SUMMARY

SUMMARY OF THE CHAPTERS The aim of the studies was to investigate: 1) whether changes in microvasculature, angiogenesis, and angiogenesis factors occur in heart failure, and affect its outcome and 2) whether neurohormonal correlates can be identified that modulate these factors, and 3) whether correction of the microvascular abnormalities with specific neurohormonal inhibitors is possible. It was shown that changes in microvasculature, angiogenesis, and angiogenesis factors occur in heart failure, although it is not established yet whether changes in microvasculature, angiogenesis, and angiogenesis factors affect the outcome of heart failure. The second aim yielded data on the impact of neurohormonal parameters, and signaling proteins that confer neurohormonal activation. We established that blockade of the AT_1 receptor, beta-receptor and ERK may be involved in microvascular abnormalities.

Heart failure is a widespread disease in developed countries with high morbidity and excess mortality. Historically, heart failure was regarded as a contractile impairment of the heart muscle, leading to hypoperfusion of the organs, especially upon demand (exercise). Nowadays, heart failure rather is considered as a clinical syndrome, in which the cardiac dysfunction is the primary trigger for activation of various counter-regulatory systems. Although on the short term these systems may uphold the circulation, on the long term they further impede cardiac function, and also adversely affect (remodel) other organ systems, especially the vasculature and the kidneys.

A specific compartment of the vasculature, the microcirculation, presents with distinct abnormalities in heart failure. The microvasculature consists of small arterioles (beyond the third order), capillaries and efferent venules. Generally, microvessels with a diameter of <150 μm are referred to as "microvessels". The abnormalities comprise a decrease in total number of microvessels, as well as a dysfunction of the remaining endothelium (endothelial dysfunction).

Although the concept of a contributory role of endothelial dysfunction to cardiac dysfunction is widely accepted, the importance of a deficient microvasculature is contested. It is not well understood what the role is of various factors that become activated in heart failure with respect to the growth and maintenance of the microvessels. Factors that are mitogenic to the endothelium have been isolated, and are thought to play a major role in the growth of microvessels. Especially vascular endothelial growth factor (VEGF) is a key factor in microvessel growth (angiogenesis). This factor is secreted by many cells upon hypoxia.

This thesis addresses issues that may clarify the presence of microvessel abnormalities. Specifically, the number of microvessels in (hypertensive) cardiomyopathy and after myocardial infarction was studied. Pharmacological modalities were evaluated for their value to restore microvessel abnormalities. In chapters **1** and **2**, definitions and terminology are provided, and aims and scope of the thesis are described. Since heart failure is a syndrome with a heterogeneous etiology, we discussed in chapter **2** the microvascular abnormalities that are present in various forms of heart failure. The knowledge that has been accumulated thus far, points towards a decrease of microvessels in most forms of heart failure.

Some specific forms of cardiac hypertrophy, like exercise- and thyreotoxicosis-induced left ventricular hypertrophy (LVH), are however accompanied by an increase in microvessels. Noteworthy, these forms of LVH rarely culminate into heart failure. The expression patterns of growth factors that mediate microvessel growth have inadequately been studied. Neurohormonal disarray, an important pathophysiological contributory factor in heart failure, affects microvascular growth, but the precise interplay between the two is not fully elucidated.

In chapter **3**, the expression pattern of VEGF in idiopathic dilated cardiomyopathy (IDC) was investigated, establishing a down-regulation of VEGF, irrespective of the severity of the disease was established. Moreover, there seems to be a differential activation pattern, with a more substantial down-regulation of the smallest VEGF isoform ($VEGF_{121}$), and a smaller decrease of $VEGF_{165}$ and $VEGF_{189}$. It is hypothesized that this downregulation of VEGF could be associated with the observed decrease of microvessels in IDC, and may entail a mechanism that, at least in part, explains the ischemia-like myocardial responses to stress.

In chapter **4**, levels of plasma VEGF were studied in patients with heart failure, and the response to treatment with the beta-blocker carvedilol. It was found that plasma levels of VEGF rise after initiation of beta-blockade. This may reflect increased myocardial VEGF production in the heart, possibly due to lowering of the heart rate. Further studies should delineate whether the beneficial effects of beta-blockers in heart failure, but also in ischemic heart disease, are explained by the upregulation of VEGF and subsequent angiogenesis.

To investigate the impact of the renin-angiotensin system (RAS) on microvascular growth in heart failure, transgenic models were used that overexpress specific elements of the RAS. In chapter **5**, a model of experimental myocardial infarction (MI) that induces left ventricular remodeling was studied, and normal rats were compared with transgenic rats (TGR), that have cardioselective overexpression of the angiotensin II type 1 (AT_1) receptor. Part of the rats was treated with the selective AT_1 receptor blocker losartan. It was shown that high levels of AT_1 receptors are associated with low levels of microvessel density in the non-infarcted, remodeled part of the myocardium. This was amenable to selective AT_1 receptor blockade. VEGF showed a differential expression pattern in these rats. Although it was increased after MI, AT_1 receptors blockade blunted rather than stimulated VEGF expression. The VEGF expression was increased post-MI. However, it was not correlated with an increase in microvessel density. Hemodynamic alterations and the level of neurohormonal activation did also not affect VEGF expression. Microvessel density seemed thus regulated by AT_1 receptor level, but possibly also by other neurohormonal parameters, and hemodynamics.

Chapter **6** evaluates the mitogen-activated protein kinases (MAPKs) pathways that may mediate AT_1 receptor signaling, using parameters of LV remodeling as outcome measures. For this, another transgenic model, the well described REN2 rat (homozygous variant) was employed. This model is homozygous for the expression of the mouse renin-2 gene. This strain has severe end-organ damage that quickly proceeds into intractable and lethal heart failure. We found that in the REN2 rat, the extracellular signal-related kinase (ERK) is activated, whereas p38 MAPK remains unactivated. Hereafter, the value of in-

hibitors of ERK was explored. It was found that ERK inhibition partially led to prevention of LVH and LV dysfunction. Furthermore, ERK-inhibition completely prevented fibrosis, and restored microvessel density to its control values. Control treatment with AT_1 receptor blockade (candesartan) was also able to prevent the development of cardiac dysfunction, and LV architectural changes. It is concluded that in this model of hypertensive heart failure, ERK is activated and ERK inhibition prevents LV remodeling. This may be a cell-specific activation, with a relative over-activation in fibroblasts and endothelial cells, since inhibition was especially efficacious in preventing fibrosis and microvessel abnormalities.

In chapter 7, the effects of AT_1 receptor signaling on *short-term* ischemia were studied. Again, the TGR model with cardioselective overexpression of the AT_1 receptor was employed, both with and without the selective AT_1 receptor blocker losartan. In the isolated rat heart, we studied the impact of ischemia-reperfusion on cardiac damage and total time of arrhythmia during reperfusion. *In-vivo*, we studied the short-term effects of MI by recording mortality and serum troponin. AT_1 receptor overexpression was not found detrimental in terms of duration of reperfusion-induced arrhythmias, however AT_1 receptor blockade limited total duration of reperfusion-induced arrhythmias, specifically in the TGR. After MI, all rats exhibited a similar elevation of serum troponin and equal infarct sizes. Mortality however, was increased in TGR, and AT_1 receptor blockade prevented the excess mortality. From the 2 studies with the TGR (chapter 5 & 7), it is concluded that the *level* of AT_1 receptor expression critically regulates ischemia-related cardiac parameters, both early after the ischemic event, as well as during the remodeling process. Reversely, AT_1 receptor blockade is able to prevent the detrimental effects of the elevated AT_1 receptor expression. This may be of clinical importance, since many clinical syndromes such as coronary artery disease with stuttering ischemia, and left ventricular dysfunction are characterized by increased expression of the AT_1 receptor. Noteworthy, these syndromes are also associated with an increased incidence of ischemic events.

In chapter 8, the efficacy of gene therapy with DNA encoding human VEGF was studied in a model of experimental MI in rats. Parameters of LV remodeling were monitored as outcome measures, with echocardiography, and in the isolated heart in a perfusion setup. Although the gene therapy led to increased VEGF gene expression and increased VEGF levels, no improvement on LV remodeling parameters could be found. Intramyocardial delivery of the gene (and the control DNA) increased coronary resistance, possibly due to myocardial swelling. It is speculated that another time point, later after MI, may ameliorate LV remodeling, since endogenous VEGF levels have declined by then. Infarct size was limited by VEGF therapy, and this may also translate into improved function later after MI. This remains to be shown in future studies.

FUTURE DIRECTIONS

MICROVASCULAR GROWTH, ANGIOGENIC FACTORS, AND HEART FAILURE There is still debate whether the decrease of microvessels is cause or consequence in heart failure. It has been established that endothelial cells, that mainly constitute microvessels, are extremely important in regulating myocardial function. The microvascular endothelium actively

controls the local circulation, and directly modulates myocyte function through local mediators[1]. Furthermore, endothelial dysfunction (endothelial denudation with Triton X-100) causes dysfunction of the myocardial musculature, with decreased cardiac performance[2]. To fully appreciate the importance microvascular abnormalities in heart failure, studies should be conducted that test the effect of a decreased number microvessels on myocardial function. Administration of anti-angiogenic compounds (like angiostatin[3], or synthetic analogues of fumagillin, like AGM-1470[4]) could put this into effect, when tested in models that are prone to develop heart failure, such as rat models with LVH (SHR, REN2). It is hypothesized that the decrease in microvessels will accelerate the progression to heart failure. If so, this provides a rationale for therapies that aim to restore microvascular abnormalities in heart failure.

ANGIOGENESIS, NEUROHORMONES, AND INTRACELLULAR SIGNALING PROTEINS This thesis describes a distinct pattern of microvessel growth and expression of factors that mediate angiogenesis in heart failure. This pattern is characterized by a lacking microvessel growth, that is mediated, amongst other factors, by the AT_1 receptor, sympathetic signaling, and elements of the intracellular signaling pathway. The AT_1 receptor was shown to be associated with lower microvessel numbers in LHV after MI, and in hypertension. VEGF release was not correlated with the observed dynamics in microvessel growth, and therefore other factors like hemodynamics, neurohormonal activation, and signaling cascades may be more important in this respect. *In-vitro*, there is compelling evidence that neurohormones, especially angiotensin II, lead to upregulation of angiogenic factors, and subsequent angiogenesis. This may go via TGR-β signaling as well[5;6]. *In-vivo*, neurohormones may have reverse effects, as was shown in this thesis. The treatment of heart failure nowadays comprises an array of neurohormonal inhibitors. It is hypothesized for the 2 mostly used therapies, ACE-inhibitors and beta-blockers, that the effect probably is pro-angiogenic. AT_1 receptor blockade also exerts pro-angiogenic effects *in-vivo*. Future studies should isolate additional factors that mediate the microvessel growth and the release of angiogenic factors. Since the observed discrepancy between *in-vitro* and *in-vivo* findings (at least when the role of the AT_1 receptor is concerned), a focus should be on (experimental and human) *in-vivo* studies. Clearly, there is a need for data that explain the discrepancy between *in-vitro* and *in-vivo* effects of neurohormones.
In the hypertensive setting, the relative contribution of mitogen-activated protein kinases (MAPKs) was studied, and the microvessel density was found to be mainly mediated by the extracellular signal-related kinase (ERK) member of the MAPKs. Knowledge on the intracellular pathways that confer the effects of neurohormonal signaling has quickly expanded the last few years[7;8]. It has become clear that these pathways are operative in the heart and are associated with the development of cardiac hypertrophy[7], atrial fibrillation[9], and myocardial ischemia[10]. *In-vitro*, substantial knowledge has been gathered on the pathways that may direct the expression of angiogenic factors. In endothelial cells, epidermal growth factor (EGF), that is directly upstream of ERK, is phosphorylated by AT_1 receptor signaling, and this in turn upregulates VEGF. It was shown that *exclusive* activation of ERK leads to strong enhancement of VEGF gene expression by means of HIF-1-dependent phopsphorylation. This points towards an important role of the ERK sys-

tem in angiogenesis. In cardiac fibroblasts, a similar pathway is operative, via the AT_1 receptor and ERK[11]. In-vivo, there are many reports on the MAPK activation in the failing heart; it remains unknown however whether each cell types activate a specific member, and what the importance is for the pathophysiology.

FUTURE THERAPIES? A promising approach would be to use models of cardiac hypertrophy with a known enhancement microvessel growth and increase of angiogenic factors (this is discussed in chapter 2), like exercise-induced and thyreotoxicosis-induced LVH. These models should be compared to LVH with a decreased microvessel content, like post-MI and hypertensive LVH. With the use of gene array techniques that are already operative in cardiovascular research[12;13], it is feasible to isolate genes that are responsible for maintenance and growth of microvessels in these distinct hypertrophic states. The thus-established genes should prompt studies with gene therapy or pharmacotherapy.

The use of therapeutic angiogenesis is promising in this respect. Although the study reported in chapter 9 failed to show a beneficial effect, possibly a better timing or repetitive administration would have yielded positive results. Although VEGF is by now the most used agent, strategies as described above would possibly reveal newer (better) agents. After MI, the injection of DNA encoding the angiogenic factor (like VEGF) can be performed in adjacent myocardial tissue, from which the transfected will diffuse to the ischemic parts ("A little VEGF goes a long way"[14]).

The application of MAPK inhibitors was beneficial with respect to restoring microvessel growth in LVH. Other parameters of LV remodeling improved as well. This promising results back up the findings by others, that also showed beneficial effects of MAPK inhibitors[10;15]. Future studies should establish the activation pattern of signaling proteins in heart failure, with a focus on the cell-specific activation. This should allow a targeted therapy that may yield a full inhibition of the deleterious signal, without an escape phenomenon, as regularly seen in "classical" neurohormonal therapy.

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