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**Diabetic complications: a role for the
prorenin – (pro)renin receptor – TGF- β_1 axis?**

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Abstract

Morbidity and mortality of diabetes mellitus are strongly associated with cardiovascular disease including nephropathy. A discordant tissue renin-angiotensin system (RAS) might be a mediator of the endothelial dysfunction leading to both micro- and macrovascular complications of diabetes. The elevated plasma levels of prorenin in diabetic subjects with microvascular complications might be part of this discordant RAS, especially since the plasma renin levels in diabetes are low. Prorenin, previously thought of as an inactive precursor of renin, is now known to bind to a (pro)renin receptor, thus activating locally angiotensin-dependent and -independent pathways. In particular, the stimulation of the transforming growth factor- β (TGF- β) system by prorenin could be an important contributor to diabetic disease complications. This review discusses the concept of the prorenin - (pro)renin receptor - TGF- β_1 axis, concluding that interference with this pathway might be a next logical step in the search for new therapeutic regimens to reduce diabetes-related morbidity and mortality.

Keywords: diabetes; cardiovascular disease; renin-angiotensin system; prorenin; (pro)renin receptor; TGF- β_1

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1. Diabetes mellitus and the renin-angiotensin system

Cardiovascular disease, including nephropathy, accounts for most of the morbidity and mortality in patients with diabetes mellitus (Grundy et al., 1999). Both macrovascular and microvascular injury, with endothelial dysfunction as the main initiating event, are thought to underlie diabetic disease complications (de Jager et al., 2006; Ladeia et al., 2005). Prospective studies have demonstrated that endothelial dysfunction is highly predictive of future cardiovascular disease (Schachinger et al., 2000).

The renin-angiotensin system (RAS) is a major contributor to the cardiovascular and renal complications of diabetes mellitus (Gilbert et al., 2003; Hollenberg et al., 2004). While the systemic RAS is undoubtedly of importance in the regulation of blood pressure and volume homeostasis, a 'local' RAS has been linked to tissue damage (Danser, 2003). Inhibition of the RAS, by blocking the generation or action of its end-product angiotensin (Ang) II, with angiotensin-converting enzyme (ACE) inhibitors and Ang II type 1 (AT₁) receptor blockers, respectively, not only improves blood pressure control but also ameliorates endothelial function and cardiovascular/renal outcomes in patients with diabetes and nephropathy (Lewis et al., 2001; Yusuf et al., 2000; Zuanetti et al., 1997). In addition, RAS inhibition is capable of reducing the incidence of new-onset diabetes (Perkins & Davis, 2008).

Remarkably, the circulating renin levels in diabetic subjects are low to normal (Danser et al., 1998; Stankovic et al., 2006). The additional, blood pressure-independent effect of RAS inhibition in diabetes may therefore point to increased tissue RAS activity in diabetic patients, e.g., in the vascular wall and kidney. This would imply that the

systemic and tissue RAS are discordant in diabetes mellitus (Gilbert et al., 2003). The underlying mechanism of this phenomenon is unknown.

2. Prorenin - (pro)renin receptor - TGF- β ₁ axis

2.1 Prorenin

The components required to synthesize Ang II in multiple tissues are generated at specific sites in the body: renin in the kidney, angiotensinogen in the liver, and ACE on the cell surface of endothelial and various other cells. With the cloning of the renin gene in 1984, prorenin was proven to be the precursor of renin (Hobart et al., 1984). A 43-amino acid N-terminal propeptide explains the absence of enzymatic activity of prorenin as compared to renin. This propeptide covers the enzymatic cleft and obstructs access of angiotensinogen to the active site of renin to generate Ang I. Circulating prorenin is mainly derived from the juxtaglomerular epithelioid cells of the kidney, although the reproductive organs, together with the adrenal, eye, and submandibular gland also generate and release prorenin (Krop & Danser, 2008; Krop et al., 2008). Prorenin release occurs constitutively, and the prorenin levels in human blood plasma are \approx 10-fold higher than those of renin (Danser et al., 1998). Renin and prorenin levels are highly correlated but do not always alter in parallel (Danser et al., 1998). This is due, at least in part, to the fact that renin, unlike prorenin, is not synthesized extrarenally. Consequently, following a nephrectomy, only prorenin is detectable in blood plasma (Krop et al., 2008).

For unknown reasons, prorenin is elevated in patients with diabetes mellitus with microvascular complications (Luetscher et al., 1985). In fact, this elevation appears to precede the occurrence of these complications (Deinum et al., 1999). A recent study

suggests that the source of the elevated prorenin in diabetes mellitus is the collecting duct. Unexpectedly, at this site Ang II stimulates the synthesis of prorenin (Kang et al., 2008), as opposed to its inhibiting effects in the juxtaglomerular apparatus. Furthermore, plasma prorenin in diabetic subjects plasma was found to correlate with the renovascular response to captopril (Stankovic et al., 2006). This would imply a direct effect of prorenin in the renal vascular bed. Taken together, these findings suggest that circulating prorenin, in contrast to what was originally thought (i.e., that it has no function), may have some function after all.

An attractive postulate is that the elevated prorenin levels in diabetic subjects are responsible for the increased tissue RAS activity in this disease, thus causing the above-mentioned disease complications (Danser & Deinum, 2005). Supporting evidence for a local role of prorenin comes from studies in transgenic rodents with (inducible) prorenin expression in the liver. These animals display increased tissue angiotensin levels, and cardiac and renal pathology independently of hypertension (Prescott et al., 2002; Véniant et al., 1996). However, no clear evidence exists for local prorenin-renin conversion (Saris et al., 2001a), and infusing prorenin does not result in the appearance of renin (Lenz et al., 1991). In the absence of extrarenal renin generation, the question then remains how prorenin might exert a local effect. The recent discovery of a receptor for both renin and prorenin offers an explanation.

2.2 (Pro)renin receptor

The heart and vascular wall do not express the renin gene. Therefore, renal renin and/or prorenin need to be taken up from the circulation to allow Ang I generation in these

organs. Simple diffusion cannot explain the renin levels at cardiac tissue sites (Danser et al., 1994; Katz et al., 1997; van Kesteren et al., 1999), and thus receptor-mediated uptake mechanisms might be involved (Danser et al., 1999). Indeed, endothelial cells, cardiomyocytes and cardiac fibroblasts are all capable of binding and internalizing renin and prorenin (Admiraal et al., 1999; van Kesteren et al., 1997). At least two candidate receptors have been proposed: the mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) (Saris et al., 2001a; Saris et al., 2001b; van den Eijnden et al., 2001; van Kesteren et al., 1997) and the (pro)renin receptor (Nguyen et al., 2002). The M6P/IGF2R non-selectively binds M6P-containing proteins like prorenin and renin. However, such binding did not result in Ang II generation, and it is now believed that the M6P/IGF2R actually is a clearance receptor for prorenin and renin (Saris et al., 2002).

This leaves the (pro)renin receptor as the most promising candidate for specific tissue uptake of circulating prorenin and renin. This receptor binds prorenin with higher affinity than renin and, unlike the M6P/IGF2R, it does not internalize these proteins (Batenburg et al., 2008; Batenburg et al., 2007). Prorenin binding to the (pro)renin receptor allows the renin precursor to become catalytically active without proteolytic cleavage of the prosegment (Batenburg et al., 2007; Nguyen et al., 2002; Nurun et al., 2007) (Figure). Such ‘non-proteolytic activation’ (involving a conformational change of the prorenin molecule) is also known to occur at low temperature (‘cryo-activation’) and low pH (‘acid-activation’) (Schalekamp et al., 2008). Apparently, binding to the receptor allows the prorenin molecule to undergo the same conformational change as exposure to low temperature or low pH. Receptor-mediated, non-proteolytic activation of prorenin solves the problem related to the absence of prorenin-renin converting enzymes at

extrarenal tissue sites: binding of prorenin to its receptor is apparently sufficient to allow the inactive precursor to gain full enzymatic activity.

The (pro)renin receptor is a 350-amino acid protein with a single transmembrane domain. Although it was first described on cultured human mesangial cells (Nguyen et al., 2002), the C-terminal part of the receptor had been described earlier as an 8.9 kDa fragment being associated with a vacuolar H⁺-ATPase (Ludwig et al., 1998). This part is highly homologous in both mammalian and non-mammalian species, suggesting that it has an important conserved function (Burcklé & Bader, 2006). Indeed, inactivation of the (pro)renin receptor gene before the end of embryogenesis is lethal in zebrafish (Amsterdam et al., 2004). The extracellular, prorenin- and renin-binding part of the receptor, retains high similarity between mammals only (Burcklé & Bader, 2006). This part of the (pro)renin receptor might therefore have developed later in evolution, for instance because there are advantages upon its binding with prorenin or renin (Bader, 2007). Interestingly, (pro)renin receptor expression appears to be upregulated under pathological conditions, e.g. at cardiac tissue sites of stroke-prone spontaneously hypertensive rats on a high salt diet (Ichihara et al., 2006a), and in the clipped kidneys of Goldblatt rats (Krebs et al., 2007).

After the discovery of the (pro)renin receptor, peptidic antagonists were designed based on the idea that the prosegment of prorenin contains a 'handle region' which binds to the receptor, allowing prorenin to become catalytically active (Ichihara et al., 2004; Nurun et al., 2007). These antagonists, known as 'handle region peptides' (HRPs), mimic the handle region and thus will bind competitively to the receptor, thereby preventing receptor-mediated prorenin activation and reducing tissue RAS activity. In support of

this, HRP infusion normalized the elevated renal Ang II content in diabetic rats (Ichihara et al., 2004), as well as the cardiac Ang II content in stroke-prone spontaneously hypertensive rats, without affecting blood pressure (Ichihara et al., 2006a).

Concomitantly, the development of diabetic nephropathy and cardiac fibrosis were prevented, suggesting that these phenomena depended on prorenin-induced tissue Ang II generation. Surprisingly however, identical renal effects of HRP occurred in diabetic AT_{1a} receptor deficient mice (Ichihara et al., 2006b), despite the fact that in these animals Ang II is no longer able to exert its normal effects (van Esch et al., 2006). This raises the possibility that (pro)renin receptor activation also results in Ang II-independent effects, in other words, that prorenin and renin act as agonists of the (pro)renin receptor, in addition to their capacity to generate Ang I when bound to the receptor.

2.3 Angiotensin-independent effects of (pro)renin receptor stimulation

Experiments with cells expressing the (pro)renin receptor have confirmed the concept of Ang II-independent effects mediated via this receptor. Both prorenin and renin were found to activate mitogen-activated protein kinases (MAPK) in the presence of RAS blockers (Huang et al., 2007b; Huang et al., 2006; Ichihara et al., 2006b; Nguyen et al., 2002; Saris et al., 2006). In neonatal rat cardiomyocytes, prorenin-induced MAPK p38 activation resulted in heat shock protein 27 phosphorylation and an alteration in actin filament dynamics, potentially affecting cardiac hypertrophy (Saris et al., 2006). In addition, in mesangial cells renin stimulated transforming growth factor β_1 (TGF- β_1) production via MAPK p42/p44, which subsequently resulted in an upregulation of profibrotic and prothrombotic molecules like fibronectin, collagen-1, and plasminogen-

activator inhibitor-1 (PAI-1) (Huang et al., 2007b; Huang et al., 2006; Nguyen et al., 2002). Increased expression of TGF- β_1 is of particular interest in relation to the disease complications of diabetes mellitus (Figure). The RAS and TGF- β system have been suggested to act together, providing an 'emergency response' to tissue injury (Border & Noble, 1998). However, when this response is inappropriate, as in diabetes, continued activation of the RAS and TGF- β system leads to progressive tissue injury with fibrosis and ultimately organ failure (Border & Noble, 1998; Gilbert et al., 2003). The TGF- β system regulates tissue remodeling by stimulation of matrix synthesis and inhibition of matrix degradation (Border & Noble, 1998). Indeed, overexpression of the TGF- β axis induces hyperplasia, apoptosis and fibrosis (Schulick et al., 1998), potentially leading to vascular and cardiac dysfunction (Chen et al., 2007). Furthermore, via upregulation of PAI-1 (Huang et al., 2007a), thrombotic cardiovascular events may occur, especially in the presence of a dysfunctional endothelium. TGF- β_1 also plays a pivotal role in the pathogenesis of diabetic nephropathy (Sharma et al., 1997; Yamamoto et al., 1993).

Results from in-vivo studies in transgenic rats overexpressing the human (pro)renin receptor are in agreement with the concept of (pro)renin-induced, angiotensin-independent effects (Burcklé et al., 2006; Kaneshiro et al., 2007). The human (pro)renin receptor binds, but does not activate rat prorenin (Kaneshiro et al., 2007), and thus rat prorenin will only induce signaling via the human (pro)renin receptor. Indeed, the plasma and tissue angiotensin levels of transgenic rats overexpressing the human (pro)renin receptor were unaltered. Yet, these animals displayed increased levels of aldosterone in blood plasma and of cyclooxygenase-2 in the renal cortex. Moreover, from the age of 7 months, they developed hypertension, proteinuria and/or glomerulosclerosis. The latter

was accompanied by increased MAPK p42/p44 phosphorylation and TGF- β_1 expression. HRP prevented the development of glomerulosclerosis in human (pro)renin transgenic rats (Kaneshiro et al., 2007). Moreover, it even reversed the glomerulosclerosis that had developed in diabetic rats (Takahashi et al., 2007)

In contrast with these observations, glomerulosclerosis did not occur in transgenic rats with inducible prorenin expression, despite the fact that such rats, following induction, displayed a 200-fold rise in plasma prorenin and were hypertensive (Peters et al., 2008). Moreover, HRP blocked neither prorenin binding to cells overexpressing the human (pro)renin receptor (Batenburg et al., 2007), nor prorenin-induced signaling in U937 monocytes (Feldt et al., 2008a). It was also ineffective in double transgenic rats expressing both human renin and human angiotensinogen (Feldt et al., 2008b), and in spontaneously hypertensive rats on a normal salt diet (Susic et al., 2008). Remarkably, in the latter model, it did decrease cardiac hypertrophy and fibrosis during high-salt conditions (Susic et al., 2008). Furthermore, HRP bound to cells which expressed a (pro)renin receptor variant lacking the transmembrane region, so that binding should actually not have occurred (Feldt et al., 2008a). Thus, at present it is uncertain to what degree the beneficial in-vivo effects of HRP are solely due to prorenin blockade. Possibly, HRP has an alternative, as yet unknown mechanism of action, unrelated to the (pro)renin receptor, and/or its effectiveness is limited to animal models with a high prorenin/renin ratio (like in diabetes).

3. Prorenin inhibition

The beneficial effects of ACE inhibitors and AT₁ receptor antagonists involve interference with the generation or effects of Ang II at tissue sites (van Kats et al., 1998; van Kats et al., 2000). Thus if, as discussed above, prorenin is a major determinant of tissue angiotensin production, prorenin is an obvious drug target. There are potentially two ways to inhibit prorenin: renin inhibitors will block the enzymatic activity of prorenin (as present after ‘unfolding’ of the prosegment (Batenburg et al., 2008; Feldman et al., 2008)), and (pro)renin receptor blockers will block both the receptor-induced prorenin activation and any receptor-mediated, angiotensin-independent effects of prorenin (Figure).

The current RAS blockers (i.e., the ACE inhibitors and AT₁ receptor blockers) act downstream of any prorenin-induced Ang I generation. Since Ang II, like prorenin, stimulates TGF- β ₁, their beneficial effects in diabetes might involve, at least in part, a reduction of TGF- β ₁. Indeed, the ACE inhibitor-induced reduction in the serum levels of TGF- β ₁ correlated with long-term renoprotection in diabetic patients (Sharma et al., 1999). Recently renin inhibitors have been introduced into the clinical arena. These drugs act directly at the start of the angiotensin generation cascade, and the question is whether they will do better than the existing RAS blockers. The first clinical results with the only renin inhibitor that is currently available, aliskiren, suggest they do. Aliskiren reduced albuminuria on top of optimal antihypertensive therapy (including AT₁ receptor blockade) in type 2 diabetic subjects with nephropathy (Parving et al., 2008), and increased renal plasma flow far greater than any other RAS blocker (Fisher et al., 2008). To explain these beneficial findings, one has to assume that renin inhibitors either block

the RAS more effectively or that they exert additional, unexpected effects, e.g. related to the (pro)renin receptor.

So far, *in vitro* studies do not reveal any blocking effects of aliskiren towards the direct effects of renin/prorenin via their receptor. Aliskiren neither affected the binding of renin/prorenin to the receptor, nor their signaling cascade following receptor activation (Batenburg et al., 2008; Feldman et al., 2008; Feldt et al., 2008a). Aliskiren however did block angiotensin generation by receptor-bound prorenin (Batenburg et al., 2008). In addition, renin inhibitors will suppress the consequences of the renin rise that occurs in response to every type of RAS blocker. During both ACE inhibition and AT₁ receptor blockade this renin rise will result in the generation of substantial amounts of Ang I, allowing an Ang II 'escape' during ACE inhibition and the stimulation of non-AT₁ receptors during AT₁ receptor blockade (Danser, 2007). During renin inhibition however, the continuous presence of the renin inhibitor prevents such Ang I generation (Danser et al., 2008), and thus the angiotensin levels will remain low (Fisher et al., 2008). Therefore, the most important reason for the effectiveness of aliskiren, particularly on top of maximum doses of other RAS blockers, appears to be that renin inhibitors offer superior RAS blockade.

RAS blockers not only cause a rise in renin, but also (although to a lesser degree) a rise in prorenin (Schalekamp et al., 2008). One might argue that this would result in increased (pro)renin receptor activation, thus potentially causing detrimental effects. However, no such detrimental effects have been observed during any of the many clinical trials with RAS blockers. A possible explanation for the lack of such effects comes from the work of Schefe et al. (Schefe et al., 2006; Schefe et al., 2008), who showed that, on

activation of the receptor, the transcription factor promyelocytic zinc finger is translocated to the nucleus and represses transcription of the (pro)renin receptor itself, thus creating a short negative feedback loop. In other words: high (pro)renin levels, as occurring during RAS blockade, will suppress (pro)renin receptor expression, thereby preventing excessive receptor activation. In fact, diabetic rats treated with aliskiren, displayed a decrease in (pro)renin receptor expression (Feldman et al., 2008), possibly due to the above described feedback mechanism. Such downregulation might also explain, at least in part, the absence of glomerulosclerosis in transgenic rats with inducible prorenin expression (Peters et al., 2008).

Given the lack of effect of aliskiren versus direct prorenin/renin-induced effects, (pro)renin receptor blockers would be expected to exert additional effects on top of renin inhibitors, e.g. they might induce a (further) reduction in TGF- β_1 levels. Although the first results with such blockers, particularly in diabetic animals, indeed look promising (Ichihara et al., 2004; Ichihara et al., 2006a; Kaneshiro et al., 2007), the lack of effect of HRP observed in animals with high-renin hypertension (Feldt et al., 2008a; Feldt et al., 2008b) suggests that more work and/or better blockers are needed. No clinical data are currently available with these drugs.

4. Conclusions

The prorenin - (pro)renin receptor - TGF- β_1 axis is an exciting new pathway that finally provides a role for the 'inactive' precursor of renin. This is of particular relevance in diabetes accompanied by microvascular complications given the high prorenin (and low renin) levels observed in this condition. Why prorenin is elevated in the first place and

whether in humans, as in rats (Kang et al., 2008), it originates in the collecting duct remains to be determined. Prorenin might be the ‘missing link’ explaining the high tissue RAS activity and low plasma RAS activity in diabetes. Its local activation does not require a prorenin-renin converting enzyme (for which there is no evidence (Saris et al., 2001a)), but simple binding to a receptor. Renin inhibitors will efficiently block any Ang I generation by receptor-bound prorenin (Batenburg et al., 2008), thus potentially explaining why they exert effects on top of optimal therapy with ‘classical’ RAS blockers like the ACE inhibitors and AT₁ receptor antagonists. Renin inhibitors do not interfere with the direct, angiotensin-independent effects of prorenin mediated via the (pro)renin receptor. Since the latter involve, among others, TGF- β ₁ production (Huang et al., 2007b), a (pro)renin receptor blocker might theoretically reduce TGF- β ₁ on top of maximal RAS blockade. This is attractive since combined RAS blockade and TGF- β inhibition already resulted in enhanced antifibrotic effects (Yu et al., 2004).

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Figure Legend

Figure. Schematic overview of the prorenin - (pro)renin receptor - TGF- β_1 axis. A 43-amino acid N-terminal propeptide normally covers the enzymatic cleft of prorenin, thus obstructing access of angiotensinogen (Aog). (Pro)renin receptor-binding however induces a conformational change in the prorenin molecule, now allowing prorenin, like renin, to generate angiotensin (Ang) I from Aog, despite the fact that the prosegment is still present. In addition, prorenin binding per se (i.e., independently of angiotensin generation) activates intracellular signaling pathways. Theoretically, a (pro)renin receptor blocker will block both the (pro)renin receptor-mediated prorenin activation (resulting in angiotensin-dependent effects) and the direct (pro)renin receptor-induced intracellular signaling (resulting in angiotensin-independent effects), whereas a renin inhibitor will only block the angiotensin-mediated effects. ACE, angiotensin-converting enzyme; AT₁R, Ang II type 1 receptor; (P)RR, (pro)renin receptor, PAI-1 plasminogen-activator inhibitor-1; TGF- β_1 , transforming growth factor β_1 .

