Chapter 8

8.1 Ang-(1-7): a versatile modulator of the renin-angiotensin system

In this thesis, it is demonstrated that Ang-(1-7) modulates the RAS activity by antagonizing Ang II-induced vasoconstrictions in a variety of ways, and inhibiting ACE activity. Inhibition of ACE, which degrades Ang-(1-7) to the inactive Ang-(1-5), by lisinopril improves the antagonizing effect of Ang-(1-7) on Ang II-induced vasoconstrictions. This chapter presents an outline of the rationales that led to the studies described in this thesis, and is a summary of the findings. In addition, some considerations for future studies are indicated.

At the start of the studies on human arteries, presented in this thesis, the effect of Ang-(1-7) on RAS activity in humans was not known. From the literature, it was known that Ang-(1-7) could antagonize Ang II-induced vasoconstrictions in rabbit aorta and inhibit the activity of isolated canine ACE. Clearly, this was not sufficient to predict the role of Ang-(1-7) in human vascular function, and a set of experiments was planned to obtain a better insight into this matter.

In Chapters 2 and 3, the effect of Ang-(1-7) on RAS activity in human arteries, plasma, and cardiac tissue was explored. The data show that Ang-(1-7) antagonizes Ang II-induced vasoconstrictions in a non-competitive way (Chapter 2 & 3). Furthermore, Ang-(1-7) inhibits plasma, cardiac tissue, and vascular tissue ACE activity (Chapter 3). Ang-(1-7) had a greater affinity for ACE than Ang I (Chapter 3). The latter result indicates that Ang-(1-7) may be an important competitor of Ang I for binding to ACE. Because ACE inhibition augments Ang-(1-7) levels, a consequence of reduced Ang-(1-7) breakdown, Ang-(1-7) may have a significant bystander effect in patients treated with ACE inhibitors. Already it has been shown that Ang-(1-7) contributes to the antihypertensive effects of blockade of the RAS in animals. This observation still has to be confirmed in patients. Measurement of plasma and tissue levels of Ang I, Ang II as well as Ang-(1-7) in studies that explore the clinical effects of ACE inhibitors in patients may provide more insight in the role of Ang-(1-7).

Ang-(1-7) antagonizes Ang II-induced vasoconstrictions. Before onset of the studies presented in this thesis, it was suggested that occupation of the \( \text{AT}_1 \) receptor or Ang-(1-7)-induced NO release be the mechanisms. However, from cell culture studies other possible mechanisms arose. We speculated that Ang-(1-7) prostaglandin release, possibly through stimulation of \( \text{AT}_2 \) or \( \text{BK} \_B_2 \) receptors, may also antagonize Ang II. As Ang-(1-7) levels are augmented by ACE inhibition, an ACE inhibitor might increase these actions of Ang-(1-7). These hypotheses were addressed in a series of studies performed on aortic segments of normotensive rats described in chapters 4 and 5.
In Chapter 4, it is demonstrated that this antagonistic action of Ang-(1-7) is mediated by prostaglandins. This could be the vasodilator prostacyclin, which is released from cultured vascular smooth muscle cells upon stimulation with Ang-(1-7). The involvement of NO in rat aorta is still doubtful. However, in other setups, with other vessels types, it was demonstrated that also NO can play a role in the effects of Ang-(1-7). This may in particular be the case in coronary vessels. Thus, each vessel type responds differently to Ang-(1-7), which may have important clinical implications for therapy with ACE inhibitors, but also for the validity of models in which the effects of ACE inhibitors are tested.

In Chapter 5 the question through which receptor types Ang-(1-7) antagonizes is involved in the antagonistic effect of Ang-(1-7) was addressed. AT$_1$-receptors, AT$_2$-receptors, non-AT$_1$/AT$_2$ receptors for Ang-(1-7), and BK B$_2$ receptors were suggested as candidates.

In the presence of the ACE inhibitor lisinopril, Ang-(1-7) decreased the responsiveness of rat thoracic aorta to Ang II through stimulation of AT$_2$ receptors and BK B$_2$ receptors. The involvement of AT$_2$ receptor can be explained, because prostaglandin release upon stimulation of AT$_2$ receptor with Ang-(1-7) has been observed in other studies. For BK B$_2$ receptors, a direct interaction of Ang-(1-7) has not been demonstrated yet. Rather, Ang-(1-7) could induce BK release through AT$_2$ receptors, thus stimulating BK B$_2$ receptors.

A second way for Ang-(1-7) to antagonize Ang II-induced vasoconstrictions is occupation of the AT$_1$ receptor. Although the literature reported that in untreated rabbit aorta this pathway for antagonism is used, in rat aorta, pretreatment with L-NMMA- and indomethacin abolished the effect of Ang-(1-7). Apparently, Ang-(1-7) acts differently on AT$_1$ receptors in rat aorta than in rabbit aorta. Therefore, the hypothesis that Ang-(1-7) antagonizes Ang II-induced vasoconstriction by binding to the AT$_1$ receptor cannot be rejected.

The third pathway for Ang-(1-7) to antagonize, namely stimulation of D-Ala$^7$-Ang-(1-7)-sensitive non-AT$_1$/AT$_2$ angiotensin receptors, is not involved in the antagonizing effect of Ang-(1-7) on Ang II in rat aortic tissue. Therefore, the effects of Ang-(1-7) on blood pressure through D-Ala$^7$-Ang-(1-7)-sensitive receptors described in the literature$^{2,3}$ probably involve an interaction in tissues other than the vessel wall. However, the already mentioned vessel type- and species-related differences may be applicable here as well. More research in different vessel types, such as coronary and resistance vessels, is indispensable to obtain full insight into the direct effect of stimulation of the non-AT$_1$/AT$_2$ receptors on vasomotor function.
The effect of Ang-(1-7) mediated by prostaglandins was increased by ACE inhibitors (Chapters 4 & 5). Therefore, the antihypertensive effects of ACE inhibitors may be facilitated by an increase in Ang-(1-7)-induced release of vasodilating prostaglandins. Recently, it was shown that there is an inverse correlation between blood pressure and Ang-(1-7) levels in patients with essential hypertension. Also it was shown that Ang-(1-7) contributes to the antihypertensive effects of RAS blockade. The interaction of Ang-(1-7), lisinopril, prostaglandins, AT2 receptors, and BK B2 receptors may therefore importantly contribute to the clinical effects of ACE inhibitors.

As indicated in Chapter 2, not all of the effects of ACE inhibitors observed in rat models for cardiovascular disease could be reproduced in patients. This is particularly the case in studies to prevention of restenosis after angioplasty. It was suggested that the presence of alternative Ang I to Ang II conversion pathways in human vascular tissue could be the cause of the lack in effectiveness of ACE inhibition. If Ang-(1-7) plays a role during ACE inhibition, it is important to understand the relation between Ang-(1-7) and alternative conversion. In this thesis, this relation was studied. The next paragraph is an outline of the data that provide relevant information.

8.2 Alternative conversion of Ang I to Ang II by tissue chymase: escape from ACE inhibitors

In Chapter 2 it was demonstrated that chymase is present in the vascular wall of human arteries. Consequently, the effect of Ang-(1-7) on contractions induced by the ACE-specific Ang I analogue [Pro10]-Ang I and the non-ACE Ang I analogue [Pro11,D-Ala19]-Ang I were compared. The results showed that Ang-(1-7) inhibits ACE but not chymase. Both the presence of an alternative Ang I conversion pathway, as the ineffectiveness of Ang-(1-7) to inhibit this conversion pathway may have consequences for the local function of Ang-(1-7).

When compared to each other, the antagonistic effect of Ang-(1-7) on Ang II-induced contractions in human arteries (Chapter 3) is stronger than in rat aorta (Chapter 4). In rat aorta, lisinopril is needed to enhance Ang-(1-7) to reach an effect that is comparable to that in human arteries. This suggests that in rats more ACE is present than in humans, which can be demonstrated in plasma (Fig. 1). In addition, Ang-(1-7) causes a rightward shift in the dose-response curve of Ang I in rat aorta (Fig. 2), which is not observed in human arteries (Chapter 3). A possible reason that humans have a lower ACE activity is may be the presence of chymase. Such a highly active alternative Ang I to Ang II conversion pathway is not present in the rat vasculature, and therefore, rats would need higher amounts of local ACE. Unfortunately, no comparative data are available on vascular ACE levels in rat and human arteries, so a definite conclusion can not be drawn. Still, chymase influences the function of Ang-(1-7). The inability of
E inhibitors may be prostanoids, L-prostaglandins. The interaction of Ang-(1-7) with the ACE pathway may therefore be observed in rat plasma, particularly that Ang-(1-7) may have suggested that there is a clear interaction between Ang-(1-7) and Ang-(1-7). The Ang-(1-7) concentration between Ang-(1-7) was studied. The

ACE inhibitor function of Ang-(1-7) is circumvented, and Ang II can still be formed from Ang I. Chymase is present in the adventitia of blood vessels, and can not be inhibited by ACE inhibitors or Ang-(1-7). This has important clinical implications. Firstly, the un-
desired overproduction of Ang II in vascular tissue will persist in patients treated with ACE inhibitor. Apart from this, less Ang I will be available for the production of Ang-(1-7) so that Ang II escapes from antagonism by Ang-(1-7).

From the above, it becomes clear that ACE inhibition alone may not be sufficient to efficiently block RAS activity in the human vessel wall. Amongst others, a sufficient increase in production of counterregulating Ang-(1-7) may not be reached. To obtain the beneficial effects of Ang-(1-7) in patients, an alternative way to increase Ang-(1-7) levels in vascular tissue should be found. Expression of peptides through transfer of genetic material (gene transfer or transfection) by vectors into the vascular tissue is a promising technique. This prompted the investigations on the ability of recombinant Semliki Forest virus vector to transfer genes into vascular tissue described in chapter 6 and 7. In the next paragraph, the data of these studies are summarized, and a possible vector for Ang-(1-7) production is presented.

8.3 Viral vectors: towards a new approach for cardiovascular therapy through increased Ang-(1-7) production

Gene transfer, or transfection, techniques offer the possibility to increase the production of proteins in cardiovascular tissue. Viral vectors currently represent the most efficient gene transfer systems, and have been successfully employed in animal models for the treatment of restenosis after angioplasty. Viral vectors have several drawbacks such as lack of specificity for cell type and a late onset of gene expression. Transfection with vectors based on the Semliki Forest virus (SFV) may overcome these drawbacks. In Chapter 6 it is shown that SFV induces a faster expression of foreign genes than adenoviral vectors in cultured VSMC. Furthermore, SFV selectively transfects VSMC as compared to endothelial cells, and, therefore, it is not cytotoxic to endothelial cells.

It was speculated that SFV selectively transfers genes into vessel segments in which the endothelium has been disrupted by an angioplasty procedure. In Chapter 7 it is demonstrated that SFV selectively transfects balloon-injured vessel segments. Theoretically, vectors based on SFV provide the possibility to intervene in the very early processes involved in hypertrophy of the vessel wall after angioplasty. Furthermore, SFV transfects VSMC, the main target cell, and leaves the endothelial cells unaffected. An intact endothelium will reduce hypertrophy and, hence, the risk for restenosis.

Once SFV has entered the target cell, the translational machinery of the host will be completely occupied for the expression of the transferred genes. The target cell will lose its physiological function and will perish eventually. Therefore, SFV is particularly suitable to increase expression of paracrine factors. Ang-(1-7) would be an inter-
patients treated with recombinant SFV-Ang-(1-7) were insulin resistant, a sufficient production of Ang-(1-7) was observed. To obtain a sufficient Ang-(1-7) production in the vessel wall, an Ang-(1-7)-coding recombinant SFV was designed, based on the genetic code for angiotensinogen (see Appendix). In a pilot study, the expression of SFV-Ang-(1-7) in cultured VSMC (rat aorta A7r5 cells) was measured with the use of a radioimmunoassay for Ang-(1-7). A control vector carrying the lacZ reporter gene already augmented the level of Ang-(1-7) immunoreactivity, but SFV-Ang-(1-7) further increased this level (Fig. 3).

Although SFV-Ang-(1-7) increased Ang-(1-7) release, it is difficult to predict whether the apparent Ang-(1-7) levels observed in this pilot were increased to a point that a clinical effect could be expected. The Ang-(1-7) level may seem rather low to expect a clinical effect. However, the SFV titers used were rather high and, therefore, may already be cytotoxic in an early phase. Lowering the virus titers may optimize the expression of Ang-(1-7). In addition to this, the viral genome may be equipped with a translational enhancer to increase foreign gene expression. Secondly, different VSMC cell types may express Ang-(1-7) to different levels. Thirdly, the identity of the Ang-(1-7) immunoreactive products has to be confirmed biochemically. These questions have to be solved first before Ang-(1-7) expressing vectors based on the present Ang-(1-7)-coding sequence can be applied in a rational manner in vivo. Once characterized, these vectors can either be used in models for cardiovascular diseases, such as restenosis, or they can be used to explore the physiological relevance of Ang-(1-7), for instance through transgenic animals overexpressing Ang-(1-7).
Chapter 8

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