The ACE (I/D) polymorphism and the RAAS in type 1 diabetes mellitus

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Chapter 1
Background

The renin angiotensin aldosterone system (RAAS) is involved in the regulation of systemic vascular tone, renal hemodynamics and sodium balance. Furthermore, angiotensin (Ang) II acts as a growth factor. Interestingly, all effects of RAAS activation (hypertension, renal efferent arteriolar vasoconstriction, sodium and fluid reabsorption and increased mesangial matrix formation) occur in patients with diabetes mellitus who develop renal complications. Together with thepressive protective actions of drugs that interfere in the RAAS, this has pointed towards the idea of involvement of this system in the development and progression of diabetic microvascular complications. In spite of the many efforts to elucidate the role of the RAAS in the propensity to develop diabetic complications, many controversies exist, partly due to lack of data, partly due to conflicting evidence.

The last decade it has become increasingly clear that the RAAS is genetically diverse. Genetic polymorphisms have been identified for most components of the RAAS, i.e. renin, angiotensinogen, ACE and the angiotensin II type 1 receptor (AT1R). This raises the possibility that RAAS function is different between individuals with different genetic profiles. If so, it would be logical to assume that such a heterogeneity might hamper the interpretation of phenotypic data on RAAS function in diabetes. Whereas association studies suggest that the genetic polymorphisms of the RAAS may indeed have phenotypic consequences, in diabetes this issue has not been explored by true physiological studies so far.

Among the genetic polymorphisms of the RAAS, most interest has been drawn by the ACE genotype, as this is associated with an increased rate of renal function loss in non-diabetic as well as diabetic renal disease. This makes the ACE genotype a logical and relevant candidate gene to study in the context of diabetes mellitus. However, a lot of discrepancies have been reported in the literature. These discrepancies can in part be attributed to the fact that the physiological context in which the RAAS exerts its diverse functions was not sufficiently taken into account. Both theoretical and empirical considerations suggest that the impact of the ACE (I/D) polymorphism should be evaluated in its (patho-)physiological context, as its impact probably is context dependent. In this respect, several factors are likely to be relevant.

First, it is well-established that RAAS activity is inversely related to sodium intake. In prior studies in non-diabetic subjects, we demonstrated that sodium intake modified the phenotype related to the ACE (I/D) polymorphism, most likely by its effect on background RAAS activity. This emphasizes the need to explicitly analyze gene-environment interactions, when exploring the effects of genetic polymorphisms in complex disease. Moreover, an
altered sodium homeostasis is present in diabetes. By evaluating different studies that are not comparable with respect to sodium intake, bias may have been introduced, which may account for discrepant results. Other environmental factors, such as the diabetic state and glycemic control can also affect the activity and function of the RAAS, and thus should be taken into account as well.

Second, many different neurohumoral systems are involved in the regulation of sodium balance, blood pressure and renal hemodynamics and, as a rule, these systems act in concert. Consequently, the RAAS should not be regarded as an isolated entity. For instance, extensive interactions between the RAAS and the autonomic nervous system have been reported. Little attention however has been paid to these interactions in the study of genetic polymorphisms of the RAAS.

Finally, many discrepancies can be introduced by heterogeneity in patient populations. Literature is very diverse with respect to the type of diabetic patients (type 1 or type 2) studied, the stage of diabetes and the presence and severity of diabetic complications. Assuming that background RAAS activity is a determinant of the impact of the ACE genotype, and assuming that RAAS activity is involved in the different stages of progression from uncomplicated diabetes towards the full picture of diabetic end organ damage, it would be highly important to stratify for the stage of diabetes studied.

The purpose of this thesis is to investigate the impact of the ACE (I/D) polymorphism on systemic and renal hemodynamics in diabetes, by physiological studies exploring its impact on RAAS function, in conditions with different background RAAS activity. The general hypotheses underlying our studies are, first, that ACE (I/D) polymorphism exerts its effects by modulating RAAS function, and second, that these effects are modulated by differences in background RAAS activity, induced by environmental factors. We studied uncomplicated type I diabetic patients to provide a well defined, homogenous population. Furthermore, by studying this specific population, the information obtained on early abnormalities in the RAAS might offer clues for prevention of diabetic complications in the future.

In the first section of this thesis, the effects of differences in sodium intake on renal hemodynamics and the RAAS cascade will be studied. The second section deals with the effects of the ACE (I/D) polymorphism and diabetes mellitus on the renal and systemic responses to exogenous angiotensin I and II. We will also study possible influences of the ACE (I/D) polymorphism on the pressor response to the effector hormone of the sympathetic nervous system, i.e. norepinephrine, to explore the effects of genetic variability in the RAAS in the context of the multifactorial regulation of blood pressure.
Interestingly, once diabetic nephropathy ensues, there is considerable similarity in the factors that promote progression of renal damage in non-diabetic kidney diseases and diabetic nephropathy, respectively. The ACE (I/D) polymorphism is one of those common progression promoters. In the final section, we describe these risk factors, in order to put this thesis in a more clinical perspective.

This chapter will give a short description of diabetic renal disease and will provide an overview of the current concepts on the function of the RAAS and the involvement of the RAAS in the development of diabetes mellitus associated complications. Several aspects of the RAAS will be highlighted, including a functional overview, environmental influences, and genetic aspects.

Diabetic nephropathy

Patients with (type 1) diabetes mellitus (DM) are prone to develop microvascular complications, including retinopathy, neuropathy and nephropathy. The development of diabetic nephropathy is one of the most severe complications of DM. It leads to renal insufficiency and carries at the same time a high risk of cardiovascular morbidity and mortality (1). Early functional abnormalities, also denoted as incipient nephropathy, include hypertension, intraglomerular hyperperfusion with ensuing hyperfiltration and microalbuminuria (30-300mg/day), which progresses to proteinuria in many patients. The clinical syndrome of overt nephropathy is characterized by persistent and progressive albuminuria, early arterial blood pressure elevation, and a relentless decline in glomerular filtration rate (GFR). Early histologic features are hypertrophy of glomerular and tubular structures, arteriolar matrix accumulation and thickening of glomerular and tubular basement membranes which finally result in the development of Kimmelstiel Wilson lesions. Ultimately, end-stage renal disease is the final common pathway.

The finding of microalbuminuria, which progresses to overt proteinuria, can identify early renal involvement and is an established predictor for the development of overt diabetic nephropathy in type 1 and type 2 diabetes mellitus (2;3). Risk factors for the development of micro- and macroalbuminuria in type 1 DM include baseline albumin excretion rate, HbA1c (glycemic control), the presence of retinopathy and smoking (4). Agressive antihypertensive treatment is able to retard the rate of deterioration of kidney function and since the introduction of ACE-inhibitors (ACEi), the natural course of diabetic nephropathy has
improved tremendously. Early treatment of microalbuminuric patients with these agents reduces the incidence of proteinuria, retards the rate of renal function loss and improves renal survival (5;6). A total of ≈ 30% of type 1 diabetic patients may develop diabetic nephropathy within 25 years of diabetes duration (7), although recent data suggest a declining incidence (8;9), probably due to improved glycemic control, lower blood pressure (in part due to early aggressive antihypertensive treatment), and reduced prevalence of smoking. Furthermore, there is a decline in the progression from microalbuminuria to overt proteinuria in type 1 DM. Previously, 80 to 90% of microalbuminuric type 1 DM patients progressed to overt nephropathy (3). In the early nineties estimates revealed that during the last 10 to 15 years only 30% of microalbuminuric patients progressed to clinical proteinuria (10). By the use of RAAS intervening therapies, marked reduction in progression to DN can be achieved (6;11).

Despite intensive insulin treatment, more strict metabolic control and the availability of effective renoprotective agents such as ACE inhibitors, diabetic nephropathy has still become the leading cause (25-30%) of end stage renal disease in Europe, the United States and Japan (1). This is mainly due to an increase of the incidence of nephropathy in type 2 DM, which is expected to double within the next 15 years. In Europe, the direct economic cost of DM is approximately 8-10% of the health care budget and nearly 80-90% of the direct costs is needed for the treatment of diabetic micro-and macro-angiopathy (12).

Why does one patient develop diabetic nephropathy early in the course of diabetes and does another patient not develop signs of diabetic nephropathy at all, sometimes even despite poor diabetes regulation? These interindividual differences, together with the observation that diabetic nephropathy clusters in affected families support the involvement of genetic factors in its pathogenesis (13-15). When there is a family history of diabetic nephropathy, type 1 DM siblings have a life long risk of 70% to develop nephropathy, whereas the risk is only 20% when there is no first degree relative with diabetic nephropathy (15)! Adults with short stature (associated with birth weight) have an increased risk of developing diabetic nephropathy (16). These findings suggest that genetic predisposition or factors operating in utero contribute to the development of diabetic nephropathy. However, the involved genes have not been identified so far, except that I/D polymorphism of the ACE-gene, which will be extensively discussed later in this chapter, has been widely investigated as a possible promotor of renal function loss once nephropathy has ensued (17).
The renin-angiotensin aldosterone system

The renin-angiotensin aldosterone system (RAAS) plays an important role in blood pressure control, renal hemodynamics and volume homeostasis and has cell proliferative effects as well (18). In the classical concept of the RAAS, renin, generated in the juxtaglomerular apparatus of the kidney, catalyzes the conversion of angiotensinogen to angiotensin I (AngI). Next, angiotensin converting enzyme (ACE), converts the inactive decapptide angiotensin I (AngI) to the octapeptide angiotensin II (AngII), the effector component of the system. AngII stimulates the production of aldosterone, a mineralocorticoid that exerts water and sodium retaining effects on the distal tubule. Figure 1 gives a schematic overview of the current concepts of the RAAS cascade, which also includes the presence of non-ACE AngII forming pathways, other effector angiotensins (Ang 1-7), and the recently discovered homologue ACE2.

Angiotensin Converting Enzyme (ACE)

ACE is a membrane-bound, zinc- and chloride-dependent dipeptidase that catalyzes the conversion of the decapptide AngI to the octapeptide AngII. Besides this particular function, ACE catalyzes a lot of different enzymatic processes in the human body, therefore it is sometimes referred to as the most promiscuous enzyme in the body. It is important to note that there are a number of non-ACE pathways, such as chymase, that contribute to the formation of AngII as well, in a species- and organ-specific manner (19;20). There are two forms of ACE, somatic ACE (sACE) and sperm-specific germinal ACE. Human sACE is expressed strongly in endothelial cells in many organs, highest in the capillaries of the lung, as well as in epithelial cells in the kidney (proximal tubule) and in the small intestine. The extracellular localization of ACE on endothelial cells creates an ideal position for the interaction with its substrate AngI and bradykinin (which ACE degrades into inactive fragments). ACE is shed from the endothelium at a high rate, leading to relatively high ACE levels in the circulation. Traditionally, renin rather than ACE activity is assumed to be the rate-limiting factor for the production of AngII (21). However, as shown by Danser et al, the greatest fraction of AngII is formed within tissues, where baseline ACE activity is much lower, but can be increased by different disease-associated triggers (22). This might point towards a possible rate-limiting role for tissue ACE in such disease conditions. This possibility was supported in experimental studies, in which infusion of increasing AngI concentration in control rat hindlimb resulted in saturably increased conversion to AngII.
When the authors upregulated ACE gene expression and ACE activity in a two-kidney, one clip hypertension model, this lead to an increased local AngI to AngII conversion (23).

The mRNA for sACE is expressed in virtually all tissues. Biochemical measurements of ACE activity illustrate that >90% of ACE is located in tissue (24). Tissue ACE is now recognized as a key factor in cardiovascular and renal diseases (25). In the healthy human kidney, ACE is mainly located at the brush border of the proximal tubule (26). Interestingly, in the kidney, ACE was reported to be absent in endothelial cells of any vessel type (27). This organ-specific lack of endothelial ACE may contribute to a relatively lower vascular resistance of the human kidney and the high renal blood flow. Furthermore, this absence may protect the renal circulation against excessive AngII formation and kinin inactivation (27). Interestingly, endothelial neoexpression of ACE was observed in different kidney diseases in man (27), which was mainly associated with interstitial fibrosis and showed some selectivity for glomerular endothelial cells in diabetes mellitus and hypertension. This suggests a pathophysiological role for kidney ACE in the pathogenesis of kidney diseases. In fact, in type 2 DM patients with diabetic nephropathy, the number of glomeruli with a positive staining for ACE was markedly increased compared to healthy subjects, again suggesting a pathophysiological role for renal ACE in the development of diabetic
nephropathy (28). Furthermore, the number of ACE transcripts in the kidney was increased in healthy Japanese subjects carrying the DD-genotype (29), suggesting that renal ACE expression is modified by ACE genotype. Whether genetic regulation – by ACE genotype – modifies renal ACE expression in diabetic nephropathy, is however unknown. In models of heart failure and atherosclerosis, both ACE activity and mRNA was increased in heart and blood vessels, respectively (30;31). In adriamycin nephrosis in rats, our group found that a high renal ACE activity correlated with a worse renal outcome (32) and that during ACE inhibition, residual renal ACE activity is associated with a poor prognosis (33). These data further illustrate the potential causal importance of tissue ACE in renal disease.

ACE2 is a recently discovered homologue of ACE, thought to counterbalance the effects of ACE (34;35). ACE2 cleaves AngI and AngII into inactive Angiotensin 1-9 (Ang 1-9) and the vasodilator and anti-proliferative Angiotensin 1-7 (Ang 1-7). The functional role of ACE2 still needs to be elucidated, but studies in ACE2 knockout mice support a functional role, as absence of ACE2 is associated with the development of heart failure (36). Yagil et al. recently hypothesized that in blood pressure control, ACE2 counterbalances the vasopressor effects of ACE through the generation of Ang 1-7 and by hydrolyzing part of AngII. ACE2 is known to be present in the human and rat kidney. In animal studies it has been shown that ACE2 localization and expression in the kidney can be altered in diabetic or hypertensive conditions (37). In man, ACE2 has been identified in the heart, kidney and testis (34;38). The first human data suggest that there is endothelial neoexpression of ACE2 in the kidney, in various native kidney disease, including diabetic nephropathy (39).

**Angiotensin II**

AngII is a potent vasopressor and mediates a broad spectrum of physiological and pathophysiological effects by binding to specific cell-membrane receptors. Two distinct types of receptors for AngII have been characterized in humans and rodents. The AngII type1 (AT1) and type 2 (AT2) receptors are heterogeneously distributed in peripheral tissues and the brain (40). The AT1 receptor is predominantly expressed in the kidneys, adrenal glands, vascular smooth muscle cells and the heart. The regulatory functions of AngII on blood pressure, renal hemodynamics and sodium and water balance are attributed to the AT1 receptor (40). AngII increases systemic arterial blood pressure and increases intraglomerular pressure, by means of vasoconstriction of the efferent arteriole. Together with many other systems this mechanism is involved in the regulation of intraglomerular pressure, thereby maintaining the glomerular filtration rate. AngII
also exerts renal tubular effects, both directly and indirectly via stimulation of adrenal aldosterone synthesis, resulting in sodium retention and potassium excretion. Via these renal and systemic vasoconstrictive effects AngII is involved in the regulation of blood pressure, renal hemodynamics and sodium balance. In particular, AngII contributes to the maintenance of systemic blood pressure and glomerular filtration, and to preservation of extracellular volume under conditions of sodium and volume deficit (see figure 1). Finally, AngII is a growth promoting factor and has profibrotic effects, resulting in an expansion of extracellular matrix and mesangial cell hypertrophy (41).

The AT2 receptor is present at high density during fetal development. During adult life, significant AT2 expression only occurs in the adrenal medulla, uterus, ovary, vascular endothelium and certain areas in the brain. The AT2 receptor is thought to counterbalance the effects mediated by the AT1 receptor: it induces vasodilation and may be involved in the control of cell proliferation, differentiation and angiogenesis (42;43).

**Aldosterone**

Binding of AngII to the adrenal zona glomerulosa AT1 receptors results in the production of aldosterone, the main mineralocorticoid in humans, that exerts water and sodium retaining effects on the distal tubule. The effects of aldosterone are mediated by a Na+/K+-ATPase pump, therefore retaining sodium induces tubular potassium excretion. Besides by AngII, aldosterone production is also influenced by extracellular potassium concentrations and adrenocorticotropic hormone (ACTH). The different natriuretic factors (ANP, BNP) are known to inhibit aldosterone secretion, which appears to be part of an integrated response to reduce sodium retention.

Aldosterone has effects on the brain, the heart, the vasculature and kidneys that lead to elevated blood pressure. These changes include enhanced sympathetic nervous system activity, reduced vascular compliance and endothelial-derived vasorelaxation, increases in volume expansion and reduced serum potassium, and increases in left ventricular mass and cardiac output (44). Within the kidney aldosterone has vasoconstrictor effects on the glomerular microcirculation (45). Aldosterone has regained interest as a potential pathogenetic factor in cardiovascular disease, as a profibrotic factor. This concept is supported by the protective effects of treatment with spironolactone in patients with heart failure (46). There is experimental evidence to suggest that aldosterone also contributes to the development of nephrosclerosis and renal fibrosis in models of diabetes and hypertension (47). Data on aldosterone levels in diabetes, however, are not consistent, as
plasma aldosterone levels have been reported to be normal (48-50), increased (51) or low (52-54) in DM. Again, differences in characteristics of the studied patients are likely to account for the discrepancies.

The RAAS in type 1 diabetes mellitus

The RAAS has since long been associated with the development and progression of diabetic complications, as the effects of RAAS activation (hypertension, efferent renal vasoconstriction, sodium and fluid reabsorption and increased mesangial matrix formation) are all observed in patients with diabetes mellitus who develop renal complications. Early abnormalities preceding overt nephropathy include microalbuminuria, a rise in blood pressure, an increase in intraglomerular pressure and consequently in glomerular filtration rate (55;56). Volume expansion may contribute to these processes, as renal sodium excretion is known to be blunted in diabetic patients (57-61), an effect that might be mediated by the sodium retaining effects of insulin (62;63).

Furthermore, a role of the RAAS in diabetic complications is supported by the marked protective effects of RAAS-blockade (64). From the early 1980’s this was first encompassed by using ACE-inhibitors, which inhibit the conversion of AngI to AngII. In the 1990’s the class of AT1R blockers (AIIRB’s) was introduced. Throughout the years, many studies on the effect of RAAS blockade in DM were performed. In patients with type 1 DM, treatment with ACE inhibitors has been shown to result in pronounced beneficial effects. It retards the occurrence of microalbuminuria (incipient nephropathy) (65), delays progression from microalbuminuria to overt nephropathy (6;11) and reduces atherosclerotic events (66) in DM patients. The renoprotective effects of AIIRB’s in type 1 DM have been mainly addressed in short-term studies. Losartan has been shown to reduce blood pressure, glomerular hyperfiltration and filtration fraction in normotensive, normoalbuminuric type 1 DM patients (67). Andersen et al. compared the short term (two months) effects of enalapril and losartan in 16 type 1 DM patients with nephropathy. They found a similar reduction in blood pressure and proteinuria with the two RAAS interfering agents (68). Recently, there is increasing evidence that addition of an AIIRB on top of maximal doses of ACE inhibition has additional renoprotective effects in diabetic and non-diabetic renal disease than the usage of either drug alone (69;70).

Interestingly, our group showed that diabetic patients that respond favorably to one
class of antiproteinuric drug also respond favorably to other classes of available drugs, supporting a main role for individual patient factors in the responsiveness or resistance to antiproteinuric intervention (71). This points toward possible genetic influences on the effectivity of RAAS intervention. Our group has studied the antiproteinuric and renal hemodynamic effects of short-term treatment with losartan in microalbuminuric type 1 DM patients. Losartan reduced albuminuria effectively and we found a close correlation between antiproteinuric effects and the renal hemodynamic effects induced by losartan (increase in ERPF with decreased FF), indicating the importance of renal hemodynamics in the pathophysiology of diabetic proteinuria (72).

**Abnormalities in the RAAS in DM**

Several RAAS abnormalities have been reported in patients with DM. The separate components (renin/plasma renin activity (PRA), ACE, AngI/II, aldosterone) were found to be lowered, unchanged or elevated in these patients (60;73). However, exchangeable sodium is consistently found to be elevated (60;74). Combining the observations of the slightly reduced, normal or elevated RAAS components with the presence of volume expansion, the concept emerged that, relative to the state of volume expansion, the RAAS is insufficiently suppressed in DM.

Furthermore, alterations in the RAAS have been suggested by studies using AngII infusions, showing increased or unchanged pressor responsiveness in diabetic patients (75-77). Most of these studies are hampered by the fact that patients were not studied during standardized sodium balance, since sodium status is an important determinant of RAAS activity and of angiotensin responsiveness (78). There is one controlled study comparing the effects of low and high sodium consumption in type 1 DM, showing that the expected increase in PRA and aldosterone during low sodium was blunted in the diabetic patients, which indicates a persistent degree of volume expansion during a sodium restricted diet. The diabetes associated renal hemodynamic abnormalities (increased GRF and RBF with a decreased RVR) were worsened by sodium restriction, whereas sodium restriction did not affect renal hemodynamics in healthy control subjects (79).

**Hyperglycemia induced RAAS activation**

As mentioned earlier, aggressive glycemic control delays the onset and/or improves the occurrence of microvascular complications in patients with type 1 DM (8;80). Several lines
of evidence suggest that hyperglycemia is involved in the diabetes-associated increased RAAS activity. Acute infusions of glucose induce elevations of GFR, ERPF and FF in patients with type 1 DM (81-83). Miller et al showed that hyperglycemia increased PRA and blood pressure. The renal hemodynamic effects of hyperglycemia included an unchanged GFR along with significant declines in renal blood flow and a rise in FF (84). These data suggest that sustained hyperglycemia activates the RAAS, thereby increasing systemic and renal vasomotor tone. Interestingly, it was shown in another study that patients carrying the II genotype were resistant to the glomerular changes induced by hyperglycemia (85). Osei et al. found an enhancement of renal vasodilation during hyperglycemia by captopril and eprosartan without alteration of PRA, which suggests activation of the intrarenal renin angiotensin system (86;87).

In vitro studies showed cellular effects of glucose. Evidence suggests that the intrarenal RAAS within glomeruli and proximal tubules may be activated by hyperglycemia, leading to stimulation of local AngII production (88). High glucose has been shown to stimulate angiotensinogen gene expression in rat proximal tubules (89). Hyperglycemia also increased mesangial AngII levels (88). When glomerular mesangial cells were cultured in high glucose, an increased formation of AngII from AngI was observed together with an increase in AngII produced from Ang(1-9) (90). This could however not directly be attributed to an increase in ACE level.

Discrepancy between local and systemic RAAS

When studies on the circulating RAAS were first undertaken, an unanticipated suppression of the RAAS was found in many patients, especially in patients with diabetic nephropathy (91). There is increasing evidence for the existence of discrete, tissue-specific renin-angiotensin systems (RAS) that are regulated independently from the ‘conventional’ circulating RAAS. In part, this is based on the finding that multiple organs and cell types express all of the components necessary to recapitulate a functional, local RAS in situ (25). Functionally relevant changes in local AngI-II conversion are not necessarily reflected by detectable changes in circulating AngII (92). With respect to the kidney, mRNA and protein with respect to all components of the RAS have been found in the kidney tubule as well as in the glomerulus (93-95). The concentrations of AngII in glomerular ultrafiltrate are significantly higher than those in plasma, suggesting glomerular AngII synthesis in vivo. Furthermore, AngII levels in the proximal tubular lumen are approximately 1000-fold higher than the concentration in plasma (96;97). It is thus believed that AngII is
synthesized by proximal tubular cells and released into the lumen, where it may act on AT1 receptors to regulate solute and water transport and cell growth. In this respect, the possible role of renal (tubular) ACE levels and the reported increased and abnormal expression of renal ACE in kidney diseases seem relevant in pathophysiologic conditions (28). A recent study in renal biopsies from patients with diabetic nephropathy showed elevated ACE and AngII expression in tubular and interstitial cells (98).

In type 2 DM, enhanced activation of kidney RAS with suppressed circulating RAAS parameters has been shown (99;100). The group of Hollenberg provided evidence for the existence of increased intrarenal RAS activation in this population by a triad consisting of blunting of renal vascular response to exogenous AngII, despite high sodium diet, an exaggerated renal vasodilatory response to ACEi and the correction of blunted RVR response to AngII by ACEi. This phenotype is more or less similar to that found in non-modulating hypertension. All characteristics are consistent with excessive action of AngII on the renal circulation, mainly during high sodium (101). This suggests that measures of circulating renin activity can be misleading, providing little insight into the state of the intrarenal RAS (99;101). Lansang et al. compared the renal hemodynamic responses to an ACEi (captopril) and an AIIRB (candesartan) in type 1 DM, to test whether the renoprotective action of ACEi is mainly due to its effects on AngII formation or to effects on other vasodilative systems (102). It was found that the renal vasodilator responses correlated very well, which indicates that reduced AngII formation is the main mechanism of action of the ACEi. The remarkable rise in RPF on both agents, despite high-salt balance, and despite suppressed PRA, supports intrarenal activation of the RAS in DM that is not reflected in plasma renin levels.

Finally, experimental studies in rats showed that AngII receptors are differently expressed in the diabetic kidney, with upregulation in the vasculature and downregulation in the tubulo-interstitial compartment, as recently reviewed (103). These studies suggest that alterations in local AngII formation and differences in receptor expression within the kidney may be of relevance in the pathogenic role of the RAAS in diabetic nephropathy.

*Genetic polymorphisms of the RAAS-components*

Polymorphisms in genes coding for components of the RAAS have been described and investigated for functional consequences. The most extensively investigated so far are the insertion/deletion (I/D) polymorphism for the ACE-gene, the M235T polymorphism for the angiotensinogen (AGT) gene and the A1166C polymorphism for the AngII type 1 receptor (AT1R) gene. The ACE (I/D) polymorphism will be extensively discussed later. The M235T
polymorphism for AGT results in 10-20% higher AGT levels in TT homozygotes than in MM homozygotes (104). Some studies in DM could not find any association between this polymorphism with diabetic nephropathy (105-107), whereas other studies found an association between the TT genotype and diabetic nephropathy (108-110). No phenotypical differences have been associated with the A1166C polymorphism for the AT1R gene. Receptor density and affinity – studied on platelets – do not seem to be affected by the AT1 (A/C) genotype (111). The functional consequences have not been fully clarified, but an increased AngII response in internal mammary arteries of CC-homozygotes has been reported in vitro (112). In vivo, the C-allele was associated with an increased sensitivity to exogenous AngII and an increased response to AIIRB (113;114). With respect to diabetic nephropathy, the CC genotype was associated with an increased incidence of albuminuria (115) and hypertension (109) although others could not find any association (116;117). An extensive review of the mentioned genetic polymorphisms within the RAAS is beyond the scope of this thesis, but we will discuss the role of the ACE(I/D) polymorphism in relation to diabetes mellitus and its complications below.

Most studies that were performed on this issue so far are based on the demonstration of an association between a gene and a disease condition. The pitfalls of association studies, as well as the possible clues to future research were summarized in several recent reviews (118-120). In brief, important limitations are the fact that associations with nearby genes are ascribed to the genes of interest. Furthermore, association studies may reflect the particularities of the studied population and thus lack generalizability. When there is a true risk conferred by the candidate gene, moreover, in an association study, there can be selection bias by competing risks associated with the genotype. With respect to the ACE genotype, when studying the impact on renal failure in diabetes mellitus, ACE genotype may affect mortality by a concomitant effect on cardiovascular risk. This may lead to an under representation of alleles promoting renal function loss in cross-sectional studies in end-stage renal failure. Finally, there may be a publication bias, as suggested by the smaller sample size in studies with positive findings than in those with negative findings, but its effect is hard to estimate.

**Genetic analysis of complex disease**

It is difficult to attribute the cause or progression of a complex disease to a single genetic factor, as multiple genes often are involved, resulting in potentially important gene-gene interactions. Furthermore, many environmental factors exert influences on the
disease, as well as on the influence of a certain genetic polymorphism, resulting in gene-environmental interactions. Obviously, taking into account multiple gene-gene, as well as gene-environment interactions, this leads to a very large set of possibly relevant variables. To entangle these by association studies, i.e. by the epidemiological approach, would require very large populations, which usually results in increasing heterogeneity in the population. This in turn hampers the possibility to draw valid conclusions. The alternative approach would be to start the other way round, i.e. to explore the function of a specific candidate gene, and to investigate, in experimental setups, whether the candidate gene polymorphism is associated with functional alterations that are relevant for (patho-)physiology. The finding of physiological effects of a certain genotype in a well-controlled experimental setting can provide valid evidence for the relevance of the genotype.

Schork et al. proposed a six-fold way to guide research in modern genetics of complex traits (121). In brief, they distinguish the following approaches, that should be considered as complementary, all providing different pieces of the puzzle:

1. Gene discovery: identification of genes (chromosomal location, boundaries etc.).
2. Structural genomics: construction and patterning of the nucleotide and amino acid sequences associated with genes in an effort to predict the function and relatedness of genes.
3. Clinical genetics: identification of important phenotypic endpoints. Most complex diseases can be subdivided into components, each with slightly different characteristics and phenotypic manifestations that are likely caused by subtle differences in their genetic determinants as well as the interaction of these determinants with environmental factors.
4. Integrative physiology: determination and characterization of physiological phenomena and processes that probably reflect the immediate correlates and outcomes of gene activity.
5. Functional genomics: determination of the association of the presence or absence of specific genes with physiological functions, for instance by studying the pathophysiology induced by knock-out models. This does not give information on the specific pathway in which the gene exerts its function.
6. Population genetics and genetic epidemiology: identification and characterization of genetic, social and environmental processes that allow disease to emerge and be maintained in the general population. In this respect, phenomena such as gene-environmental interaction should be considered.
The fact that many factors influence complex diseases (i.e. diabetes and hypertension) creates situations in which the effect of a single factor may be more or less pronounced in the presence of others, i.e. they are “context dependent”. As apparent from the above considerations, this context dependency forces researchers to conduct either large epidemiological studies, suitable to reveal the robustness of the impact of a certain genetic polymorphism, or by physiological experiments varying the relevant environmental factors one-at-a-time. This thesis aims to do so for the ACE (I/D) genotype as a candidate gene in patients with DM during controlled environmental circumstances. With this design, the ACE (I/D) genotype is studied in the “integrative physiology” step of the mentioned six-fold way, in particular the physiological consequences of the increased ACE level induced by the D-allele of the genetic polymorphism. The next paragraph will provide a review of the current knowledge on the effects of the ACE (I/D) polymorphism in DM.

The ACE-(I/D) polymorphism in diabetes mellitus

The variance in plasma ACE levels is explained for 47 % by a polymorphism in the ACE gene. This polymorphism consists of a 287 base pair insertion (I) or deletion (D) of intron 16 of the ACE gene and influences plasma ACE and tissue ACE activity (122). It is a common polymorphism, with marked ethnical differences in distribution (123). In healthy Caucasian populations the D allele frequency ranges between 0.50 and 0.63 (124). The D-allele is associated with the highest plasma ACE levels. There is evidence to suggest that renal (29) and vascular tissue ACE also relate to ACE genotype (22;125). Interestingly, patients with diabetic nephropathy carrying the DD genotype showed a more rapid decline in kidney function, compared to patients with the ID and II genotype (126), also found in non-diabetic kidney disease (127). Many association studies on effects of the ACE genotype on the progression of diabetic nephropathy have been performed. Table 1 shows an overview. If an association is present, it is always with the DD-genotype and a poor prognosis, but there are also many studies that could not confirm this. These inter study discrepancies may be accounted for by methodological flaws, such as inhomogeneous patient groups, selection bias by competing risks of the DD-genotype on cardiovascular mortality, lack of adjustment for diabetes duration, glycemic control (HbA1c) or the presence of diabetic complications, and by the weaknesses of association studies in general. One prospective study found an association mainly in patients with poor glycemic control (128). The association between the D-allele and an increased propensity to end-organ damage is attributed to an increased AngII formation in subjects with the DD genotype, as supported
by studies in non-diabetic healthy subjects. These studies suggest that DD homozygotes have an increased conversion of AngI to AngII. Thus, ACE levels and the ACE gene polymorphism might play a role in the conversion of AngI to AngII. The functional significance of the elevated plasma ACE levels in physiology and patho-physiology, however, is uncertain so far. In a pharmacological setup in normal volunteers, our group (129) and others (130) found evidence that elevated ACE levels in the DD-genotype can have functional consequences, as in the DD genotype infusion of pharmacological doses of AngI leads to an enhanced response of blood pressure and renal function as compared to the II genotype, consistent with enhanced conversion of AngI. In line with these findings, an attenuated response of renal vascular resistance and plasma flow after captopril administration in healthy volunteers with the DD genotype was reported, suggesting that the ACE genotype might affect the activity of tissue RAAS (131), but data on the impact of the ACE genotype on the renal hemodynamic effects of RAAS blockade are not consistent (132). We also found an effect of sodium intake on the impact of the ACE genotype on the antiproteinuric response to ACE inhibition in non-diabetic renal patients, consistent with interaction between sodium status and ACE genotype (133). Interestingly, the enhanced response of blood pressure and renal hemodynamics to AngI in DD homozygotes that was reported by our group was blunted by a low sodium diet, supporting the possible gene-environment interaction between the ACE (I/D) genotype and sodium intake (129). Failure to account for the role of sodium intake may partly explain the discrepancies between studies on the impact of ACE genotype on therapy response to RAAS blockade.

The enhanced AngI response in DD homozygotes opens the possibility that the elevated ACE levels modulate RAAS-function. In diabetic patients the functional consequences of higher ACE levels in the DD genotype are unknown. This question is even more interesting when one takes in mind that diabetes as such is associated with elevated ACE levels (134;135). Several reports mentioned elevated plasma ACE levels in DM compared to healthy controls (136). Whether this has functional consequences by increased AngII formation is unknown. It has to be noted here that the mechanisms and functional consequences of diabetes-associated increases in plasma ACE levels, may not be similar to those of genetically determined elevations in plasma ACE levels. It has been suggested that high ACE levels in diabetes merely reflect increased shedding of ACE from cell membranes, due to impaired endothelial anchoring of this ecto-enzyme, as a feature of endothelial dysfunction (135), whereas the genetically elevated plasma ACE level in DD genotype reflects elevated tissue ACE as well (125). In this respect, there are reports on a familial increase in plasma ACE-level due to increased endothelial shedding of ACE. In
these subjects, plasma ACE levels are up to 5 times the normal levels, whereas no increased cardiovascular morbidity or mortality was observed (137). Studies on functional effects were not performed, but this observation strongly suggests that the functional effects of elevated plasma ACE-levels based on increased ACE expression (i.e. the ACE (I/D) polymorphism, with cellular and circulating ACE increased) might differ from elevations in circulating ACE levels that are due to an increased endothelial ACE shedding.

Table 1. Overview of studies on ACE (I/D) genotype and progression of diabetic nephropathy in type 1 DM.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Design</th>
<th>N</th>
<th>Outcome parameter</th>
<th>Effect genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marre (138)</td>
<td>Cross-sectional</td>
<td>494</td>
<td>Creatinine, albuminuria</td>
<td>Worse in DD</td>
</tr>
<tr>
<td>Barnas (139)</td>
<td>Association</td>
<td>122</td>
<td>Albuminuria</td>
<td>Worse in DD</td>
</tr>
<tr>
<td>Tarnow (140)</td>
<td>Case-control</td>
<td>390</td>
<td>Albuminuria</td>
<td>NS</td>
</tr>
<tr>
<td>Chowdhury (141)</td>
<td>Association</td>
<td>936</td>
<td>Nephropathy</td>
<td>NS</td>
</tr>
<tr>
<td>Jacobsen (142)</td>
<td>Prospective</td>
<td>59</td>
<td>GFR decline</td>
<td>NS</td>
</tr>
<tr>
<td>Vleming (143)</td>
<td>Association</td>
<td>161</td>
<td>ESRD</td>
<td>Worse in DD</td>
</tr>
<tr>
<td>Schmidt (144)</td>
<td>Association</td>
<td>247</td>
<td>Albuminuria</td>
<td>NS</td>
</tr>
<tr>
<td>Marre (145)</td>
<td>Association</td>
<td>124</td>
<td>Albuminuria</td>
<td>Worse in DD</td>
</tr>
<tr>
<td>Ringel (146)</td>
<td>Association</td>
<td>360</td>
<td>Albuminuria</td>
<td>NS</td>
</tr>
<tr>
<td>Hadjadj (128)</td>
<td>Prospective</td>
<td>310</td>
<td>Progression of DN</td>
<td>Worse in DD</td>
</tr>
<tr>
<td>Freire (147)</td>
<td>Cross-sectional</td>
<td>166</td>
<td>Albuminuria</td>
<td>NS</td>
</tr>
<tr>
<td>De Azevedo (148)</td>
<td>Prospective</td>
<td>30</td>
<td>Albuminuria, GFR decline</td>
<td>Worse in DD</td>
</tr>
<tr>
<td>Pfohl (149)</td>
<td>Cross-sectional</td>
<td>210</td>
<td>Albuminuria</td>
<td>NS</td>
</tr>
</tbody>
</table>

ESRD = end-stage renal disease, DN = diabetic nephropathy.
Scope of this thesis

The deletion genotype of the ACE (I/D) polymorphism is a risk factor for progressive renal function loss in diabetic renal disease. The mechanism of this increased risk is poorly understood. In healthy DD homozygotes, increased responses of blood pressure, GFR and aldosterone to exogenous AngI have been reported, which could be normalized by sodium restriction. This elicits the hypothesis that background RAAS-activity, influenced by environmental factors (i.e. sodium intake), is a determinant of the impact of the D-allele.

In the presence of type 1 diabetes mellitus, associated with increased plasma ACE levels, investigating the functional effects of the ACE genotype is even more complex. As mentioned earlier in this chapter, extracellular volume is reported to be increased in DM. Furthermore, diabetes-induced hyperglycemia has effects on the RAAS. Finally, other systems involved in the regulation of blood pressure and volume homeostasis might be affected by diabetes mellitus as well. Figure 2 gives a simplified representation of this theoretical framework.

So far, no systematical data on the (patho-)physiology of the ACE genotype in type 1 DM are available. Our aim is to provide such a systematical exploration in uncomplicated type 1 DM, to test the general hypothesis that ACE genotype affects RAAS (patho-)physiology in type 1 DM, and that its impact is modified by factors affecting background RAAS activity.

Outline of this thesis

The studies described in this thesis are divided in three sections.

Section I addresses the impact of sodium intake on the RAAS and renal hemodynamic function in uncomplicated type 1 diabetes mellitus. In chapter 2 we describe the PRA and aldosterone levels during high sodium intake, in response to sodium restriction (endogeneous RAAS stimulation) and in response to infusion of AngI (exogenous stimulation) during both sodium intakes, compared with healthy subjects. chapter 3 describes the renal hemodynamic and systemic effects of a restriction in dietary sodium in patients with uncomplicated type 1 DM compared with healthy subjects.

Section II addresses the functional consequences of the ACE (I/D) polymorphism. Numerous studies investigated the role of the ACE (I/D) polymorphism in renal disease and DM. Evidence for functional consequences of increased plasma ACE levels associated
Figure 2. Schematic representation of our working hypothesis on the relationship between the ACE (I/D) genotype, the RAAS and diabetic nephropathy in the context of diabetes associated environmental influences. The interrelationships within the square are explored in this thesis.

with carriage of the D-allele has been obtained by our group and others. Van der Kleij et al. found an increased conversion of AngI to AngII in healthy subjects during high sodium intake. The functional effects of the ACE (I/D) polymorphism have never been studied in patients with diabetes mellitus. Therefore in chapter 4, we study the possible effects of this genotype on the systemic and renal responses to infusion of AngI and AngII in uncomplicated type 1 DM. To investigate the possible gene-environment interaction with sodium intake in DM, patients were studied both during high and low sodium intake.

Increases in plasma ACE level have been reported in DM. Its mechanism and functional consequences are unknown. Furthermore, alterations in systemic and renal sensitivity to angiotensins have been reported. chapter 5 describes a comparison of the diabetic patients studied in chapter 4 with healthy subjects matched for ACE genotype. The impact of the ACE genotype on AngI responses was compared between diabetic and healthy subjects, allowing to assess the functional effects of the diabetes-associated increase in plasma ACE level, in the context of the genetic effects on ACE, conferred by ACE genotype. Furthermore, this approach allows analysis of angiotensin sensitivity in uncomplicated type 1 DM and of the adaptation of angiotensin sensitivity to a change in sodium intake.
The RAAS regulates blood pressure in close interaction with other systems, of which the autonomic nervous system is the most important. Extensive interactions between RAAS and autonomic nervous system have been described. On theoretical grounds, an increased AngII availability, which might occur in subjects carrying the D-allele, could facilitate sympathetic nervous system functioning. So far, the impact of the ACE(I/D) polymorphism on neurohumoral systems adjacent to the RAAS has not been studied. The ACE(I/D) polymorphism is mainly studied using the approach of strategies that interfere with the RAAS. In chapter 6, therefore, we study the effects of the ACE (I/D) polymorphism on the blood pressure and heart rate response to increasing doses of norepinephrine in healthy subjects. Furthermore, non-invasive analysis of heart rate variability (spontaneous fluctuations in heart rate) and baroreflex sensitivity (baroreceptor-mediated heart rate responses that occur with changes in blood pressure) were performed to study the autonomic responses to norepinephrine in these subjects.

Section III deals with diabetic nephropathy. The main body of this thesis deals with diabetic patients that have not developed microvascular complications, and aims to gain knowledge on pathophysiological processes which might offer clues for protective measures. Unfortunately, in the future, some of the patients will probably do develop complications. Interestingly, once diabetic renal disease occurs, the risk factors for progressive kidney function loss shows impressive similarities with those present in non-diabetic kidney disease, including the role of ACE (I/D) genotype as a risk factor for progressive renal function loss. In chapter 7, an overview will be given which describes these risk factors and summarizes the possible therapeutic approaches aimed at these risk factors.
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INTRODUCTION


INTRODUCTION


