than those of Ang II (Kobori et al., 2007a).

Recent attention has been focused on findings that local Ang II levels are differentially regulated in the kidney. Because there often is not clear evidence for markedly elevated circulating renin or Ang II concentrations, identification of local RAS activity is essential for understanding the mechanisms mediating pathophysiological functions. In particular, the Ang II contents in renal tissues are much higher than can be explained on the basis of equilibration with the circulating concentrations (Navar, 1997; Navar & Nishiyama, 2004). Furthermore, the demonstration of much higher concentrations of Ang II in specific regions and compartments within the kidney indicates selective local regulation of intrarenal Ang II (Ichihara et al., 2004; Navar & Nishiyama, 2004). Thus, it is now apparent that intrarenal Ang II levels are regulated in a manner distinct from circulating Ang II concentrations. It has also been revealed that Ang II produced locally in the kidney exerts an important regulatory influence on renal hemodynamics and functions as a paracrine factor (Paul et al., 2006). Further studies have demonstrated that reduced renal function and its structural changes are associated with inappropriate activation of the intrarenal Ang II, leading to the development of hypertension and renal injury (Navar, 2005).

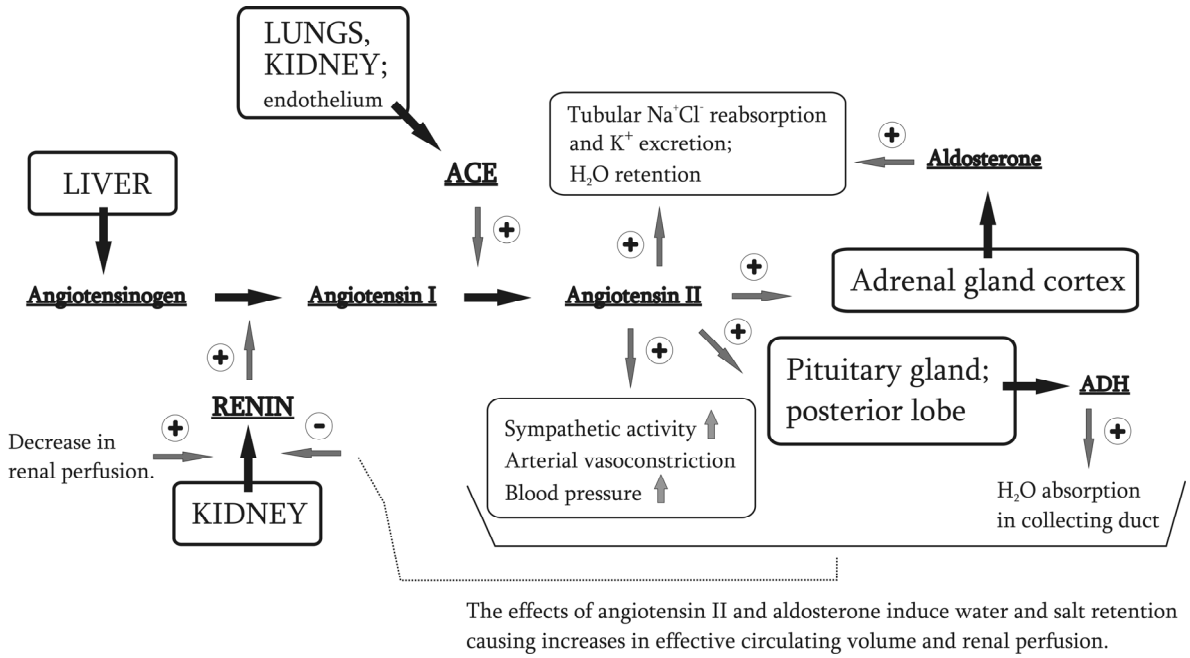

Figure 2.1 *Simplified overview of the renin-angiotensin system:* Kidneys respond to a decrease in renal perfusion by increasing renin secretion. This leads to increased formation of angiotensin I from the vast stores of angiotensinogen in plasma. Angiotensin-converting enzyme (ACE) catalyzes the formation of angiotensin II (Ang II), while Ang II acts in cooperation with aldosterone to increase renal perfusion by increasing circulating blood volume. This increase acts as an inhibitor of renin secretion effectively completing the feedback loop. Figure by the author with data by Navar, Paul et al., and Kobori et al. (Navar, 1997; Paul et al., 2006; Kobori et al., 2007a)

2.3.2. *The intrarenal renin-angiotensin system*

In recent years, the focus of interest on the role of the RAS in the pathophysiology of hypertension and organ injury has changed to a major emphasis on the role of the local RAS in specific tissues. In the kidney, all of the RAS components are present and intrarenal Ang II is formed by independent multiple mechanisms (Navar & Nishiyama, 2001). Proximal tubular angiotensinogen, collecting duct renin, and tubular AT_Rs are positively augmented by intrarenal Ang II. In addition to the classic RAS pathways, prorenin receptors and chymase are also involved in local Ang II formation in the kidney (Prescott et al., 2002). Moreover, circulating Ang II is actively internalized into proximal tubular cells by AT_R-dependent mechanisms (Higuchi et al., 2007). Consequently, Ang II is compartmentalized in the renal interstitial fluid and the proximal tubular compartments with much higher concentrations than those existing in the circulation. Recent evidence

has also revealed that inappropriate activation of the intrarenal RAS is an important contributor to the pathogenesis of hypertension and renal injury (Kobori et al., 2007a). Thus, it is necessary to understand the mechanisms responsible for independent regulation of the intrarenal RAS.

The RAS has been acknowledged as an endocrine, paracrine, autocrine, and intracrine system, and because of that, it has been almost impossible to distinguish the different contributions of the classic RAS versus the local RAS (Navar et al., 2002; Kobori et al., 2007a). Emerging evidence suggests that local formation is of major significance in the regulation of the Ang II levels in many organs and tissues. For example, there is substantial evidence that the Ang peptide levels in the brain are regulated in an autonomous manner (Kobori et al., 2007a). Although every organ system in the body has elements of the RAS, the kidney is unique in having every component of the RAS with compartmentalization in the tubular and interstitial networks as well as intracellular accumulation. Recent attention has been focused on the existence of unique RASs in various organ systems. Various studies have demonstrated the importance of the tissue RAS in the brain, heart, adrenal glands, and vasculature as well as in the kidney (Navar et al., 1995). There is substantial evidence that the major fraction of Ang II present in renal tissues is generated locally from angiotensinogen delivered to the kidney as well as from angiotensinogen locally produced by proximal tubule cells. Ang I delivered to the kidney can also be converted to Ang II (Komlosi et al., 2003). Renin secreted by the juxtaglomerular apparatus cells and delivered to the renal interstitium and vascular compartment also provides a pathway for the local generation of Ang I (Hackenthal et al., 1990, Kobori, 2007 #332). ACE is abundant in the rat kidney and has been located in the proximal and distal tubules, the collecting ducts, and renal endothelial cells. Therefore, all of the components necessary to generate intrarenal Ang II are present along the nephron (Paul et al., 2006; Kobori et al., 2007a).

Although most of the circulating angiotensinogen is produced and secreted by the liver, the kidneys also produce angiotensinogen (Kobori et al., 2007b). Intrarenal angiotensinogen mRNA and protein have been localized to proximal tubule cells, indicating that the intratubular Ang II could be derived from locally formed and secreted angiotensinogen. The angiotensinogen produced in proximal tubule cells seems to be secreted directly into the tubular lumen in addition to producing its metabolites

intracellularly and secreting them into the tubule lumen (Lantelme et al., 2002). Proximal tubule angiotensinogen concentrations in rats have been reported in the range of 300 to 600 nM, which greatly exceed the free Ang I and Ang II tubular fluid concentrations (Navar & Nishiyama, 2001). Because of its substantial molecular size, it seems unlikely that much of the plasma angiotensinogen filters across the glomerular membrane, further supporting the concept that proximal tubule cells secrete angiotensinogen directly into the tubule (Kobori et al., 2007a).

2.3.3. *Plasma renin and aldosterone*

The plasma renin concentration or activity is often used as a measure of the overall activity of the RAS. In most species, renin synthesized by the juxtaglomerular apparatus cells is the primary source of both circulating and intrarenal renin levels. The secreted active form of renin contains 339 to 343 amino acid residues after proteolytic removal of the 43-amino acid residue at the N terminus of prorenin. Circulating active renin and prorenin are released mainly from the kidney, but other tissues also secrete prorenin into the circulation (Sealey et al., 1986). Besides serving as the precursor for active renin, it has been suggested that circulating prorenin is taken up by some tissues where it may contribute to the local synthesis of Ang peptides (Prescott et al., 2002). In the heart under normal conditions, renin is not produced and its transcript is undetectable or extremely low (Kobori et al., 2007a). Although there have been suggestions that renin or prorenin may directly induce cellular effects - independent of the generation of Ang II - the well established role of renin is to act on angiotensinogen, a protein with a glycosylated weight of 52 to 64 kDa and synthesized primarily by the liver to form Ang I. However, the renin receptor may also initiate intracellular signaling to activate extracellular signal-regulated kinases (Nguyen et al., 2002). In the heart and kidney, the renin receptor binds renin and prorenin, leading to an increase in the catalytic efficiency of Ang I formation from angiotensinogen (Nguyen et al., 1996). It has also been reported recently that the binding of prorenin to an intrinsic prorenin-binding receptor plays a pivotal role in the development of diabetic nephropathy by a mechanism that involves the receptor-associated prorenin system (Ichihara et al., 2004).

The mineralocorticoid aldosterone is synthesized by a series of enzymatic reactions from cholesterol in the zona glomerulosa of the adrenal gland. In addition, extra-adrenal

synthesis of aldosterone has been reported in different tissues (Takeda et al., 2000; Cachofeiro et al., 2008). The extra-adrenal production of aldosterone appears to be regulated by the same stimuli that regulate adrenal synthesis. At the vascular level, its production has been reported in both endothelial and smooth muscle cells. However, the physiological relevance of this production is under discussion (Cachofeiro et al., 2008). High circulating aldosterone concentration is acknowledged as a significant cardiovascular risk factor (Schmidt & Schmieder, 2003; Schmidt et al., 2006), and independently of whether the aldosterone origin is circulating or local, it exerts actions in the vascular wall through genomic and non-genomic effects.

Genomic actions imply the binding of aldosterone to cytoplasmatic mineralocorticoid receptors – a member of the nuclear receptor superfamily - and involve transcription and protein synthesis (Fuller & Young, 2005). These receptors have been found in both endothelial and smooth muscle cells and their expression can be increased in certain pathological situations such as hypertension (Fuller & Young, 2005); Cachofeiro, 2008 #434}. The direct targets for aldosterone are the late distal convoluted tubules, the connecting tubules, and the principal cells of the cortical and medullary collecting ducts. Aldosterone binds to the receptor and translocates into the nucleus. The ligand-receptor complex then forms a homodimer, binds to the specific DNA sequences and stimulates the transcription of its target genes (Nagase & Fujita, 2008).

The non-genomic effects of aldosterone are observed quickly after secretion or administration and are insensitive to transcription inhibitors. To date, two different non-genomic responses have been reported (Cachofeiro et al., 2008). One appears to involve mineralocorticoid receptors, whereas the second involves an unidentified membrane receptor. The signalling pathways include the modulation of intracellular calcium, Na^+ / H^+ exchanger activity, and phosphorylation of signalling molecules, including protein kinase C, epidermal growth factor receptor, mitogen-activated protein (MAP) kinases, and cellular Src tyrosine kinase (c-Src) (Cachofeiro et al., 2008; Nagase & Fujita, 2008). In fact, it was reported recently that aldosterone-induced activation of c-Src, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and p38 MAP kinase is increased in spontaneously hypertensive rat vascular cells, suggesting that aldosterone plays an important role through the upregulation of c-Src signalling in vascular alterations observed in hypertensive rats (Cachofeiro et al., 2008).

2.3.4. *Angiotensin-converting enzymes and Ang II receptors*

Ang I is rapidly converted into the major effector of the RAS, Ang II, by ACE, which is located on endothelial cells in many vascular beds and on membranes of various other cells including brush border membranes of proximal tubules (Mezzano et al., 2003). The localization of ACE within the kidney in various species has been well characterized. However, there are some important differences between humans and commonly used experimental animals. Indeed, it has been reported that kidneys from normal human subjects predominantly expressed ACE in the brush border of proximal tubular segments, and very little ACE expression was observed on vascular endothelial cells. ACE was not detectable in the vasculature of the glomerular tuft or even in the basolateral membranes of epithelial cells. In contrast, there was intense labeling on the endothelial cells of almost all of the renal microvasculature of rats (Kobori et al., 2007a). It has been reported that less than 10% of arterially delivered Ang I is converted to Ang II, which along with the reduced ACE expression on renal vascular endothelial cells in humans implies that the influence of intrarenal Ang II formed from circulating precursors may not be of major significance (Paul et al., 2006).

In 2000 the RAS gained a new member as angiotensin-converting enzyme 2 (ACE2) was discovered and characterized (Tipnis et al., 2000). ACE2 is expressed predominantly in vascular endothelial cells of the heart and kidney. ACE2 and ACE have different biochemical activities, and Ang I is converted to Ang 1–9 with nine amino acids by ACE2, whereas ACE converts it to Ang II with eight amino acids. Ang II is a potent bloodvessel constrictor. Ang 1–9 has no known effect on blood vessels but can be converted by ACE to a shorter peptide, Ang 1–7, which is a blood-vessel dilator. Thus, it has been suggested that ACE2 functions as one of the protective component of the RAS preventing the formation of the vasopressor Ang II (Boehm & Nabel, 2002).

Most of the actions of Ang II on renal function are the consequence of activation of Ang II receptors, which are widely distributed in various regions and cell types of the kidney. Two major categories of Ang II receptors, type 1 (AT_{1R}) and type 2 (AT_{2R}), have been described, pharmacologically characterized, and cloned (Kobori et al., 2007a). Additionally, AT_{1R} features two well characterized subtypes: 1a (AT_{1aR}) and 1b (AT_{1bR}), although recent evidence suggests that humans only express AT_{1aR} (Higuchi et al., 2007).

In literature, most of the hypertension-causing actions of Ang II are generally attributed to the AT_{1R}s, marking it as the bad receptor (Ito et al., 1995). AT_{1R} transcript has been localized to proximal tubules, the thick ascending limb of the loop of Henle, glomeruli, arterial vasculature, vasa recta, arcuate arteries, and juxtaglomerular cells (Tufro-McReddie & Gomez, 1993). In rodents AT_{1aR} is the predominant subtype in all nephron segments, whereas AT_{1bR} is more abundant than AT_{1aR} only in the glomerulus. In mature kidneys, AT_{1aR} have been localized to the luminal and basolateral membranes of several segments of the nephron, as well as on the renal microvasculature in both cortex and medulla, smooth muscle cells of afferent and efferent arterioles, epithelial cells of the thick ascending limb of Henle, proximal tubular apical and basolateral membranes, mesangial cells, distal tubules, collecting ducts, and macula densa cells (Harrison-Bernard et al., 1999). This evidence is consistent with the localization of the transcript for the AT_{1R} subtypes in all of the renal tubular and vascular segments in rats (Miyata et al., 1999).

The AT_{2R} is highly expressed in human and rodent kidney mesenchyme during fetal life and decreases dramatically after birth (Kobori et al., 2007a). AT_{2R} protein has been localized to the glomerular epithelial cells, proximal tubules, collecting ducts, and parts of the renal vasculature of the adult rat (Miyata et al., 1999). Although the role of AT_{2R} in regulating renal function remains uncertain, it has been suggested that AT_{2R} activation counteracts AT_{1R} effects by stimulating formation of bradykinin and nitric oxide, leading to increases in interstitial fluid concentration of cyclic guanosine monophosphate (Siragy & Carey, 1999). AT_{2R} activation seems to influence proximal tubule sodium reabsorption either by a cell membrane receptor-mediated mechanism or by an interstitial nitric oxide-cyclic guanosine monophosphate pathway (Jin et al., 2001). Ang II infusion into AT_{2R}-knockout mice leads to exaggerated hypertension and reductions in renal function, probably due to decreased renal interstitial fluid levels of bradykinin and cyclic guanosine monophosphate available that counteract the direct effect of Ang II (Siragy & Carey, 1999). AT_{2R} can thus be regarded as a protective component of RAS inhibiting vasoconstriction.

2.4. URIC ACID METABOLISM AND HYPERURICEMIA

UA is generated during the catabolism of purines obtained by ingestion or from the breakdown of DNA, RNA and ATP (Watanabe et al., 2002; Johnson et al., 2008b). UA is a weak acid (pK_a=5.75) distributed throughout the extracellular fluid compartment as sodium urate and cleared from the plasma mainly by glomerular filtration (Waring et al., 2000). The immediate precursor enzyme is xanthine oxidoreductase, which promotes the last metabolic steps by converting hypoxanthine to xanthine and xanthine to UA (Doehner & Anker, 2005). Substrates used for hypoxanthine formation by the enzyme adenine deaminase are adenine and adenosine, whereas the enzyme inosine phosphorylase uses inosine as substrate. Guanine, on the other hand, is metabolised straight to xanthine by the guanine deaminase (Waring et al., 2000).

Xanthine oxidoreductase is a flavoprotein that contains both iron and molybdenum and uses NAD^+ as an electron acceptor. It exists in two interconvertible forms: xanthine dehydrogenase and xanthine oxidase (XO). In its XO form, this enzyme can transfer the decreasing equivalent to molecular oxygen as redox partner generating free oxygen radicals - superoxide anion and hydrogen peroxide, which can be converted to free hydroxyl radicals (Doehner & Anker, 2005). In 1968, the cytosolic XO was the first documented putative biologic generator of oxygen-derived free radicals, and since then, it has been established that XO is a major source of free oxygen radical production in the human body (Terada et al., 1992; Harrison, 1997). This metabolic pathway is of particular significance in conditions of tissue hypoxia and ischemia / reperfusion because the increased degradation of adenosine triphosphate via adenosine leads to increased substrate load for XO. Accordingly, an increase in serum UA level has been observed in hypoxic states such as obstructive pulmonary disease, neonatal hypoxia, cyanotic heart disease, and acute heart failure (Doehner & Anker, 2005). Simultaneously, in ischemia and hypoxia, xanthine dehydrogenase is increasingly converted to XO, which further adds to accelerated radical production (Terada et al., 1992).

In most mammals UA is further degraded to allantoin by the enzyme uricase (urate oxidase), resulting in serum UA levels in the range of 30-90 μmol/l (Watanabe et al., 2002; Johnson et al., 2008b). Uricase is localized predominantly in liver and is associated with the peroxisome as a tetramer with a subunit molecular mass of 32-33 kDa (Wu et al.,

1989). Depending on the species, allantoin may be further degraded by allantoinase to generate ammonia (Johnson et al., 2008b). The serum UA concentration of hominoids - apes and humans - and some New World monkeys is, however, higher than in other mammals. This increase is due to distinct mutations in the uricase gene that made it non-functional during the Miocene era (Wu et al., 1992). Elevated serum UA concentration is called hyperuricemia when it exceeds 360 μmol/l or 420 μmol/l in women and men, respectfully (Johnson et al., 2003). Hyperuricemia may be best known for its association with gout, which is a disease recognized for centuries and currently the most common inflammatory arthritis in the Western world (Terkeltaub, 2006). Gout is caused by deposition of urate crystals in the joints in which they induce chronic inflammation and tissue damage. However, hyperuricemia is also known to be present without crystal formation in many CKD and CVD patients. Characteristically, patient groups with the highest cardiovascular risk coincidentally have highest mean serum UA concentrations. These include heavy alcohol users (increased UA generation and decreased excretion), diuretic users (decreased UA excretion), and black race (unknown reason – possibly lower nephron count) (Johnson et al., 2003). Additionally, serum UA is typically higher in men and postmenopausal women than premenopausal women because of the uricosuric (UA excretion increasing) actions of estrogen, whereas high insulin concentration has in turn been reported to decrease UA excretion (Muscelli et al., 1996).

In humans, the chimpanzee, and the gorilla, three uricase mutations have been identified. These include a nonsense mutation of codon 33, a nonsense mutation of codon 187, and a splice mutation in exon 3, while the human uricase gene totals 304 codons – 8 exons and 8 introns. The mutation at codon 33 is also found to be present in the orangutan, whereas none of these mutations are present in the gibbon, in which a separate 13 bp reading-frame-changing deletion was identified between codons 72 and 76 of exon 2 (Wu et al., 1992). Based on the phylogeny of hominoid evolution as assessed by DNA-DNA hybridization, it was proposed that the nonsense mutation affecting the hominoid lineage at codon 33 occurred between 24 and 13 to 16 million years ago, and the 13 bp deletion in exon 2 occurred sometime after the split of the gibbon from the other hominoids, which occurred around 22 to 24 million years ago (Wu et al., 1992; Watanabe et al., 2002). The current evidence now suggests that the loss of uricase in humans may have been stepwise, with a progressive loss in activity due to mutations in the promoter region followed by complete silencing of the gene (Oda et al., 2002; Johnson et al., 2008b).

The biological reason for the loss of urate oxidase activity in hominoids is unknown, but there are a few reasonable hypotheses that are in agreement with the existent UA evidence. An evolutionary fact is that when a mutation completely silences the function of an enzyme, it either hampers its carrier, or gives the carrier an advantage over the wild-type population. When this kind of mutation sweeps all over the population and becomes the wild-type, we can safely assume that the mutation was highly advantageous in the contemporary environment. During the Miocene this was the situation that happened with the uricase gene. Non-functional uricase presented a new kind of survival advantage that eventually lead to its complete precipitation in later hominoids (Watanabe et al., 2002; Johnson et al., 2008b).

The most quoted hypothesis explaining the advantage associated with the non-functional uricase is that originally proposed by Ames et al. (Ames et al., 1981), who suggested that the uricase mutation may have occurred as a means to replace serum antioxidant activity after the loss of ascorbate (vitamin C) synthesis. Indeed, UA is a water-soluble antioxidant that can help maintain ascorbate levels and is also considered to be one of the most important antioxidants in the plasma (Ames et al., 1981; Johnson et al., 2008b). However, UA is not solely an antioxidant as it can also function as a pro-oxidant on a variety of cell types and *in vivo* (i.e. reactions with peroxynitrite) (Santos et al., 1999). According to the hypothesis, it was also suggested that the reason humans have longer life expectancy compared to other mammals may relate to the antioxidant benefits provided by the higher UA levels. Challenging this hypothesis, however, is the observation that neither ascorbate nor UA levels correlate with maximum life span in vertebrates (Lopez-Torres et al., 1993; Johnson et al., 2008b).

Another hypothesis is that the increase in UA resulted in better reaction time and higher mental performance due to its putative neurostimulant properties based on its similarity in chemical structure with caffeine (Johnson et al., 2008b). While some epidemiological and experimental studies have supported this, the evidence has been weak at best. Other alternative hypotheses have linked the increase in UA with improved innate immune function and the ability to ward off infections or tumors. Specifically, UA is reported to aid in the immune recognition of dying cells, help activate the inflammasome critical for interleukin-1 β release, and participate in the immune rejection of tumor cells (Shi et al., 2003; Hu et al., 2004; Johnson et al., 2008b).

The latest uricase hypothesis was postulated by Watanabe et al. in 2002 (Watanabe et al., 2002) and it has generated mixed opinions on different medical fields (Ellman & Becker, 2006; Johnson et al., 2008a). According to this hypothesis, elevated UA would have benefited the hominoids of the Miocene by causing sodium retention, concurrently helping to maintain BP during the times of low sodium ingestion. Hominoids that could better conserve sodium and maintain BP might have prevailed during the harsh environment of the Miocene (Watanabe et al., 2002). In fact, there is evidence that during the early Miocene there was a marked increase in the number of ape species (Begun, 2003). However, by the mid Miocene there was global cooling - the Miocene Disruption - associated with the extinction of numerous species, likely including many species of apes. During this period, large rain forests dried out leaving only deserts and grasslands, therefore forcing early hominoids to develop knuckle walking and to modify their diet (Johnson et al., 2008b). The Paleolithic diet was low in sodium (Eaton & Konner, 1985), and hence survival would have been better with those species that could maintain BP and salt sensitivity. The increase in serum UA acutely increases BP and maintains sodium conservation because of the action of UA to enhance activation of the RAS in response to a low-salt diet. However, UA can also induce renal microvascular disease by stimulating smooth muscle cell proliferation with the activation of MAP kinase and stimulation of platelet-derived growth factor (PDGF), cyclooxygenase-2 (COX-2), and the RAS (Watanabe et al., 2002). Microvascular disease, preglomerular arteriolar disease, and interstitial inflammation are associated with narrowing of renal afferent arteriolar lumens, thereby causing local vasoconstriction, glomerular hypertension, and salt sensitivity with a chronic increase in BP (Sanchez-Lozada et al., 2002). The activation of these 2 main pathways – the RAS and microvascular disease – results in a persistent elevation in BP with concurrent maintenance of sodium balance (Watanabe et al., 2002) (Figure 2.2). Indeed, there is also clinical evidence that high serum UA levels are associated with increased proximal tubular sodium reabsorption in men (Cappuccio et al., 1993), while sodium sensitivity is considered a characteristic feature in hyperuricemia (Ward, 1998).

Extensive studies showing that hyperuricemia independently predicts the development of hypertension (Johnson et al., 2005a; Sundstrom et al., 2005) have been carried out recently. Hyperuricemia has been shown to be prevalent in early hypertension, and in one study it was present in almost 90% of hypertensive adolescents (Feig & Johnson, 2003). Furthermore, recent clinical trials have found that lowering UA lowers BP in both

adolescents and adults with hypertension (Feig et al., 2004; Feig & Johnson, 2007). In addition to elevating BP, recent studies support UA as having a role in insulin resistance and obesity (Nakagawa et al., 2006; Sanchez-Lozada et al., 2007). It has been reported that fructose, which rapidly raises UA, induces metabolic syndrome in animals and this can be ameliorated by lowering serum UA (Nakagawa et al., 2006). The mechanism by which UA mediates features of the metabolic syndrome may result from the ability to block some of insulin's actions by reducing endothelial nitric oxide synthesis as well as due to direct effects of UA on the glomerular adipocytes (Sautin et al., 2007; Johnson et al., 2008b).

How is it possible that a mutation once presenting a crucial advantage now plays a role in the cardiovascular epidemic? The consequence of the uricase mutation is that humans not only have higher serum UA levels than most other mammals, but they also can not regulate UA levels as effectively (Johnson & Rideout, 2004; Johnson et al., 2005d). Interestingly, because the current Western diet is high in meats and fructose - both of which generate UA - humans today have higher UA levels (range 240-600 μmol/l) compared to primates that lack uricase (typically 180-240 μmol/l range) (Johnson et al., 2005d). However, the most compelling finding is the elevation of mean serum UA levels in the USA during the last century. In men, mean serum UA levels reportedly increased from <210 μmol/l in the 1920s to approximately 300 μmol/l in the 50s, and to 350-390 μmol/l in the 70s (Nakagawa et al., 2008). Recent preliminary studies have shown that the Yanomamo Indians living in their original habitat and with their primitive diet have serum UA levels in the 120-240 μmol/l range, suggesting that primitive humans had even lower UA levels than those in the beginning of the twentieth century (Johnson et al., 2008b). In today's society we are ingesting significantly more fructose-containing sweeteners and purine-infested meats than in the past, and those who obtain the highest UA levels could end up developing hypertension, kidney disease, insulin resistance and obesity (Johnson et al., 2008b), while hyperuricemia reportedly correlates with increased cardiovascular mortality (Fang & Alderman, 2000).

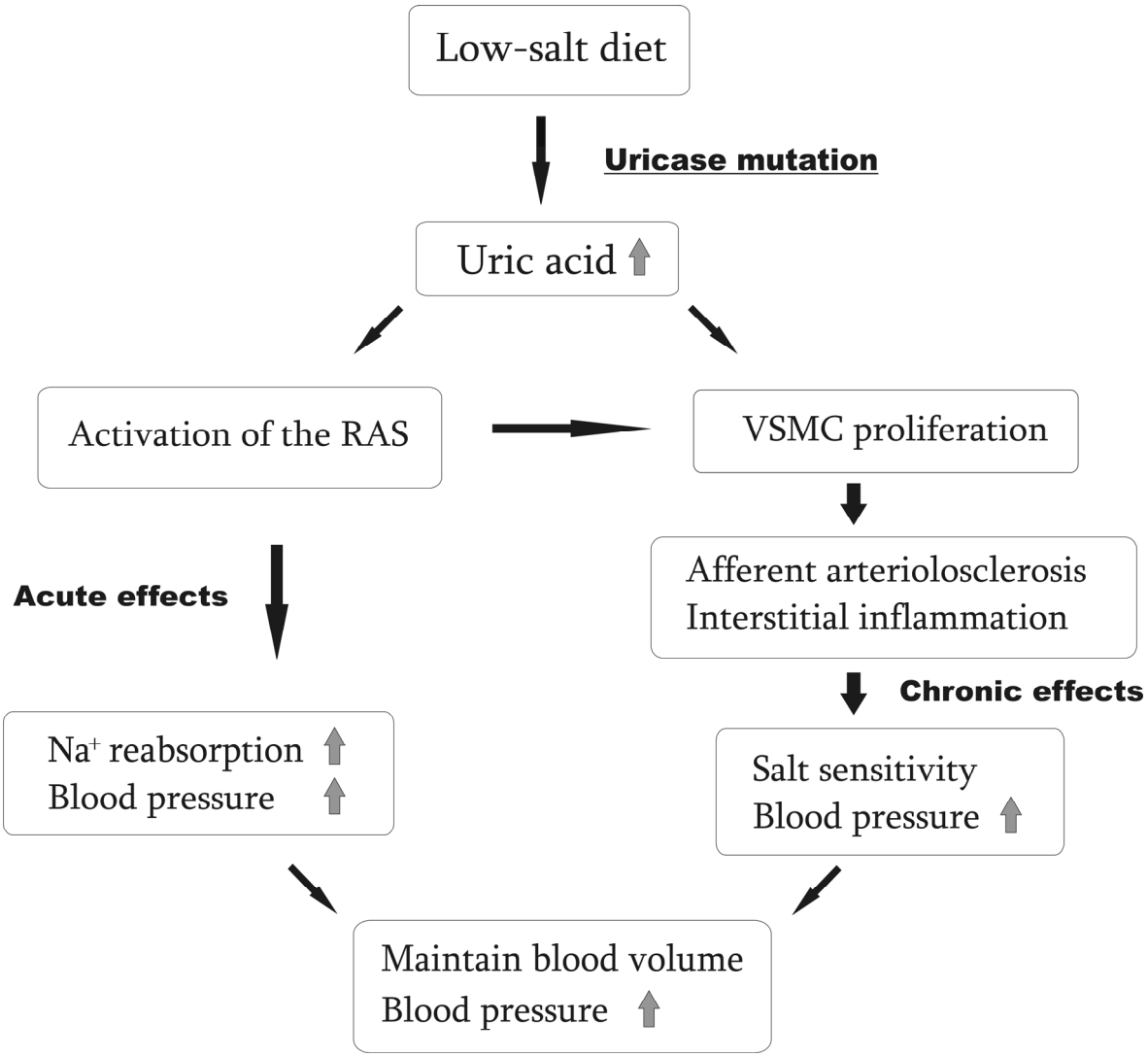

Figure 2.2 *Salt sensitivity hypothesis:* Environmental pressures during the Miocene resulted in a survival advantage for hominoids that could better conserve sodium and maintain blood pressure (BP). The increase in uric acid (UA) acutely increases BP and causes sodium retention because elevated UA enhances the activation of the the renin-angiotensin system (RAS) in response to a low-salt diet. UA also induces renal microvascular disease by stimulating vascular smooth muscle cell (VSMC) proliferation with the activation of MAP kinase and stimulation of PDGF, COX-2, and the RAS. The development of renal microvascular disease and interstitial inflammation causes salt sensitivity and a chronic increase in BP. These two main pathways result in a permanent elevation of BP along with sodium retention. Figure adapted from Watanabe et al. (Watanabe et al., 2002).

In humans, UA is mainly excreted through glomerular filtration, although bacterial degradation in the intestine also plays a small role in the elimination. The regulation of urinary UA excretion is complex. According to the classic notion, UA is freely filtrated at glomerulus, but almost completely reabsorbed in the proximal tubulus. As a consequence, only 10% of urate is excreted in urine (Nakagawa et al., 2008) (Figure 2.3). Recent studies have focused on the specific transporters in the proximal tubules that are involved in reabsorption, and uric acid transporter 1 (URAT1) seems to be one of the major UA transporters present in the luminal border of human proximal tubular cells (Anzai et al., 2007). URAT1 is an organic anion transporter that exchanges organic anions for urate. A key role for URAT1 in regulation of serum UA levels has been suggested, since mutation of URAT1 is known to cause hypouricemia in humans (Nakagawa et al., 2008).

Altered serum UA concentrations, both above and below normal levels, have been linked to a number of other disease states as well. As mentioned above, hyperuricemia has been correlated with gout, hypertension, cardiovascular disease, and renal disease, whereas lower than normal UA concentration, hypouricemia, has been linked to Parkinson's disease, Alzheimer's disease, and optic neuritis (Kutzing & Firestein, 2008). Several recent studies have also reported lower levels of UA in multiple sclerosis patients, while other studies found no such correlation (Spitsin & Koprowski, 2008). Historically, elevated UA has been considered a marker of the above disease states, but recently published studies have provided evidence that UA may actually play a role in the development or progression of such diseases (Kutzing & Firestein, 2008). As a result, the manipulation of UA concentrations is now either included in, or being investigated for, the treatment of a variety of disease states (Kutzing & Firestein, 2008).

The current treatment of hyperuricemia is mainly aimed at gout patients, as there is no agreement on whether lowering of UA in CVD is necessary. Several drugs are known to lower UA. These drugs either increase UA excretion (uricosuric drugs), block the final step in UA production via XO inhibition, or lead to UA breakdown. The most effective uricosuric drugs are probenecid and sulfinpyrazone, while fenofibrate and losartan (an Ang II antagonist) also have uricosuric activity (Dawson & Walters, 2006). Rasburicase, on the other hand, is a recombinant uricase which converts UA to allantoin. It is used in association with some anticancer treatments and is unsuitable for repeated dosing. There are also two commercially available XO inhibitors - allopurinol and oxypurinol - which

both are purine analogs. Allopurinol is rapidly metabolized to oxypurinol that binds to XO thereby inhibiting its activity (Dawson & Walters, 2006). A new non-purine XO inhibitor called febuxostat is also being developed (Sanchez-Lozada et al., 2008). It differs from allopurinol because it does not inhibit other enzymes in purine and pyrimidine metabolism pathways. Moreover, the XO inhibiting effect exerted by febuxostat *in vitro* and *in vivo* is more potent than that of allopurinol (Takano et al., 2005). Studies have shown that febuxostat inhibited the activity of XO simply by obstructing substrate binding and this inhibition was not influenced by changes in the redox status of the system (Okamoto et al., 2003; Sanchez-Lozada et al., 2008).

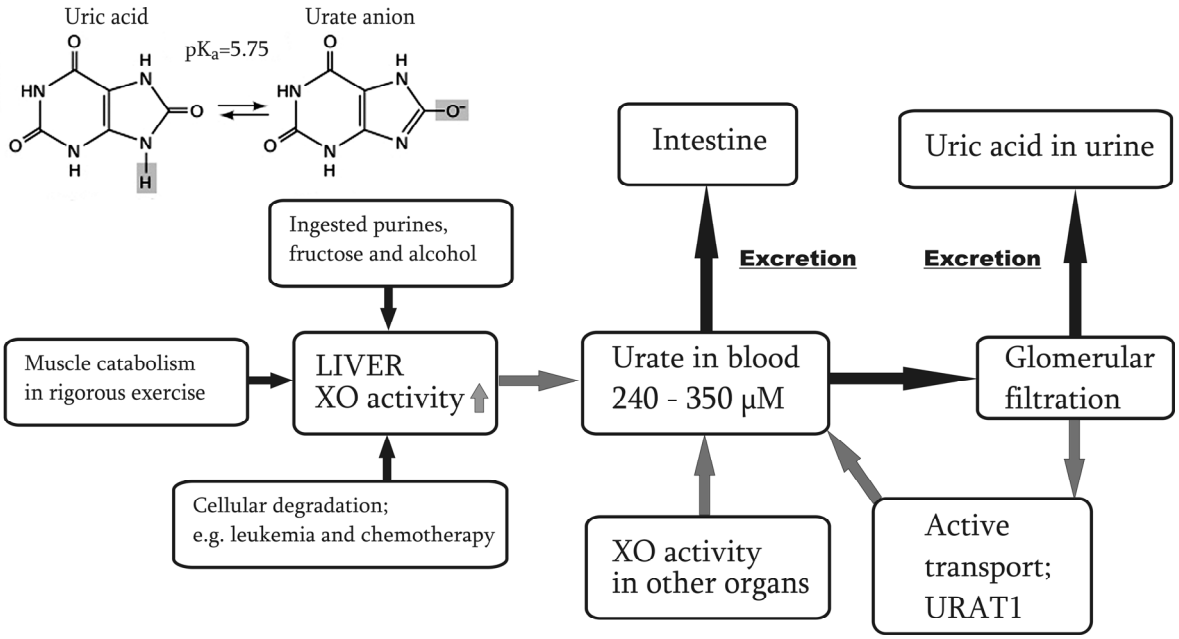

Figure 2.3 *Simplified overview of the uric acid metabolism:* In blood, uric acid (UA) is present as urate anion. It is produced by the enzyme Xanthine oxidase (XO) mainly in the liver. Additionally, XO in other organs (lung, intestine etc.) contribute to the urate reservoir in blood. XO uses purines obtained from the diet or from the degradation of nucleic acids and proteins as its substrate. UA is excreted mainly through glomerular filtration, while bacterial degradation in the intestine also plays a role in the elimination. UA is actively reabsorbed from the proximal tubule by uric acid transporter 1 (URAT1), which exchanges organic anions for UA. As a consequence, only 10% of UA is normally excreted. Figure by the author with data by Johnson et al. and Nakagawa et al. (Johnson et al., 2008b; Nakagawa et al., 2008)

2.5. EXPERIMENTAL MODELS IN THE PRESENT STUDY

2.5.1. *Experimental chronic renal insufficiency*

The 5/6 nephrectomized remnant kidney model, NX (Morrison & Howard, 1966), has been extensively studied to investigate CKD and CRI. In this model, removal of one kidney and two-thirds of the remaining kidney results in progressive hyperperfusion, hyperfiltration, and hypertrophy (Morrison & Howard, 1966; Shimamura & Morrison, 1975; Hostetter et al., 1981). Direct micropuncture studies have demonstrated that single nephron function is increased after subtotal nephrectomy (Olson & Heptinstall, 1988). When renal mass is reduced, the remaining nephrons undergo functional as well as structural hypertrophy. Adaptations in the microcirculation of remnant glomeruli result in an increased mean driving force for filtration and, therefore, a marked increase in filtration rate (Hostetter et al., 1981). The magnitude of increase in single nephron GFR correlates closely with the amount of renal mass that has been lost. That is, greater degrees of removal result in greater increases of single nephron GFR in the residual nephrons. This functional hypertrophy is generally considered beneficial in the sense that it minimizes the reduction in total GFR that would otherwise occur. Studies have, however, shown that a pathological process of sclerosis eventually occurs in the glomeruli of these residual nephrons (Morrison & Howard, 1966; Shimamura & Morrison, 1975; Purkerson et al., 1976). These studies showed that after NX, eventual sclerotic destruction of remnant glomeruli was accompanied by progressive proteinuria and arterial hypertension. With severe reduction in renal mass, alterations in glomerular structure were detected as early as 2 wk. By 7 wk more than 50% of glomeruli exhibited morphological changes, and all animals died by 90 days (Purkerson et al., 1976).

The NX model is suitable for studying different stages of CKD, as the duration of disease progression can easily be adapted to meet the specific needs of the study. Early stages of CKD are present in NX immediately after recovery from the renal ablation. Subsequent glomerulosclerosis then causes disease progression and concomitant loss of renal function with the same mechanisms that come to play in the human CKD (Morrison & Howard, 1966). Even though kidneys have a remarkable degree of functional reserve, a decrease in number of functioning nephrons beyond a certain limit leads to a an overload on the

remaining functioning nephrons, causing disease progression irrespective of the cause of the renal disease (Shimamura & Morrison, 1975).

2.5.2. *Experimental hyperuricemia*

Experimental crystal-independent hyperuricemia model based on uricase inhibition was first developed in 2000 by Mazzali et al. (Mazzali et al., 2001). Since then it has been used numerous times to model the events associated with non-gouty hyperuricemia. Experimental studies, where rats were made hyperuricemic by the ingestion of uricase inhibitor oxonic acid (2.0% in diet for 4-7 weeks) have suggested that high UA may play a causal role in the development of hypertension (Mazzali et al., 2001; Kang et al., 2002; Mazzali et al., 2002; Sanchez-Lozada et al., 2002; Nakagawa et al., 2003; Sanchez-Lozada et al., 2005). The oxonic acid model of hyperuricemia has been shown to induce preglomerular arteriolar disease leading to tubular ischemia, interstitial infiltration of lymphocytes and macrophages, oxidant generation, and local vasoconstriction. These changes are associated with decreased GFR and thus sodium filtration and increased sodium reabsorption, so resulting in salt sensitivity (Johnson et al., 2002).

The initial increase in BP in hyperuricemic rats is associated with increased number of renin-positive cells in the juxtaglomerular apparatus (Johnson et al., 2005b), and a direct correlation of serum UA with the percentage of renin-positive juxtaglomerular cells has been reported in this model (Mazzali et al., 2001)**.** Treatment of hyperuricemia with XO inhibitor allopurinol and uricosuric agents such as benziodarone have been repeatedly shown to prevent the pathological and pathophysiological changes induced by oxonic acid feeding (Mazzali et al., 2001; Kang et al., 2002; Mazzali et al., 2002; Sanchez-Lozada et al., 2002; Nakagawa et al., 2003; Sanchez-Lozada et al., 2005). This effectively validates the model, as any possible harmful changes induced independently by the oxonic acid feeding would not have been prevented with UA treatment.

3. AIMS OF THE STUDY

The present experimental study featured two principal aims:

1. The first aim was to scrutinize the effects of hyperuricemia on the circulating components of the RAAS and on the K^+ / Na^+ balance. The main focus was to gauge potential changes in PRA and aldosterone concentration, as neither of these have been previously reported in association with the oxonic acid-induced hyperuricemia model. Hyperuricemia-associated increase in juxtaglomerular renin-positive cells communicated by Mazzali et al. (Mazzali et al., 2001) suggested that circulating RAAS could have a role in elevated UA.

2. The second aim was to examine the local RAS components of the kidney. Local RAS activation is known to play an important role in CKD progression and hypertension, while data of its importance in experimental hyperuricemia is scarce. Therefore ACE, ACE2, AT_{1R}, and AT_{2R} were chosen for closer inspection using autoradiography and real-time quantitative RT-PCR.

4. METHODS

4.1. ANIMALS AND TREATMENT

Male Sprague Dawley rats, housed two per cage, were used (n=48) with free access to water and food pellets (Lactamin R34, AnalyCen, Lindköping, Sweden). The rat chow contained 0.9% calcium, 0.8% phosphorus, 0.27% sodium, 0.2% magnesium, 0.6% potassium, 12550 kJ/kg energy, 16.5% protein, 4.0% fat, 58% nitrogen-free extract, 3.5% fibre, 6.0% ash, and 10% water. Surgery was performed under ketamine / diazepam anesthesia (75 mg/kg and 2.5 mg/kg, respectively) at 8 weeks of age. NX was carried out by removal of upper and lower poles of the left kidney, and the whole right kidney (Jolma et al., 2003; Kööbi et al., 2003), whereas sham-operation was performed by kidney decapsulation. Antibiotics (metronidazole 60 mg/kg, cefuroxim 225 mg/kg) were given after surgery, and pain was relieved with buprenorphine (0.2 mg/kg, three times daily for three days) (Kööbi et al., 2003). Systolic BPs were measured at 28°C with a tail-cuff BP meter (model 129; IITC; Woodland Hills, California, USA) (Jolma et al., 2003).

Three weeks after NX (rat age 11 weeks), the animals were divided into four groups so that systolic BPs and body weights in the Sham and Sham+Oxo, and NX and NX+Oxo groups, respectively, (n=12 in each) were similar. After division, the Sham+Oxo and NX+Oxo rats were switched to chow containing additional 2.0% oxonic acid. These diets continued for 9 weeks (Figure 4.1), and 24-hour fluid consumption and urine output were measured during the last study week. The rats were anesthetized (urethane 1.3 g/kg) and blood samples from cannulated carotid artery were drawn with ethylene diamine tetraacetic acid (EDTA) and heparin as anticoagulants, as appropriate. Blood samples were not obtained from one NX and three Sham rats due to cardiac arrest during anesthesia. The hearts and the kidneys were removed and weighed. A kidney half from each rat was snap-frozen in isopentane at -40°C and stored at -80°C. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland. The investigation conforms to the Guiding Principles for Research Involving Animals.

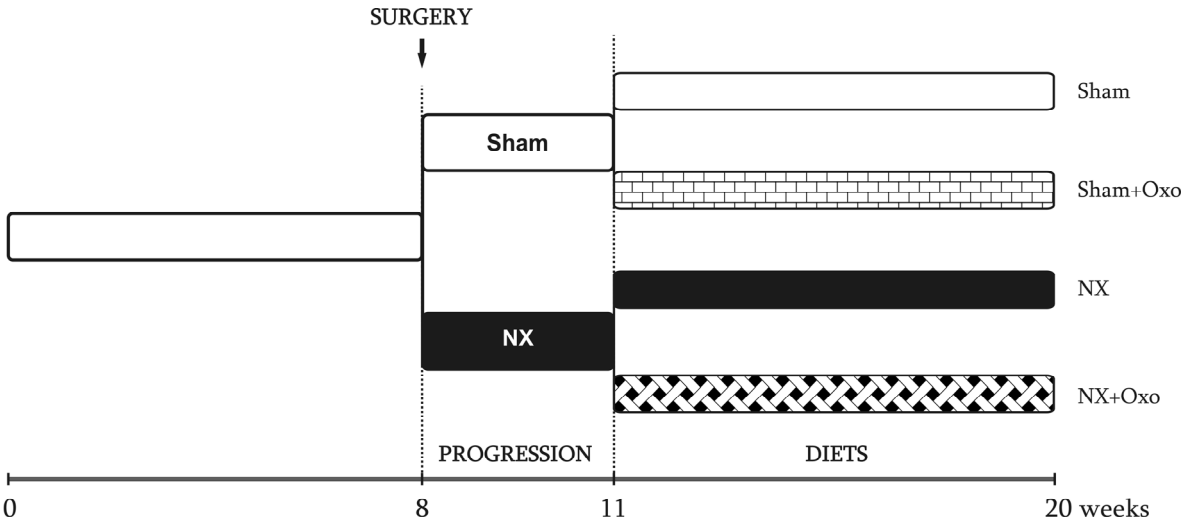

Figure 4.1 *Flowchart of the study:* Surgery was performed at rat age of 8 weeks. The 2.0% oxonic acid diets started for Sham+Oxo and NX+Oxo groups at week 11 after 3 weeks of disease progression / recovery. The total duration of the diets was 9 weeks. Blood and tissue samples were obtained at the end of the experiment at week 20. Sham=Sham operated, NX=5/6 nephrectomy, Oxo=2.0% oxonic acid diet.

4.2. IN VITRO AUTORADIOGRAPHY

Frozen kidney sections (20 μm thick) were cut on a cryostat at -17°C, thaw mounted onto Super Frost R Plus slides (Menzel-Gläser, Braunschweig, Germany), 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).

4.2.1. *ACE autoradiography*

A tyrosyl residue of lisinopril (MK351A, Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania, USA) was iodinated by chloramine T method, purified on SP-Sephadex C-25 column (Pharmacia, Piscataway, New Jersey, USA), and then a previously described technique was applied (Kohzuki et al., 1991; Bäcklund et al., 2001). Kidney sections were pre-incubated for 15 minutes at room temperature in 10 mmol/l sodium phosphate buffer, pH 7.4, containing 150 mmol/l NaCl and 0.2% bovine serum albumin (BSA), followed by incubation for 1 hour at room temperature in fresh volume of the same buffer containing 0.3 μCi/ml of ^{125}I-351A. Non-specific binding was determined in parallel incubations in the same buffer containing 1 mmol/l Na_2-EDTA. After incubation,

the sections were washed four times for 1 minute in ice-cold buffer without BSA and ^{125}I-351A to remove unbound radioligand and dried under a stream of cool air. For quantification of ACE binding, the sections were placed on a Fuji Imaging Plate BAS-TP2025 (Tamro, Finland) for three hours. The optical densities were quantified by an image analysis system (AIDA 2D densitometry) coupled to FUJIFILM BAS-5000 phosphoimager (Tamro, Finland) from four kidney sections per rat, six representative areas per section, altogether 24 analyses per each kidney. Specific binding was calculated as total binding minus non-specific binding.

4.2.2. *AT_{1R} and AT_{2R} autoradiography*

Sar^1,Ile^8-Ang II (Sigma, St. Louis, Missouri, USA) was iodinated by chloramine T method and purified on a Sep-Pack C18 cartridge with methanolic gradient elution. Autoradiographic quantification of angiotensin receptors with [^{125}I]-Sar^1,Ile^8-Ang II was performed using a published modification (Stewen et al., 2003) of a previously described method (Zhuo et al., 1999). Kidney sections were pre-incubated for 15 minutes at room temperature in 10 mmol/l sodium phosphate buffer, pH 7.4, containing 150 mmol/l NaCl, 5 mmol/l Na_2-EDTA, and 0.2 % BSA, followed by a 1 hour incubation at 37°C in fresh volume of the same buffer containing 0.2 µCi/ml of ^{125}I-[Sar^1,Ile^8]Ang II. Non-specific binding was determined in the presence of 1 µmol/l unlabelled Ang II (Sigma). The density of AT_{1R} was determined in presence of the AT_{2R} antagonist PD 123,313 (10 µmol/l), and the density of AT_{2R} in presence of the AT_{1R} antagonist losartan (10 µmol/l). After incubation, the sections were washed four times for 1 minute in ice-cold buffer without BSA and radioligand and dried under stream of cool air. The optical densities of angiotensin receptor binding from 10 kidney sections per rat were quantified as described above for ACE from six representative cortical and six medullary areas per each section: four sections were used for total specific binding, two sections for AT_{1R} binding, two sections for AT_{2R} binding, and two sections for non-specific binding.

The outcome in each group was related to the mean value of the Sham group, except for the medullary to cortical AT_{1R} density ratio, in which the respective densities in each rat kidney were related to each other.

4.3. REAL-TIME QUANTITATIVE RT-PCR

For the extraction of RNA, small pieces of tissue were cut from each frozen kidney sample in a way that equally represented both medulla and cortex. Samples were homogenized mechanically, and total RNA was isolated from these kidney homogenates using Trizol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically at 260nm, whereas the quality of RNA was checked by gel electrophoresis. Reverse transcription of RNA was performed using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

The expressions of ACE, ACE2, AT_{1aR}, and AT_{2R} mRNAs were studied using real-time quantitative RT-PCR. PCR was performed either with SYBR Green chemistry (ACE and AT_{1aR}) or TaqMan chemistry (ACE2 and AT_{2R}) using ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, California, USA). PCRs for ACE and AT_{1aR} were performed in duplicate in a 25 µl final volume containing 1X SYBR Green Master mix (Applied Biosystems) and 300 nmol/l of primers (Table 4.1). PCRs for ACE2 and AT_{2R} were performed in duplicate in a 25 µl final volume containing 1X TaqMan Master mix (Applied Biosystems), 300 nmol/l of primers and 100 nmol/l of ACE2, 150 nmol/l of AT_{2R} TaqMan probe (Table 4.1). PCR cycling conditions were 10 min at 95ºC and 40 cycles of 20 seconds at 95ºC and 1 min at 60ºC. Data were analyzed using the absolute standard curve method, and the amplification of a housekeeping gene 18S was used for normalizing the results. The unnormalized expression of 18S mRNA did not differ between the experimental groups enabling its use as the control housekeeping gene in the present study. The intra-assay coefficient of variation percentage was determined from the threshold cycle values of duplicate samples in one PCR run, while the inter-assay coefficient of variation was determined from the threshold cycle values of duplicate samples in six replicate PCR analyses. The intra-assay and inter-assay coefficients of variation for the studied mRNAs were ≤1.2% and ≤2.8% respectively (Table 4.2).

Table 4.1 *Primers and probes used in real-time RT-PCR amplification*

Gene	Primer nucleotide sequence
ACE	Forward 5'-GGAGACGACTTACAGTGTAGCC-3' (Harada et al., 2001)
	Reverse 5'-CACACCCAAAGCAATTCTTC-3' (Harada et al., 2001)
AT_{1aR}	Forward 5'-GGCAGCCTCTGACTAAATGGC-3'
	Reverse 5'-ACGGCTTTGCTTGGTTACTCC-3'
ACE2	Forward 5'-ACCCTTCTTACATCAGCCCTACTG-3' (Tikellis et al., 2003)
	Reverse 5'-TGTCCAAAACCTACCCCACATAT-3' (Tikellis et al., 2003)
AT_{2R}	Forward 5'-TGTCTGTCCTCATTGCCAACA-3'
	Reverse 5'-TTCATTAAGGCAATCCCAGCA-3'
Gene	Probe sequence
ACE2	5'FAM-ATGCCTCCCTGCTCATTTGCTTGGT-TAMRA (Tikellis et al., 2003)
AT_{2R}	5'FAM-TCAGAACCATTGAATACTT-MGB

Table 4.2 *Intra-assay and inter-assay coefficients of variation for real-time RT-PCR*

Gene	Intra-assay CV* of duplicate samples	Inter-assay CV* of 6 replicate PCR runs
18S	≤1.1%	≤1.4%
AT_{1aR}	≤1.1%	≤1.8%
AT_{2R}	≤0.8%	≤2.8%
ACE	≤0.8%	≤1.4%
ACE2	≤1.2%	≤2.5%

* coefficients of variation

4.4. RADIOIMMUNOASSAYS

Plasma aldosterone concentration and PRA were determined using radioimmunoassays (GammaCoat® PRA RIA kit and ALDOCTK-2 RIA kit, Diasorin S.p.A., Saluggia, Italy) according to the manufacturer's instructions.

4.4.1. *Aldosterone RIA*

For the measurement of aldosterone concentration, deep frozen (-80ºC) EDTA plasma samples were rapidly thawed to room temperature before assaying in duplicate. The assay is based on competition for a limited number of fixed antibody binding sites between the ^{125}I-labelled aldosterone and the aldosterone contained in calibrators or samples to be assayed. 200 µl per sample and 500 µl of ^{125}I-labelled aldosterone tracer were dispensed on the bottom of an anti-aldosterone antibody coated tube, then mixed thoroughly on vortex, and incubated overnight at room temperature. Zero and control calibrators were subjected to the same protocol as the measured samples. After the overnight incubation, all tubes were carefully aspirated so that no liquids were present during the actual measurement. Radioactivity measurements were performed on a gamma counter (Wallac Wizard 1470, PerkinElmer, Massachusetts, USA), and the aldosterone concentrations were calculated using the obtained calibration curve. In this assay, the amount of ^{125}I-labelled aldosterone bound to the rabbit anti-aldosterone antibody on the tube walls is inversely related to the concentration of unlabelled aldosterone present in calibrators or samples, while the use of coated tubes gives the advantage of bound / free separation, because only the bound aldosterone is present after a complete aspiration of the incubation mixture.

4.4.2. *Plasma renin activity RIA*

For the PRA measurement, deep frozen (-80ºC) EDTA plasma samples were rapidly thawed to room temperature before assaying in duplicate. The principle of this assay is that PRA is measured via its Ang I producing capability. Therefore an incubation for Ang I generation was performed before the actual measurement. One ml of plasma per sample was dispensed in an uncoated plastic tube, marked accordingly, and mixed well with 10

µL phenylmethylsulfonyl fluoride (PMSF) solution and 100 µl maleate buffer (pH 6.0). All samples were then divided in two tubes (500 µl aliquots), after which one tube was placed in an ice bath (marked 4) and the other in a 37ºC water bath (marked 37) for 90 minutes of Ang I generation. After 90 minutes, the tubes in water bath were transferred to ice bath in order to stop Ang I generation for the beginning of the actual PRA assay. This part of the assay is based on competition for a limited number of fixed antibody binding sites between the ^{125}I-labelled Ang I and the Ang I contained in calibrators or samples. Rabbit anti-Ang I antibody coated tubes were used in this measurement. 100 µl of each sample and 1.0 ml of tracer were mixed thoroughly in a coated tube, and incubated for three hours in room temperature. All controls were subjected to the same protocol as the measured samples. After incubation, the liquid in all the tubes were aspirated completely, and the radioactivity in each tube was measured using a gamma counter (PerkinElmer). PRA values for each sample were obtained by first deducting the measured value of each 4 tube from the corresponding 37 tube, and then calculating correct value using the calibration curve as reference.

4.5. PLASMA AND URINE DETERMINATIONS

Plasma potassium and sodium concentrations were measured by potentiometric direct dry chemistry, while urea nitrogen was measured by colorimetric enzymatic dry chemistry (Vitros 950, Johnson & Johnson Clinical Diagnostics, Rochester, NY). Plasma protein was determined by colorimetric endpoint measurement according to Biuret (Cobas Integra analyzer) and plasma creatinine by the kinetic colorimetric assay according to Jaffe (Jaffe et al., 1987; Jolma et al., 2003). Plasma pH was measured with the use of an ion-selective electrode (model 634 pH Analyzer, Ciba Corning Diagnostics, Sudbury, UK), and hemoglobin by photometric analysis with the use of a cyanide-free hemoglobin reagent (H*2, Technicon Instruments, Tarrytown, NY) (Jolma et al., 2003).

4.6. DATA PRESENTATION AND ANALYSIS OF RESULTS

The amounts of ACE and AT_{1R} in renal tissue were depicted in relation to the mean value of the Sham group. Statistics were by one-way and two-way analyses of variance (ANOVA), and the least significant difference test was used for post-hoc analyses (SPSS 11.5, SPSS Inc., Chicago, Illinois, USA). If the distribution of the variables was skewed, the Kruskal-Wallis test was applied, and post-hoc analyses were performed with the Mann-Whitney U-test, the P-values being corrected with the Bonferroni equation. Results were expressed as mean ± SEM, and differences were considered significant when $P<0.05$.

5. RESULTS

5.1. ANIMAL DATA

Systolic BPs at the beginning of the oxonic acid feeding period did not differ between the groups, although at study week 9 BP in the NX+Oxo group was higher than in the Sham group (Table 5.1). No significant difference in BP was detected between the other groups. Final body weight in the NX+Oxo group was slightly lower when compared with the NX group, and when analyzed using two-way ANOVA a significant lowering influence on body weight was associated with oxonic acid feeding (P=0.004). The heart to body weight ratio and 24-hour urine output were higher in both NX groups when compared with Sham rats (Table 5.1).

5.2. LABORATORY FINDINGS

The oxonic acid feeding elevated plasma UA concentration by 80-90 μmol/l in both Sham and NX rats, as expected (Table 5.1). Creatinine clearance was similarly reduced by approximately 60% in both NX groups and also by about 25% in hyperuricemic Sham rats (Figure 5.1A). Plasma concentration of urea was over two-fold higher in the NX group when compared with Sham rats, whereas no difference between the NX and NX+Oxo groups was observed (Table 5.1). Blood pH was slightly lower in the NX group than in Sham rats, while hemoglobin was decreased in the NX+Oxo group when compared with Sham rats.

Hyperuricemia had a clear elevating effect on PRA (Figure 5.1B) and plasma aldosterone concentration (Figure 5.2A). In the Sham+Oxo group PRA increased 1.2-fold and plasma aldosterone 1.4-fold, whereas in the NX+Oxo group the increases were 2.5-fold and 2.3-fold, respectively. The plasma aldosterone to renin ratios in the experimental groups were (mean ± SEM) 12 ± 2, 14 ± 2, 360 ± 208* and 213 ± 110* in the Sham, Sham+Oxo, NX and NX+Oxo groups, respectively (*P<0.001 both NX groups vs. both Sham groups). A clear K^+ loss / Na^+ retention-effect was observed in both hyperuricemic groups (Table 5.1). Subsequently, urine K^+ to Na^+ ratio was elevated two-fold in the Sham+Oxo group and 1.6-fold in the NX+Oxo group (Figure 5.2B). The 24-hour urinary calcium

excretion was 1.7-fold increased in the Sham+Oxo group, whereas no significant changes were observed in the NX groups (Table 5.1).

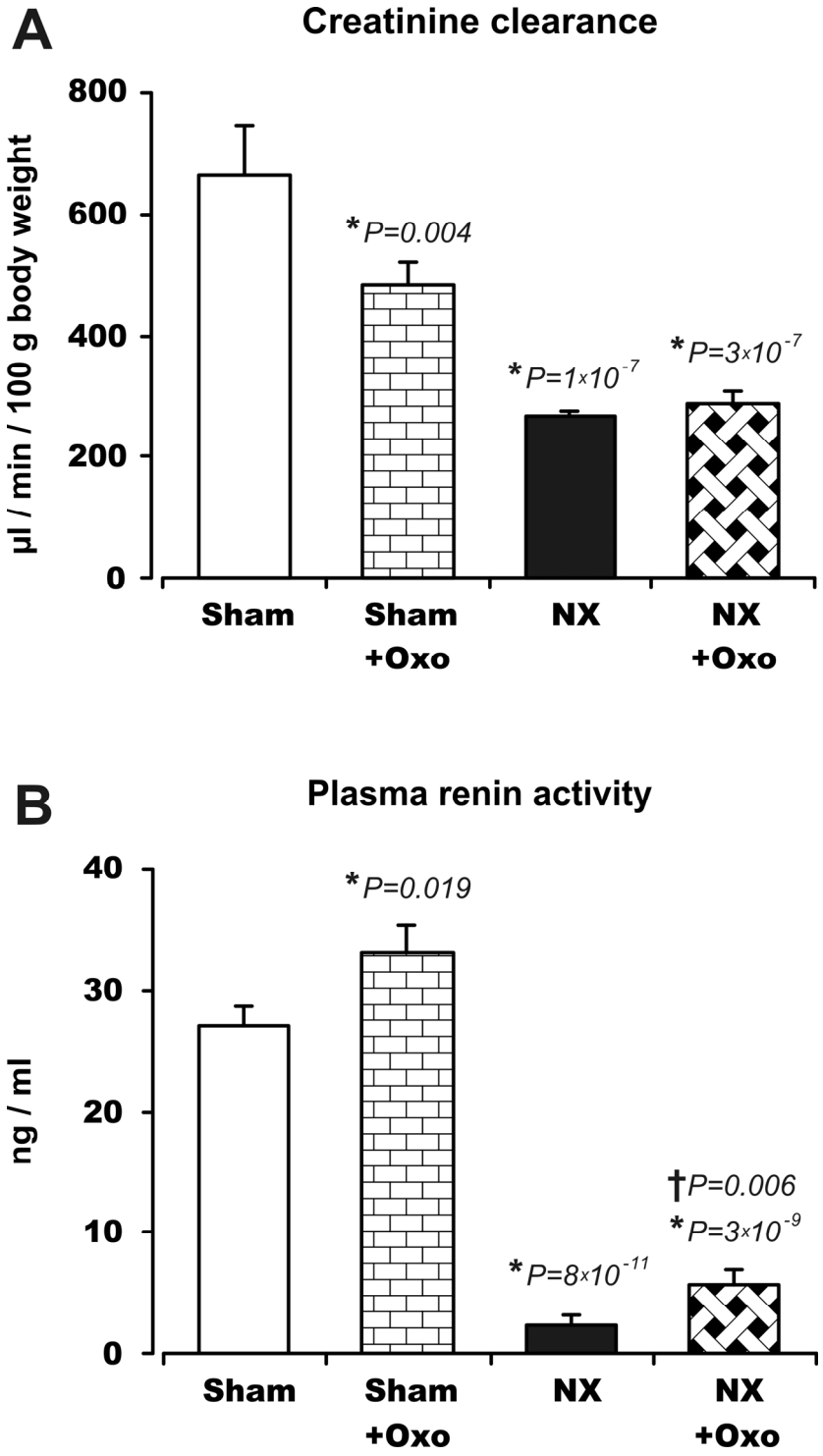

Figure 5.1 Bar graphs show creatinine clearance (panel A, *n*=9-12 in each group) and plasma renin activity (panel B, *n*=8-12) in sham-operated (Sham) and 5/6 nephrectomized (NX) rats ingesting either normal or 2.0% oxonic acid diet (Oxo); mean ± SEM; *P<0.05 versus Sham, †P<0.05 versus NX. Exact P-values given after the corresponding symbol.

Table 5.1 *Experimental group data and laboratory findings*

	Sham (n=9-12)	Sham+Oxo (n=12)	NX (n=11-12)	NX+Oxo (n=12)
Systolic BP at week 0 (mmHg)	120 ± 4	121 ± 5	127 ± 5	125 ± 5
Systolic BP at week 9 (mmHg)	134 ± 7	136 ± 5	142 ± 6	152 ± 4*
Body weight at week 0 (g)	339 ± 6	338 ± 7	333 ± 8	332 ± 7
Body weight at week 9 (g)	433 ± 8	412 ± 11	448 ± 10	411 ± 8†
Heart weight / body weight (g/kg)	3.97 ± 0.05	4.12 ± 0.09	4.95 ± 0.27*	5.18 ± 0.33*
Urine volume (ml/24h)	25.2 ± 1.7	25.8 ± 1.8	53.3 ± 3.8*	49.3 ± 3.9*
Blood and plasma determinations				
Uric acid (µmol/l)	36 ± 11	117 ± 21*	63 ± 19	152 ± 19*†
Urea (mmol/l)	6.62 ± 0.34	8.33 ± 0.42	13.54 ± 0.87*	14.54 ± 2.00*
pH	7.42 ± 0.028	7.37 ± 0.023	7.34 ± 0.034*	7.37 ± 0.023
Hemoglobin (g/l)	167.5 ± 3.2	168.5 ± 2.7	157.9 ± 4.7	149.8 ± 4.1*
Potassium (mmol/l)	4.12 ± 0.13	3.79 ± 0.08	4.28 ± 0.19	4.42 ± 0.18
Sodium (mmol/l)	136.5 ± 0.5	137.3 ± 0.6	136.7 ± 0.9	137.0 ± 0.5
Urine determinations				
Potassium (mmol/24h)	2.84 ± 0.14	4.56 ± 0.20*	2.93 ± 0.09	3.73 ± 0.21*†
Sodium (mmol/24h)	8.47 ± 0.41	6.60 ± 0.25*	7.62 ± 0.25	6.44 ± 0.65*
Calcium (µmol/24h)	28.65 ± 4.47	47.45 ± 5.47*	40.03 ± 6.18	34.79 ± 4.38

Values are mean ± SEM, groups as in figure 5.1, *P<0.05 compared with the Sham group, †P<0.05 compared with the NX group

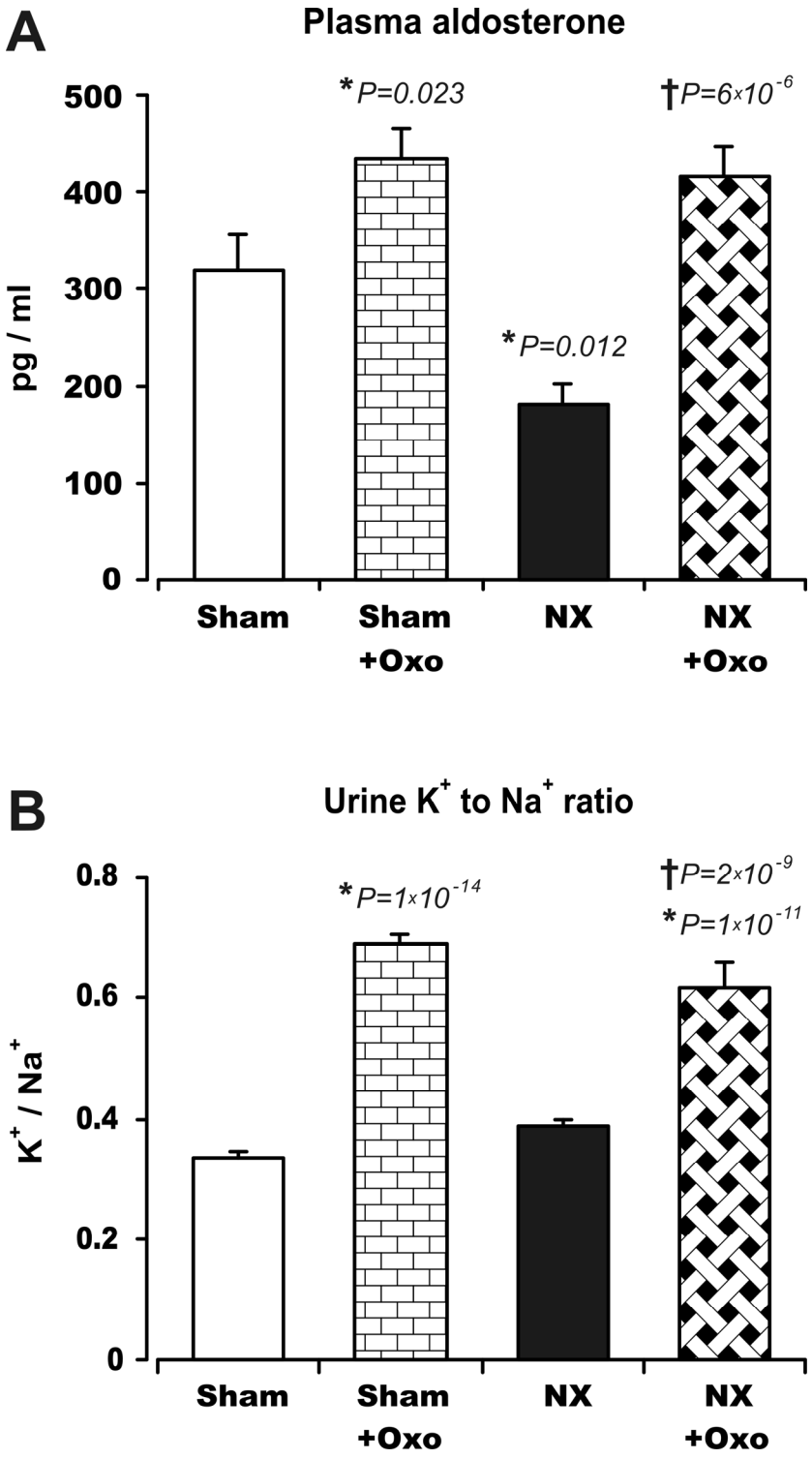

Figure 5.2 Bar graphs show plasma aldosterone (panel A, *n*=8-12 in each group) and urine K^+ to Na^+ ratio (panel B, *n*=12) in sham-operated (Sham) and 5/6 nephrectomized (NX) rats ingesting either normal or 2.0% oxonic acid diet (Oxo); mean ± SEM; $^*P<0.05$ versus Sham, $^\dagger P<0.05$ versus NX. Exact P-values given after the corresponding symbol.

5.3. AUTORADIOGRAPHY AND REAL-TIME QUANTITATIVE RT-PCR

When kidney tissue ACE content was analyzed using quantitative *in vitro* autoradiography, which measures binding to the active site of ACE protein (Zhuo et al., 1997), no difference was observed in the NX groups when compared with Sham rats (Figures 5.3A and 5.3B). Highest ACE signal was detected in a circular fashion in the inner cortex and outer medulla in the Sham-operated groups, whereas ACE was more widely distributed in the remnant kidneys of the NX groups (Figure 5.3A). However, when determined using real-time quantitative RT-PCR, both NX groups showed significantly lower ACE mRNA levels than the respective Sham rats (Figure 5.4A). In addition, kidney tissue ACE2 mRNA levels were also lower in both NX groups than in the Sham rats (Figure 5.4B).

When analyzed using autoradiography, AT_{1R} densities in kidney cortex did not differ from Sham in the NX groups. However, cortical AT_{1R} density was approximately 25% higher in NX+Oxo than NX rats (Figure 5.5A). In renal medulla, no difference in AT_{1R} density between the two NX groups was observed, while the density was about 43% higher in the NX+Oxo group when compared with Sham rats (Figure 5.5B). The Sham+Oxo rats featured no changes in renal AT_{1R} density. In all groups, AT_{1R} density in the kidney medulla was higher than in the cortex (data not shown). In addition, the ratio of medullary to cortical AT_{1R} density was increased in both NX groups, the ratios being 2.27 ± 0.08, 2.00 ± 0.15, 3.25 ± 0.34*, 3.04 ± 0.24* in the Sham, Sham+Oxo, NX and NX+Oxo groups, respectively (*$P<0.02$ both NX groups vs. both Sham groups).

Hyperuricemia had no effect on kidney tissue AT_{1aR} mRNA content, the levels of which were significantly lower in both NX groups than in Sham rats (Figure 5.6A). AT_{2R} densities in cortex and medulla were similar in all study groups (not shown), and the AT_{2R} binding comprised only 1.2-2.1% of all AT receptor binding in the cortex and 0.7-1.2% of all binding in the medulla. Kidney tissue AT_{2R} mRNA level was also lower in the NX+Oxo group than in the Sham rats and was not significantly affected by hyperuricemia (Figure 5.6B).

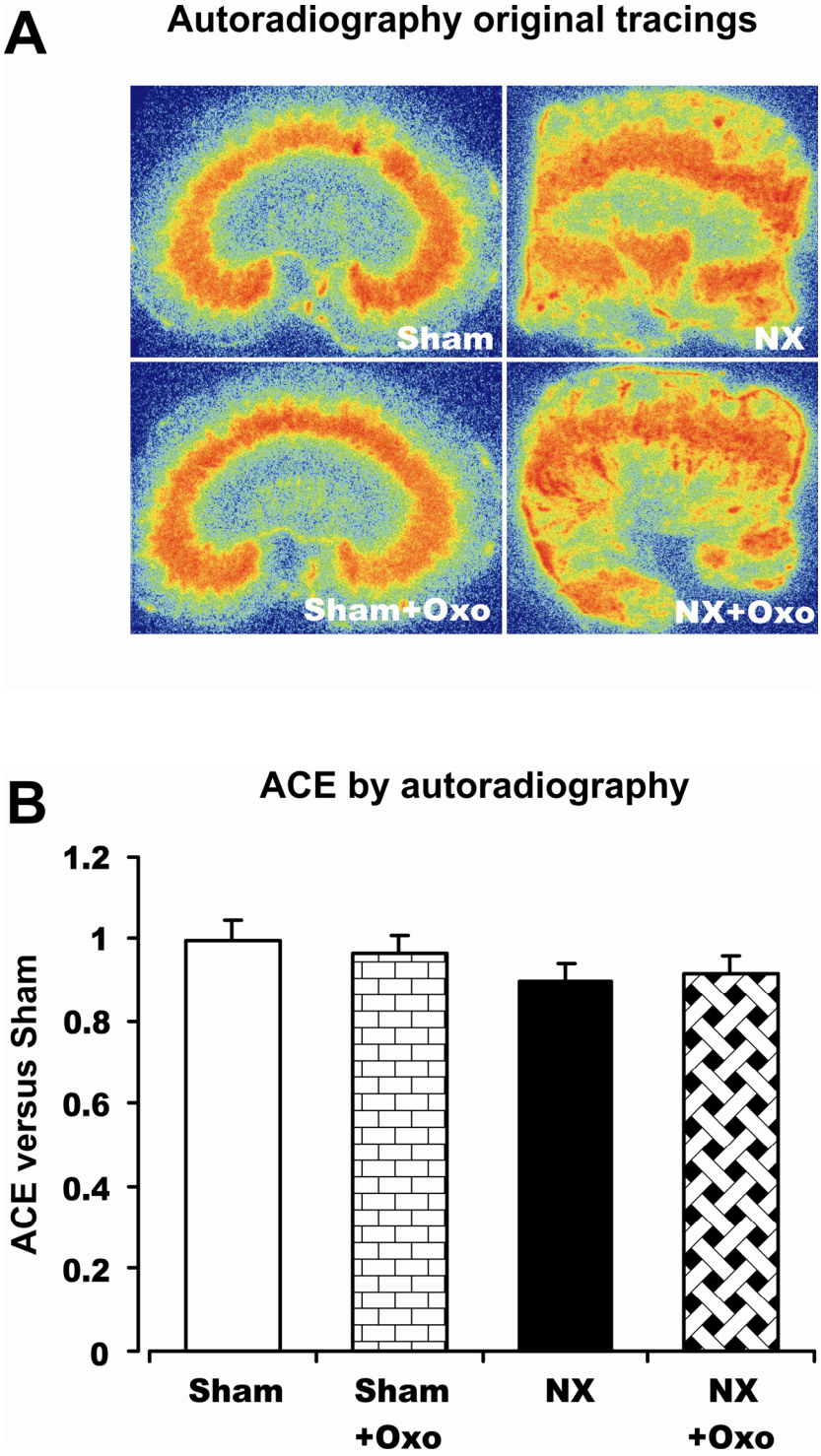

Figure 5.3 Representative original tracings (panel A, intensity of red colouring reflects active binding to ACE) and the bar graph show ACE determined using autoradiography (panel B, values related to the mean value of the Sham group) in sham-operated (Sham) and 5/6 nephrectomized (NX) rats ingesting either normal or 2.0% oxonic acid diet (Oxo), *n*=11-12, mean ± SEM.

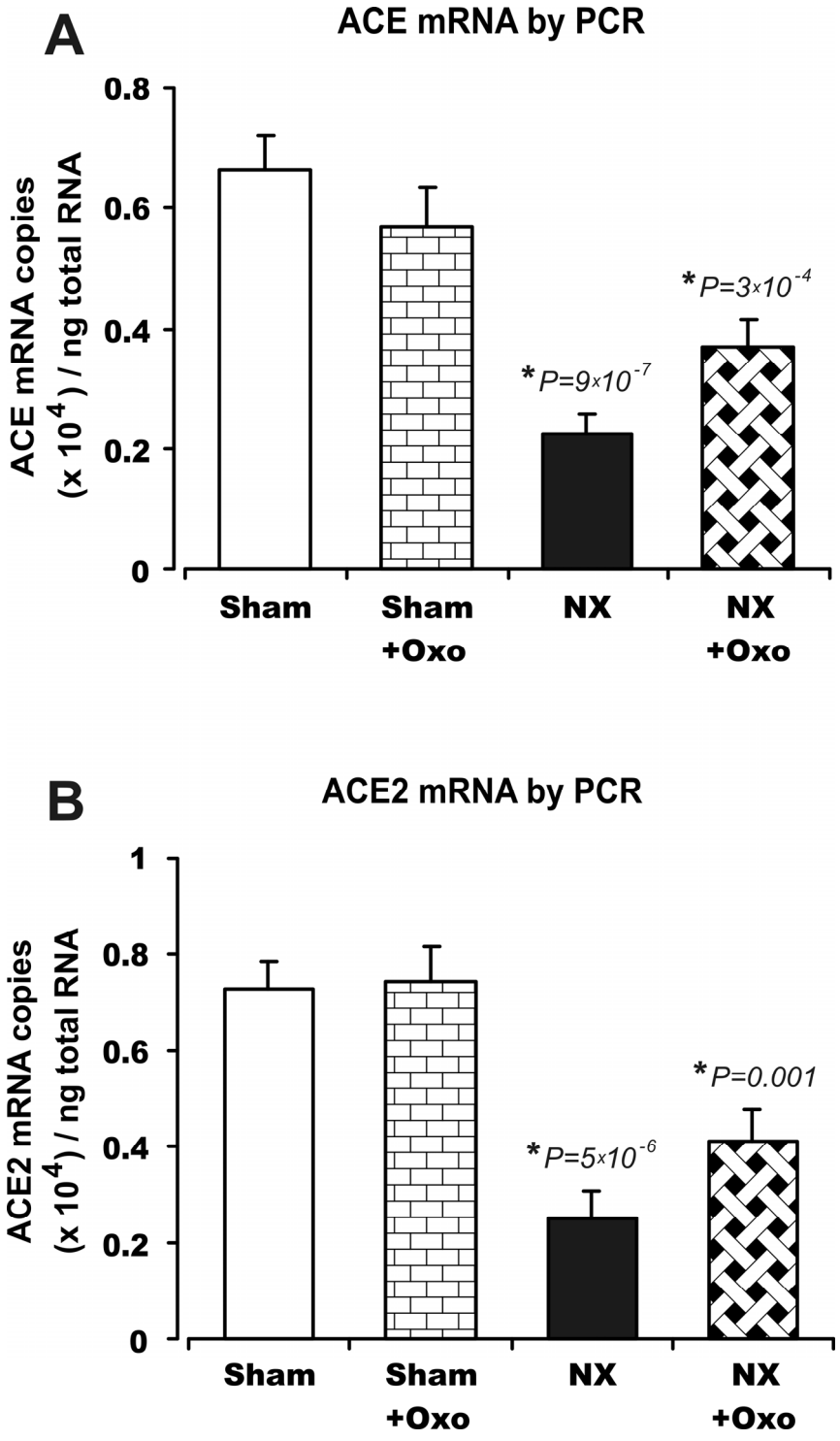

Figure 5.4 Bar graphs show ACE mRNA (panel A) and ACE2 mRNA (panel B) determined using PCR; groups as in Figure 5.3, *n*=11-12, mean ± SEM; $^{*}P<0.05$ versus Sham. Exact P-values given after the corresponding symbol.

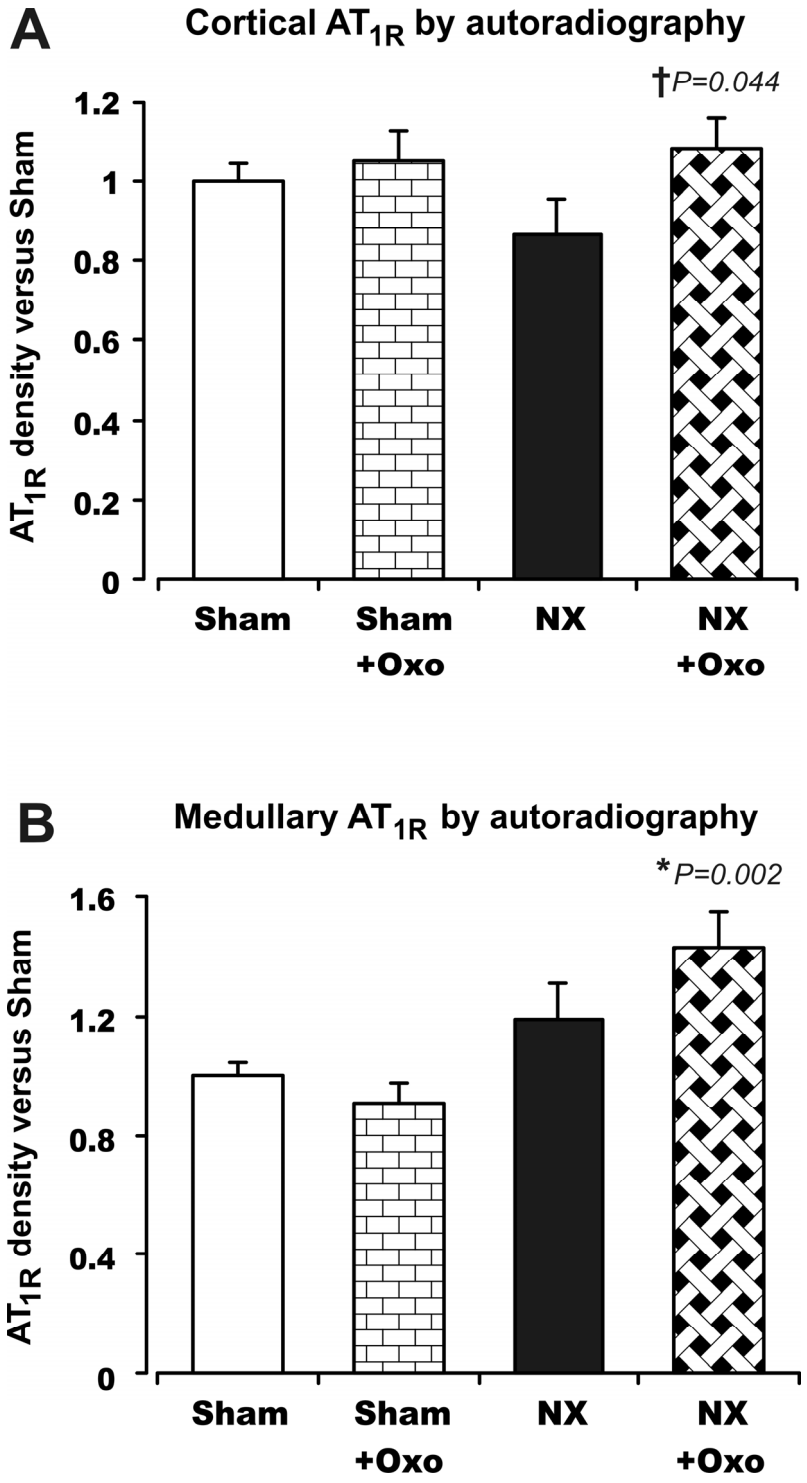

Figure 5.5 Bar graphs show AT_{1R} densities in kidney cortex (panel A) and medulla (panel B) determined using autoradiography; groups as in Figure 5.3, n=11-12, mean ± SEM; $^{*}P<0.05$ versus Sham, $^{\dagger}P<0.05$ versus NX. Exact P-values given after the corresponding symbol.

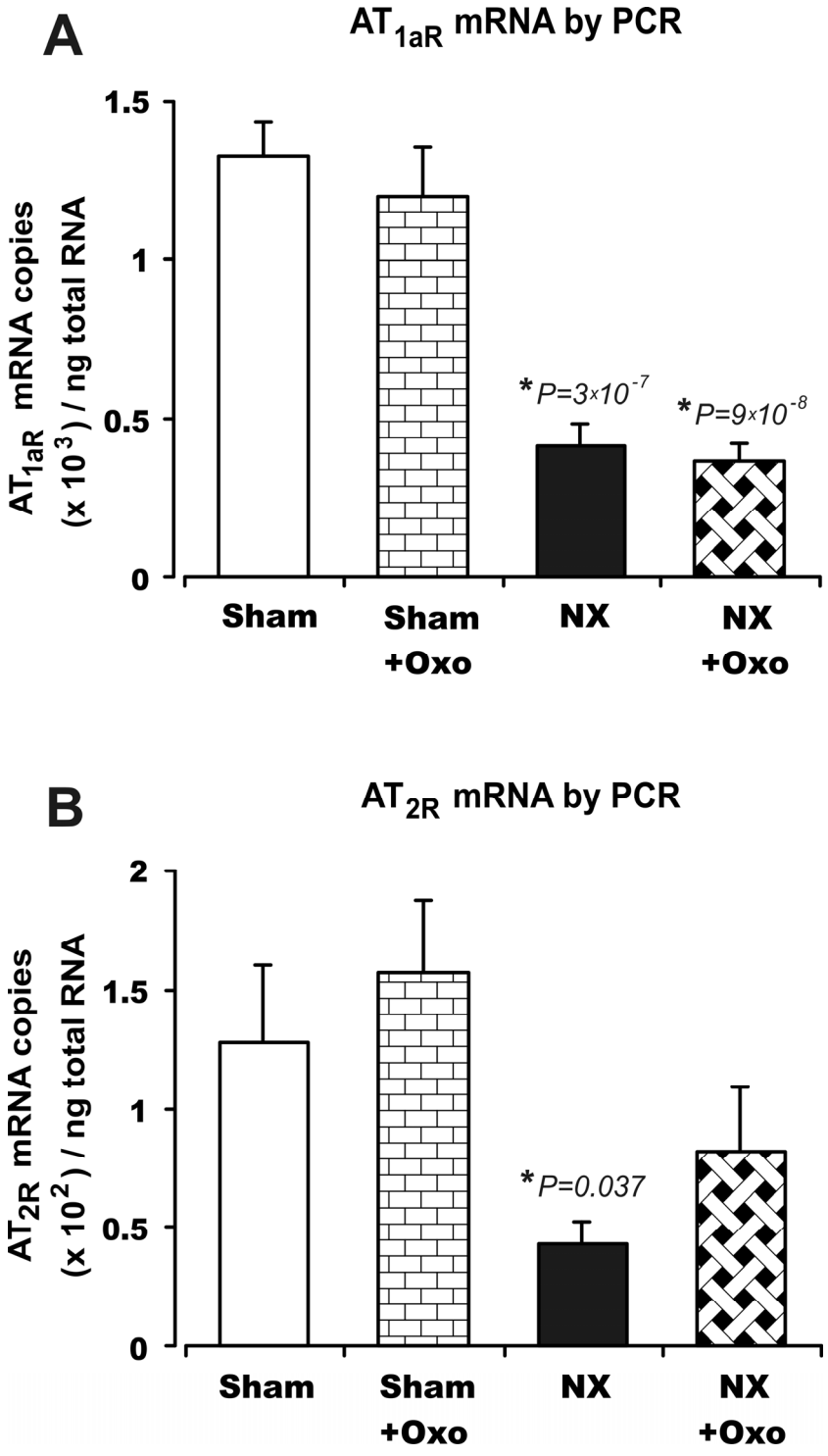

Figure 5.6 Bar graphs show AT_{1aR} (panel A) and AT_{2R} (panel B) mRNA determined using PCR; groups as in Figure 5.3, *n*=11-12, mean ± SEM; $^{*}P<0.05$ versus Sham. Exact P-values given after the corresponding symbol.

6. DISCUSSION

The present study demonstrated that hyperuricemia, induced by 2.0% oxonic acid diet for 9 weeks, was associated with increased PRA and plasma aldosterone concentration. Hyperuricemia also increased K^+ excretion and reduced Na^+ excretion, leading to elevated K^+ to Na^+ ratio in the urine. This may be largely explained by the elevation in plasma aldosterone levels and the subsequent increase in Na^+ reabsorption in the distal nephron.

6.1. DISCUSSION OF THE METHODS

The methods used in the present study were found to be adequate and suitable for the testing of the underlying hypotheses, and no significant problems were encountered during the experiments or laboratory determinations. The surgical procedures, 5/6 nephrectomy and sham-operation, succeeded in producing viable rats without animal losses, and the experimental models functioned as expected. The RIA determinations also yielded results that were in agreement with earlier observations (Ramsay et al., 1975; Mazzali et al., 2001). Overall, the general reliability of the results appears to be high, although some differences were observed between the determinations of the RAS components at the protein level (autoradiography) and at the mRNA level (RT-PCR).

The *in vitro* autoradiography is a method providing information about the quantity and localization of the assayed proteins in a histological sample. Its drawback is the same as with any other histology-based assay, as the fixing of samples may cause artefacts hindering the reliability of the obtained results. In this study, no artefact-causing fixatives were used, as the samples were cut frozen, thaw mounted, and then desiccated. It can be questioned how well the 20 μm thick sections of the kidney tissue represented the whole kidney. Altogether 24 analyses per each kidney were performed, and this approach can be argued to have increased the reliability of the autoradiography results.

The real-time quantitative RT-PCR, in turn, is a reliable method of determining gene expression levels via amplification of the gene transcript. Obviously, the reverse transcription and amplification procedures can be prone to errors if the conditions are not optimized properly, or if primer dimers or other inappropriate products are amplified

along with the measured transcript. These problems, however, can be overcome by routinely observing the product length with gel electrophoresis. The RT-PCR measurements in this study were trustworthy, as the intra-assay and inter-assay coefficients of variance were under 1.2% and 2.8%, respectively. Still, the reason for the observed protein / mRNA differences may be found by analysing these RT-PCR results. The intangible step is the total RNA extraction stage, since the original macroscopic localization of the extracted RNA is not possible. We can only assume that the result represents the mean transcriptional level of the whole cell population. However, a marginal number of cells with thousands of times higher or lower transcription levels may shift the result, and subsequently the outcome may indicate a higher or lower overall transcription level in the whole cell population. This makes RT-PCR determinations using tissue samples difficult, and therefore the method should be used in combination with another method that provides information about the quantity and macroscopic localization of the gene product.

6.2. DISCUSSION OF THE RESULTS

As expected, the 2.0% oxonic acid diet was successful in raising plasma UA values in both oxonic acid-treated groups (Kang et al., 2002; Mazzali et al., 2002; Sanchez-Lozada et al., 2005). Treatment of hyperuricemia was not included in the present study protocol, as allopurinol and uricosuric agents have been repeatedly shown to prevent the pathological and pathophysiological changes induced by oxonic acid feeding (Mazzali et al., 2001; Kang et al., 2002; Mazzali et al., 2002; Sanchez-Lozada et al., 2002; Nakagawa et al., 2003; Sanchez-Lozada et al., 2005). Renal insufficiency was induced by surgical 5/6 nephrectomy, and the NX rats showed several characteristic findings of CRI (Table 4.1). Creatinine clearance was reduced, and blood urea was increased in both NX groups. Corresponding to previous findings showing preglomerular arterial disease after oxonic acid diet (Johnson et al., 2002), we found that hyperuricemia decreased creatinine clearance in Sham rats. It should be noted that hyperuricemia is also associated with decreased renal blood flow in humans (Messerli et al., 1980). However, the present hyperuricemia did not reduce creatinine clearance in NX rats. An explanation may be the higher PRA values in Sham rats resulting in higher Ang II, which is the major modulator of glomerular blood flow and filtration rate. Both NX groups developed polyuria, and exhibited two-fold increases in 24-hour urine volume. Increased volume diuresis and the

subsequent kaliuresis (Gennari, 1998) is a very likely explanation for the absence of hyperkalemia in the NX groups.

The 12 weeks of CRI in this study did not unearth a significant BP elevation in the NX rats on the normal diet. Previous studies have shown that after an additional 8 weeks (total follow-up of 20 weeks) the NX rats developed clear hypertension, so that systolic BP was elevated by 30 mmHg (Kööbi et al., 2006). However, the NX+Oxo rats presented with elevated BP after 9 weeks of diet, which probably resulted from the hyperuricemia-associated sodium retention. Sodium sensitivity is considered a characteristic feature in hyperuricemia (Ward, 1998). Both NX and NX+Oxo groups exhibited increased heart to body weight ratios, an apparent result of the increased volume load in CRI (Kööbi et al., 2006).

As experimental hyperuricemia has been associated with increased number of renin-positive cells in the juxtaglomerular apparatus (Mazzali et al., 2001; Johnson et al., 2003), we scrutinized the circulating and renal components of RAS. Immunohistochemical staining of renin-positive cells has been used previously as a marker of the tissue-level renin expression (Mazzali et al., 2001), but knowledge about circulating renin activity in hyperuricemia is scarce. Experimental models of hyperuricemia are characterized by preglomerular arteriolar disease, leading to tubular ischemia and local vasoconstriction. As the severity of the arteriolar disease varies between nephrons, some nephrons will be underperfused and ischemic, whereas others may be overperfused. This leads to heterogeneity in renin expression and a failure to suppress renin release for the degree of sodium intake, resulting in sodium sensitivity (Sealey et al., 1988; Johnson et al., 2005b). Because of the putative nephron heterogeneity, we chose to measure PRA instead of renin staining to examine possible changes in the renin status during hyperuricemia.

The 5/6 NX rat is a low renin CRI model (Pörsti et al., 2004), but clear increases in PRA were observed in both Sham and NX rats after the oxonic acid diet. This corresponds well with the results of previous clinical studies (Saito et al., 1978; Gruskin, 1985) suggesting a correlation between hyperuricemia and elevated PRA. Previously, adrenal aldosterone production has been found to be increased 8 days after 5/6 nephrectomy in rats despite a decrease in PRA; the putative stimulus for the aldosterone synthesis being up-regulated adrenal renin synthesis (Endemann et al., 2004). In concert with these findings, subtotal

nephrectomy was associated with a marked increase in plasma aldosterone to renin ratio in the present study. However, this ratio was not affected by oxonic acid feeding, which suggests that increased PRA was the probable explanation for the observed increase in plasma aldosterone. High circulating aldosterone concentration is acknowledged as a significant cardiovascular risk factor (Schmidt & Schmieder, 2003; Schmidt et al., 2006), but during hyperuricemia, its role has received little attention. It should be noted that the present rats were not subjected to a metabolic balance study, and claims of a constant Na^+ retention and K^+ loss cannot be presented from these results. The possibility exists that the influence of plasma aldosterone on electrolyte balance became more evident in the unfamiliar environment of a metabolic cage. However, our results imply that increased plasma aldosterone may mediate the Na^+ retention associated with high serum UA.

The knowledge of the possible hyperuricemia-associated changes in renal tissue components of RAS is limited. Our aim was, therefore, to examine if some of the detrimental effects of elevated serum UA could be accounted for by alterations in renal RAS components. Kidney ACE, ACE2, AT_{1R}, and AT_{2R} were measured at protein and mRNA levels using *in vitro* autoradiography and real-time quantitative RT-PCR, respectively. The autoradiography analyses did not unearth any changes that could explain the hyperuricemia-induced alteration in Na^+-K^+ balance. There were no differences in kidney ACE content between the study groups, while changes in cortical and medullary AT_{1R} densities were only observed in the NX+Oxo group: about 25% higher cortical AT_{1R} density than in the NX group, and 43% higher medullary AT_{1R} density than in the Sham group. Thus, hyperuricemia may be associated with increases in renal AT_{1R} density in CRI, but neither of these changes could explain the altered Na^+-K^+ balance, as increased renal K^+ wasting was also observed in the Sham+Oxo group in the absence of alterations in renal AT_{1R} density.

When determined using RT-PCR, both NX groups showed significantly lower ACE and ACE2 mRNA levels than the respective Sham rats. Kidney AT_{1aR} and AT_{2R} mRNA contents were also lower in both NX groups than in Sham rats. Importantly, the oxonic acid diet did not influence ACE, ACE2, AT_{1aR} or AT_{2R} mRNA in kidney tissue of either Sham or NX rats. As the RT-PCR analyses were performed from kidney extracts, no macroscopic localization of the mRNA molecules could be obtained. In the case of AT_Rs, the *in vitro* autoradiography provided information about distribution at the tissue level, in

effect its localization in the cortex or medulla. Experimental CRI was also associated with an approximately 40% increase in the medullary to cortical ratio of AT_{1R} density, which was not influenced by 2.0% oxonic acid feeding. Moreover, highest ACE labelling was detected in the corticomedullary region in the sham-operated rats, but in the 5/6 nephrectomized rats kidney ACE showed patchy and wider tissue distribution than in normal kidneys (Pörsti et al., 2004). Altogether, the RAS components investigated showed no changes at the mRNA level that would explain the influence of hyperuricemia on Na^+-K^+ balance. As the mRNA levels, but not the corresponding protein contents, were reduced in the two NX groups, the above RAS components were probably subject to post-transcriptional modulation (i.e. more effective translation) or slower protein turnover (i.e extended half-life) in experimental CRI. This may be related to the lower PRA and reduced circulating RAS activity in this form of renal insufficiency.

7. CONCLUSIONS

In conclusion, hyperuricemia induced by 2.0% oxonic acid diet did not induce changes in the local components of RAS in renal tissue, but activated the circulating RAS, reflected as increased PRA and aldosterone in both Sham and NX rats. The functional significance of these hormonal findings is clearly evidenced by the subsequent increase in K^+ excretion and reduction in Na^+ excretion in the hyperuricemic rats, leading to a marked elevation of the K^+ to Na^+ ratio in the urine. The present results emphasize the role of aldosterone in the hyperuricemia-induced sodium retention and BP elevation.

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